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Aberrations in the Development of Dendritic Cells in the Autoimmunity-Prone Non-Obese Diabetic Mouse

Tatjana Nikolic

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Afwijkende ontwikkeling van dendritische cellen in de autoimmuniteit-gevoelige non-obese diabetische muis

Proefschrift

ter verkrijging van de graad van doctor aan de
Erasmus Universiteit Rotterdam
op gezag van de
Rector Magnificus

Prof. dr. S.W.J. Lamberts

en volgens besluit van het College voor Promoties

De openbare verdediging zal plaatsvinden op
woensdag 3 november 2004 om 15:45 uur

door

Tatjana Nikolic

geboren te Zenica (Joegoslavië)

Promotiecommissie

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The studies presented in this thesis were performed at the Department of Immunology of the Erasmus MC in Rotterdam, The Netherlands, with financial support of the Dutch Diabetes Research Foundation – DFN (Grant 96.606). Printing of this thesis was financially supported by Dutch Diabetes Research Foundation; Immunology Dept, Erasmus MC; Erasmus University and Mrs. A. Fekec

Lay-Out: A.Vasic/T. Nikolic
Figures: T. van Os/T.Nikolic
Cover and illustrations: A.Vasic
Printing: Offsetdrukkerij Ridderprint, Ridderkerk

to my parents....

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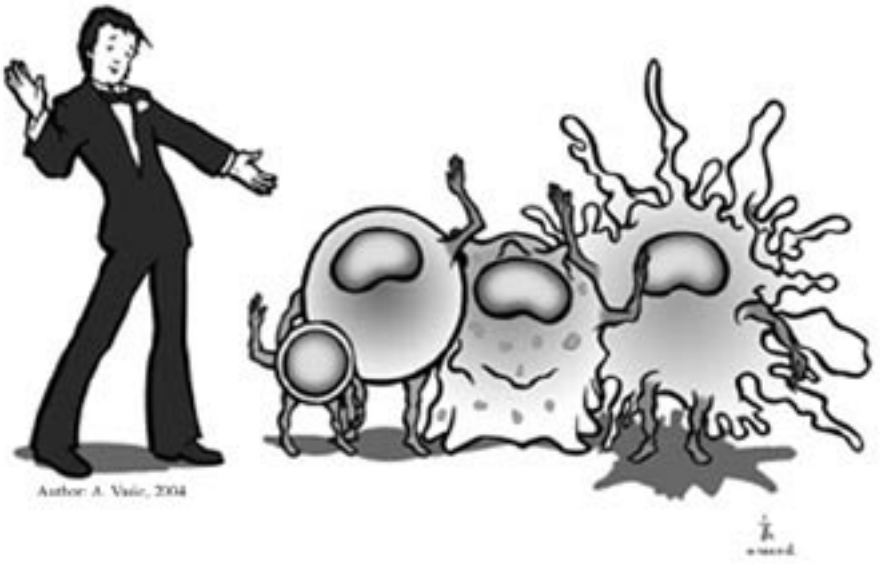
Introduction



1.1

Heterogeneity of the Mononuclear Phagocyte System

To be published in modified form as a chapter:
Histiocyte function and development in the normal immune system
Tatjana Nikolic and Pieter J.M. Leenen
In: **Histiocyte Disorders of Children and Adults**; ed. S.Weitzman & M. Egeler;
Cambridge University Press

The mononuclear phagocyte system (MPS) consists of hematopoietic cells with diverse characteristics, which were originally recognized on the basis of a common lineage derivation and a primary function of endocytosis [1, 2]. In general, cells of the MPS originate in the bone marrow (BM) and migrate as monocytes through the blood to peripheral tissues, where they develop into mature cells with different features depending on tissue-specific environmental conditions. Macrophages (Mϕ) occur in virtually all organs of the body [2]. Moreover, each organ mostly contains multiple, phenotypically different Mϕ subpopulations. For example, the lung hosts at least two different Mϕ types: alveolar Mϕ in the alveolar space and interstitial Mϕ integrated in the tissue. Microglia are Mϕ of the nervous tissue, osteoclasts are phagocytes of the bone and Kupffer cells of the liver. All these cells belong to the group of resident Mϕ. Since many of them were identified before their relationship to the MPS was established, their names often do not categorize them as Mϕ. Apart from resident, exudate Mϕ develop at acute inflammation sites [3]. In chronic inflammation, granuloma Mϕ and epitheloid cells are present, which can fuse to form multinucleated giant cells [4, 5].

Soon after the identification of the dendritic cell (DC), by Steinman and co-workers [6, 7], members of this family were considered as candidates for the MPS [2, 8]. However, initially this notion has not created a broad audience. The more recent observation that DC as well as Mϕ can be generated *in vitro* from peripheral blood monocytes has increased the awareness that both cell types are closely related and are justly unified in a single system [9-13].

A conceptual difficulty, discussed further in this review, is raised by the relatively recent findings that DC as well as Mϕ may originate from either myeloid- or lymphoid-related progenitors [14, 15]. In addition, some populations of mononuclear phagocytes may be maintained in the steady state independently from BM precursors [16], while others are strictly BM-dependent. Thus, despite the similarities in morphological and molecular phenotype observed in the various MPS populations, a common developmental origin might be an incompletely fulfilled property for all cells that belong to the MPS.

Similar to their developmental diversity, the various members of the MPS have widely diverse functions and cannot be unified by a single functional characteristic. The most common feature of mononuclear phagocytes is their significant ability to endocytose different substances, hence their name. However, DC are only strongly endocytic in an immature state and downregulate this activity upon final maturation. Furthermore, the functions that DC and Mϕ fulfill by performing endocytosis are different. Macrophages use their endocytic potential for the primary function of clearance of pathogens or other unwanted material with least consequences for the host. Dendritic cells, however, employ the endocytic process as a mean to collect antigens in order to present them to cells of the adaptive immune system. In other words, although these two cell types share the capacity to engulf substances of foreign or of self-origin, they employ this feature in different manners to perform distinct functions.

The most well-known functions ascribed to Mϕ on the one hand - clearance by phagocytosis and digestion - and to DC on the other hand - primary activation of the adaptive immune system - have long been the leading principle in distinguishing between these cell types. Therefore, this convention will be retained in the description below. However, it should be realized that this distinction is strongly biased by the initial, historical characterization of these cell types, while the biological reality is much more complex. Hence, mononuclear phagocytes should be considered as a large family of functionally and/or developmentally related cells that form a continuum in which the cells that are recognized as prototypical “Mϕ” and “DC” are the

extremes of a spectrum.

Given the lack of selection criteria that characterize all cells of the MPS unequivocally, we propose that mononuclear phagocytes are leukocytes that lack specific criteria found in other leukocytes such as polymorphonuclear morphology, specific granularity or unique antigen-specific receptor expression realized by gene rearrangement. Mononuclear phagocytes have specialized in certain functions that are shared by large groups in the family, such as endocytosis, antigen presentation, and sentinel function to exogenous stimuli. Their functional diversity, however, goes far beyond these tasks and justifies the notion that mononuclear phagocytes are crucial cells in the maintenance of homeostasis.

Taken together, the heterogeneity of mononuclear phagocytes can be defined at several levels. They are extremely versatile and heterogeneous in function, depending on factors such as cell type, tissue localization, maturation or activation status. In conjunction with their functional heterogeneity, the phenotype of the different mononuclear phagocytes, reflected in morphology, ultrastructural features, and surface and intracellular molecule expression, varies significantly. This has enabled the demarcation of a plethora of cell types that belong to the MPS. Finally, cells can be segregated based on their developmental relationship and hematopoietic origin. In the subsequent parts, these aspects of mononuclear phagocyte heterogeneity will be elaborated.

Phenotypic heterogeneity of the mononuclear phagocyte system

The difficulty to develop uniform criteria to define mononuclear phagocytes stems from their extreme phenotypic heterogeneity in different tissues. This relates to the morphology of the cells at light microscopic and electron microscopic level, as well as to molecular features of the cells visualized by cytochemical and immunocytochemical methods. Nonetheless, a number of morphological generalizations at the subcellular level can be made for mononuclear phagocytes. In addition to this, subtypes may show distinctive morphological features, such as the racket-shaped Birbeck granules that characterize the Langerhans cells [17]. In the last decades, the hybridoma technology provided a wealth of monoclonal antibodies against molecules on the surface or in the cytoplasm. Tagging these antibodies with fluorescent dyes or other detecting agents in conjunction with advanced flow cytometric and immunohistological technology made immunophenotypic labeling of mononuclear phagocytes the most commonly used method for identification. Any of the methods used revealed not more than a static view of the MPS as they only allowed taking a snap-shot picture in time, preventing the follow-up during cell development, migration or activation. Therefore, it is important to realize that, due to the lack of a dynamic dimension, our notion of the developmental and functional relationship between the different mononuclear phagocytes is still limited. Despite this restriction, morphological and (immuno)phenotypic identification have brought important insights into the heterogeneity of mononuclear phagocytes, which is depicted in the following parts.

Phenotypic heterogeneity of macrophages

The extensive diversity of Mϕ is illustrated by the lack of a universal, common Mϕ marker, despite the vast amount of monoclonal antibodies that has been generated against

these cells. In the mouse, F4/80 was introduced as a prototypical Mϕ marker [18, 19] but this monoclonal antibody fails to recognize some subpopulations such as Mϕ of the lung and lymphoid organs. Similarly, CD14 and CD68 are widely expressed on human Mϕ, but cannot be considered as truly universal markers. Expression of the receptor for M-CSF, the essential growth factor for Mϕ recognized by CD115 antibodies, has been proposed as a common marker for the MPS [13, 20]. Indeed, mice deficient in CSF-1 or the CSF-1R show a deficit in several Mϕ subpopulations including monocytes, peritoneal Mϕ and osteoclasts [21, 22]. However, the Mϕ deficiency is not complete in these mice, implying that also CD115 is not a universal marker for all Mϕ.

The array of markers that has become available for phenotypic labeling allowed a detailed analysis of Mϕ in steady state as well as in inflammatory conditions. For instance, the study of Mϕ in the steady-state spleen in human and mouse has revealed a significant number of subpopulations differing in phenotype and anatomical location [23, 24]. Such initial studies have increased our conscience of Mϕ heterogeneity considerably, but left many questions about the functional implications. Approaching these questions has resulted more recently in a better understanding of the phenotype - function-link of Mϕ. Multiple ways of activation have been identified that are all characterized by a distinct marker profile [25-27]. However, much remains to be learned in this respect. Current developments in molecular profiling by array technology undoubtedly will contribute significantly to this field in the near future [28].

Phenotypic heterogeneity of dendritic cells

The highly increased awareness of the role of DC in the steering of the immune system and their possible clinical applications has resulted in a wealth of information about the phenotypic heterogeneity of DC. However, unequivocal interpretation of these findings in developmental and functional terms has proven to be difficult, especially because of the high turn-over of DC, their significant mobility between peripheral and immune organs, and the phenotypic volatility of these cells in response to environmental conditions. Thus, the application of the static phenotypic labeling created confusion by definition of many “new” DC types in different organs, without a clear view on the possible developmental relationships between these and already known DC types. Given these restricting considerations, different classifications can be made of phenotypically defined DC types. Figure 1 provides an exemplary overview of only some of the DC populations identified in lymphoid and non-lymphoid tissues.

To illustrate the complexity of the DC field: in human thymus, three distinct DC populations were found [29], up to five populations in lymph nodes [30] and tonsils [31] and at least three populations in blood [32]. The situation is similarly complex in mice, since up to six different DC types were found in mouse lymph nodes and spleen [33]. The generation of an overview of lymph node DC is complicated by the fact that, apart from resident DC, the lymph node hosts varying populations of DC that migrate from the periphery upon triggering. Depending on the tissue drained by the different lymph nodes, different subtypes of DC have been observed, such as in mesenteric and subcutaneous lymph node DC [34]. This points to tissue-specific differences between DC in peripheral organs, as has been observed for Mϕ before.

Dendritic cells in non-lymphoid organs represent a sizeable cell population of immature cells. For example, in the skin, Langerhans cells are located in the epidermis in close proximity

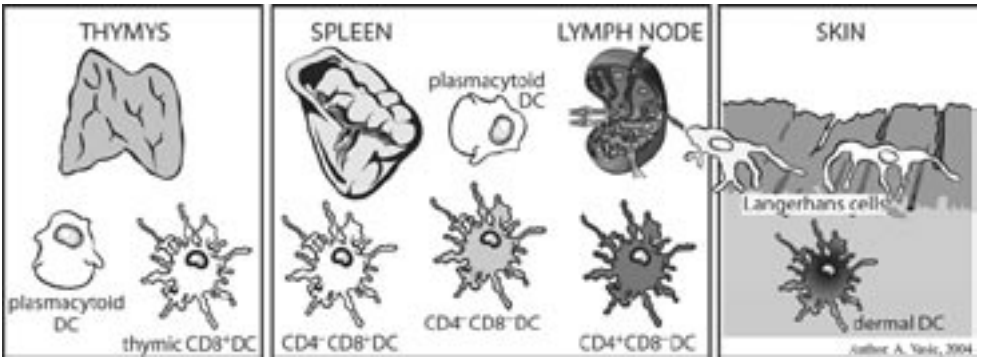


Figure 1. Exemplary classification of main DC subtypes in lymphoid and non-lymphoid organs. The phenotypic distinction of DC according to their expression of CD4 and CD8 markers is based on studies in the mouse. Depending on the marker combinations used, different, and often more, subtypes can be distinguished in both mouse and human tissues (see text for details).

to the basal membrane. Upon antigenic or irritant triggering, they migrate to the skin-draining lymph nodes and present accumulated antigens to T cells. A unique feature of Langerhans cells is the possession of cytoplasmic Birbeck granules that makes them distinguishable from other cells at the subcellular level. Related to this, Langerhans cells can be uniquely identified by their expression of Langerin, a C-type lectin that is functionally involved with the induction of Birbeck granules [35]. Next to the Langerhans cells, which are primarily located in the epidermis, a distinct population of skin DC has been identified in the dermis. In humans, these cells are characterized by the expression of factor XIIIa and DC-SIGN [36, 37]. Likewise, virtually all other non-lymphoid tissues contain at least one DC population in the steady state.

Recent studies on phenotypic variability of DC have revealed considerable promiscuity of DC to express molecules previously considered characteristic for other hematopoietic lineages. Typical examples are the expression of CD4, CD8 and B220 (CD45R) molecules by particular mouse DC subpopulations. Conversely, phenotypic features thought to characterize DC uniformly had to be reconsidered. While classic DC in the mouse all have a high level expression of CD11c, the recently discovered plasmacytoid DC (PDC) express only low levels, in combination with the previous B-cell marker B220 [38, 39] and, in some mouse strains, the granulocyte marker Gr-1 [40]. Realization of this phenotypic promiscuity and flexibility led to identification of additional DC subtypes, such as the new “tolerogenic” DC [41]. These cells could be generated *in vitro* by using GM-CSF with IL-10 and were also found in the spleen expressing a $CD11b^{+}$ $CD11c^{low}$ $CD45RB^{+}$ phenotype. Time will tell whether this is yet another new DC type or an unrecognized developmental stage of already known DC or Mϕ.

Functional heterogeneity of macrophages and dendritic cells

The functional diversity of the mononuclear phagocytes is reflected in the wide-ranging actions in the innate immune response, for the support of the adaptive immune system, but also for the organogenesis, hematopoiesis, wound-healing; maintenance of homeostasis in general. Mϕ and DC have long been considered separate cell types, specialized in particle uptake and degradation on the one hand, and antigen presentation and immune activation on the other. However, the dichotomy between Mϕ and DC regarding these primary functions has proven to be unrealistic: DC may phagocytose as good as Mϕ, while Mϕ may also activate the immune system. Moreover, Mϕ and DC perform many more homeostatic functions (Table 1). In general, these can be separated into functions related to endocytosis and digestion, and those related to tissue regulation. The production of cytokines and other mediators plays an important role in these regulatory functions.

Table 1. Overview of mononuclear phagocyte functions

function	cell type (example)
endocytosis and digestion	
effete cells	splenic red pulp Mϕ
bone and cartilage degradation	osteoclasts
killing and degradation of microorganisms[1]	monocytes / inflammatory Mϕ
tissue regulation	
regulation of inflammation	resident tissue Mϕ
regulation of immune responses	mature DC in the lymph node
regulation of hematopoiesis	hematopoietic island Mϕ in bone marrow
regulation of endocrine function	folliculo-stellate cells in pituitary
tissue remodeling	embryonic Mϕ
wound healing and tissue regeneration	histiocytes (connective tissue Mϕ)
killing or support of tumor cells	Mϕ and DC in the tumor

[1] depending on the activation stage of mononuclear phagocytes, they will also support microbial growth.

Endocytosis

The common characteristic of Mϕ and DC is their extensive ability to endocytose particulate and soluble substances. Based on the nature of the endocytosed substance and the mechanism of uptake, endocytosis has been subdivided into pinocytosis and phagocytosis (reviewed in [42]). Phagocytosis starts with recognition of the material via receptors that are present on the surface of phagocytes. These receptors facilitate the full enclosure of the particle by the phagocyte membrane in a zipper-like fashion. Though mononuclear phagocytes lack antigen-specific receptors as expressed by T- and B-lymphocytes, they display an impressive array of less selective receptors that enable them to sample their environment continuously. These receptors are involved in endocytosis, but also lend the cells their sentinel function, depending on the signaling cascade triggered by occupation of the receptor. Phagocyte receptors can be segregated into several groups depending on their ligands, their overall molecular structure, the structure of the recognition site or a common function that phagocytes performs

by employing them (reviewed in [43]).

Different *lectin*-like receptors recognize carbohydrate structures and play an important role in host defense [44, 45]. They include distinct families of receptors, which enable complex interactions with pathogens as well as (modified) self molecules. The most extensive family comprises the C-type lectins that require calcium ions for carbohydrate recognition. A number of these C-type lectins, such as CD205 (DEC-205), CD207 (Langerin) and CD209 (DC-SIGN) are expressed more specifically on DC and are used as markers for these cells [46]. The versatility of these lectins is well illustrated by the variety of functions identified for CD209 (DC-SIGN). This lectin is involved in the recognition of a variety of microorganisms, including HIV and mycobacteria, but also in transendothelial migration of DC precursors as well as in interaction with T cells [47].

The *Toll-like receptors* (TLRs) represent another family of structurally homologous receptors that mediate the recognition of primarily microbial structures (reviewed in [48]). The most thoroughly studied is TLR4 that is essential in binding of LPS and initiating the subsequent signaling cascade. TLRs are also present on DC and provide a means for diversification of different DC types as they express specific sets of TLRs differentially [48, 49]. Interestingly, this differential expression of TLRs by different mononuclear phagocytes thus implies that different cell types respond depending on the nature of the infective agent.

Additional mononuclear phagocyte receptors that are involved in recognition of potentially hazardous substances are *Fc receptors* which bind immunoglobulin-opsonized particles [50, 51], *complement receptors* [52] or *scavenger receptors*, which recognize a wide variety of mostly poly-anionic molecules [53]. Together with a phosphatidylserine receptor, these receptors also mediate phagocytosis of apoptotic cells [54]. This homeostatic clearance function is probably the primary role of these receptors. Uptake of apoptotic cells does not activate mononuclear phagocytes, unless they are simultaneously triggered by a danger signal, such as those delivered by TLRs. In addition to the role in clearance, engagement of some of the endocytic receptors is important for anti-tumor activity of mononuclear phagocytes, which could be further exploited in anti-tumor therapy [55].

The fate of the endocytosed substance depends highly on its nature, as well as on the mononuclear phagocyte type that engulfs it. While prototypical scavenger Mϕ have extensive lysosomal machinery that mostly facilitates elimination, DC have different subcellular features. These allow DC to contain many microbes, such as mycobacteria, in a live but inactive stage and extend their ability to process microbial molecules for presentation to the adaptive immune system [56].

Regulation of homeostasis

As prime cells of the innate immune system, mononuclear phagocytes take part in all phases of the acute and chronic inflammatory responses. Resident Mϕ and DC act as sentinel cells in peripheral tissues on the basis of their versatile receptor expression (see above) and become activated upon encountering danger. In short time they produce and release large amounts of proinflammatory cytokines, such as TNF-α and IL-1, and chemokines, such as IL-8 and CCL2/MCP-1, and other mediators that will attract inflammatory cells of the innate immune system [57, 58]. However, depending on the tissue site of inflammation and the eliciting agent, the type of infiltrating mononuclear phagocyte may differ significantly. For instance, at mucosal sites, myeloid DC are rapidly recruited in response to bacterial challenge

[59]. Furthermore, in viral infection a specific role has been identified for PDC, as these cells are major producers of IFN-α upon activation [60, 61]. Finally, at a certain point in the inflammation process, mononuclear phagocytes that develop at the site of inflammation will start producing more IL-10 and suppressor molecules that induce apoptosis of cells in the inflammatory infiltrate [25, 57]. Thus, these cells initiate the resolution of inflammation before too much damage occurs at the affected site.

Since inflammation takes place primarily in connective tissues, it is not surprising that mononuclear phagocytes have a close relationship to connective tissue and its components. These cells are involved in maintaining homeostasis by their sentinel function and production and degradation of extracellular matrix components. Together with their impressive ability to recognize and engulf apoptotic cells, this makes mononuclear phagocytes, such as connective tissue histiocytes, lung Mϕ and osteoclasts, to prime cells in tissue remodeling in steady state, during wound healing and in embryonic development [62-65].

Tissue regulation by mononuclear phagocytes is most prominently illustrated by their interaction with the endocrine and hematopoietic systems. Both male and female gametogenesis are crucially dependent on the proper function of Mϕ and DC [66-68]. Not only do they have a role in clearance and endocrine tissue remodeling, but they also modulate hormonal secretion by endocrine cells, primarily by production of cytokines such as IL-1 and IL-6. In essence, all endocrine organs contain populations of mononuclear phagocytes, which appear intimately involved with regulation of endocrine function [66, 69, 70]. Also in hemato- and lymphopoiesis, mononuclear phagocytes play important roles as integral constituents of the hematopoietic stroma in BM and thymus. They are central to erythro- and myelopoiesis in the BM as well as to selection of developing T cells in the thymus [71, 72].

Antigen presentation

The support of the adaptive immune response essentially involves presentation of an antigen. DC and Mϕ share, together with B cells, the function of antigen presentation that leads to the activation or the suppression of T cells. In general, DC are claimed to be unique in their capacity to activate naïve T cells, while Mϕ and B cells primarily stimulate memory responses [73]. This view is supported by findings in *in vivo* models in which DC were depleted experimentally, leading to a significant decrease in immune activation [74, 75]. However, this generalization should be valued with caution, since multiple studies show that also cells with the unequivocal features of Mϕ may activate naïve T cells [76, 77].

Activation of T cells requires that at least two signals are transferred by the antigen-presenting cells: (1) antigen in the form of peptide bound to MHC molecules (reviewed in [78]), (2) costimulation, especially provided by molecules of the B7 and TNF-receptor families [79, 80]. Virtually all cells express on their surface MHC class I molecules in which peptides of cytoplasmic, endogenous molecules are incorporated. In contrast, MHC class II molecules primarily contain peptides from exogenously obtained antigens. To enable activation of naïve MHC class I-restricted cytotoxic T cells, DC are specialized in so-called cross presentation of exogenous antigens on MHC class I molecules (see [81] for a review). CD1 family members, which are expressed by Langerhans cells and some other leukocyte types, may be considered alternative antigen-presentation molecules as they present (glyco-) lipids to (NK)T cells with a restricted T cell receptor repertoire [82]. Cellular adhesion molecules and cytokines support activation of T cells that have made a productive interaction with antigen-presenting- and co-

stimulatory molecules. For instance, DC produce IL-2 upon microbial stimulation, which is thought to play an important role in activation of naïve T cells [83].

In addition to the well-characterized antigenic and costimulation signals, antigen-presenting cells (APC) convey additional signals that imprint the developing T cells. A third signal relates to the skewing of T cells into a polarized phenotype, such as T helper-1 (Th1) or T helper-2 (Th2) [84]. Cytokines produced by APCs, such as IL-12 or IL-4, mediate this process in which transcription factors GATA-3 and Stat6 versus Stat1 and T-bet promote the Th2 versus Th1 differentiation, respectively [85-88]. In addition, T cell skewing was recently also ascribed to Notch ligands, Delta and Jagged, which separately induce Th1 or Th2 differentiation and are present on DC [89]. Furthermore, depending on the tissue of origin of the APC, stimulated T cells may be endowed with corresponding tissue-specific homing capacities [90, 91]. This can be considered a fourth signal delivered by APC to T cells.

Together, the quantity and quality of the distinct signals delivered by APC determine the outcome of the ensuing T cell response [92]. This may lead to a strong effector cell response, activating T helper or cytotoxic T cell subsets that are polarized in certain directions. Alternatively, APC also are crucial in the downregulation and tolerization of the adaptive immune system. It is now more appreciated that the primary function that APC perform is the induction and maintenance of immune tolerance rather than activation of effector responses [93].

Tolerance induction - regulation of the adaptive immunity

Mononuclear phagocytes play central roles in the regulation of the adaptive immune system by virtue of stimulation, but also by specific silencing and constant tolerization. Tolerance is defined as lack of an effector immune response against a particular antigen. Depending on the origin of the antigen, we can differentiate tolerance for self-antigens and tolerance for antigens that originate in the environment. In addition, induced tolerance for alloantigens after transplantation is the goal for the transplantation immunology. Brake of tolerance leads to pathology in all cases. Reaction of the acquired immunity against alloantigen leads to the transplant rejection; against foreign antigens that are normally tolerized to allergy while the immune response against self-originating antigen is identified as the autoimmunity.

There are different mechanisms by which immune tolerance can be achieved: by mechanisms that ensure removal of T cells with unwanted specificity, by inducing a balance between Th1 and Th2 types of immunity or by action of specific T cells (regulatory T cells - T_{reg}) that can induce tolerance. With regard to the control of the immune response directed against self-antigens, the available knowledge does not clarify whether all mechanisms contribute equally to tolerance or different antigens preferentially employ a particular tolerogenic process.

A superior ability of DC to interact with naïve and experienced T cells makes them the most likely candidates for tolerance induction; they contribute to all mechanisms of immune tolerance. DC are involved in the deletion of potentially dangerous autoreactive T cells in the thymus, as a part of the central tolerance. Furthermore, in the periphery they prevent the destructive action of self-reactive T cells, that had escaped the selection in the thymus, by mechanisms of peripheral tolerance (reviewed in [93]). Stimulation of Th1 and/or Th2-type of the immune response, which is hypothesized to balance the immunity, is also critically

dependent on action of DC [89, 94, 95]. Finally, a cross-talk between DC and T_{reg} is active. Induction and maintenance of T_{reg} depends on DC while T_{reg}, in addition to the influence on effector T cells, employ their regulatory action also through direct interaction with DC [96-99]. All these tolerogenic mechanisms essentially employ the antigen-presenting function of DC.

Heterogeneity of tolerance-inducing dendritic cells: Do tolerogenic dendritic cells represent a distinct subset?

In general, two schools of thinking have arisen in the past decade; one that proposes tolerogenicity to be a functional property of DC at a particular, immature stage in development and another that hypothesizes that a specific tolerogenic DC lineage exists.

In favour of the first proposal, immature DC were able to induce anergic T cells, T cells with regulatory properties as well as T cells that secrete immunomodulatory cytokines [100, 101]. However, it may be questioned whether this holds true *in vivo*, i.e. whether DC in the lymph nodes that originate from the immature DC in the periphery are still able to perform such tolerogenic function. Internal signals that induce DC migration towards the draining lymph node simultaneously induce their maturation [102], which according to the hypothesis, would lead to the loss of their tolerogenic capacity. Therefore, the initial hypothesis has been somewhat modified and an additional maturation stage of "semi-mature" DC has been introduced [103]. These DC would represent the steady state DC that carry self antigens and differ in maturation state from the DC that migrate from the periphery in case of inflammation. Semi-mature DC are characterized by a mature cell surface marker phenotype, but lack high level pro-inflammatory cytokine production, in contrast to fully mature DC. Cells with a semi-mature phenotype could be generated *in vitro* and their repetitive injection prevents autoimmunity in a mouse model of experimental autoimmune encephalomyelitis [104]. The spontaneous ability of DC to induce tolerance in an immature (or a semi-mature) state explains tolerance in steady-state conditions. Infections or other inflammatory signals, in general induce maturation of DC. Therefore, a special DC would be needed to preserve a non-destructive autoimmunity in the settings of an activated immune system.

The hypothesis about a specific lineage of DC that induces tolerance has been widely accepted as well (reviewed in [105]). The first likely candidates for the tolerogenic DC lineage was the $CD8\alpha^+$ DC population. Thymic DC in the mouse were almost exclusively $CD8\alpha^+$ and were shown to originate from lymphoid precursors. These cells were involved in the negative selection of T cells in the thymus [72]. Also in the periphery, $CD8\alpha^+$ DC were found to be involved in induction of tolerance [108], so they were a logical candidate for a special tolerogenic DC lineage. $CD8\alpha^+$ and $CD8\alpha^-$ DC have been implicated in the regulation of immunity also by their ability to stimulate Th1 vs. Th2 responses, differentially [109, 110]. However, it has been shown that the potential of DC to stimulate a particular type of helper T cells depends on the type of the stimulatory signal they encounter and is not related to the expression of CD8α [111, 112]. The discovery that $CD8\alpha^+$ and $CD8\alpha^-$ DC may share the origin and that DC express CD8α at a specific stage in maturation (see later), made them less attractive as candidates for a separate tolerogenic DC lineage. Still, their capacity to mediate tolerance has not been discarded.

The title of the tolerogenic DC in the mouse was next attributed to the mouse PDC, shortly after their discovery [113]. Recently, it has been demonstrated that PDC also control

the allergic inflammation in the periphery [114], showing indeed their significant role in the down-regulation of the immune system. Yet, PDC are also able to stimulate both Th1 and Th2 immunity as well as $CD8^+$ T cell responses, indicating therefore that they can perform distinct functions depending on the sum of factors that had influenced the maturation and activation of PDC [115, 116].

Interestingly, PDC as found in the blood, BM or other peripheral organs represent the immature stage of this cell type. This speaks in favor of the notion that "immature" DC (plasmacytoid or myeloid) are able to downregulate immunity until they encounter an activation stimulus and become immunostimulatory APC. This again strengthens the view that all DC are in principle tolerogenic in a steady state situation, unless triggered by a "danger signal" in the inflammatory context.

In summary, DC that mediate tolerance induction exist without any doubt. The question which DC do what *in vivo* is still incompletely answered. Immune tolerance can be achieved through different mechanisms, so there are probably several DC types involved in tolerance induction. Moreover, induction of tolerance by one mechanism, for instance by stimulation of naïve regulatory T cells, might well be performed by other DC than those maintaining the tolerant state. Whether DC able to mediate a particular mechanism belong to a separate lineage or represent a particular maturation stage is a matter of semantics. Most likely, this issue will be solved once the development of the DC system is understood and we are able to track DC throughout their life without influencing their functionality.

Criteria to distinguish dendritic cells from macrophages: is this distinction real?

The vast majority of studies published on DC and Mϕ might leave the impression that both cell types are separate entities that can be well distinguished on the basis of phenotypic and functional criteria. This partition primarily stems from the early characterization of DC as the professional APC, restricted to thymus and secondary lymphoid organs. These cells are indeed easily distinguished from the Mϕ, located in peripheral tissues, and defined as professional phagocytes that eliminate debris and microorganisms. A more extensive list of criteria that separate these prototypical Mϕ and DC is provided in Table 2.

Before DC were better understood, all phagocytic cells in the periphery were designated Mϕ. But, were they all Mϕ? We know now that immature DC reside as phagocytic cells in the periphery and would be characterized as Mϕ by many accepted criteria [118, 119]. Thus, in many instances the distinction between Mϕ and DC appears to become a matter of definition and semantics, rather than a biological reality.

Multiple surface markers are shared between DC and Mϕ and even a combination of several markers does not provide sufficient indication for a clear separation. Both Mϕ and myeloid DC in the mouse may express CD11b, both can express CD11c, F4/80, MHC class II, costimulatory molecules, etc. It was thought that especially markers related to antigen presentation are expressed constitutively on DC and only upon activation on Mϕ. However, this difference was mostly based on a restricted definition of DC as resident cells in secondary lymphoid organs. Furthermore, this criterion is difficult to use in practice, applying mostly non-dynamic phenotypic characterization. The notion that both cell types are sensitive to environmental conditions and change their phenotype extensively during maturation and activation, makes a general phenotypic distinction between DC and Mϕ a treacherous enterprise.

Table 2. Phenotypic and functional criteria used to distinguish between prototypical Mϕ and DC, based on early characterization of the cell types.

	Mϕ	**DC**
phagocytosis	++	–/±
adhesion	+	–/±
acid phosphatase	++	± (spot)
non-specific esterase	+	–
killing of microbes	++	–
MHC class II expression	inducible	constitutive
costimulation	±	++
APC for 1° response	–	++
APC for 2° response	+	++

At the level of function, DC can phagocytose significantly *in vitro* as well as *in vivo* [120]. They also share with Mϕ the potential to produce several cytokines and other soluble factors, which provide them with the potential to perform similar regulatory functions. Conversely, as argued before, some Mϕ may activate naïve T cells, at least in *in vitro* assays. Thus, also in these prime functions, no clear-cut difference between DC and Mϕ can be made.

The *in vitro* generation of DC and Mϕ has been a similar source of confusion. For example, the same cytokine, granulocyte-macrophage colony stimulating factor (GM-CSF) has been used for the generation of cells that were either designated DC or Mϕ, depending on the definition and characterization [11, 121-123]. In general, GM-CSF has now been accepted as a growth factor of choice to generate myeloid DC in both mouse and human, but this may be related to a wider appreciation of the DC-entity.

Taken together, there are no solid criteria to distinguish between DC and Mϕ in general. Nevertheless, multiple subtypes of cells can be recognized as either DC or Mϕ, based on the expression of prototypical features and historic conventions. Given the resulting Babylonic confusion, it is no surprise that a number of studies have reported the conversion from Mϕ into DC and vice versa, depending on experimental conditions [124-126]. We do not expect that unequivocal separating features between DC and Mϕ will be found in the future, based on the extensive phenotypic and functional overlap between the cells. Developmental relationship between cell types could have been an alternative and more definitive criterion for distinction. However, in the next section, the comparably heterogeneous and confusing developmental origin of DC and Mϕ will be elaborated.

Heterogeneity in the origins of dendritic cells and macrophages

In the original concept of the MPS, the members are all considered to have a myeloid nature and originate from a common progenitor in the BM [1]. According to this view, the shared direct precursor of Mϕ in the periphery is a circulating monocyte that, upon passage through the endothelium of the blood vessel, undergoes final differentiation into a macrophage [1, 127, 128]. The wealth of more recent studies that have shown that monocytes also can develop into DC [11, 129-131] support inclusion of DC in the MPS. Moreover, several studies have demonstrated that DC originate from the BM and can be derived from BM precursors *in vitro* using different myeloid-related growth factors [40, 105, 132-134]. However, the view that all Mϕ and DC are myeloid in origin and continuously depend on replenishment by BM precursors may be too simplistic.

Studies on early development of DC in both mouse and human suggest that subsets of DC may be developmentally closer related to lymphocytes than to the myeloid lineage [135, 136]. In particular, thymus-derived common T/NK/DC precursors appear to mature into distinctive DC, independent of myeloid development. Human lymphoid-related DC were found to be identical to the enigmatic plasmacytoid cells, the major IFN-α producers upon viral stimulation [60, 137]. Identification of a mouse equivalent of PDC underlined the evolutionary importance of this cell type [38, 39, 138]. The lack of the myeloid marker CD11b on their surface, their morphology and other features resembling human PDC, point towards their lymphoid origin. However, mouse PDC, unlike human PDC, can develop independently of T cells [139]. This study did not exclude the possibility that, in the mouse, PDC may be more related to B cells, and recently a lymphoid heritage for PDC has been demonstrated by the presence of immunoglobulin heavy chain gene rearrangements, CD3 chain mRNA expression, and pre-Tα mRNA expression [140].

Interestingly, similar lymphoid-related molecular markers were observed in thymic $CD8\alpha^+$ DC, but not in DC from peripheral organs, indicating a closer lymphoid relationship of thymic DC [140]. Previously, CD8α expression was proposed as a marker for lymphoid-related DC [107], but this concept was left after finding induction of CD8α on myeloid-related DC when stimulated under different conditions [132, 141-143]. These findings, together with specific localization of $CD8\alpha^+$ DC in the T cell zone of the spleen or the lymph nodes, imply that the expression of CD8α on DC probably marks their functional state rather than origin.

The recent characterization of DC precursors has indicated an impressive plasticity of hematopoietic progenitors to generate different DC types. For example, both lymphoid- and myeloid-committed progenitors isolated from mice have the ability to generate similar DC types: both develop into $CD8\alpha^+$ and $CD8\alpha^-$ DC *in vitro* or *in vivo* [144, 145]. The frequency of DC that originate from myeloid or lymphoid progenitors varies per organ, with general predominance of a myeloid derivation.

In contrast to the proposed dual lymphoid and myeloid origin of DC, Ardavin and collaborators proposed distinct lineage derivation for DC [146]. They have isolated a precursor population that can give rise to $CD8\alpha^+$ DC, $CD8\alpha^-$ DC as well as PDC. Similarly, it has been shown that the $CD31^+Ly\text{-}6C^+$ population in mouse BM can develop into all three types of DC [147]. Both studies, however, lack the formal proof that this is possible at single cell level. In fact, further analyses of the respective DC precursor populations revealed heterogeneity and, thus, possible contamination with other progenitors [148, 149].

An alternative explanation for the confusing lineage derivation of DC has been offered by Canque and Gluckman [150]. According to their model, hematopoietic progenitors might retain the potential to develop into DC for an extended period during their development, depending on environmental signals. Thus, DC might be generated by a lineage-independent developmental program that has evolved relatively recently and, once switched on, elicits the rapid transformation of distinct leukocytes into professional APCs (namely DC). Recent studies lend support to such a view as they suggest that common lymphoid progenitors are flexible cells that can be reprogrammed to develop into myeloid cells, including DC, depending on specific cytokine signals [151, 152]. Even pro-B cells might develop into DC, when triggered appropriately [153].

In a similar manner, Mϕ might arise from progenitors that have a lymphoid past, in contrast to the usual myeloid origin of Mϕ. Reprogramming of developing B cells into Mϕ was shown by the lack of Pax-5 or enforced expression of transcription factors of the C/EBP family [154, 155]. It could be argued that such genetically manipulated systems bear little resemblance to normal *in vivo* situations. However, bipotential cells capable of developing into B-cells and Mϕ have also been demonstrated in unmanipulated mice [15, 156]. In fact, the human Hodgkin's lymphoma cells might represent similar transitional stages, as they derive from germinal center B cells and carry immunoglobulin gene rearrangements, but lack most classic B-cell markers, and express some myeloid features instead [157]. Interestingly, more than a decade ago Brown and colleagues proposed an alternative model for hematopoietic development in which the Mϕ pathway would represent a default choice in case developing cells failed to fulfill other pathways [158].

In addition to a fixed myeloid origin of Mϕ and DC, also the obligatory replacement of mature cells by precursors derived from the BM has been under scrutiny. During embryonic development, the first CSF-1R expressing cells appear in the yolk sac and spread through the developing embryo. These are primitive Mϕ that play an important role in phagocytosis of dying cells, and therefore in tissue remodeling and organogenesis. Embryonic Mϕ do not originate from hematopoietic islands, they do not pass through the monocyte stage, lack F4/80 expression and seem to be independent of PU.1, a key transcriptional regulator in adult Mϕ development [65, 160]. It has been argued before that they might remain in the adult as BM-independent populations, but this has not yet been resolved definitively (discussed in [24]). A problem in these studies is that adequate monitoring of the fate of transplanted genetically marked BM cells requires conditioning of the host, and thus disturbance of the steady state, including putative local precursors. However, even in this potentially biased setting, lung and liver Mϕ would remain mostly of host origin even after one year and microglia were even less likely to be donor-derived [161, 162].

Taken together, the ground plan of the MPS has been profoundly confirmed and completed since its establishment. However, a large number of studies have urged to revise the concept of a single lineage of cells that is continuously replenished from BM precursors. The view is emerging that plasticity at the precursor level may allow immature cells from different origins to adopt a developmental program that leads to the generation of mononuclear phagocytes with indistinguishable phenotypes and functions. A good example of this putative multiple origin is provided by the development of Langerhans cells, residing in epithelial tissue (Fig. 2).

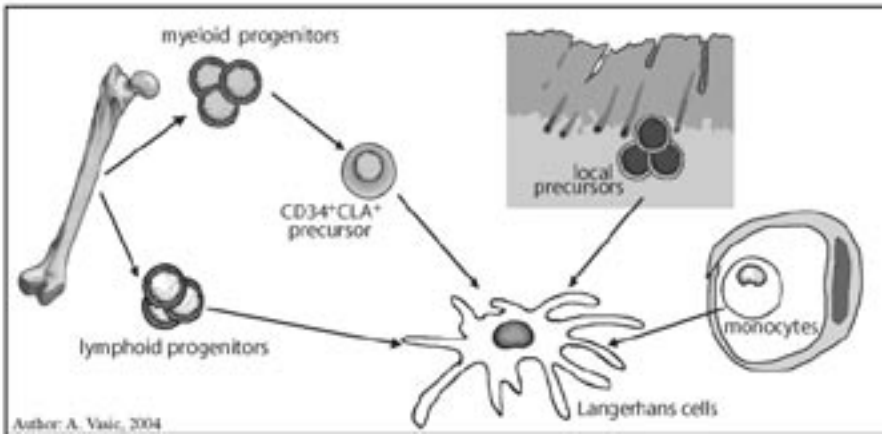


Figure 2. Proposed origins for Langerhans cells. In different experimental settings, Langerhans cells were shown to derive from a subset of myeloid progenitors, lymphoid progenitors, circulating monocytes or local precursors (see text for details). CLA = cutaneous lymphocyte antigen, a homing molecule for the skin.

In man, Langerhans cells have been derived *in vitr*o from a distinct myeloid $CD34^+$ precursor population characterized by expression of the skin-homing molecule CLA [163, 164]. Alternatively, Langerhans cells may develop from monocytes under the influence of GM-CSF and TGF-β [165]. *In vivo* studies in mice have suggested that Langerhans cells may have a lymphoid rather than a myeloid origin [166]. Last but not least, experiments using parabiotic mice have shown that Langerhans cells in the steady state are maintained independently from the BM [16], possibly by local precursors residing in the skin [167]. Only when depleted collectively by an inflammatory trigger, BM-derived cells will reoccupy the niche and develop into Langerhans cells. Thus, while the former studies show the potential of different precursor cells to develop into Langerhans cells, the latter experiments represent the best indication that Langerhans cells are essentially a BM-independent population of mononuclear phagocytes.

Concluding remarks

Mononuclear phagocytes are characterized by their functional and phenotypic heterogeneity. As outlined above, Mϕ and DC have crucial functions in multiple homeostatic processes. This is reflected in their phenotypic characteristics, which are so diverse that no clear-cut phenotypic definition of mononuclear phagocytes can be given. The heterogeneity may originate from different developmental processes.

First, mononuclear phagocytes can have different precursors. The vast majority of mononuclear phagocytes probably stems from the myeloid hematopoietic lineage, but also lymphoid progenitors may give rise to mature DC and Mϕ that may either be indistinguishable from their myeloid counterparts (e.g. Langerhans cells) or constitute a specific subset of cells (e.g. PDC). Even mesenchymal cells may be direct progenitors of adult mononuclear phagocytes, if, as some studies suggest, fetal Mϕ may persist throughout adult life.

Second, environmental factors crucially influence the generation of diversity among mononuclear phagocytes throughout the different phases of their development. At the precursor level, cells will commit to a certain lineage under the influence of local developmental factors. Subsequently, numerous exogenous factors influence the maturation of precursor cells to peripheral Mϕ and DC, with obvious functional consequences. Finally, the nature of Mϕ and DC as peripheral sentinel cells makes them sensitive to their environment par excellence. These cells carry peripheral messages not only to the immune system, but also to other, such as the hematopoietic and neurendocrine systems.

Since the adaptive immune system is vital to host defense and strongly depends on the proper function of the MPS, it is no wonder that the observed heterogeneity and redundancy exists. Complete absence of Mϕ and DC has, to our knowledge, never been reported in humans or in experimental settings, and is probably incompatible with life.

Here, we propose the MPS as a large continuum of cell types of different functions, different phenotypes and even different origins. The flexibility and limits in developmental heterogeneity of the MPS is just now starting to be explored. This research is boosted by the awareness that modulation of the function of the MPS, in either a stimulating or inhibiting manner, may have great clinical potential in a wide variety of diseases. However, the study of various functions of mononuclear phagocytes has remained underdeveloped, primarily because of the pre-occupation of viewing DC as cells only specialized in immune interaction and Mϕ only as scavengers and destroyers of invaders. Given the recent interest in the mononuclear phagocytes, and DC especially, it may be anticipated that before too long more light will be shed on these “dark spots” of the mononuclear phagocyte system.

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1.2

The Autoimmune Diabetes of the Non-Obese Diabetic (NOD) Mouse

Immune reaction directed against self-antigens is called autoimmunity. Eliciting factors of autoimmunity lead to a malfunction of immune regulation and brake of tolerance. Autoimmune destruction of insulin-producing β-cells in the pancreas causes type 1 diabetes (T1D), an organ-specific autoimmune disease clinically detected by insulin insufficiency. After Banting and Best had discovered insulin, and this hormone was applied as a treatment of diabetic patients, their life could be significantly prolonged, however, not without adverse consequences. Several complications would develop, like damage to the circulatory and nervous system, which lead to an increased risk of heart attacks, strokes, blindness, kidney failure and foot and leg amputations. These complications usually begin to occur approximately 10 years after the diagnosis of T1D, unless the person's blood glucose has been well controlled.

The causes of T1D are multiple. Both environmental and genetic factors contribute to type 1 diabetes [1-6]. Genetic susceptibility has been related mostly to the genes of the major histocompatibility complex (MHC) [7, 8] but many other non-MHC-associated genes contribute to the propensity for the disease. Environmental factors like infections, stress, diet and hygienic circumstances contribute to the autoimmunity by increasing the risk for developing the autoimmune disorder [9-13]. Neither genetic susceptibility nor environment alone will lead to the disease. Combined, however, genetically determined susceptibility creates the playground at which the environmental factors determine the course of the autoimmune response. It has been shown that at early ages, genetic factors contribute to the diabetes more critically than later in life, while in adults, environmental factors contribute more actively to the susceptibility [14, 15].

T1D is clinically recognized only when more than 80% of the islet mass is destroyed. At that point, the self-directed immune response is already full-blown and it is impossible to trace the preceding steps and factors. Therefore, animal models play an important role in the investigation of the pathogenesis of diabetes.

Animal models for type 1 diabetes

Several animal models for T1D exist [16]. In the animal models, the disease develops either spontaneously (the BB rat and the NOD mouse model) or is induced (by cyclophosphamide or via transgenes e.g. the RIP-LCMV mice). Animal models provide means to follow the initiation and progress of the disease prior to onset. Furthermore, they enable research of potential treatments that can be applied at early stages of the autoimmune process. Also, recent progress in NOD mice shows that animal models are very helpful for the development of prognostic tools [17, 18].

Inbred mice and rats used as animal models for T1D are genetically identical. A lack of genetic variability may be considered a disadvantage of the inbred animal models when the findings need to be extrapolated to a human situation (for a larger group of patients). Furthermore, experimental animals are bred in specific pathogen free conditions, in which the environmental factors play a minor role, so the research mainly focuses on the genetic determinants of the disease. This might be an important drawback considering that T1D is both genetically or environmentally driven.

From the point of view of basic investigations, the fact that inbred animals are genetically identical is an advantage for it provides reliable studies in an properly controlled setting. The

lack of variability when using a single inbred mouse strain as a disease model can be improved by using different transgenic animals, which may enlarge the animal pool of different genetic backgrounds and/or with different etiologies of the same disease. Yet, generation of new transgenic animals is time consuming and not widely available.

Much of the knowledge about the course of the pancreatic β-cell destruction in T1D has been obtained in the past two decades from two spontaneous animal models: the non-obese diabetic (NOD) mouse and the BioBreeding (BB) rat.

Bio-breeding (BB) rat

The BB rat strain has been derived from an outbred Wistar strain in Canada [19, 20]. After a few generations of inbreeding, two lines have been selected, a diabetes-resistant (BB-DR) rat and a diabetes-prone (BB-DP) rat. In the diabetes-resistant rat colony less than 1% animals develop the disease while 50-80% of BB-DP rats become diabetic with the mean age of onset of about 14 weeks. Similar to humans, there is no difference in diabetes incidence between males and females in the BB-DP rat. In addition, similar to the human and the mouse, an important genetic determinant that greatly contributes to susceptibility is the MHC locus [21]. However, BB-DP rats differ from human diabetics since they are severely lymphopenic due to the inheritance of the lymphopenia (Lyp) gene [22]. Although both MHC and Lyp genes are important for diabetes development, these two are not sufficient to produce susceptibility [23-25]. In addition, the pre-clinical period between the evident initiation of insulitis and the clinical onset is relatively short [20]. This points to a fast progression of the autoimmune process.

Since the actual autoimmune process cannot be followed in patients, it is hard to establish with certainty the course the autoimmune process has taken in a patient with overt diabetes. Given that the variety of factors and mechanisms can lead to T1D, the rapid disease onset in the BB-DP rat might reflect one of many possible routes.

Non-obese diabetic (NOD) mouse

The similarities between a presumed scenario for the pathogenesis of human T1D and the observed islet inflammation in the NOD mouse made this model one of the earliest and most extensive models studied.

The NOD mouse has been generated in Japan by selection from a mixture of outbred mice prone to develop cataract (a complication of diabetes) [26]. Interestingly, the NOD mouse was derived as a "by-product" of a selective breeding for an obese mouse: a model for type II diabetes [27]. So, the researchers were looking for a fat mouse that would develop diabetes and they ended up with a slim mouse, which would spontaneously develop insulin dependent diabetes. At first, this mouse was exclusively kept in Japan, but later several colonies around the world have been started. Although all NOD mice come from the same original source, years of isolated breeding have led to varying levels of diabetes incidence and possibly other differences with regard to some cellular defects as well [28, 29]. Importantly, while the diabetic phenotype appears with different incidence among colonies, virtually all NOD mice develop insulitis [29].

Similar to the human, environmental factors such as diet, infections or the temperature contribute to diabetes development in NOD mice [30-32]. Different breeding conditions

influence the diabetes incidence. NOD mice bred in a microbiologically "clean" environment have in general a higher diabetes incidence. Remarkably, even in the same animal facility, different caging of mice will influence the incidence, demonstrating a multifactorial contribution to the outcome of the autoimmune process.

NOD mice are not only used as a model for autoimmune diabetes but also as a model for other autoimmune diseases. Besides chronic inflammation in the pancreas that leads to autoimmune diabetes, these mice develop autoimmunity of the salivary glands (Sjogren's syndrome) [33, 34] and thyroid autoimmunity in a specific setting i.e. when kept on a high iodine diet and with the appropriate MHC class II genes [35, 36]. NOD mice also develop hemolytic anemia and anti-nuclear antibodies, indicating that they suffer from systemic autoimmunity as well [37]. Around 12 weeks of age, NOD mice start to develop a spontaneous hearing loss, which also might be autoimmune-related [38]. The NOD mice are susceptible to induced autoimmune models like experimental allergic encephalomyelitis (EAE) [39, 40] and have been used for studies on some features of systemic lupus (SLE) [41, 42]. They can develop an extremely strong Th2 allergic response against OVA, therefore are susceptible for experimentally induced asthma as well [43]. Remarkably, however, NOD mice are resistant to collagen-induced arthritis (CIA), a model for reumathoid arthritis (RA) [44].

Taken together, a susceptibility to different types of autoimmune disease coexists in the NOD mice. Since the genetic factors play a greater role in the specific-pathogen free conditions, under which they develop these diseases, NOD mice are a good model to study genetic factors and gene clusters that are shared between T1D and other autoimmune disorders.

Genetic control of diabetes in NOD mice

Investigation of genetic traits can help significantly to understand genetic defects that increase the susceptibility to an autoimmune disease as well as to determine which molecules are essential for the pathological mechanisms. Identifying polymorphisms that enlarge the propensity to autoimmunity in general or to a particular autoimmune disease can help in prognostic analysis. However, this knowledge does not provide direct means to fight the disease.

Studies in which NOD mice were crossed with diabetes-resistant mouse strains have unveiled several loci contributing to insulitis or diabetes. These loci are termed *Idd's* (for insulin-dependent diabetes) and they are regions in the mouse genome which provide a partial or a full protection from diabetes, when replaced by the same region from the diabetes-resistant mouse. Up to now, 24 *Idd* loci distributed across 14 chromosomes in the genome have been found to be associated with the insulin-dependent diabetes (IDDM) [45-47]. Overview of the *Idd* loci, their location and contribution to the autoimmune diabetes in the NOD mouse is given in Table 1.

It is not yet clear for all *Idd* loci by which means they contribute to diabetes. Of them, *Idd1* located on chromosome 17, which contains the NOD-specific MHC genes, is the major locus associated with IDDM. Replacement of this locus fully protects NOD mice from diabetes development [48]. Together with the MHC, the *Idd3* locus on chromosome 3 has the strongest control of the autoimmune diabetes in the mouse [49, 50]. Interestingly, replacement of some *Idd* loci in the NOD mouse shows minor contribution to protection from diabetes, yet

combination of them provides a strongly increased resistance. This underlines the complexity of the defects in the immune system in which several small faults, that on their own would not be able to do much harm, combined in the NOD mouse lead to an inevitable and irreparable destruction of β-cells and disease.

The genes encoded by the different *Idd* loci are not all known yet. Despite the uncertainties, the congruence and the candidate-gene approach have taught us that besides the MHC-locus, genetic predisposition is closely linked to the genes that encode the costimulatory molecules CD28 and CTLA-4 on chromosome 1, genes encoding molecules from the TNF family on chromosome 4, and possibly the IL-2 gene on chromosome 3 [51-53]. Thus, it seems that genes correlated to the diabetes susceptibility that have been identified thus far are often associated with immune regulation and with molecules expressed in APC in particular.

Genetic loci that contribute to other autoimmune diseases in the NOD mouse have been traced as well [45]. Some of them are shared by several disorders and probably influence the common aspects of autoimmunity, like inflammatory effects. However, many remain specific for a particular disease, indicating that each disease is controlled by a unique set of disease-promoting genes. The genetic control of autoimmune disorders might be more distinct in the NOD mouse than previously suggested [54, 55]. In the NOD mouse, the various spontaneous diseases use different pathogenic pathways, which also importantly depend on the initial trigger, microbiological status, stress etc.

Table 1. Currently available information regarding *Idd* loci, their contribution to insulitis or diabetes and candidate genes found in these loci

Locus	Chromosome	Role of NOD allele in Disease Phenotype	Candidate Genes at Locus
Idd1	17	**Diabetes**: required but not sufficient diabetes	MHC class II genes (IA^{g7}, IE)
Idd2	9	**Diabetes**: confers susceptibility; Insulitis: no linkage	undefined
Idd3	3	**Diabetes and insulitis**: confers susceptibility	*Il-2* (Interleukin-2), *Fgf2* (fibroblast growth factor-2)
Idd4	11	**Diabetes**: confers susceptibility; Insulitis: no linkage	undefined
Idd5.1	1	**Diabetes**: confers susceptibility	*Casp8*, *Cd152* (CTLA4), Cflar (FLIP), *Cd28*
Idd5.2	1	**Diabetes**: confers susceptibility	*Nramp1*, *Cmkar2* (CXCR2, IL-8 receptor α)
Idd6	6	**Diabetes**: confers susceptibility; **Insulitis**: no linkage	undefined
Idd7	7	**Diabetes**: partially protective; **Insulitis**: no linkage	undefined
Idd8	14	**Diabetes**: partially protective; **Insulitis**: no linkage	undefined
Idd9.1	4	**Diabetes**: confers susceptibility; **Insulitis**: no linkage	may co-localize with *Idd11*, *Lck* is a candidate gene
Idd9.2	4	**Diabetes**: confers susceptibility; **Insulitis**: no linkage	*Tnrf2*, *Cd30* (receptors for tumor necrosis factor family)
Idd9.3	4	**Diabetes**: confers susceptibility; **Insulitis**: no linkage	*Cd137* (receptor for tumor necrosis factor family)
Idd10	3	**Diabetes and insulitis**: confers susceptibility	region includes *Csfm*, *Cd53*, *Kcna3*, *Nras*, *Rap1a*
Idd11	4	**Diabetes**: confers susceptibility	may co-localize is a candidate with *Idd9.1*; *Lck* gene
Idd12	14	**Diabetes**: confers susceptibility	undefined
Idd13	2	**Diabetes**: confers susceptibility; **Insulitis**: no linkage	beta 2 microglobulin gene
Idd14	13	**Diabetes**: confers susceptibility; **Insulitis**: no linkage	undefined
Idd15	5	**Diabetes**: confers susceptibility	undefined
Idd16	17	**Diabetes and insulitis**: confers susceptibility	MHC Class I K gene
Idd17	3	**Diabetes**: confers susceptibility	undefined
Idd18	3	**Diabetes**: confers susceptibility	region includes *Csfm*, *Cd53*, *Kcna3*, *Nras*, *Rap1a*
Idd19	6	Protective against diabetes	undefined
Idd20	6	**Diabetes**: confers susceptibility	undefined
Idd21	18	**Diabetes**: confers susceptibility	undefined

Which cells are the players in the diabetes pathogenesis?

All types of immune cells seem to be implicated in the self-directed inflammation in the pancreas. However, some contribute to the initiation while others play a predominant role in the progression phase or the final precipitation of the disease.

In essence, diabetes pathogenesis in NOD mice can be divided into two phases. In the first phase, a gradual accumulation of leukocytes in the close proximity of islets of Langerhans takes place. This phase is called the insulitis-phase. The second phase involves the actual destruction of the β-cells in the pancreas leading to overt diabetes (schematic overview of the diabetogenic process is given in the Figure 1).

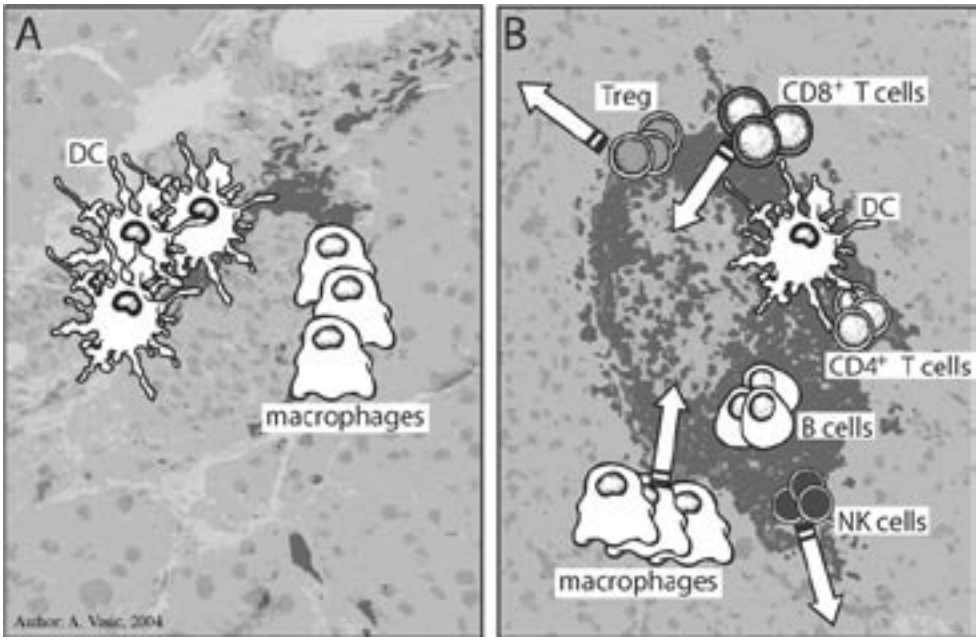

Figure 1. Two phases of the diabetogenic process. A. The earliest event related to the initiation of the autoimmunity in the NOD mouse is the accumulation of DC and Mϕ in the close proximity of Langerhans islets. B. Many cells of the immune response are present in the insulitic infiltrate: Mϕ, DC, effector $CD8^+$ and $CD4^+$ T cells, B cells, NK cells, regulatory T cells. Arrows demonstrate the changes in the cell composition during the progression from the non-invasive towards the invasive insulitis. This process is marked by the infiltration of Mϕ and $CD8^+$ T cell into the islet and the departure of NK cells and regulatory T cells from the islets or loss of their function.

Congenic NOD mice, in which a part or a complete *Idd* locus has been replaced, have provided much insight concerning the genes that are involved in these different disease phases. Some congenic strains are protected from both insulitis and diabetes, while others are protected from diabetes only but still develop insulitis (a complete overview of congenic NOD mice is provided in [56]). In addition, gene expression profiles using cDNA microarray analysis of spleen cells has enabled defining an immunological checkpoint at 5 weeks of age in the NOD mouse, at which a switch of the expression profiles of about 70 genes (among them seven genes map to the diabetes susceptibility regions) are consistent with increased antibody production,

antigen presentation, and cell proliferation associated with an active autoimmune response [57]. The functional studies will reveal whether these genes contribute to the progression towards insulitis and diabetes.

The first signals of the insulitis-phase involve the increased presence of DC and Mϕ around the islets. Already in neonatal NOD mice a slightly raised number of DC and Mϕ is found in the target organ [58]. However, it is not known whether at this point in time the cells are involved in the developmental changes of the pancreas or already in initiating autoimmunity, probably in both [59]. An additional accumulation of DC and Mϕ around the islets at 4-5 weeks of age precedes the peri-insular infiltration of lymphocytes [60, 61]. Subsequently, DC and Mϕ in the NOD pancreas probably are involved in the production of the chemokines necessary for the formation of the high-endothelial venules and the lymphocyte attraction. Activated pancreatic endothelium enables the extravasation of lymphocytes into via integrins [62, 63]. At this stage, lymphocytes create large non-destructive peri-insular infiltrates.

An important checkpoint in the initiation of autoimmunity is the propagation of autoreactive T cells in the pancreatic lymph nodes (PLN). This occurs in the first three to four weeks after weaning, as the removal of PLN at this time significantly prevented diabetes [64]. This demonstrated the essential role of PLN in the stimulation of autoreactive lymphocytes. However, the antigens that elicit the autoimmune response might not be delivered solely by the pancreas via pancreatic DC that traffic to PLN. The initial priming of diabetogenic T cells is suggested to take place in the gut-associated lymph nodes and the PLN would serve for the amplification of the autoimmune response [65]. It is predictable that DC mediate the priming of diabetogenic T cells but no direct evidence exists that DC originating from pancreas present antigen in the PLN. Peripheral DC continuously migrate from the periphery to the draining lymph nodes, where they present antigen [66, 67]. This notion is highly probable for pancreatic DC as well. A transmigration of DC in the pancreas through the lymphatic endothelium has been demonstrated, although at later stages of the pathogenic process [68].

Activated autoreactive T cells migrate to the pancreas where they accumulate and form infiltrates located in the near proximity of the islets (peri-islet accumulation). Infiltrates contain DC, Mϕ, $CD4^+$ and $CD8^+$ lymphocytes and are initially benign [60]. $CD4^+$ T cells are at this stage of both Th1 and Th2 type. The β-cell destruction is mediated mostly by $CD8^+$ lymphocytes and Mϕ, which relocate into the islets [60, 61]. So far it is incompletely understood which event(s) trigger the progression of a non-destructive towards the destructive form of insulitis. Several events take place at this point. Progression from insulitis to diabetes could be due to the shift toward Th1 type inflammation [69, 70], which can be traced as a downregulation of IL-10 and an increase of IL-12 in serum [71]. A candidate cytokine that could drive this Th1-shift could be IL-15. An acceleration of insulitis progression by cyclophosphamide downregulates the expression of prostaglandin H synthase 2 and arginase (an antagonist enzyme of the inducible NO synthase) and induces a strong concomitant upregulation of IL-15 that precedes lymphocyte invasion of islets and a rise of IFN-γ mRNA levels [72]. B cells have also been implicated in overcoming the checkpoint towards destructive insulitis since mice in which $I\text{-}A^{g7}$ was replaced specifically in B cells did not spontaneously convert a non-invasive into destructive insulitis [73]. Conversely, regulatory T cells and NK cells might provide the necessary keys that hold the insulitis under control, and loss of these cells or loss of their function enables other cells to start the destruction [74-76].

Which cellular defects lead to autoimmunity?

The knowledge of the biology and physiology of cells involved in insulitis is still insufficient to enable a straightforward translation of the scarce genetic polymorphisms found in diabetes-related loci into functional defects. Therefore in parallel with the research of the NOD mouse genetics, many groups have focused on investigations of aberrant function of different cells involved in the diabetogenic process. The goal of this research is to find intrinsic defects in β-cells or in cells of the innate or the adaptive immune system, which could contribute to the dysregulation of the immune system, leading to the initiation and progression of the autoimmune response and finally causing diabetes.

Defects in the target organ

Abnormalities in the pancreas could contribute to the sensitivity for an autoimmune attack or even to an elicitation of an autoimmune response. Indeed, aberrant islets and β-cells have been found in the NOD mice. Malformations in the development of the pancreas have been traced [59]. These probably find their origin already at the fetal stage. In the perinatal pancreas, Fas^+ nerves are present, which concomitantly express IP-10, a chemokine that attracts leukocytes [77]. Indeed, others have suggested that not only β-cells but also neuronal elements might be the targets in the NOD pancreas [78]. In addition, β-cells in the NOD mouse are hypersensitive to oxidative stress [79, 80]. All the architectural aberrancies and/or the hypersensitivity to oxidative stress might induce "danger" signals that stimulate local cells of the innate immune system to start and perpetuate the autoimmune response.

Defects in the cells of the adaptive immune system

Effector T cells recognizing auto-antigens – In all mouse models as well as in human diabetes, disease onset critically depend on the existence of auto-reactive effector T cells. Lymphocyte-deficient NOD mice (NOD-scid or NOD-Rag knock-out mice) are protected from insulitis and diabetes. The reconstitution of these mice with the T cell pool from diabetic NOD mice will in short time lead to diabetes [81, 82]. Both $CD4^+$ and $CD8^+$ autoreactive T cells exist in the NOD mouse. Unlike $CD4^+$ T cells that mediate their destructive activity by cytokines, $CD8^+$ T cells are themselves actively cytoxic cells that enter the islet and destroy β-cells.

Reconstitution experiments demonstrate the important role of T cells in the diabetes pathology but do not imply that these cells have possible functional defects. The existence of autoreactive T cells is considered to reflect a defect in the negative selection in the thymus [83]. Faulty negative selection might originate in an intrinsic defect of the thymocyte itself or in the cells that mediate negative selection, in particular the thymic DC. An intrinsic defect of NOD lymphocytes relevant to defective negative selection could be their hyporesponsivness to normal TCR stimulation [84, 85]. However, such poor reactivity could not explain the expansion of autoreactive T cells in the periphery. Later studies demonstrated that a reduced apoptosis might be the defect in NOD thymocytes by which autoreactive T cells escape negative selection [83, 86-89]. However, Villunger et al have challenged this view as they could induce a proper apoptosis in NOD thymocytes [90]. Also for circulating T cells, an incomplete activation induced cell death (AICD) or a prolonged proliferation due to a low apoptosis *in vitro* has been demonstrated [91, 92]. However, from the latter two studies it cannot be clearly deduced

whether the defective apoptosis comes from an abnormality in the lymphocyte or from the APC that does not stimulate the T cell sufficiently to go into apoptosis. Interestingly, it has been reported that some $CD8^+$ T cells from the NOD mouse are able to re-activate the VDJ-recombination machinery in the periphery [93]. This might explain why some high-avidity autoreactive lymphocytes can develop again in the peripheral pool of selected $CD8^+$ T cells in the NOD mouse [94].

Regulatory T cells – An important cell type that contributes to the prevention of autoimmune disease is the regulatory T cell [95]. Cells that can execute regulatory activity are a heterogeneous group (reviewed in [96, 97]). There are in principle two types of T cells that propagate suppressive activity: T cells that produce the suppressive cytokines IL-10 or TGF-β (called Tr1 or Th3 cells) and so called regulatory T cells (T_{reg}) that also work via cell-cell interaction. Cells of the T-regulatory system express a diverse TCR repertoire and recognize foreign as well as self-antigen. They also have a different development: T_{reg} are generated and selected in the thymus while Tr1/Th3 derive from the naïve peripheral T cell pool that is specifically stimulated in the lymphoid organs in an active immune response [96, 97].

The precise phenotype of T_{reg} cells has not been revealed completely and nowadays they are primarily defined on the basis of their functionality. Most of the T_{reg} cells are $CD4^+CD25^+$, but this phenotype is not an exclusive characteristic of T_{reg} cells. Activated $CD4^+$ T cells also express CD25 (the IL-2-receptor α chain). Additional markers expressed by T_{reg} have been reported recently: CD44, CD62L, GITR, CD134 and Foxp3 (reviewed in [98, 99]).

The important role of T_{reg} in the maintenance of tolerance is well accepted. A plethora of studies have demonstrated that depletion of these cells leads to autoimmunity [98, 100] or that the selective supplementation or *in vivo* expansion of these cells prevents autoimmunity [101-103]. The number of $CD4^+CD25^+$ T cells is reduced in NOD mice and these cells have been shown to be functionally deficient as well [102]. The decrease in the suppressive activity of these cells in NOD mice has been reported to be age-dependent [70]. Interestingly, a defect in STAT-1 has been shown to lead to an impaired development and function of regulatory T cells, which renders STAT-1 deficient mice susceptible to autoimmune disease [104]. Potentially, defective function of STAT-1 might contribute to the regulatory T cell deficiency in the NOD mouse as well.

Balance between the effector and regulatory T cells - Autoreactive T cells have been found in healthy individuals [105], which do not necessarily have a higher risk to develop autoimmunity. Furthermore, some self-reacting T cells might work as regulators and ensure tolerance, unless imbalanced by inflammatory conditions. High frequency of autoreactive T cells may exist without causing autoimmune disease due to a proper equilibrium between effector and regulator cells [60, 74, 106]. Too many effectors or too few regulators might become dangerous when an altered function of one of them disturbs the balance.

The altered response of T cells to apoptotic signals and/or defects in the inhibitory signaling pathways of the NOD T cells contribute to the dysbalance and autoimmunity (reviewed in [107]). However, the balance can be disturbed by an altered function of other players in the immune response as well. Likely candidates are APC, which may allow for the propagation of a dangerous self-reactive immune response either by an unnecessary activation of effectors or by a deficient support of the suppressive forces.

B cells – The contribution of B cells to autoimmunity was previously attributed exclusivelly to the production of auto-antibodies. However, islet-specific antibodies are of little importance for the initiation of autoimmunity. T cells transferred without B cells to NODscid mice were able to induce diabetes, implying that B cells are not essential in the effector response. New attention to B cells has been drawn by studies indicating that they may play a role as antigen-presenting cells in the autoimmune diabetogenic process. B cell-deficient NOD mice did not develop diabetes and a depletion of B cells also abrogated the disease [108, 109]. The antigen-presenting function of B lymphocytes is important in later phases of the autoimmune process, at the switch from a non-destructive to a destructive insulitis [73]. Recently, a defective deletion of B cells that recognize some soluble antigens has been described, which could be an important intrinsic defect leading to a high frequency of B cells reactive to auto-antigens in the NOD mouse [110, 111]. In addition, an expanded pool of B cells has been found in NOD mice that express a high level of CR1/CR2 complement receptors and have a high CR3 binding activity. Prevention of the emergence of these cells conferred resistance to autoimmune diabetes in NOD mice [112].

Defects in the cells of the innate immune system

NK and NK T cells - The function of NK cells is mainly killing of recognized tumor targets. Interaction of NK cells with self-MHC class I molecules provides inhibitory signals that keep NK cells in an inactive state. If MHC class I molecules start presenting antigens other than self (e.g. derived from viruses), NK cells will sense that "self is missing" and start producing proinflammatory cytokines, which are able to activate DC and in that way stimulate an adaptive immune response [113-115]. NK T cells represent a subset of T lymphocytes that also express the NK-specific molecule (NK1.1). These cells have a very limited TCR repertoire and the majority of them recognize glyco-lipid antigens presented by the MHC class I-like molecule (CD1d) [116].

A reduced number and function of NK and NK T cells in NOD mice has been reported soon after the initiation of the analysis of cellular defects in this mouse strain [117, 118]. Further investigation was hindered by the fact that NOD mice lack the NK1.1 molecule, the only available marker that separates NK cells from other leukocytes in mice. Generation of transgenic NOD mice that express NK1.1 facilitated cytometric and functional analysis of these cells [119]. In these mice, NK and NKT cells were reduced in the number in the periphery and increased in the BM, suggesting a defect in NK cell export from the BM. Furthermore, they produced less IL-4 upon stimulation when compared to normal NK cells.

A severe but partially restorable killing defect in the NK cells of NOD mice has been reported recently [120]. Additionally, NOD NK cells to fail to modulate the activating receptor NKG2D, which implied an unwanted killing activity [121], and the blockade of this molecule prevented autoimmunity in NOD mice [122].

NK T cells are normally present in pancreatic islets of both female and male NOD mice. Their number starts to decline specifically in female mice at the time of diabetes progression, therefore they might be important in the regulation of autoimmune diabetes [123]. Modulation of the NK T cell pool by adoptive transfer, endogenous recovery or stimulation with αGalCer prevented spontaneous diabetes development in NOD mice [124-126]. Stimulation of NK T cells with αGalCer induced their accumulation in the pancreas and PLN, supporting their putative regulatory role.

Macrophages – At least two subsets of Mϕ can be found in the pancreas, ER-MP23$^+$ and BM8$^+$ Mϕ. ER-MP23$^+$ are resident Mϕ, normally present in the pancreas while BM8$^+$ Mϕ are often associated with inflammation and are critically involved in the destruction of β-cells [60]. Several defects of Mϕ have been identified in NOD mice. Abnormal Mϕ development and underlying intracellular defects have been found in the BM cells [127]. Furthermore, isolated NOD Mϕ show a reduced phagocytosis of apoptotic cells and a reduced cytokine production in response to apoptotic cells [128, 129]. In general, phagocytosis of apoptotic cells is a mechanism to sustain tolerance and this defect could abnormally promote inflammation in NOD mice [130, 131]. The molecular defect underlying the increased ability to mediate proinflammatory response might be a hyperactivation of NF-κB, the transcriptional activator of many cytokines [132, 133]. Indeed, NOD Mϕ produce increased levels of IL-12 when stimulated with LPS or Hsp60 [134, 135]. In addition, NOD Mϕ also produce high amounts of nitric oxide (NO), which contribute to the destructive phase of the insulitis process [136, 137].

Taken together, defects found in NOD Mϕ point to a preferential and elevated proinflammatory response when stimulated. Potentially, this could have detrimental consequences for the pancreas in which resident Mϕ could "misunderstand" the signals from the environment and induce inflammation there where it normally should not be induced.

Dendritic cells – Together with Mϕ, DC are the first leukocytes that accumulate in the pancreas during the islet inflammatory response. They are present in the pancreas throughout the course of the autoimmune destruction of β-cells. Because of their important role in the acquisition and maintenance of tolerance as well as in induction of immunity, it is likely that a faulty function of DC will contribute to the development of autoimmune diabetes (Figure 2). Therefore, the search for defects in DC has been a focus of investigation for many groups.

The interplay between DC and T cells is essential for the initiation, propagation and regulation of the T cell function. The defects in T cells were not evident indisputably, so investigations of problems with their partners (DC) were performed as well. A candidate explanation for the faulty selection of T cells was a defective function of DC in the thymus or in the peripheral lymphoid organs (the spleen or the lymph nodes).

At first, a defect in the function of DC in NOD mice was associated with MHC molecules. It has been shown that a unique I-A^{g7} allele, specifically expressed in NOD mice, shows a poor binding to certain peptides [138-142]. According to the hypothesis, due to an instable MHC-peptide complex, the duration of the DC-thymocyte contact would be inefficient and auto-reactive cells would escape negative selection [139, 140]. Also in the periphery, where a strong interaction with DC enables the AICD of T cells, the short interaction with the NOD DC would disable proper deletion of auto-reactive effectors. However, auto-reactive cells also need a good interaction with peripheral DC to be activated and an instable MHC-peptide complex could not explain the propagation of these cells in the periphery (reviewed in [143]). Furthermore, the contribution of the MHC defect to autoimmunity does not necessarily involve DC, since other cells (e.g. B cells, Mϕ, endothelial cells) also express MHC II molecules. The role of the MHC molecules in providing susceptibility to diabetes is certain [48, 142, 144], yet whether it implicates the functional defect in DC is not clear.

Problems in antigen presentation by DC might also involve the non-MHC-linked genes that contribute to the failure to delete the high avidity autoreactive cells in the NOD thymus [87]. Defects in Tap1 and Tap2 molecules important for antigen presentation through MHC

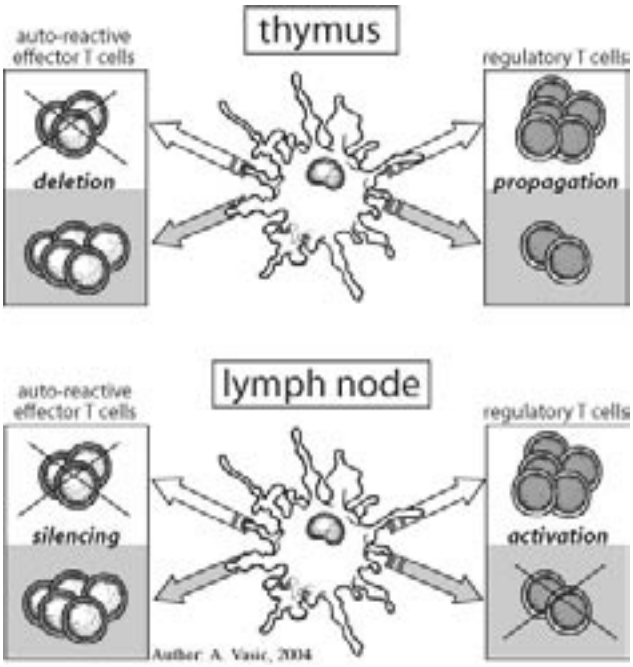

Figure 2. Checkpoints at which DC could be involved in the faulty tolerance regulation. A "healthy" scenario is represented with white arrows while grey arrows point to the autoimmunity-related scenario. To preserve tolerance, in the thymus or in the lymph node, autoreactive effector T cells are deleted (or silenced) and regulatory T cells generated (or activated). Both faulty removal of autoreactive effector T cells and deficient propagation of regulatory T cells contribute to the break of tolerance. Importantly, DC critically regulate both processes

class I molecules to $CD8^+$ T cells could enable faulty selection of potentially auto-reactive cytotoxic clones [143]. However, this also does not necessarily need to be a DC problem.

Modest conclusive data exist on the actual defects of DC in NOD mice. *In vitro* generation of DC from the NOD BM appears to be defective [145-149]. When stimulated with GM-CSF, BM cells from the NOD mouse yield low numbers of DC, which are also poor cytokine producers and poor stimulators of T cells. Unlike cells from other mouse strains, BM cells from the NOD mouse need to be stimulated with GM-CSF together with IL-4 in order to fully mature into capable antigen presentign cells. However, when sufficiently stimulated, the NOD BM cells tend to show an increased DC generation and an elevated Th1 skewing capacity of the cells generated *in vitro* [150, 151].

Many of these defects could not be clearly traced *in vivo*. In a congenic NOD mouse strain, an imbalance in the *in vivo* development leading to an over-representation of $CD8\alpha^-$ DC in the spleen has been demonstrated [152]. However, DC isolated from the spleen and lymph nodes of the genuine NOD mouse did not reveal major phenotypic nor functional defects [92, 149]. Of note is also that the functional studies focused on the ability of *ex vivo* isolated DC to stimulate, not to silence T cells, so the hypothesis that the NOD DC fail to silence autoreactive T cells is neither rejected nor confirmed. Furthermore, the possibility that peripheral DC fail to maintain T_{reg} also remains.

A central role of a DC dysfunction in the development of autoimmunity is expected. However, a specific aberrant function of NOD DC has not been traced. Moreover, opposite conclusions drawn from different studies make it difficult to reach a definite conclusion on whether a particular deviation in the NOD DC exists.

Aims of this thesis:

The great heterogeneity of the DC and Mϕ with regard to their phenotype, active functional communication with other cells of the immune system and the functions as APC and regulators of the immune homeostasis assure the central place for DC and Mϕ in the immune response. Fine-tuning of DC and Mϕ in central and peripheral lymphoid organs is of essential importance in determining the type of immune response that will be induced.

Dysfunction of DC may also be central to development of autoimmunity. A role of DC in the induction and maintenance of tolerance, early presence of DC and Mϕ in the NOD mouse pancreas and functional aberrancies of the *in vitro* generated DC from the NOD mouse BM, underscore the importance of further studies of DC in the autoimmune diabetes.

Therefore, given the suggested aberrations in the development of DC in the NOD mouse and the uncertainties about their reality, the putative role of DC and Mϕ in the initiation, propagation, faulty regulation of the autoimmune response and the final β-cell destruction leading to diabetes and given the heterogeneity but a close developmental relationship of the DC/Mϕ system, we aimed:

- to establish relationships between Mϕ, DC and lymphocytes in the pancreas of NOD mice and investigate the modulation of the phagocytic cell compartment and the effects on insulitis and diabetes development in the NOD mouse;

- to characterize the *in vivo* developmental stages of DC and Mϕ in mice;

- and to try to clarify inconsistent findings about the faulty *in vitro* development and investigate potential aberrations in the *in vivo* DC differentiation in NOD mice.

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Chapter 2

Dendritic cells and macrophages in the NOD mouse pancreas

Dendritic cells and macrophages are essential for the retention of lymphocytes in (peri)-insulitis of the non-obese diabetic mouse: a phagocyte depletion study

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Dendritic cells (DC) and macrophages (Mϕ) are present in higher numbers in the pancreas of the NOD mouse during the diabetogenic process from very early stages onwards. In this study we used clodronate-loaded liposomes to mediate the temporary systemic depletion of these phagocytic cells and monocytic precursors in order to modulate the pancreatic inflammation. Two intra-peritoneal injections given with a 2-day interval to 8-week old NOD mice depleted monocytes from the circulation and monocytes, DC and Mϕ from the spleen within the first days after the injections. Monocytes, DC and Mϕ re-appeared in the circulation and the spleen within one week and had an unchanged phenotype and antigen presenting function. Interestingly, this treatment caused a delayed, long lasting disappearance (7–21 day post-injection) of DC and Mϕ from the endocrine pancreas at a time when monocytes, DC and Mϕ had already repopulated the circulation and spleen. The depletion of DC and Mϕ from the endocrine pancreas was accompanied by a total disappearance of lymphocytes from the pancreas. DC, Mϕ and lymphocytes re-appeared in the pancreatic inflammatory infiltrates in treated mice from 28 days post depletion onwards. Importantly, the treatment significantly postponed the onset of diabetes, leading to a strongly decreased incidence by 35 weeks of age.
Taken together, our data show an essential role of phagocytic cells, i.e. DC and Mϕ, in the recruitment of lymphocytes to the pancreatic islets in NOD mice.

Type I diabetes is an auatoimmune disease in which a self-destructive immune process against the pancreatic β-cells leads to insulin deficiency. In the non-obese diabetic (NOD) mouse, a widely used animal model for autoimmune diabetes, dendritic cells (DC) and macrophages (Mϕ) have been proposed to be important for the initiation, progression and final destruction of the β-cells [1]. Histological studies show slightly raised numbers of DC and Mϕ already in the pancreas of neonatal NOD mice [2]. A further accumulation of DC and Mϕ around islets at 4-5 weeks of age precedes the peri-insular infiltration of lymphocytes. A relocation of lymphocytes and Mϕ into the islets characterizes the initiation of the final β-cell destruction [3, 4]. The continuous presence of elevated numbers of DC and Mϕ in the NOD pancreas from birth onwards strongly suggests an important role of these cells in the diabetogenic process. Yet, these histological studies are descriptive and therefore inherently inconclusive regarding the functional role of these cells.

Functional approaches have involved the blocking of the intra-islet infiltration of inflammatory cells by an intervention of leukocyte-adhesion to the pancreas endothelium [5-7] or by inducing a long-term depletion of phagocytic cells [8-10]. These methods protected mice from diabetes, further strengthening the concept of an essential role of Mϕ and DC in the development of autoimmune diabetes in the NOD mouse. However, previous studies mainly focused on the depletion of Mϕ and did not consider the effects of DC depletion. We have previously demonstrated that clodronate-loaded liposomes (lip-CL_2MDP) not only deplete Mϕ, but also DC in liposome targeted-tissues [11].

We here report an investigation on the intra-pancreatic and peripheral changes in the monocyte, DC and Mϕ compartment after a short-term lip-CL_2MDP treatment of NOD mice. We show that two intra-peritoneal (i.p.) injections of lip-CL_2MDP given with a 2-day interval in 8-week-old pre-diabetic NOD mice significantly delayed diabetes onset for more than 20 weeks in the majority of treated mice. Considering the short-term disappearance of monocytes, Mϕ and DC from the circulation and the spleen, and the rapidly regenerated normal function of the newly recruited spleen DC, the beneficial effects could not be attributed to a modulation of cells from this compartment. However, the same treatment induced a delayed and prolonged disappearance

of DC and Mϕ from the endocrine pancreas. The absence of DC and Mϕ directly influenced the presence of lymphocytes and lymphocytic (peri-) insulitis resolved completely. Our observations point to an essential role of DC and Mϕ in the continued persistence of lymphocytic infiltrates the pancreas and subsequent β-cell destruction.

Material andMethods

Animals

Female NOD/Ltj and C3HeB/FeJ mice were bred at the animal facility of the ErasmusMC, Rotterdam, The Netherlands. C57BL/6j, BALB/c and NOR female mice were obtained from Harlan (Horst, The Netherlands). All strains were kept under specific pathogen-free conditions and fed *ad libitum*. By 30wks of age, the incidence of diabetes in our NOD colony is 90% for females and 60% for males. For all experiments, mice were sacrificed at postnatal ages of 7-12wks or at 35wks of age. Glycosuria was tested with the Gluketur test (Roche Diagnostics GmbH, Mannheim, Germany). A minimum of 3 animals per age/strain/time point was used for all studies. Animal handling followed the ethical rules of the European Union and was approved by the Erasmus University Animal Welfare Committee.

Liposome preparation and *in vivo* application

Multilamellar liposomes containing clodronate (dichloromethylene bisphosphonate, a gift from Roche Diagnostics GmbH) (lip-CL_2MDP) in the aqueous phase were prepared as described previously [12, 13]. Liposomes consisted of phosphatidyl choline and cholesterol in 6:1 molar ratio. After washing, the liposomes were resuspended in PBS. A volume of 0.2ml, containing about 2mg of liposome-entrapped clodronate was injected twice intraperitoneally (i.p.) with a two-day interval between two consecutive injections into 8-week old mice (in a stage of well advanced peri-insulitis). This approach was chosen in order to obtain a complete depletion of phagocytic cells in the spleen. Mice were sacrificed (by CO_2 exposure) at various time points after the second injection (days 2, 4, 7, 14, 21, 28) and indicated organs were removed for analysis.

Diabetes incidence after treatment with clodronate-loaded liposomes

Mice were followed until 35wks of age and the presence of glucose and ketones in the urine was measured weekly. A group of non-injected mice of the same age were used as control. At 35wks of age, mice were sacrificed and organs removed. Insulitis was characterized by analyzing the composition of islets with respect to the presence of insulin- and glucagon positive cells, T cells, DC or Mϕ in histological examination of sequential sections stained for each cell type, according to the staging established previously [3]. In addition, a numerical score was ascribed to the four stages as follows: stage 0 (unaffected islets)–score 0, stage I+II (early para-insular accumulation of DC and Mϕ, no T cells present, insulin$^+$)–score 1, stage III+IV (para- and peri-insular accumulation of DC, Mϕ and T cells, insulin$^+$)–score 2, stage V (infiltration of T cells into islets, insulin$^+$)–score 3, stage VI (end stage, insulin-)–score 4. In the latter case, islet remnants were identified by glucagon-labeling. A score was assigned to each counted islet and an average of all scores per 100 counted islets was calculated.

Preparation of single cell suspension of circulating leukocytes and spleen

Blood was obtained by heart puncture after exposing the organ, and collected in heparin-coated tubes or in syringes containing 1ml PBS with 8mM EDTA. Erythrocytes were eliminated using lysing solution (BD Biosciences, San Diego, CA, USA) and leukocytes were washed twice by centrifugation at 400g in phosphate-buffered saline (PBS) supplemented with 0.5% BSA and 20nM NaN_3 (further referred to as the FACS buffer).

The spleen was cut into two parts. The larger part was used for histological analysis. The smaller part of the spleen was cut into small pieces and incubated for 1h at 37°C with collagenase-D (Roche Diagnostics GmbH; 0.27 U/mg) at a final concentration of 1mg/ml in RPMI-1640 medium supplemented with 60mg/ml penicillin and 100mg/ml streptomycin. The resulting digested tissue suspension was pushed through a 100mm cell strainer using a rubber-end of a 5ml syringe plunger and centrifuged. Subsequently, the cell suspension was washed with RPMI-1640 medium supplemented with antibiotics and 10% fetal calf serum (FCS).

Spleen DC isolation by MACS procedure

DC were sorted from the spleen cell suspension by magnetic enrichment using auto-MACS. Cells were centrifuged, resuspended to obtain a concentration of 10^8 cells in 400ml MACS buffer (PBS supplemented with 1% FCS and 2mM EDTA) and incubated with 100ml CD11c microbeads (Miltenyi Biotec GmbH, Germany) for 30min on ice. Subsequently, cells were washed twice and then resuspended in MACS buffer to obtain a concentration of 10^8 cells/ml. The program for high purity of cells with low frequency was used on the autoMACS machine (Miltenyi Biotec GmbH) and a positive fraction was collected. In all experiments, the purity was higher than 95% as determined by subsequent flowcytometric analysis.

Phenotypic analysis

The list of antibodies used in this study is given in the Table 1. Aliquots of $2x10^6$ cells were incubated with the prepared mix of monoclonal antibodies. Each incubation step was performed at room temperature for 10min. All biotinylated antibodies were detected by streptavidin-APC (BD Biosciences). Anti-CD71 was detected by R-PE labeled goat-anti-rat IgG (mouse-absorbed; GaRa-PE) purchased from Caltag Laboratories, San Francisco, CA.

Table 1. Monoclonal antibodies used in this study

marker	monoclonal antibody	conjugate	origin
Ly-6G	1A8	PE	BD Biosciences[a]
Ly-6C	ER-MP20	FITC	own laboratory
CD11b / Mac-1	M1/70	PerCP	BD Biosciences
CD11c	N418; HL3	∅[b]; FITC, PE	own laboratory; BD Biosciences
CD43	S7	FITC, PE	BD Biosciences
CD8α	53-6.7	FITC, APC	BD Biosciences
F4/80	F4/80	FITC	Caltag Lab, San Francisco, CA, USA
CD31	ER-MP12	bio	own laboratory
CD45RB	16A	FITC	BD Biosciences
MHC class II ($I\text{-}A^b$)	ER-TR3	bio	BMA Biomedicals AG, Switzerland
MHC class II ($I\text{-}A^{g7}$)	10.2.16	bio	own laboratory
CD80	16-10A1	PE	BD Biosciences
CD86	GL1	FITC	BD Biosciences
CD4	RM4-5	APC	BD Biosciences
insulin		∅[b]	DAKO,Carpinteria, USA
glucagon		∅[b]	DAKO,Carpinteria, USA
CD3	KT3	∅[b]	own laboratory
ER-MP23	ER-MP23	∅[b]	own laboratory
BM8	BM8	∅[b]	BMA Biomedicals AG, Switzerland
CD71	ER-MP21	∅[b]	own laboratory

[a]San Diego, CA, USA; [b]unconjugated

Mixed leukocyte reaction

The capacity of sorted spleen DC to activate allogeneic lymph node (LN) cells was measured as previously described [14]. Briefly, responder T cells, isolated from LN of C3H mice, and stimulator cells (DC) were resuspended in RPMI-1640 (with Hepes), 10% FCS (heat inactivated; 0.2 mm filtered), 60 mg/ml penicillin, 100 mg/ml streptomycin, 20 mg/l sodium pyruvate and 50 mM 2-ME (further referred to as MLR medium). T cells were resuspended at a concentration of 10^6/ml. The concentration of stimulator cells varied depending on the desired stimulator: responder cell ratio. The cells (100ml of each cell suspension) were incubated in round-bottom 96-well plates for 4 days at 37°C in 7% CO_2. Stimulator and responder cells, incubated separately in MLR medium, were used as negative controls. Mitogenic stimulation of T cells by concanavalin A (final concentration 1.25 mg/ml) (Sigma Chemical Co., St. Louis, MO, USA) was used as a positive control for cell proliferation.

After 4 days of co-culture, cells were harvested and analyzed by flow cytometry. Triple labeling of cells with antibodies against CD4, CD8 and CD71 (anti-transferrin receptor) was performed and 1.5×10^4 events were acquired within the live cell gate. Data analysis was performed using Cell Quest Pro analysis software. The percentage of CD71-positive T cells, as a measure of proliferating cells, was determined within the $CD4^+$ and $CD8^+$ population.

Immunohistochemistry

Pancreases were embedded in Tissue-Tek (Miles, Elkart, IN, USA) and frozen in liquid nitrogen. Cryostat sections (5mm thick) were cut at 100 or 200mm intervals. Series of 5 or 6 pancreas sections cut at different levels were fixed for 10min in acetone containing 0.03% hydroxyperoxide to block endogenous peroxidases. PBS with 0.05% Tween-20 was used for the washing steps. Unconjugated primary antibodies specific for insulin, glucagon, T-cells (CD3), dendritic cells (CD11c) or subsets of Mϕ (ER-MP23 or BM8 [15]) were subsequently detected with appropriate peroxidase-coupled- (DAKO, Glosstrup, Denmark) secondary antibodies in the presence of 1.5% normal mouse serum. The 3-amino-9-ethylcarbazole substrate (Sigma Co., St.Quentin Fallavier, France) dissolved in 50mM sodium acetate/0.02% hydroxyperoxide was used to detect peroxidase activity. The resulting labeling was examined by light microscopy.

Results

Phagocyte depletion with lip-CL_2MDP in 8 week-old pre-diabetic NOD mice, significantly delays the onset of diabetes.

To investigate the modulating effects of temporary phagocyte depletion on pancreatic inflammation and diabetogenesis, we treated NOD mice with two i.p. injections of lip-CL_2MDP. This treatment

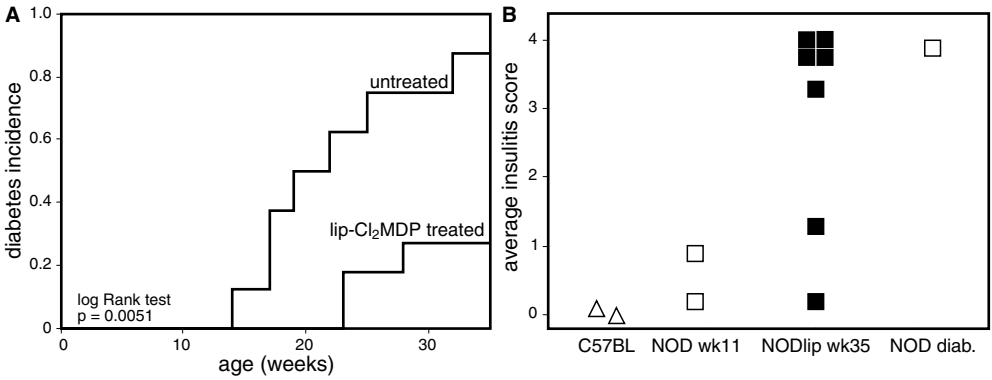

Figure 1. A short-term treatment of 8-week-old NOD mice with lip-CL_2MDP induced a significant delay of diabetes onset, resulting in strongly reduced incidence at 35 weeks of age. A. Kaplan-Meier analysis of the diabetes incidence in treated and untreated mice. The Log Rank statistical test was performed to compare the Kaplan-Meier curves ($p<0.01$). B. Insulitis score of control untreated C57BL mice (empty triangles), untreated NOD mice at age 11 weeks and 17 weeks (diabetic) (empty squares) and treated NOD mice at 35 weeks of age (filled squares). Symbols represent the values of the insulitis score of mice, individually. For a description of scoring, see the Material and Methods section

caused a significantly delayed onset of diabetes ($p<0.01$) (Fig. 1A). At the endpoint of our studies (35 weeks of age) the majority of treated NOD mice (73%) was still normo-glycemic. However, immunohistological examination of the pancreases of these mice showed that none of the mice had been permanently protected from insulitis. Leukocyte accumulations in the endocrine pancreas were present in all treated mice at 35 weeks of age. The infiltrates comprised DC, Mϕ and T cells and the infiltrates composition varied per individual islet, depending on the stage of the destructive process, as has been described before for non-treated mice [3]. The insulitis severity score (Fig. 1B) of the non-diabetic 35wk old treated NOD mice was comparable either to that of non-treated pre-diabetic (11wks old) or to the diabetic NOD mice. Hence, a two-fold injection of lip-CL_2MDP strongly reduced the progression of the destructive process and significantly delayed the diabetes onset in majority of treated mice for 18-24 weeks.

Monocytes, DC and Mϕ return rapidly to the spleen of NOD mice after the depletion.

We examined the effects of lip-CL_2MDP on the disappearance of monocytes, DC and Mϕ from the circulation and the spleen of NOD mice as well as the kinetics of their repopulation and compared these parameters to those of identically treated C57BL mice.

Blood monocytes were quantified using a recently established analysis [16] in which monocytes are gated as $SSC^{lo}CD11b^{hi}$ cells. Blood monocytes consist of an immature pool (Ly-$6C^{high}$), readily recruited into inflammations, and mature monocytes (Ly-$6C^{low}$), a resident DC and Mϕ precursor pool [17]. For the quantification of DC and Mϕ in the spleen by a flow cytometric analysis, monoclonal antibodies against CD11c, CD8α, CD11b and F4/80 were applied simultaneously. This enabled definition of four distinct cell populations (Fig 2A). $CD11c^+$ cells (gate: R1) were subdivided into $CD11c^+CD11b^{hi}CD8\alpha^-$ (myeloid DC) and $CD11c^+CD11b^{lo}CD8\alpha^+$ cells ($CD8\alpha^+$ DC). A third population, $CD11c^-CD11b^{hi}F4/80^+$ (gate: R2), represent the red pulp Mϕ as verified by immunohistochemistry. The F4/80-negative fraction in the gate R2 were granulocytes. Finally, the $CD11c^{low}CD11b^{hi}$ cells (gate: R3) formed a fourth population; these cells were $F4/80^{low}$, Ly-$6C^{low/med}$, $CD43^+$, $CD31^+$ and correspond to the mature blood monocytes as we have recently defined [16].

Treatment with lip-CL_2MDP (i.v.) causes the virtually complete depletion of circulating monocytes during the first hours, followed by a rapid return [16]. Starting from the day 2 after the i.p. treatment, the pool of Ly-$6C^{high}$ monocytes did not change. In contrast, the Ly-$6C^{low}$ monocyte population was almost completely depleted at

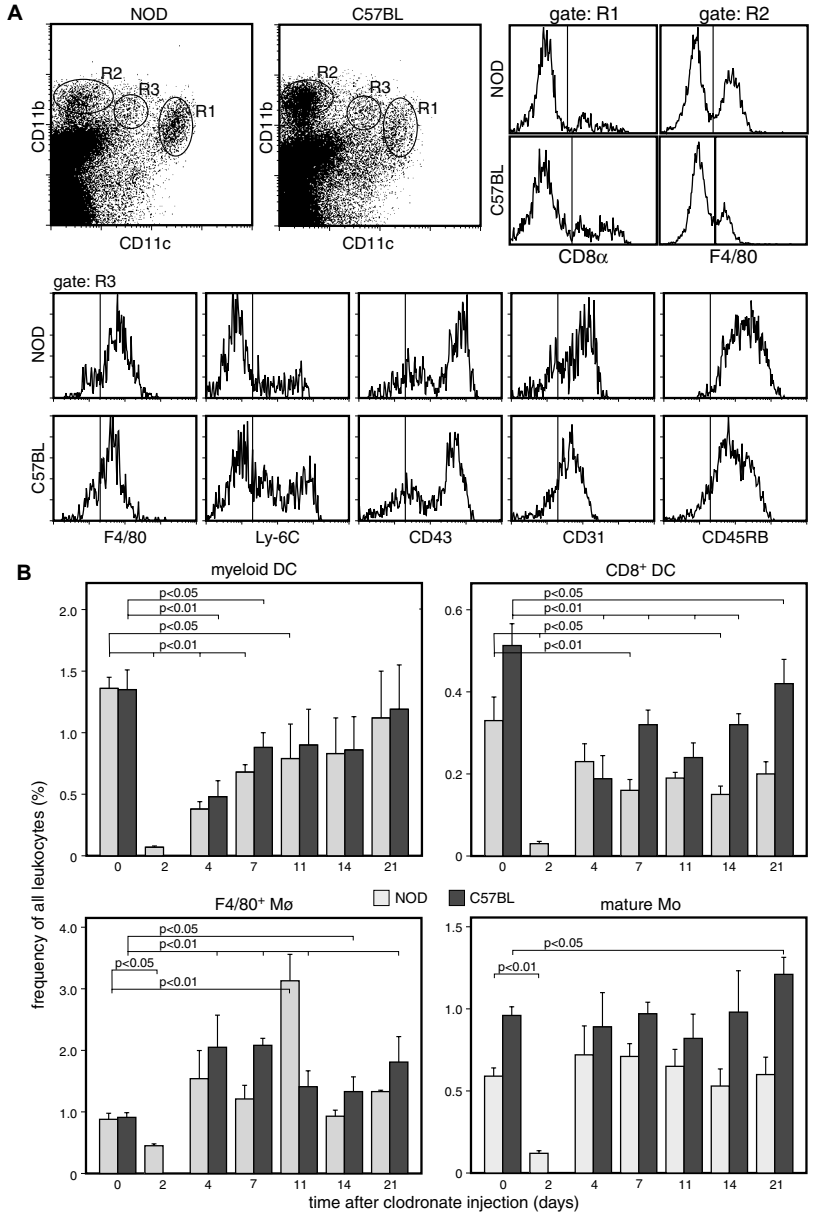

Figure 2. Normal maturation kinetics of the NOD DC and Mϕ compartment after lip-CL_2MDP in the spleen. A. Phenotypic definition of spleen DC and Mϕ populations according to CD11b and CD11c expression. Histograms represent the expression profile for a given marker of cells that belong to the indicated gate. CD11c^{hi} cells (Gate:R1) can be divided into CD8$^+$ DC and myeloid DC (CD8$^-$). The F4/80$^+$ population within CD11c$^-$CD11b^{hi} cells (Gate:R2) represent the red pulp Mϕ (verified by immunohistochemistry). The CD11c^{lo}CD11b^{hi} (gate: R3) cells correspond to mature blood monocytes and are F4/80^{lo}, Ly-6C$^{lo/med}$, CD43$^+$, CD31$^+$ and CD45RB$^+$. The vertical line represents a negative control limit. B. Time course of the depletion and repopulation of the myeloid cells in the spleen as defined by phenotype in A. Each bar represents an average value of a minimum of 3 mice per mouse strain per time point. P-values indicated graphs represent the statistical significance (Student's t-test) of the indicated time point vs. the point 0 in a given mouse strain.

day 2 and significantly reduced until the end of the observation period (day 28) (Table 2). Similar kinetics were found for the C57BL control mice (not shown).

Table 2. Kinetics of the blood monocytes repopulation in NOD mice after lip-CL_2MDP treatment

Time point (days)	All monocytes	Inflammatory Ly-6C^{hi} monocytes	Mature Ly-6C^{lo} monocytes
0	13.03 ± 1.36 [a]	4.47 ± 0.33	8.56 ± 1.05
2	5.83 ± 0.57*	5.28 ± 0.61	0.55 ± 1.07**
4	9.74 ± 0.81	5.87 ± 1.72	3.87 ± 0.04*
7	10.87 ± 1.49	5.97 ± 1.04	4.90 ± 0.74*
14	14.04 ± 3.51	9.73 ± 2.68	4.31 ± 0.93*
28	12.26 ± 1.59	7.37 ± 1.57	4.89 ± 0.83*

[a] data represent mean ± SEM
*p<0.05; **p<0.01 vs. 0 time point

Mature monocytes, DC and Mϕ in the spleen were clearly depleted at day 2 after the last injection of lip-CL_2MDP (Fig 2B). All four investigated cell groups were present in the spleen of the treated mice, starting from day 4 in both mouse strains. Myeloid DC reached steady state levels in the second week post-treatment, while CD8α$^+$ DC needed more than two weeks to normalize (Fig. 2B - upper row). Similar kinetics were found for the NOD and C57BL mice. Red pulp Mϕ were restored almost instantly: despite a short initial drop, the rate of NOD red pulp Mϕ remained stable at the steady state level with even a slight increase at day 11. However, in C57BL mice, the frequency of red pulp Mϕ remained significantly higher till the end of the observation period. Splenic mature monocytes were also depleted at day 2, but returned to normal levels shortly thereafter and remained stable from day 4 onwards in both mouse strains (Fig. 2B - lower row).

Observations in flow cytometric analysis are confirmed by the immunohistological analysis

Immunohistological analysis of CD11c$^+$ (myeloid DC and CD8$^+$ DC) and F4/80$^+$ cells (mature monocytes and Mϕ) in the spleen indicated the same kinetics as the flow cytometric analysis. As shown in Figure 3, treatment with lip-CL_2MDP induced a complete depletion of both Mϕ and DC from the spleen of NOD mice: 2 days after the second injection, the red pulp was devoid of viable F4/80 or CD11c positive cells (Fig. 3C and 3D). The remaining F4/80 and CD11c positive labeling in the white pulp at day-2 resembled non-viable pycnotic cells that lacked a typical membrane staining as present in the spleen of non-treated control mice (Fig. 3A and 3B).

F4/80$^+$ cells started to return to the red pulp from day 4 post-injection onwards (Fig. 3F) and at day 7 (Fig. 3H) the number and the distribution of the F4/80 positive population were comparable to non-depleted control mice. The repopulation of the spleen by CD11c$^+$ cells also started at day 4 (Fig. 3E) and at day 7 CD11c$^+$ cells in NOD mice had regained their normal staining pattern as compared to the non-treated controls (Fig. 3G). In addition, other Mϕ populations like metalophillic (MOMA-1$^+$) and marginal zone (ER-TR9$^+$) Mϕ were depleted in NOD mice by lip-CL_2MDP in the same fashion as has been reported for other mouse strains [18]. These cells, however, only reappeared several weeks after depletion (data not shown).

Taken together, the kinetics of depletion and repopulation by different populations of splenic DC and Mϕ in NOD mice is fully comparable with the repopulation in C57BL mice.

A normal phenotype and function of re-populated spleen DC in NOD mice after the depletion

Despite the rapid return, we argued that the treatment with lip-CL_2MDP may have caused a modulation of the function of the returning phagocytes, contributing to an altered role in diabetogenesis. Therefore, we compared the phenotype and the function of spleen CD11c$^+$ cells, isolated by an autoMACS procedure, from treated (day 7 post-injection) and from non-treated NOD and C57BL mice. Although we utilized a high purity separation program, both CD11c^{high} and CD11c^{low} cells were isolated, based on the phenotypic analysis (Fig. 4A). We found no difference in the cell yield between NOD and C57BL mice from both untreated and treated mice. Also, the isolated CD11c$^+$ cells from treated mice displayed the same expression of MHC class II and of the co-stimulatory molecules CD80 and CD86 in comparison to such cells isolated from non-treated mice (Fig. 4A). In addition, the T-cell

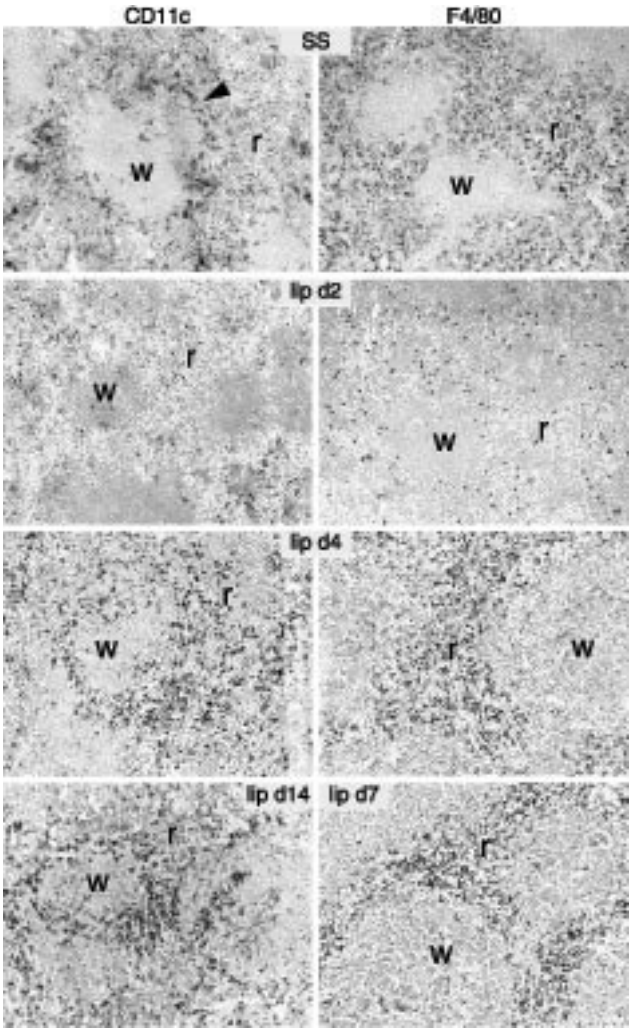

Figure 3.
Immunohistochemical analysis of the distribution of CD11c$^+$ and F4/80$^+$ cells in the NOD spleen at different time points after lip-CL$_2$MDP treatment. A-B. Distribution of cells before the treatment. C-D. At day 2 after injection DC and Mϕ were completely depleted from the spleen red pulp. E-F. The return of F4/80$^+$ cells in particular, is evident already at day 4 after injection. G-H. Normal distribution of CD11c$^+$ and F4/80$^+$ cells was observed from days 14 and 7 after the depletion, respectively.

stimulating capacity of the isolated DC co-cultured with allogeneic LN cells was similar before and after the treatment, in both mouse strains (Fig. 4B). Similar proportions of allogeneic CD4$^+$ or CD8$^+$ T cells had up-regulated CD71 (transferrin-receptor), irrespective of the fact that the cells isolated from treated mice had a higher proportion of mature monocytes than before treatment.

Lip-CL$_2$MDP treatment leads to a late disappearance of DC and Mϕ from the NOD pancreas

To examine the effect of lip-CL$_2$MDP treatment on the target organ inflammation, pancreases were removed before and after the injections (at days 0, 2, 4, 7, 14 and 28 post-treatment), and the presence of the different cell populations was examined by immunohistochemical staining.

Untreated (8wk old) NOD mice had a considerable lymphocytic peri- and intra-islet infiltration. BM8$^+$ and ER-MP23$^+$ Mϕ (Fig. 5) were found scattered throughout the pancreas and most likely represent resident Mϕ. Unlike Mϕ, DC (CD11c$^+$) were localized only in close proximity

to the islets and not in the intra- and interlobular septa of the exocrine pancreas (Fig. 7A).

The treatment with lip-CL_2MDP had no immediate effect on pancreas Mϕ and DC, unlike hitherto observed in the spleen and the circulation. However, starting from day 4 after the treatment, we observed reduced numbers of $BM8^+$ and ER-$MP23^+$ Mϕ in both exocrine and endocrine pancreas. The decline in the Mϕ number further continued and many islets were clear from Mϕ (Fig. 5C and 5D). Quantification of Mϕ-containing islet-inflammations at day-7 post-treatment showed that about 95% of islets were free of Mϕ (Fig. 6A) and the frequency of islets that contained Mϕ was almost as low as in C57BL mice of the same age. Additionally, at day 14 after injection, many islets and exocrine lobes were devoid of Mϕ (Fig. 5E, 5F and 6A). The distribution of the few remaining Mϕ had also drastically changed as they were found only in confined areas such as the inter-lobular connective tissue, but not associated with endocrine or exocrine cells. At 28 days after treatment Mϕ had reappeared again at the islet periphery (Fig. 5G and 5H).

Similar to Mϕ, the number of pancreatic DC declined from day 4 after treatment onwards (Fig. 6B). At days 7 and 14, the short-term injection of lip-CL_2MDP had almost completely depleted DC from NOD pancreases. When quantified, the number of DC-free islets was similar to the number of Mϕ-free islets (Fig. 6B). Also like Mϕ, DC were again present in islets at day 28 after lip-CL_2MDP

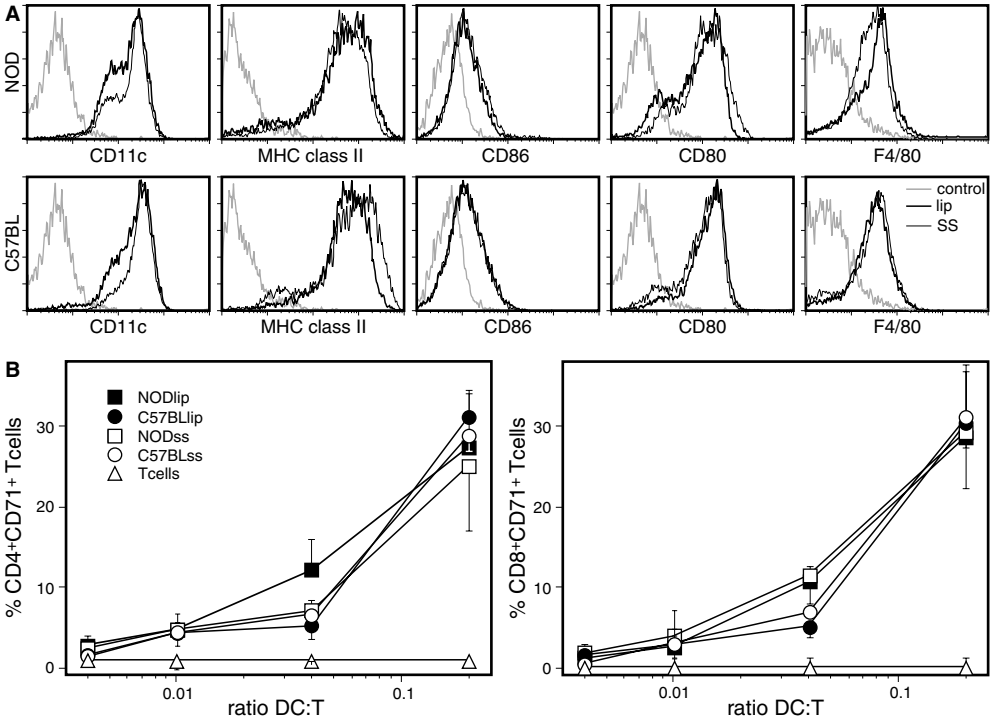

Figure 4. Phenotype and T cell stimulatory capacity of DC isolated from spleens from treated or untreated NOD and C57BL mice. A. Isolated $CD11c^+$ cells from the spleens of treated or untreated NOD and C57BL mice showed a normal mature DC phenotype. Histograms display the marker expression in the autoMACS-isolated population. The gray line represents the negative isotype control, the thin-black line the marker expression by cells isolated from untreated mice and the thick-black line the expression pattern of cells isolated from treated mice at day 7 after treatment. B. The stimulation capacity of isolated DC was the same in NOD and C57BL mice irrespective of whether DC were derived from treated or untreated mice. Stimulation of $CD4^+$ and $CD8^+$ cells was measured as a percentage of cells that have upregulated expression of transferrin-receptor (CD71) on their surface. Representative histograms (in A.) and average values ± SEM (in B.) of three separate pools from two independent experiments for each group are shown in the figure.

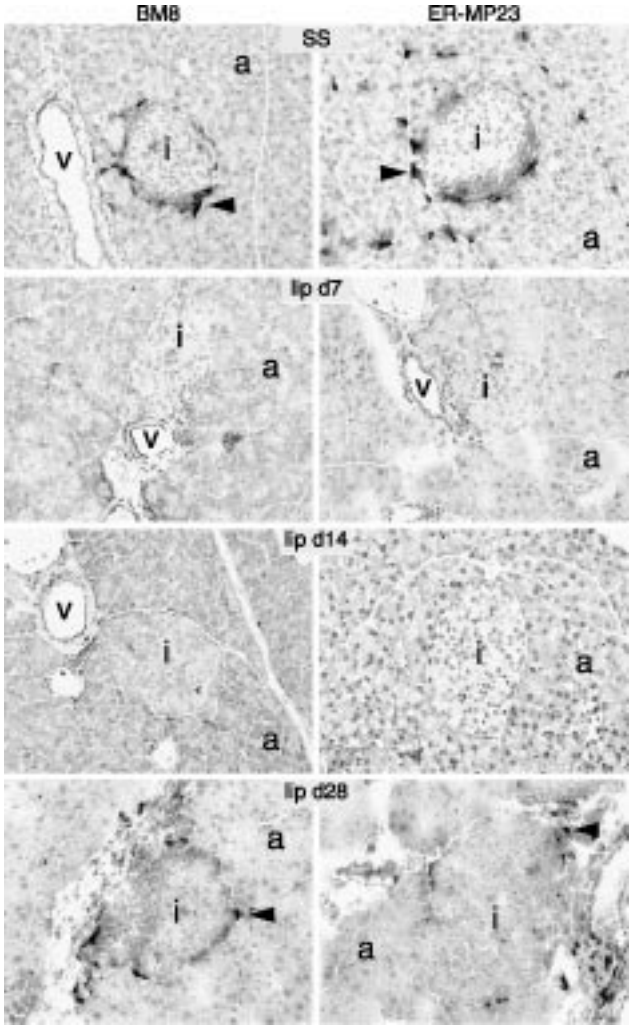

Figure 5.
Prolonged disappearance of Mϕ from the pancreas of NOD mice after lip-CL_2MDP treatment. A-B Peri- and intra-islet infiltrates from untreated 8wk old NOD mice contain $BM8^+$ and $ER\text{-}MP23^+$ Mϕ. Both subsets of Mϕ were also found scattered throughout the connective tissue of the exocrine pancreas. C-D. From day 7 after lip-CL_2MDP treatment a considerable amount of resident Mϕ had disappeared from the endocrine pancreas. E-F. At day 14, the pancreas is found similarly devoid of Mϕ as at day 7. G-H. Mϕ were again detectable at day 28 after the lip-CL_2MDP treatment and present in the close proximity of pancreatic islets.

injection (Fig. 7).

The loss of Mϕ and DC from NOD pancreas leads to a prolonged disappearance of lymphocytes

In parallel with the loss of Mϕ and DC, we observed a disappearance of lymphocytes from the pancreases of treated mice (Fig. 6B). The decline in DC and Mϕ numbers at day 4 already led to the loss of lymphocytes from the pancreatic infiltrates (not shown). This phenomenon was more prominent at days 7 and 14 after treatment and observed in parallel with the absence of DC (Fig. 7 C-F). At day-28, when a substantial number of DC and Mϕ had returned to the pancreas, T cells also re-appeared. B cells demonstrated a similar kinetics as T cells. At day-4, the numbers of B cells started to decline and the cells had completely disappeared by day 7. Interestingly, B cells seemed to require other conditions to infiltrate the pancreas again, as they did not reappear by day-28 but returned to the pancreas much later, i.e. at day-49 post-treatment (data not shown).

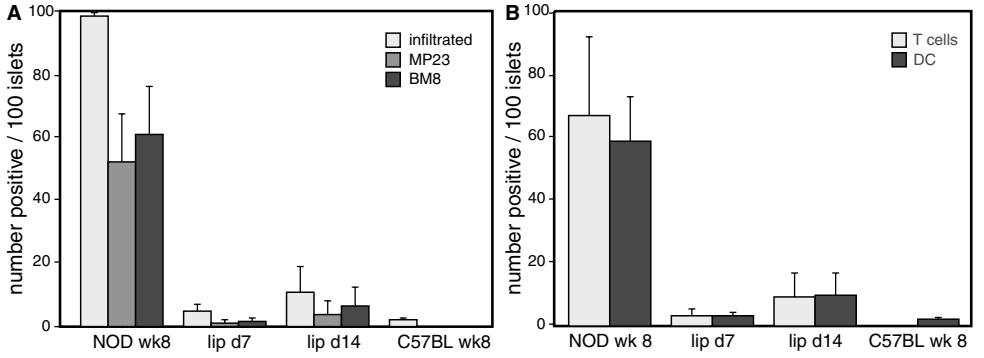

Figure 6. Differential analysis of the islets with inflammatory infiltrations before and after lip-CL_2MDP treatment. The treatment significantly decreased the frequency of islets infiltrated with Mϕ and DC, which directly correlated to the disappearance of lymphocytes from the pancreas. **A.** Correlation of the Mϕ presence with islet infiltration, at different time points. Light-grey bars represent the percentage of infiltrated islets, determined by morphological examination of glucagon-positive islets. Dark-grey bars show the frequency of islets associated with ER-MP23$^+$ Mϕ and black bars represent values for the BM8$^+$ Mϕ; before the treatment, at different post-treatment time points and in untreated C57BL mice. B. Correlation of a DC loss with the departure of T cells, in the pancreas of treated mice. Black bars represent the percentage of islets that contained CD11c$^+$ DC and light-grey bars the percentage of islets that contained T cells in NOD mice, like in A. All bars show the average value (±SEM) calculated from 3 or 4 mice per group for each strain and each time point.

Discussion

In this study we bring to light three important findings related to the role of dendritic cells (DC) and macrophages (Mϕ) in the development of insulitis and diabetes in the NOD mouse model.

First, a short-term treatment with lip-CL_2MDP given in the stage of a progressing peri-insulitis (8 weeks of age) was sufficient to induce a significant decline in the number of Mϕ and DC in the islet vicinity for a period of at least three weeks in NOD mice. Second, the lasting loss of DC and Mϕ from the pancreas revealed an essential role of these cells in the local recruitment and retention of lymphocytes. Lymphocyte infiltrations dissolved completely when the DC/Mϕ pool was absent and reappeared only upon return of DC and Mϕ in close proximity to the endocrine tissue. Third, the depletion of the DC and Mϕ did not happen concomitantly in the pancreas and in the circulatory compartment. Monocytes, DC and Mϕ were depleted and rapidly returned to the circulation and the spleen within one week with similar kinetics in NOD and control mice, whereas in the pancreas their disappearance started at the end of the first week. Furthermore, in line with the observed changes in the pancreas, the diabetes onset and incidence were strongly delayed and decreased, respectively, after short-term lip-CL_2MDP-treatment of NOD mice at 8 weeks of age.

It is well known that an i.p. injection of lip-CL_2MDP depletes Mϕ from the spleen, peritoneal cavity and the omentum [19], but the pancreas has not been included in such previous depletion studies. Our study shows that i.p. injections of lip-CL_2MDP also influence the accumulation of monocytes, Mϕ and DC in the pancreas. Remarkably, however, when we compared the effects of depletion in the pancreas with that of the spleen/circulation, we observed different kinetics of disappearance and return of the phagocytes: DC and Mϕ took a week longer to disappear from the pancreas. Unfortunately, we cannot readily explain these differential disappearance patterns.

The return of mature monocytes to the circulation and the spleen, preceded the return of DC and Mϕ. This supports the view that spleen APC originate from blood monocytes. Treatment with lip-CL_2MDP ineffectively deplete phagocytes in the connective tissue [20]. Indeed, the delayed disappearance of pancreatic DC and Mϕ upon treatment strongly suggests that liposomes do

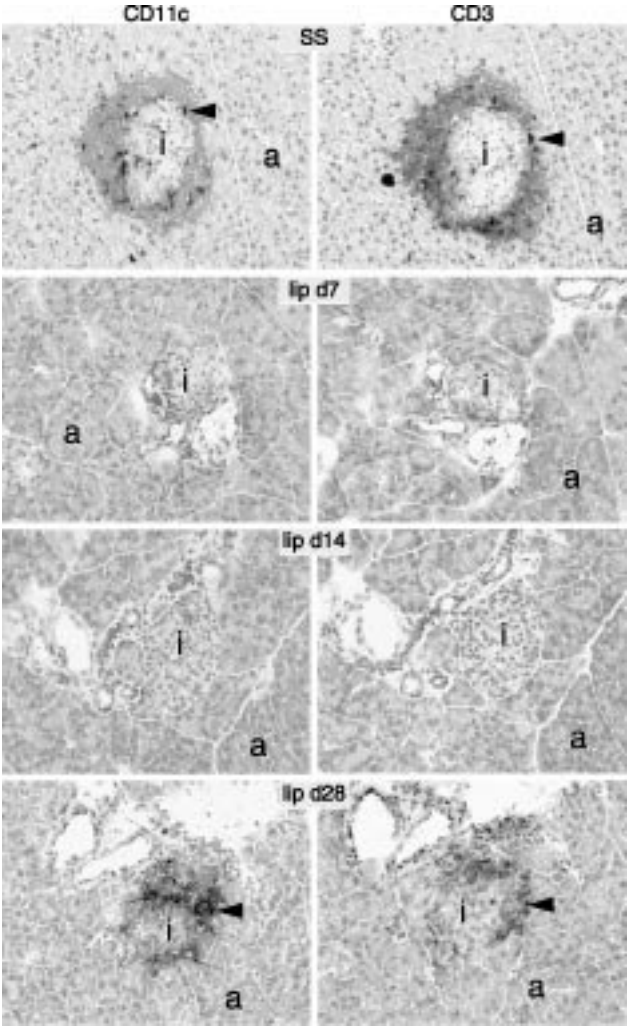

Figure 7.
The presence of T cells correlated directly with the DC in the pancreas of NOD mice. A-B. Before the treatment with lip-CL_2MDP, DC were found in the close proximity of T cells as judged by the immunohistochemical analysis of serial sections of the NOD mouse pancreas. C-D. At day 7 after the treatment, DC had disappeared from the pancreas and T cells as well. E-F. Islets that were free of DC were also free of lymphocytic accumulations in the pancreas, at day 14 after the treatment. G-H. At 28 days after the treatment, return of DC was found in a few islets, paralleled by the return of T cells.

not reach these phagocytes directly. Instead, it is more likely that the natural turnover of the cells, under conditions when the precursors might be lacking, causes their decline. It is however difficult to understand why monocytes did not rapidly replenish DC and Mϕ in the pancreas, like they do in other target organs such as spleen and liver, since the disappearance of pancreas DC and Mϕ started when monocytes were back in the circulation.

One possible explanation is that pancreas DC and Mϕ might originate from other precursors than those found in the circulation. The origin of pancreas resident Mϕ and DC is not known. They might develop from intra-organ precursors, like in the skin [21], or from precursors that reside in the vicinity of the pancreas. In this context it is noteworthy that i.p. application of lip-CL_2MDP to rats induced a depletion of Mϕ in the peritoneum and omentum within two days, but the repopulation of the omental Mϕ was not seen within the next 23 days [22]. This “postponed” pattern of re-appearance overlaps with the here described slow pattern of re-appearance of Mϕ and DC in the pancreas and this might either point to the omentum as a source of precursors

for pancreas DC and Mϕ, or to a shared precursor of omental and pancreas DC and Mϕ. Further investigation is needed to evaluate whether such peripheral precursors might be affected by the i.p. lip-CL_2MDP treatment more extensively than the circulating monocytes. Alternatively, the lip-CL_2MDP treatment does interrupt the continuous influx of monocytes into the pancreas for several days and thereby also the production of appropriate chemoattractants for other inflammatory cells [23, 24]. In this case, the dynamic inflammatory process might be transiently interrupted and subsequently slowly restored, similarly to what has been demonstrated previously [25].

A role for DC in the NOD lymphocyte accumulation has been suggested previously. The administration of streptozotocin (STZ) to C57BL/6 mice, an induced diabetes model, led to an accumulation of DC in the pancreas, followed by an enhanced expression of adhesion molecules on the pancreatic endothelium and an increased adherence and infiltration of lymphocytes [26]. Furthermore, lymphocyte adhesion and infiltration decreased upon silica-mediated depletion of the Mϕ additionally suggesting that the Mϕ were involved in the recruitment of lymphocytes [27, 28]. The DC and Mϕ possibly act by inducing the addressins VCAM-1, MAdCAM-1 and ICAM-1 on the pancreatic endothelium [5, 29, 30]. The dependence of lymphocyte infiltration on Mϕ and DC has also been shown in other situations. Elimination of phagocytic cells by lip-CL_2MDP reduced the lymphocytic infiltration of transplanted fetal pig pancreas xenografts in NOD mice [31]. In addition, the loss of marginal zone Mϕ as the result of chronic *Leishmania* infection severely abrogated normal trafficking of lymphocytes into the white pulp of the spleen [32].

The responsible Mϕ or DC-derived factor that initiates the infiltration and maintenance of lymphocytes in the pancreas has not been identified thus far. Several chemokines like IP-10, MCP-1 and RANTES have been implicated in the attraction of monocytes and Th1-cells to the pancreatic islets during the progression of autoimmune diabetes [33-36]. Although the expression of some of these chemokines was found to be islet-specific, DC and Mϕ are also able to make other suitable chemokines that possibly attract and retain lymphocytes to the pancreas.

In conclusion, a depletion of DC and Mϕ via a regimen of two injections of lip-CL_2MDP in the stage of a progressing peri-insulitis (at 8 weeks of age) was sufficient to clear DC and Mϕ almost completely from the pancreas, and to delay the re-appearance of these cells considerably. The presence of DC and Mϕ in the pancreas dictated the presence of lymphocytes, which should be taken in account for developing possible new treatments for diabetes.

Acknowledgements

The investigation presented here was made possible due to the financial support from the Dutch Diabetes Research Foundation (Grant 96.606) and grants from the European committee (QLRT-1999-00276 - "MONODIAB"). We would like to acknowledge the contribution of B. Beukenkamp and M. Aoulad-Ahmed, for the technical assistance. In addition, we thank Tar van Os for his important contribution in preparing the figures.

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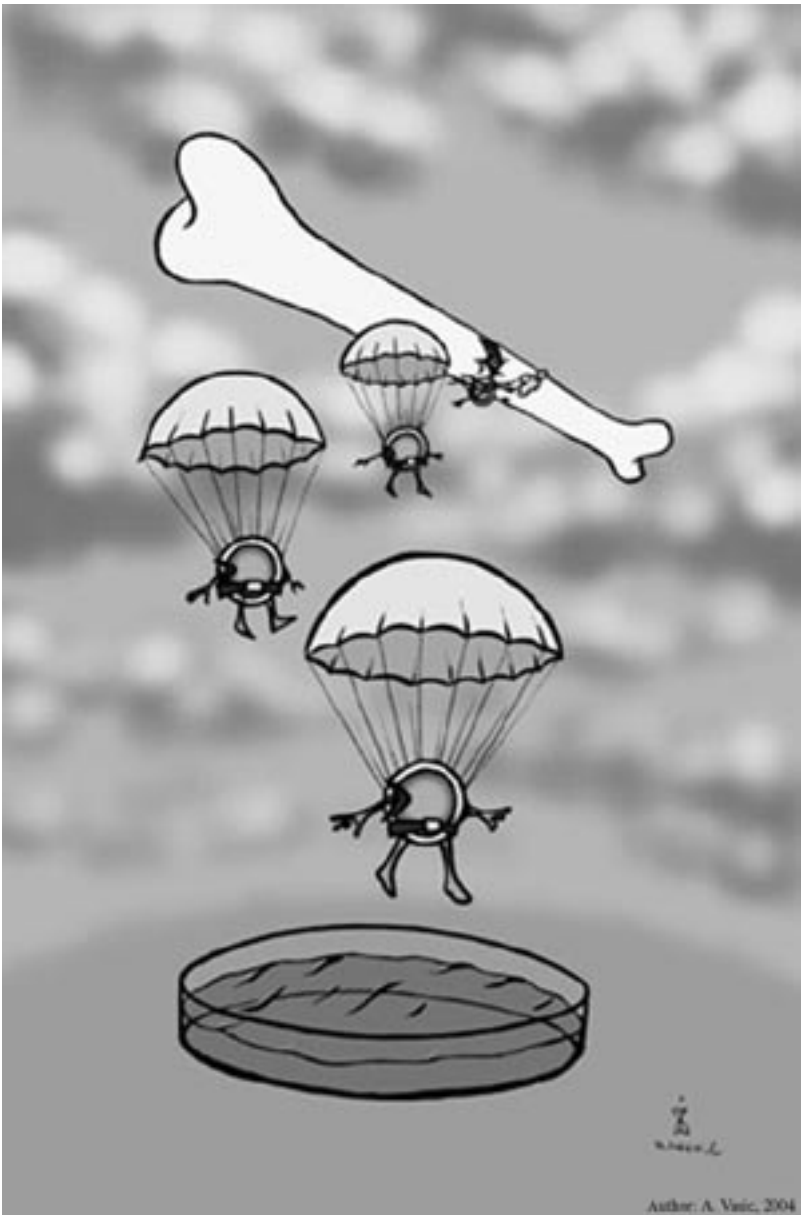
Chapter 3

Dendritic cell progenitors in the bone marrow



3.1

Developmental stages of myeloid dendritic cells in mouse bone marrow

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The lineage relationship of dendritic cells (DC) with other hematopoietic cell types has been studied extensively, resulting in the identification of different bone marrow (BM) progenitors that give rise to distinct DC types. However, the identity of the different maturation stages of DC precursors in the BM remains unclear. In this study we define the *in vivo* developmental steps of the myeloid DC lineage in mouse BM. To this end, BM cells were separated according to their expression of CD31 (ER-MP12), Ly-6C (ER-MP20) and ER-MP58 antigens, and stimulated to developinto myeloid DC, using granulocyte macrophage colony stimulating factor as a specific growth factor. DC developed from three BM subpopulations: ER-MP12^{high}/20^{-} (early blast cells), ER-MP12^{+}/20^{+} (myeloid blasts) and ER-MP12^{-}/20^{high} (monocytes). The kinetic and phenotypic features of DC developing *in vitro* indicate that the three populations represent successive maturation stages of myeloid DC precursors. Within the earliest ER-MP12^{high}/20^{-} population, DC precursors exclusively occurred in the myeloid-restricted ER-MP58^{high} subset. By using switch cultures, we show that these BM precursor subpopulations, when stimulated to develop into macrophages using macrophage colony stimulating factor, retain the ability to develop into myeloid DC until advanced stages of maturation. Together, these findings support a common ER-MP12/20-defined differentiation pathway for both macrophages and myeloid DC throughout their BM development.

Dendritic cells (DC) are bone marrow (BM)-derived cells that have the unique capacity to initiate a primary immune response by efficient antigen presentation to naive T cells. The DC system is widely distributed throughout the body, and comprises Langerhans cells in epithelial locations, veiled cells in lymph, interdigitating cells in lymphoid organs and interstitial DC in connective tissue of non-lymphoid organs [1]. Many studies in the past few years have addressed the lineage relationships of DC with other hematopoietic lineages. Mainly, DC of two possible origins, lymphoid and myeloid, have been identified in human and mouse. Both types have been shown to originate from the BM from which they can be derived *in vitro* by stimulation with different growth factors [2-4]. Myeloid DC have been generated *in vitro* from early common myeloid progenitors in BM and from monocytes in peripheral blood using granulocyte macrophage colony stimulating factor (GM-CSF) as a growth stimulus. In contrast, using Flt-3 ligand (Flt-3L), both myeloid and lymphoid DC can be generated *in vitro* from BM [5-7]. A progenitor population for human lymphoid DC, which also can give rise to lymphoid cells, has been identified in BM and thymus [3]. However, data about this DC lineage in the mouse are inconclusive since the identity of the mature lymphoid DC has been unclear. An earlier study on mouse thymic DC precursors has suggested CD8α as a marker of the lymphoid-related DC lineage [8]. More recent reports, however, show that CD8α is not a reliable marker for lymphoid DC since it can be expressed also on myeloid DC [9] and that CD8α^{+} DC can be derived from myeloid progenitors [10]. A new candidate for the lymphoidrelated DC in the mouse has been proposed [11-13] which shows characteristics equivalent to those of human plasmacytoid DC (PDC).

The developmental plasticity inherent to the DC system has been demonstrated in different studies, showing that both common lymphoid and common myeloid progenitors are able to differentiate into different types of DC [14-16]. The studies by Manz et al. and Mebius et al. indicated that both types of progenitors give rise to both CD8α^{+} and CD8α^{-} DC *in vitro* and *in vivo* [14,15], while Izon et al. showed a previously unrecognized link between early B cell and DC ontogeny [16]. Furthermore, recent experimental evidence suggests the existence of a common DC precursor in the BM [17] as well as in the peripheral blood [18] for CD8a^{+}, CD8α^{-} and presumed PDC.

The studies cited above were mainly directed to define the origins of the different DC types and

to identify their precursors in the BM. However, the identification of the different developmental stages of DC precursors in the BM still remains unclear. In this study, we have investigated in mouse BM the *in vivo* differentiation pathway of myeloid DC progenitors identified by differential ER-MP12/CD31 and ER-MP20/Ly-6C expression. In addition, we addressed the developmental relationship of myeloid DC precursors with macrophage precursors.

Material and Methods

Mice

Female C57BL6/J and C57BL6/Ly-5.1-Pep mice between 11 and 13 weeks of age were used in this study. Female C3HeB/Fej mice were used as a source of allogeneic responder T cells. Animals were specific pathogen free, and kept with free access to food and water in the animal care facility at the Erasmus University Rotterdam under the institutional guidelines for usage of experimental animals.

mAb and conjugates

mAb used for cell sorting, flow cytometric analysis, immunocytochemistry and read out of T cell activation in the mixed leukocyte reaction (MLR) were either generated in ourlaboratory or obtained from BD PharMingen (Alphen aan de Rijn, The Netherlands). These were: biotinylated ER-MP12 (anti-CD31); FITC-ER-MP20 (anti-Ly6C); undiluted hybridoma culture supernatants ER-MP58 (19,20), ER-MP21 (anti-CD71/transferrin R) and M5/114 (anti-MHC class II), obtained in our laboratory; and 53-6.7-FITC (anti-CD8a), RM4-5-PE (anti-CD4) and HL3-phycoerythrin (PE) (anti-CD11c) purchased from BD PharMingen. Biotinylated ER-TR3 (anti-MHC class II) antibody was kindly provided by BMA Biomedicals (Augst, Switzerland). PE-streptavidin, TriColor-streptavidin and PE-goat anti-rat IgG (mouse absorbed) were used as conjugates (Caltag, San Francisco, CA).

Cell suspensions

Single-cell suspensions of BM, isolated from femora and tibiae, were prepared as described previously [20,21]. Similarly, single-cell suspensions of spleen and lymph nodes were prepared and used for T cell isolation as described [22]. Cultured BM-derived DC were isolated from Teflon culture bags or from culture plates. Cells were washed in PBS supplemented with 5% FCS (heat inactivated), 60 mg/ml penicillin and 100 mg/ml streptomycin when sterile suspensions were required. Prior to phenotypic analysis, cultured cells were washed with PBS supplemented with 0.5% (v/v) BSA (Organon Teknika, Boxtel, The Netherlands) and 20 mM sodium azide.

Cell sorting and flow cytometric analysis

For cell sorting, BM cells were labeled with two (ER-MP12 and ER-MP20) or three (ER-MP12, ER-MP20, and ER-MP58) mAb as described previously (20,21). Prior to sorting (FACS Vantage; Becton Dickinson, Amsterdam, The Netherlands), cell suspensions were Æltered over a 30-mm sieve (Polymon PES; Kabel, Amsterdam, The Netherlands) to avoid clogging of the nozzle. After sorting, the purity of the cell suspensions was checked by re-running sorted samples. Purity was >95%, unless stated otherwise. Suspensions were kept at 4°C throughout the staining and sorting procedure. Sorted cells were counted in a Bürker hemocytometer.

For flow cytometric analysis, samples of cultured cells ($\geq 2x10^4$ cells) were aliquoted into 96-microwell plates (round bottom; Nunc, Roskilde, Denmark) and labeled with antibodies as described before [21]. Samples were analyzed on a FACSCalibur flow cytometer using CellQuest analysis software (Becton Dickinson).

Immunocytochemistry on poly-L-lysine-coated slides

For immunocytochemical analysis, aliquots of cultured cells were placed at 37°C, 7% CO_2 in complete medium for 1 h to adhere to poly-L-lysine-coated microwell slides (Nutacon, Schiphol-Oost, The Netherlands). Next, cell preparations were gently washed once with PBS (37°C), fixed in 1% paraformaldehyde in PBS for 10 min at room temperature, and washed with PBS and with PBS supplemented with 0.5% BSA. Cells were incubated with primary mAb, followed by rabbit anti-rat IgG conjugated to horseradish peroxidase (HRP; Jackson ImmunoResearch, West Grove, PA) (both incubations 30 min at room temperature). Diaminobenzidine (DAB; Sigma, St Louis, MO) was used as substrate. Between incubations, cell preparations were washed in PBS supplemented with 0.5% (v/v) Tween 20. After the DAB reaction was completed (3-5 min at room temperature), the cell preparations were dehydrated and coverslipped using Entallan (Merck, Darmstadt, Germany). Antibody reactivity was determined under a light microscope. Culture supernatant of the Y3 myeloma followed by the second stage antibody was used as negative control.

Growth factors

Conditioned medium of LADMAC cells was used as a source of mouse macrophage colony stimulating factor (M-CSF) [23]. Conditioned medium was prepared as described elsewhere (19). Recombinant mouse GM-CSF

(rGM-CSF) was purchased from Biosource International (Camarillo, CA).

rGM-CSF- and M-CSF-stimulated BM cultures

DC were generated by culturing total BM or isolated subsets in Teflon culture bags [24] or 24-well plates (Nunc) in RPMI-1640 (no HEPES; Biowhittaker, Verviers, Belgium) supplemented 516 Myeloid DC development in mouse BM with 10% FCS (heat inactivated; 0.2 mm filtered), 60 mg/ml penicillin, 100 mg/ml streptomycin and 20 ng/ml rGM-CSF. Cells were cultured at 37°C, 7% CO_2 for various periods of time, as indicated, for up to 14 days. Differentiation of isolated BM subsets, to macrophages, was induced by culturing in Teflon culture bags (24) in IMDM (with glutamax I; Gibco, Invitrogen BV, Breda, The Netherlands) supplemented with 20% FCS (heat inactivated; 0.2 mm filtered), 15% LADMACconditioned medium as a source of M-CSF, 100 mM 2-mercaptoethanol, 60 mg/ml penicillin and 100 mg/ml streptomycin. Cells were cultured for 4 or 5 days at 37°C, 7% CO2 as indicated.

MLR

In vitro generated DC (stimulator cells) and T cells (responder cells) isolated from the spleen and lymph nodes were resuspended in RPMI 1640 supplemented with 25 mM HEPES, 10% FCS (heat inactivated; 0.2 mm filtered), 60 mg/ml penicillin, 100 mg/ml streptomycin, 20 mg/l sodium pyruvate and 50 mM 2-mercaptoethanol (further referred to as MLR medium). T cells were resuspended at a concentration of 2 x 10^6/ml and the concentration of DC varied depending on the desired ratio of DC:T cells. The cells (100 ml of each cell suspension) were incubated in round-bottom 96-well plates for 4 days at 37°C, 7% CO_2. DC and T cells, incubated separately in MLR medium, were used as negative controls. Mitogenic stimulation of T cells by concanavalin A (final concentration 1.25 mg/ml) (Sigma) was used as a positive control.

After 4 days of co-culture, cells were harvested and analyzed by flow cytometry. Triple labeling of cells with anti-CD4, anti-CD8 and ER-MP21 antibodies was performed, and 15,000 events within the living gate was acquired. Analysis was performed using CellQuest analysis software. The percentage of ER-MP21 (CD71/transferrin R) positive cells, as a measure of proliferating cells, was determined within the $CD4^+$ and $CD8^+$ population. This method of analysis provides comparable results to those obtained with the [^{3}H]thymidine incorporation method (unpublished results) and in addition allows us to analyze, in more detail, the characteristics of the responding T cell population.

Results

Distinct DC precursors in the BM can be identified by ER-MP12/20 labeling

Based on ER-MP12/CD31 and ER-MP20/Ly-6C expression, we can fractionate BM into six separate, morphologically and phenotypically distinct subsets (Fig. 1) as we showed previously [25,26]. We also demonstrated that in this way the different hematopoietic lineages (lymphoid, erythroid, granulocytic and monocytic) and three different stages of macrophage precursors could be distinguished [21,26]. To examine in which of the cell populations DC precursors were present, BM subsets were sorted and cultured with GM-CSF. At day 10 of culture, the development of DC was evaluated by examining the cultures under an inverted light microscope (Table 1). DC were clearly visible in cultures derived from ER-$MP12^{high}20^-$, ER-$MP12^+20^+$ and ER-$MP12^-20^{high}$ subsets. No DC or other cell types could be grown from the ER-$MP12^{med}20^-$, ER-$MP12^-20^-$ and ER-$MP12^-20^{med}$ subsets by GM-CSF stimulation. The latter BM subsets consisted of morphologically identifiable cells of the lymphoid, erythroid and granulocytic lineage respectively [21,26].

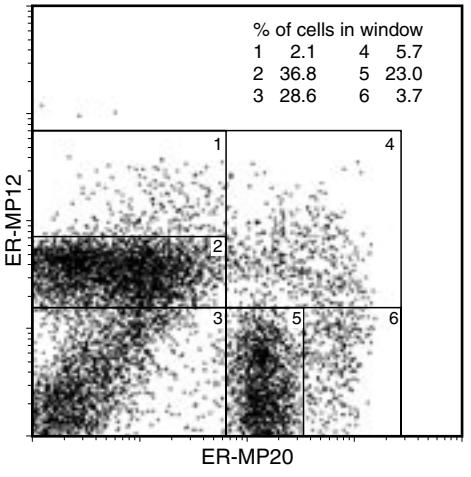

Figure 1. Flow cytometric dot-plot of ER-MP12/20-labeled BM cells. Upon double labeling of BM cells with these mAb, six distinct subsets can be discerned. Gates used for cell sorting are shown. Percentages are the mean of five experiments.

Table 1. DC-like cells generated from ER-MP12/20 bone marrow subsets

BM subset	Overall cell growth[a)]	DC-like cells visible in culture[b)]		% of MHC II^{++} cells with DC morphology (immunocytochemistry)			
		day 6	day 10	day 6		day 10	
				Exp. I	II	I	II
ER-MP12^{hi}20^{-}	+	±	+	6	ND	15	9
ER-MP12^{med}20^{-}	–	–	–	–	–	–	–
ER-MP12^{-}20^{-}	–	–	–	–	–	–	–
ER-MP12^{+}20^{+}	+	+	+	20	43	16	ND
ER-MP12^{-}20^{med}	–	–	–	–	–	–	–
ER-MP12^{-}20^{hi}	+	+	+	23	26	40	33
TBM	+	+	+	19	ND	23	ND

[a]During entire culture.
[b]Results are for Experiments I and II as they were identical.
ND, not enough cells present on the poly-L-lysine-coated slides to determine percentage MHC class II+ cells.

The presence of DC in cultures was also examined by assessing MHC class II expression in immunocytochemistry (Fig. 2). DC derived from different precursor populations showed the same typical DC morphology with MHC class II molecules present preferentially on the cell surface. However, the frequency of MHC class II^{hi} DC at day 6 (Table 1) in the ER-MP12^{high}20^{-} culture was lower than that in the ER-MP12^{+}20^{+} and ER-MP12^{-}20^{high} cultures (an average of 6 versus 33 and 25% respectively), suggesting that precursors from the ER-MP12^{high}20^{-} population needed more time to develop into DC. This was in agreement with the results from visual examination of the cultures. By day 10, the frequency of DC had increased in the cultures derived from the ER-MP12^{high}20^{-} subset (on average 12%), but was still lower than that in the ER-MP12^{+}20^{+} (16%) and ER-MP12^{-}20^{high} (on average 37%) subsets. In conclusion, on the basis of differential ER-MP12 and ER-MP20 expression, we can distinguish three subsets in mouse BM from which DC can be derived with GM-CSF.

DC derived from different ER-MP12/20 populations develop into fully mature cells

Immature and mature DC differ in the level of MHC class II molecule expression on their surface. To determine the maturation state of DC derived with GM-CSF from different BM subpopulations, we double-stained cells at the end of culture with CD11c and anti-MHC class II antibodies. On the gated CD11c^{+} population, we measured the expression of MHC class II molecules (Fig. 3A): 30, 76, 76 and 91% CD11c^{+}MHC class II^{hi} mature DC were present in cultures derived from ER-MP12^{high}20^{-}, ER-MP12^{+}20^{+}, ER-MP12^{-}20^{high} and TBM respectively. In addition, all cultures contained a relatively low percentage of CD11c^{+}MHC class II^{low} immature DC, except for the culture derived from the ER-MP12^{high}20^{-} DC precursors in which a significant amount of DC was still immature (50%). These immature cells responded very well to overnight lipopolysaccharide (LPS) stimulation as this induced a significant increase of the percentage of mature CD11c^{+}MHC class II^{hi} cells (Fig. 3B). All cultures contained also some CD11c^{-}MHC class II^{-} cells (not shown), which are mainly granulocytes also developing *in vitro* with GM-CSF.

To investigate the functionality of DC derived from different precursor subpopulations, we assessed their antigen-presenting capacity by co-culturing GM-CSF derived cells with purified T cells (Fig. 3C). DC derived from all three BM subpopulations were good activators of T cells. The MLR stimulatory capacity was enhanced after LPS stimulation, particularly in the culture derived from the ER-MP12^{high}20^{-} subpopulation when tested at lower DC:T cell ratios.

Three ER-MP12/20-defined subpopulations represent successive maturation stages of DC precursors in the BM

Morphological and functional analysis of DC cultures showed that different BM subpopulations develop with different kinetics into similar DC,

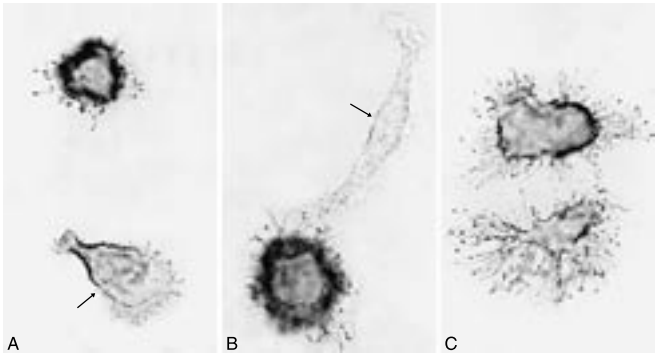

Figure 2. Immunocytochemical MHC class II staining on cells from day 6 GM-CSF-stimulated cultures. DC (identified as MHC class IIhi and typical dendritic morphology) from cultures grown from (A) ER-MP12^{hi}20^{-}, (B) ER-MP12^{+}20^{+} and (C) ER-MP12^{-}20^{hi} BM cells are shown. Mϕs were also present in the cultures (arrow) and differed from DC in morphology and MHC class II expression (lower levels). Original magnification x590.

which suggests that precursors in these populations might be in a different maturation stage at the time of isolation. To investigate this, we sorted the ER-MP12^{high}20^{-}, ER-MP12^{+}20^{+} and ER-MP12^{-}20^{high} subpopulations from the BM, stimulated them with GM-CSF *in vitro*, and analyzed the expression of ER-MP12 and ER-MP20 by cultured cells at various time points (Fig. 4).

At day 2 of culture, cells from the ER-MP12^{high} 20^{-} subpopulation had up-regulated ER-MP20 on their surface and about a half of them were double positive for both markers. By day 5 all cells derived from this population were ER-MP20^{+} while most of them showed no ER-MP12 expression anymore. By day 8, the culture contained ER-MP12/20 double-negative cells and those that expressed medium levels of ER-MP20. The ER-MP12^{+}20^{+} population followed a similar pathway of development. At day 2, most of the cells had down-regulated ER-MP12 expression and were ER-MP12^{-}20^{+}. At day 5 of culture, some cells also down-regulated ER-MP20 and were double negative. By day 8 almost all cells had lost both markers on their surface and were ER-MP12^{-}20^{-}. Finally, the ER-MP12^{-}20^{high} -derived cells also down-regulated ER-MP20 on majority of the cells by day 5 of culture. By day 8 most of the cells in this culture had died (they probably were fully differentiated already before day 8) so no phenotypical analysis could be performed at this time point. Importantly, in all investigated cultures cells successively pass through the ER-MP12^{+}20^{+} and ER-MP12^{-}20^{high} stages and finally become ER-MP12^{-}20^{-}.

In the same cultures we followed the development of DC by using the DC marker CD11c. A common pattern of *in vitro* DC development was observed in cultures from all three fractions: first CD11c expression was induced followed by a prompt down-regulation of ER-MP20 (Fig. 5). CD11c^{+} cells were present already at day 2 in the culture derived from the ER-MP12^{-}20^{high} subpopulation. CD11c^{+} cells were also present at day 2 in cultures derived from the ER-MP12^{+}20^{+} subpopulation, but at much lower frequency. The percentage of CD11c^{+} cells was increased in both cultures at day 5 and continued to rise in cultures derived from the ER-MP12^{+}20^{+} subpopulation to reach 91% at day 8 of culture. In contrast, in cultures derived from the ER-MP12^{high}20^{-} precursors, CD11c^{+} cells were present only from day 5 (10%) and reached 45% at day 8 ofculture, implying that these precursors are indeed the earliest precursors able to differentiate into DC when stimulated with GM-CSF.

The maturation sequence of BM DC precursors, indicated by these phenotypic studies, is consistent with the decreasing proliferative capacity of the cells with increasing maturity. GMCSF-stimulated culture of the distinct subsets in semi-solid medium demonstrated that the ER-MP12^{high}20^{-} subset generated predominantly large colonies (>50 cells), the ER-MP12^{+}20^{+} subset predominantly clusters (<50 cells) and some smaller colonies, while the ER-MP12^{-}20^{high} subset gave rise primarily to small clusters. Liquid culture recoveries were in line with these findings. In a representative experiment, 1x10^{5} cells from the ER-MP12^{high}20^{-} population produced 4x10^{6} cells after 7 days of culture, thus multiplying their starting number 40 times. From

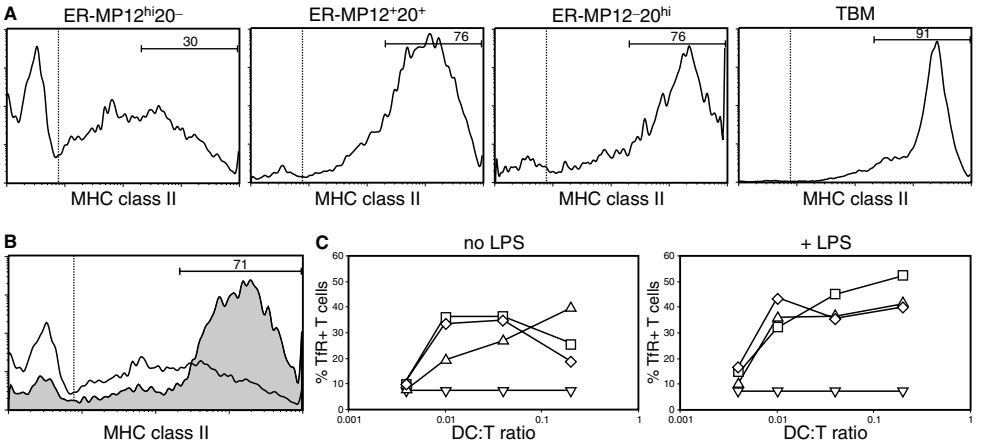

Figure 3. Flow cytometric and functional analysis of DC generated from BM precursors. (A) Cells derived from the total BM and three different precursor populations are double stained with MHC class II and CD11c antibodies. Histograms show the MHC class II expression by CD11c$^+$- gated cells. (B) DC derived from the ER-MP12^{hi}20$^-$ population matured upon 24h LPS stimulation as measured by enhancement of MHC class II expression. The profile indicated by a solid line only represents the expression before LPS stimulation; the filled surface is after 24 h LPS stimulation. The dotted line marks the isotype control and numbers represent the percentage of MHC class II^{hi} cells. (C) Potential of DC, either or not treated with LPS, derived from different BM precursors to stimulate T cells in the MLR. T cell activation is measured by expression of transferrin R (ER-MP21) on their surface.

4x10^5 ER-MP12$^+$20$^+$ cells, 3.6 x 10^6 progeny cells (9-fold) were generated, while 4x10^5 ER-MP12$^-$20^{high} cells gave rise to only 2.8 x 10^5 cells after culture (0.7-fold).

The earliest ER-MP12^{high}20$^-$ DC precursors have the phenotype of myeloid-committed cells

Previously, we showed that the same ER-MP12/20-defined subpopulations in the BM contain macrophage precursors as we found now for DC precursors [21]. In addition, we also showed that within the ER-MP12^{high}20$^-$ subpopulation, early myeloid-committed cells could be purified from the other hematopoietic differentiation capacities on the basis of differentialexpression of a third marker, ER-MP58 [20]. Since we found that DC could also be generated from the ER-MP12^{high}20$^-$ BM subset, we asked whether the earliest DC precursors also have a myeloid-committed phenotype by examining their ER-MP58 expression. For this purpose, ER-MP12^{high}20$^-$ BM cells were fractionated according to their level of ER-MP58 expression (Fig. 6) and cultured in the presence of GM-CSF. Cultures were examined microscopically for the presence of DC at different time points. Starting from day 7 (Table 2), morphologically mature DC could only be detected in cultures of the ER-MP58^{hi} subset within the ER-MP12^{high}20$^-$ population. This onset of DC appearing in culture was similar to what was observed when the total ER-MP12^{high}20$^-$ subset was cultured (see above). No DC-like cells could be grown from the ER-MP58^{med} and ER-MP58$^{-/low}$ subsets under these culture conditions. Together, these data show that, like macrophage precursors, GM-CSF-responsive DC precursors present in the ER-MP12^{high}20$^-$ BM subset are characterized by a high level Fig. 3. Flow cytometric and functional analysis of DC generated from BM precursors. (A) Cells derived from the total BM and three different precursor populations are double stained with MHC class II and CD11c antibodies. Histograms show the MHC class II expression by CD11c$^+$-gated cells. (B) DC derived from the ER-MP12^{high}20$^-$ population matured upon 24 h LPS stimulation as measured by enhancement of MHC class II expression. The profile indicated by a solid line only represents the expression before LPS stimulation; the filled surface is after 24 h LPS stimulation. The dotted line marks the isotype control and numbers represent the percentage of MHC class II^{hi} cells. (C) Potential of DC, either or not treated with LPS,

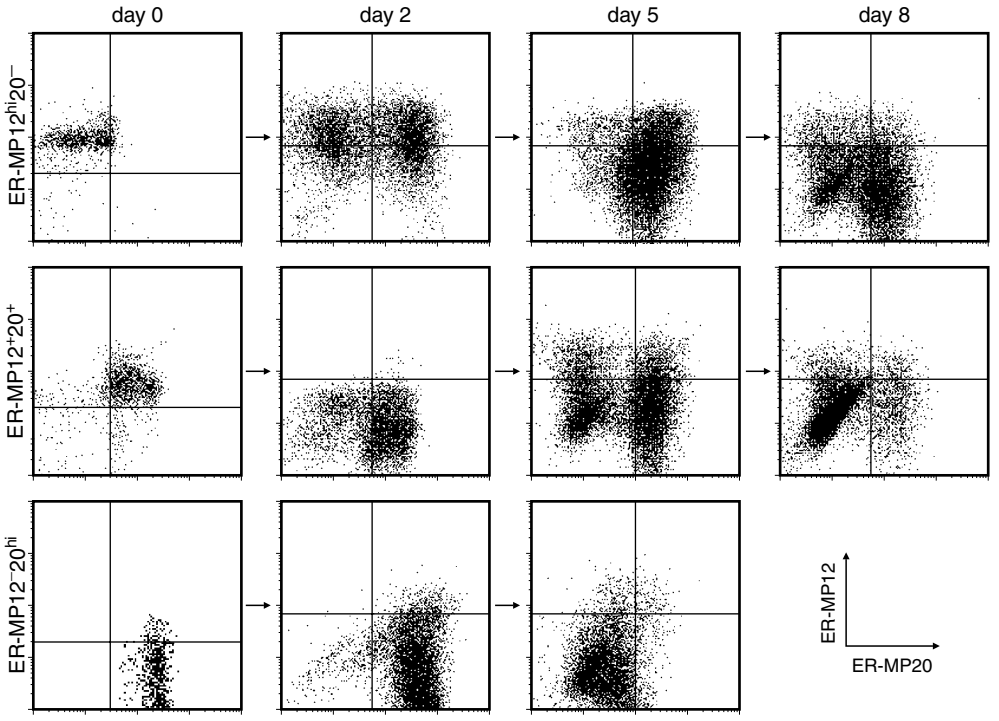

Figure 4. Successive expression of ER-MP12 and ER-MP20 during the *in vitro* culture of BM precursor populations. Sorted populations were cultured with GM-CSF, and at various time points double-labeled with ER-MP12 and ER-MP20. Starting from the earliest precursor population, ER-MP12^{hi}20^{-}, all cells show the same pattern of ER-MP12/20 expression, indicating that they follow the same ER-MP12^{hi}20^{-} →ER-MP12^{+}20^{+} → ER-MP12^{-}20^{hi} developmental pathway.

derived from different BM precursors to stimulate T cells in the MLR. T cell activation is measured by expression of transferrin R (ER-MP21) on their surface. Myeloid DC development in mouse BM of ER-MP58 expression and thus have a myeloid-committed phenotype.

BM progenitors maturing along the macrophage lineage *in vitro* maintain the capacity to generate DC

To approach the question whether macrophages and DC share progenitors throughout their BM development, we cultured BM subsets sequentially with M-CSF ('primary culture') and GM-CSF ('secondary culture'). ER-MP12^{high}20^{-}58^{high} (early myeloid precursors) and ER-MP12^{+}20^{+} (myeloid blasts) were induced first to develop along the macrophage lineage *in vitro* (21). M-CSF was then replaced by GM-CSF and the potential of macrophage precursors to form DC was measured.

After 4 days in M-CSF-stimulated culture of the ER-MP12^{high}20^{-}58^{high} subset, ~90% of the cultured cells expressed the ER-MP20 antigen at a high level (Fig. 7A), representing BM monocytes as previously shown [21]. Changing the growth stimulus to GM-CSF at this point resulted in the appearance of DC only 1 day later as determined by morphological inspection. The more mature ER-MP12^{+}20^{+} subset developed further along the macrophage pathway *in vitro*, since after 5 days of M-CSF-stimulated culture approximately half of the cells still expressed the ER-MP20 antigen, whereas the other cells in the culture had lost their ER-MP20 expression (Fig. 7B). When these cells were further stimulated by GM-CSF, similarly to ER-MP12^{high}20^{-}58^{high}-derived cultures, DC were visible after only 1 day of secondary culture and

in increased numbers after 3 days of culture. Apart from DC, macrophages (ER-MP20$^-$) also developed in cultures from both precursor fractions as indicated by the presence of cells with (i) high forward and perpendicular light scatter (data not shown), indicative of a complex cell type like the mature macrophage with its numerous vacuoles, etc., and (ii) a high autofluorescence level, also characteristic of mature macrophages. After 9 days of GM-CSF-stimulated secondary culture, 28 and 71% DC developed from ER-MP12^{high}20$^-$58^{high} and ER-MP12$^+$20$^+$ derived cultures respectively. These cells expressed MHC class II molecules at a high level and had typical DC morphology, as determined in immunocytochemistry (Fig. 7C and D). Thus, myeloid precursors from the BM developing for 4 or 5 days along the macrophage lineage in M-CSF-stimulated culture still have the potential to generate DC.

Discussion

In the past few years a significant amount of data has been generated about different DC types and their relationship to other hematopoietic lineages.

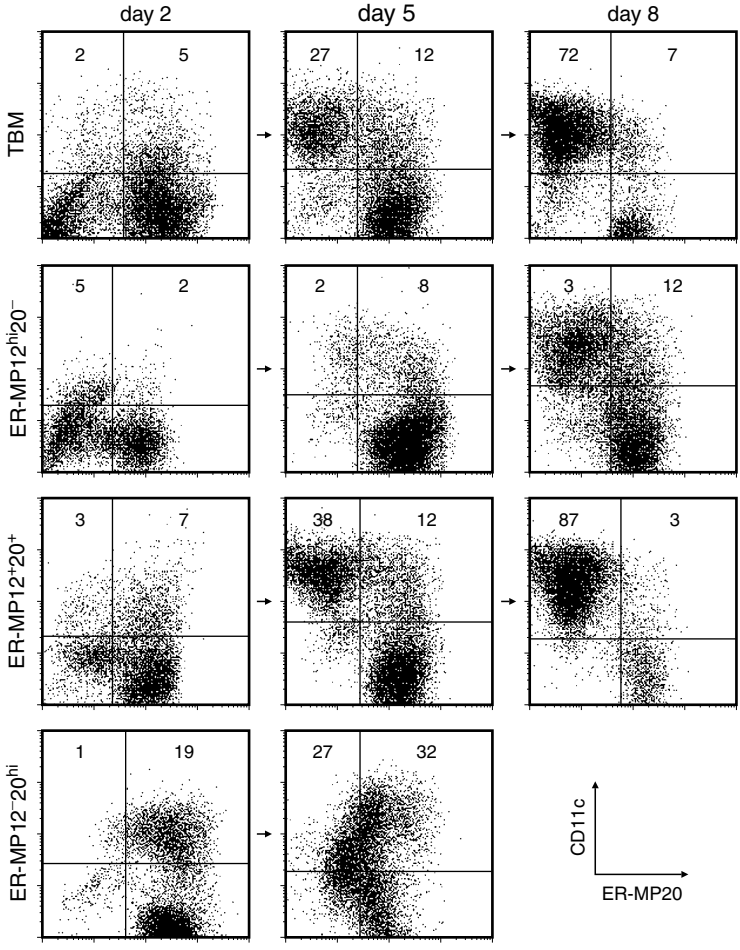

Figure 5. Kinetics of the *in vitro* DC development from different BM precursors. Subpopulations were sorted from the BM and cultured for 8 days in the presence of GM-CSF. At different time points cells were labeled with ER-MP20 and CD11c. The DC development is marked by upregulation of CD11c on the cell surface.

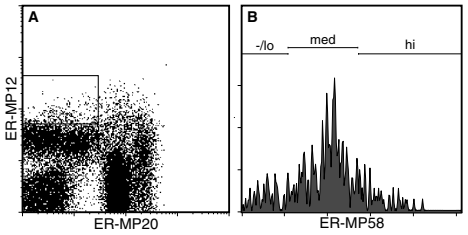

Figure 6. ER-MP58 expression within the ER-MP12^{hi} 20^{-} gate. Cells were triple labeled with ER-MP12, ER-MP20 and ER-MP58. ER-MP12^{hi}20^{-} cells were gated (A) and sorted on the basis of their ER-MP58 expression as indicated (B).

Recently, it has been shown that both common myeloid and common lymphoid precursors can give rise to different DC types both *in vitro* and *in vivo* [14-16]. Mature myeloid and lymphoid DC have been identified phenotypically in human and also in mouse [3,4]. However, the exact maturation pathways of either of the types have remained unclear.

In this study we have examined the different stages of BM DC precursor development *in vivo*, as defined by the expression of ER-MP12/CD31, ER-MP20/Ly-6C and ER-MP58 antigens. DC are derived *in vitro* with GM-CSF only from the ER-MP12^{high}20^{-} (early blasts), ER-MP12^{+}20^{+} (myeloid blasts) and ER-MP12^{-}20^{high} (BM monocytes) [21] subpopulations, while no cell growth could be detected from the other three sorted populations, ER-MP12^{med}20^{-}, ER-MP12^{-}20^{-} and ER-MP12^{-}20^{med}. These populations represent subsets containing morphologically identifiable precursors and mature cells of the other hematopoietic lineages (lymphoid, erythroid and granulocytic respectively). Our results show that GM-CSFresponsive DC precursors reside in the same ER-MP12/20-defined BM subsets as M-CSF-responsive macrophage precursors [19-21].

Table 2. DC generated from ER-MP12^{hi}20^{-} bone marrow subsets

BM subset	Overall cell growth[a)]	DC visible in culture		
		day 5	day 7	day 14
ER-MP12^{hi}20^{-}58^{hi}	+	–	+	+
ER-MP12^{hi}20^{-}58^{med} [b)]	±	–	–	–
ER-MP12^{hi}20^{-}58$^{-/lo}$	–	–	–	–

[a)] During entire culture
[b)] From this subset only macrophagess and immature cells were generated

Earlier reports show that mature DC develop from the BM within 8 days in GM-CSF-stimulated culture [27]. In agreement with this we observe that the majority of DC derived from any of the BM subpopulations express significant amounts of MHC class II molecules, which, together with their good potential to stimulate allogeneic naive T cells *in vitro*, indicates that mature DC develop in these cultures. However, in cultures derived from the ER-MP12^{high}20^{-} cells, immature DC, expressing medium levels of MHC class II, were also present in significant numbers after 7 days of culture. These cells mature upon LPS stimulation, suggesting that they need an extended period of time to develop into mature DC since they develop from early precursors.

This delay in reaching a mature state is in agreement with the observation that during the *in vitro* development, a difference in time of appearance of CD11c^{+} cells exists in cultures grown from the ER-MP12^{high}20^{-} versus the ER-MP12^{+}20^{+} and ER-MP12^{-}20^{high} BM subsets (day 5 versus 2 respectively). This further implies that ER-MP12^{high}20^{-} DC precursors are less mature than the other two subsets, and need more time to develop into DC than ER-MP12^{+}20^{+} and ER-MP12^{-}20^{high} cells. In addition, the percentage of CD11c^{+} DC derived from the GM-CSF-stimulated ER-MP12^{+}20^{+} cells is 2-fold higher than that found with the ER-MP12^{high}20^{-} BM subset as a starting population (90 versus 45%). Probably the higher proliferative capacity of the ER-MP12^{high}20^{-} population contributes to the observed difference in maturation kinetics. Both the size and extent of colony/cluster formation and culture recoveries indicate that ER-MP12^{high}20^{-} cells have the highest potential to proliferate, which then decreases to be the lowest among ER-MP12^{-}20^{high} cells. The high recoveries obtained from the ER-MP12^{high}20^{-} and ER-MP12^{+}20^{+} fractions make it highly unlikely that contaminating populations (<5%) in the sorted fractions account for the observed results. Together, these findings reinforce the notion that ER-MP12^{high}20^{-}, ER-MP12^{+}20^{+} and ER-MP12^{-}20^{high} populations represent different stages of DC development, since they show a reciprocal relationship between the ability to proliferate and the ability to develop into mature DC in a short

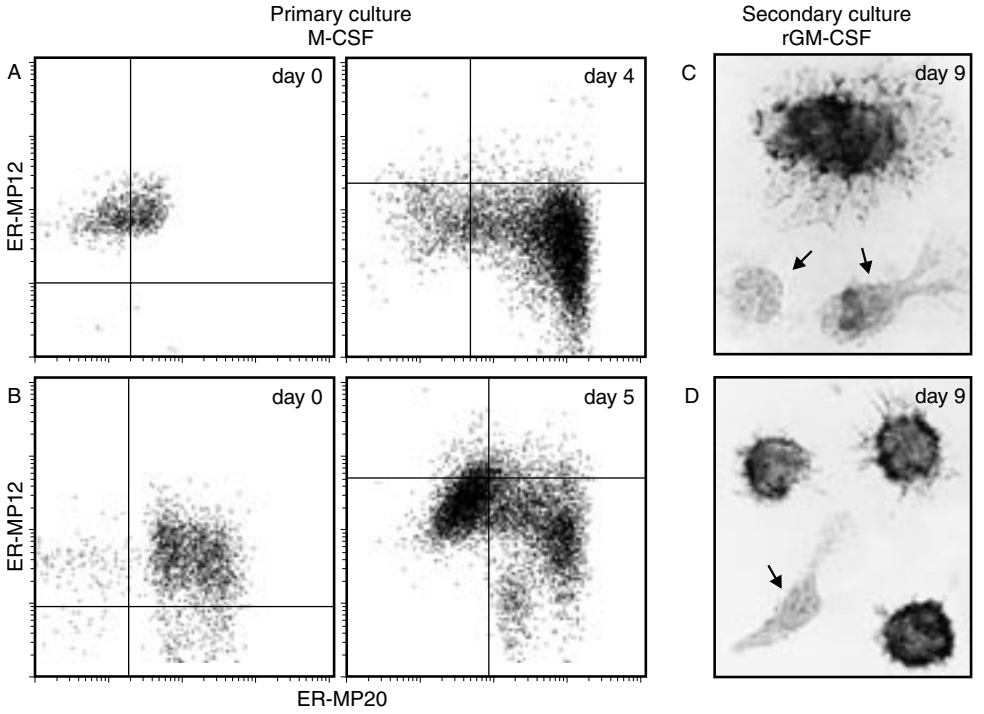

Figure 7. DC development from precursor populations stimulated previously along the macrophage pathway. Bone marrow subsets ER-MP12^{hi}20^{-}58^{hi} (A) and ER-MP12^{+}20^{+} (B) were sorted and cultured for 4 and 5 days respectively in the presence of M-CSF to stimulate macrophage development. At the end of the primary culture, ER-MP12 and ER-MP20 expression was determined in two-color flow cytometry to assess the maturity of the cultured cells. Next, cells were grown for 9 days in secondary rGM-CSF-stimulated cultures. After this period cultures were analyzed for the presence of DC by immunocytochemical staining for MHC class II (C and D). Macrophages were also present in these secondary cultures (arrow). Original magnification (C and D) x670.

period of time.

The successive expression of ER-MP12 and ER-MP20 antigens during the *in vitro* culture with GM-CSF strongly suggests that the three DC precursor populations, which we defined in the BM, differentiate along the pathway with the order ER-MP12^{high}20^{-} → ER-MP12^{+}20^{+} → ER-MP12^{-}20^{high}. Furthermore, this same sequence was previously shown for the macrophage precursors [21] suggesting that GM-CSF-responsive myeloid DC and macrophage precursors follow a similar ER-MP12/20-defined developmental pathway.

The population that contains the earliest DC precursors in the BM, ER-MP12^{high}20^{-}, can be further divided into three subpopulations based on the expression of the early myeloid marker ER-MP58 [20]. Of these three subsets, only ER-MP12^{high}20^{-}58^{high} BM cells are able to differentiate into DC in rGM-CSF-stimulated cultures. Since high level expression of ER-MP58 within the ER-MP12^{high}20^{-} BM population has been shown to mark the earliest myeloid committed precursors, able to give rise to macrophages with M-CSF [20], we conclude that macrophages and myeloid DC most probably originate from the same precursors in the BM. Recently, Akashi et al. have described the existence of a common myeloid progenitor in mouse BM that gives rise to all myeloid lineages [10]. The comparison of its colony forming ability and further characteristics with those of the ER-MP12^{high}20^{-}58^{high} population strongly suggests that the latter represents a similar, if not identical, multipotent myeloid precursor population.

In a recently published paper [7], Gilliet et al. show that the mouse PDC (CD11c^{+}CD11b^{-}B220^{+}) can be generated *in vitro* from the total BM, when

Flt-3L is used as the growth factor. They also show that addition of GM-CSF to the culture medium completely blocks generation of PDC and increases myeloid DC development. Since we used GM-CSF alone, this is in agreement with the observation that in our cultures, no plasmacytoid, but only myeloid DC, were generated as all cells in the culture expressed high levels of CD11b (data not shown). An interesting question arises regarding the potential of our three populations to generate PDC with Flt-3L. For the BM monocyte population (ER-MP12$^-$20^{high}) we regard it unlikely that they may give rise to lymphoid-related PDC, as we have shown previously that BM monocytes are all CD11b$^+$ and thus myeloid cells [21], Further, it has been reported that the CD31$^+$/Ly-6C$^+$ (i.e. our ER-MP12$^+$20$^+$) population contains precursor cells capable of differentiating into different cell types: CD8α$^+$ DC, CD8α$^-$ DC, PDC and macrophages, depending on the experimental conditions [17]. Our current results support the concept that developing myeloid DC, which might eventually express CD8α, and macrophages progress through the same precursor stages in the BM including the CD31$^+$/Ly-6C$^+$ stage. However, as we have shown earlier, the CD31$^+$Ly-6C$^+$ population in BM is phenotypically heterogeneous [21] and might contain cells with different developmental capabilities; clonal assays are essential to prove whether PDC and myeloid DC derive from single precursors. Finally, we define the DC precursor, at the stage prior to the one previously reported, which is capable of differentiating in both myeloid DC and macrophages (CD31^{hi}/Ly-6C$^-$). As mentioned above, since GM-CSF directs cell development specifically to myeloid lineage, it is unclear from our data whether PDC might originate from the same population.

Taken together, in this report, we define different stages of GM-CSF-responsive DC precursors present in the BM and show their *in vivo* developmental pathway. Furthermore, we show that, throughout their BM development, myeloid DC precursors progress through the same stages as macrophages. Even when cells are induced *in vitro* with M-CSF to follow the macrophage developmental pathway, they can still be redirected to develop into DC when M-CSF is replaced with GM-CSF. This is in line with our hypothesis that over a prolonged stretch of development, progenitors of the mononuclear phagocyte system may either develop into macrophages or DC, depending on local conditions. However, additional clonal experiments are required to confirm this hypothesis. Finally, we feel that the ER-MP12/20/58 identification of distinct BM subsets provides a powerful means to study the developmental pathway of lymphoid related PDC as opposed to the presently studied myeloid DC.

Acknowledgements

We thank Begona Grana Suares for her enthusiastic input in the initial phase of this study, and Willem van Ewijk and Paola Ricciardi- Castagnoli for stimulating discussions and support. This study was made possible by research grants from the Netherlands Organization for Scientific Research and the Dutch Diabetes Research Foundation.

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3.2

Myeloid bone marrow precursors of non-obese diabetic mice show reduced proliferation and accelerated maturation into dendritic cells *in vitro*

Dendritic cells and macrophages play an essential role in the pathogenesis of type 1 diabetes in the non-obese diabetic (NOD) mouse. *In vitro* differentiation of DC from the NOD BM demonstrated quantitative and qualitative abnormalities, but whether such defective development correlated to the problems in the myeloid progenitor pool has not been investigated. Using a combination of monoclonal antibodies against CD31 (ER-MP12) and Ly-6C (ER-MP20) molecules, we defined three successive developmental stages of common myeloid DC and Mϕ precursors in the NOD mouse BM and investigated the capability of these three successive precursor populations to develop into DC, when stimulated with GM-CSF *in vitro*. All three populations contained DC precursors in both the NOD and C57BL control mice. However, during GM-CSF-induced maturation, NOD DC precursors did not follow the sequence of CD31 and Ly-6C expression as seen in the C57BL mouse. We found an accelerated maturation of NOD BM precursors, characterized by an almost immediate transition of the earliest precursors into DC accompanied by a virtual absence of proliferation of these early precursors.

This preferred maturation over proliferation of NOD myeloid precursors probably points to an intrinsic defect in the signaling pathways of the cells. If existing, such an intrinsic precursor defect likely also causes differentiation errors of NOD DC from precursors at the local level in the pancreas.

An essential role of dendritic cells (DC) and macrophages (Mϕ) in the pathogenesis of type 1 diabetes has been demonstrated on several occasions in the non-obese diabetic (NOD) mouse, a widely used animal model for organ-specific autoimmune diseases. Blocking leukocyte migration into the pancreas or depletion of APC from the pancreas significantly delays and even prevents diabetes in NOD mice ([1-4] and Chapter 2).

Bone marrow (BM) precursor cells are able to give rise to DC and Mϕ. *In vitro* differentiation of NOD DC from BM precursors demonstrated quantitative and qualitative abnormalities; i.e. a lower yield of cells and a poor T cell stimulatory capacity of the yielded DC when sub-optimally stimulated with GM-CSF alone [5, 6]. Other investigations point to a defective expression of the Ly-6C molecule on NOD BM precursor cells [7]. However, whether such defective expression correlates to the potential of myeloid progenitors to develop into DC has not been investigated.

Using a combination of monoclonal antibodies against Ly-6C and CD31, we previously defined three successive maturation stages of common myeloid DC and Mϕ precursors in the BM of C57BL mice, i.e. the $CD31^{high}$Ly-$6C^{-}$, $CD31^{+}$Ly-$6C^{+}$ and $CD31^{-}$Ly-$6C^{high}$ progenitor populations, respectively, which were all able to give rise to DC and Mϕ [8, 9]. In this study, we used the same methodology and found that also in the NOD mouse, a combination of Ly-6C and CD31 defined the same three successive DC progenitor populations. However, when cultured *in vitro*, in the presence of GM-CSF, BM precursors of the NOD mouse demonstrated an accelerated maturation to DC together with a low expansion of early precursors. Taken together, our findings uncover defects in early myeloid progenitors of the NOD mouse that are unrelated to the Ly-6C expression defect but cause an aberrant maturation course of progeny.

Material and methods

The same experimental methods were used in this study as previously described in Chapter 3.1. [8]

Results and Discussion

Morphology and phenotype of DC progenitors in the NOD mouse BM

The double labeling of fresh isolated BM from NOD mice, with monoclonal antibodies against Ly-6C and CD31, resulted in a different staining pattern as compared to that of the the C57BL mouse (Fig. 1-upper row). In the NOD mouse BM, we could define 5 BM subsets, instead of 6. There was a conspicuous absence of population 5. In the C57BL mice, monocytes and granulocytes reside in populations 5 and 6.

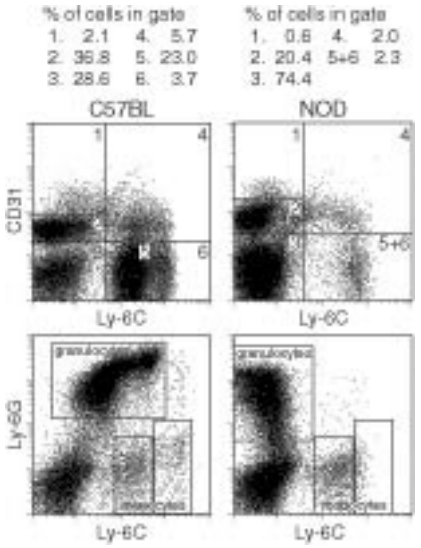

Figure 1. Different CD31/Ly-6C expression pattern in the NOD mouse BM. Analysis of the CD31, Ly-6C and Ly-6G expression by BM cells in the NOD and C57BL mouse. The CD31/Ly-6C double labeling (upper row) showed different distribution of BM cells within six populations previously defined in the C57BL mouse. In addition, the Ly-6G/Ly-6C staining (lower row) demonstrated that the Ly-6C$^+$ cells in the NOD mouse do not express Ly-6G, therefore cells of the granulocyte and not of the monocyte lineage are Ly-6C deficient.

Previously, a recombination in the 5' region of the Ly-6C gene has been found to lead to an impaired expression of the molecule on the BM cells of NOD, NZB and ST mice [10]. Since cells of both the granulocyte and monocyte lineages express Ly-6C in the BM, we investigated the contribution of the two myeloid lineages to the loss of population 5 in the NOD mouse BM. For that purpose, we made use of a recently generated antibody that recognizes the Ly-6G, also a molecule from the Ly-6 family, which distinguishes granulocytes (Ly-6G$^+$) from monocytes (Ly-6G$^-$). The simultaneous labeling with Ly-6C and Ly-6G (Fig. 1-lower row) clearly demonstrated that specifically Ly-6G$^+$ cells (i.e. the granulocytes) were Ly-6C$^-$ in the NOD mouse. In addition we sorted the Ly-6C$^+$ and Ly-6C$^-$ BM populations and analyzed these morphologically. We found that granulocytes in the NOD mouse indeed fail to express the Ly-6C molecule and therefore represent the CD11b$^+$ population within the CD31$^-$Ly-6C$^-$ niche of the NOD mouse BM.

In conclusion, granulocytes and not monocytes of the NOD mouse show the Ly-6C deficiency for. This deficiency is complete and probably stems from the granulocyte-commited progenitors. This also indicated that the Ly-6C marker can be used to distinguish the monocyte-like DC and Mϕ precursor BM populations in the NOD mouse. Indeed the remaining Ly-6C$^+$ positive cells in the NOD BM demonstrated a normal staining pattern and we could define both the CD31$^+$Ly-6C$^+$ and CD31$^-$Ly-6C^{high} populations. However, we repeatedly found between 40-50% reduction in frequency of these two populations (Fig. 1 upper row).

In C57BL mice the Ly-6C$^+$CD31$^+$ subset is a heterogeneous population and contains monoblasts, granulocytic progenitors, plasmacytoid DC and other cells [9, 11]. The lower frequency is probably caused by the absence of granulocytes and PDC, which lack Ly-6C expression in the NOD mouse (Fig. 1 and unpublished observation) and therefore reduce the percent of cells present in this population. The same holds true for the NOD CD31$^-$Ly-6C^{high} population, in which a cell population was absent that has been defined as "ring-monocytes" [12]. These cells were morphologically recognizable in the CD11b$^+$ fraction of the CD31$^-$Ly-6C$^-$ subset in the NOD mouse (not shown). Importantly CD11c$^+$ cells did not developed from the CD31$^-$Ly-6C$^-$ subset, when stimulated with GM-CSF (Fig. 2), therefore ring-monocytes lack a DC differentiation capacity.

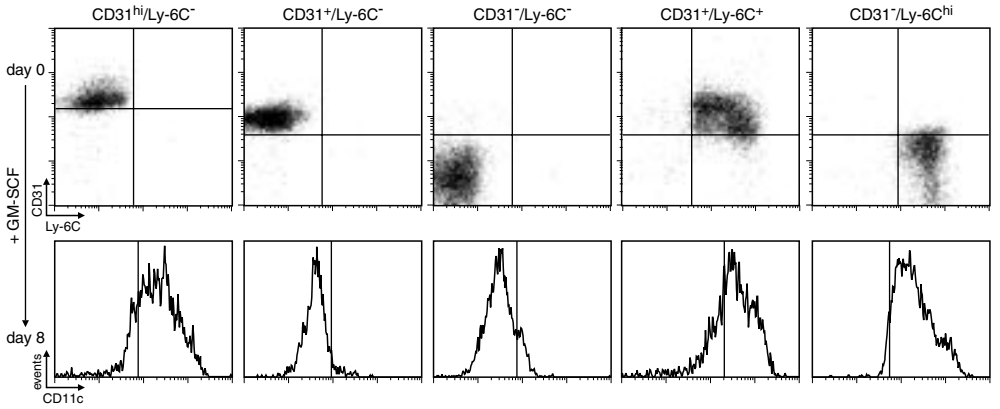

Figure 2. The same populations in the NOD and the C57BL BM contain progenitors with a DC differentiation potential. Sorted BM progenitors from the NOD BM were stimulated with GM-CSF in vitro. After 8 days, generated cells were labeled with the CD11c antibody. CD11c$^+$ cells were present only in the cultures derived from CD31^{hi}Ly-6C$^–$, CD31$^+$Ly-6C$^+$ and CD31$^–$Ly-6C^{hi} populations.

Taken together, phenotypic and morphological analysis of freshly isolated BM and of the sorted subsets of the C57BL and NOD mouse provided information with regard to the expression of the Ly-6C molecule. Cells of the monocyte lineage in the NOD mouse remain capable of expressing the Ly-6C molecule, although with a lower intensity when compared to such cells of the C57BL mouse. The NOD mouse cells of the granulocyte and possibly other lineages were unable to express the Ly-6C molecule. The absence of population 5 in the NOD mouse in Figure 1 is thus most certainly due to an allocation of granulocytes to the Ly-6C$^–$ populations (and other cells unrelated to mononuclear phagocytes) .

DC progenitors reside in the same three BM populations in NOD and control mice

Abnormal expression of the Ly-6C molecule in the BM of the NOD mouse has been related to a defective myelopoiesis [7]. Although cells with a morphology and phenotype of DC precursors were present in NOD in the same three CD31/Ly-6C subsets as in the C57BL mouse, the question whether the modifications in the Ly-6C gene might have influenced the capacity of precursor cells to differentiate into CD11c$^+$ DC was unanswered.

When isolated from the BM and stimulated with GM-CSF *in vitro*, DC developed exclusively from the CD31^{high}Ly-6C$^–$, CD31$^+$Ly-6C$^+$ and CD31$^–$Ly-6C^{high} subsets in the NOD mouse, similarly to what we have already demonstrated for the C57BL mouse BM (Fig. 2.). Hence, the DC differentiation capacity was confined to equivalent progenitor populations in the NOD BM as demonstrated for the C57BL mouse (Chapter 3.1).

An accelerated maturation of DC from NOD BM progenitors.

As we did for the C57BL mouse (Chapter 3.1 - Fig. 4 and 5), we followed the expression pattern of the CD31 and Ly-6C molecules in the NOD BM subsets when stimulated with GM-SCF *in vitro*. The CD31^{high}Ly-6C$^–$ cells sequentially up- and down- regulated the Ly-6C molecule within 8 days of *in vitro* culture in both the NOD and C57BL mouse. However, the full elution of the Ly-6C molecule from the NOD cells occurred faster than in the C57BL mouse (Fig. 3A and 3B). Also, cells of the CD31$^+$Ly-6C$^+$ and CD31^{hi}Ly-6C$^–$ subsets of the NOD mouse were all Ly-6C$^–$ before day 5 of culture while in the C57BL mice some cells remained Ly-6C$^+$ till day 8 and longer (not shown).

The accelerated loss of Ly-6C expression was accompanied by a rapid acquisition of the DC specific marker CD11c on the surface of the NOD cells (Fig. 3B). This rapid change of phenotype happened between days 2 and 5 of culture. At day 5, the majority of the cells of all three fractions had a CD31$^+$CD11c$^+$Ly-6C$^–$ phenotype in the NOD cultures. In addition, acquisition of CD11c

was accompanied by the up regulation of MHC class II (not shown), indicating that the NOD cells rapidly gain DC characteristics. Due to a low cell yield obtained in these experiments, we were not able to test whether these DC were also functionally competent APC. However, our focus in this study was the capacity of the NOD BM cells to enter the DC developmental pathway, which is probably driven by different mechanisms than the final DC maturation. In addition, functional analysis in this setting could bias our conclusions, since we found that GM-CSF alone cannot ensure proper maturation of NOD BM cells into DC *in vitro* (Chapter 3.3),

A further obvious divergence from the previously seen CD31/Ly-6C expression pattern in C57BL was an absence of the CD31 down regulation by DC progenitors in the NOD mouse (Fig. 3A). Even more so, the NOD monocytes isolated as $CD31^{-}Ly\text{-}6C^{high}$ cells, regained CD31 and remained positive throughout the observation period (not shown).

In conclusion, during *in vitro* maturation to DC, NOD BM precursors did not follow the same sequence of acquisition and reduction of Ly-6C, CD11c and CD31 molecules as we previously found for the C57BL mouse. A rapid loss of Ly-6C and acquisition of CD11c and MHC class II show an accelerated maturation/differentiation of NOD BM progenitors to DC at least at the phenotypic level. The importance of the difference in CD31 expression levels in the NOD progenitors cannot be assessed from our current data.

Early DC progenitors in NOD BM fail to expand when stimulated with GM-CSF

Early progenitors change not only their phenotype

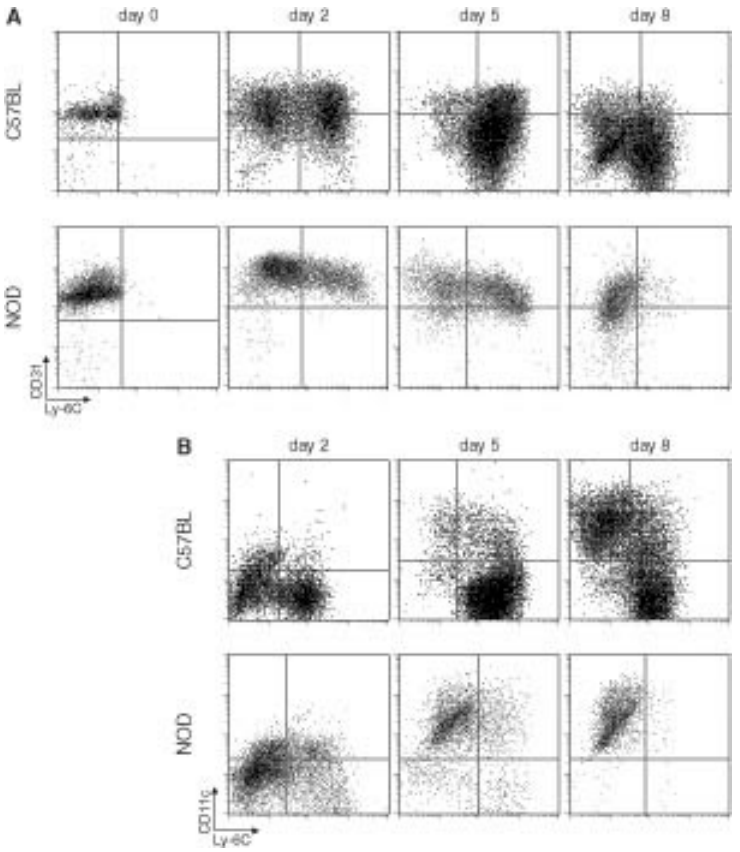

Figure 3.
Different maturation sequence of NOD BM progenitors. Expression of CD31, Ly-6C and CD11c molecules was examined during the cultivation of sorted BM populations, at different time points. A. Cells isolated from the NOD BM lost Ly-6C much faster than the control. In addition, they continued to express CD31 till the end of the culture period. B. Rapid downregulation of Ly-6C happened simultaneously with the rapid expression of CD11c on the surface, indicative of a differentiation to DC.

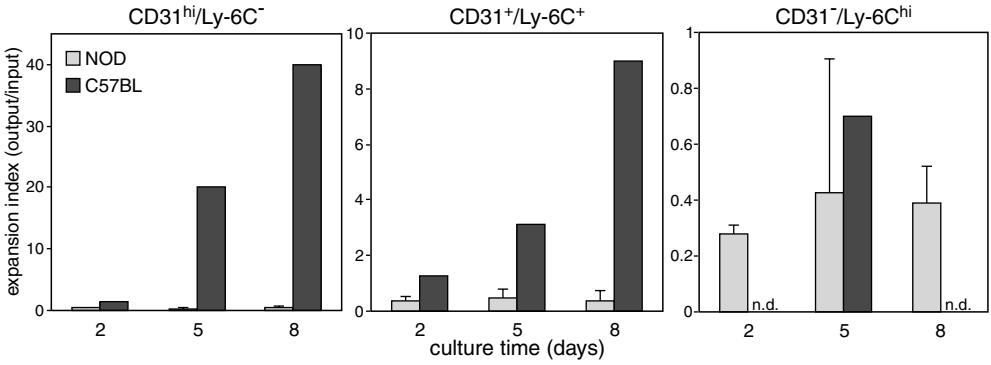

Figure 4. NOD BM progenitors fail to expand when stimulated in vitro. Three BM populations (that contain DC progenitors) were isolated from the BM: the early myeloid progenitors (CD31^{hi}Ly-6C^{-}), the myeloid blasts (CD31^{+}Ly-6C^{+}) and BM monocytes (CD31^{-}Ly-6C^{hi}) populations. Expansion index is calculated as an output/input ratio of cells stimulated with GM-CSF for a given time period. The highest proliferation index was found in the least differentiated population and it gradually decreased with the differentiation in the C57BL cultures (black bars). In contrast, the expansion was virtually absent in anu of the NOD cultures (grey bars).

in the course of maturation to DC but also proliferate (Chapter 3.1). The further the cells are in the differentiation, the less they expand when stimulated. As shown in Figure 4, at the end of the culture period the expansion of the earliest BM progenitors of C57BL mice was 40-fold, of the myeloid progenitors about 10-fold, while the differentiated monocytes did not expand when stimulated with GM-CSF *in vitro*. In contrast, all precursor cells (including the earliest progenitors) obtained from NOD BM had virtually no proliferation capacity GM-CSF (Fig 4).

In the culture of the sorted populations of the NOD BM precursors we did not observe a spontaneous generation of appreciable numbers of macrophages, so it is unlikely that macrophages had influenced the proliferation rate. Therefore, the failure to proliferate when stimulated *in vitro*, probably comes from an intrinsic defect of NOD BM precursors to respond to the cytokine stimulus. A similar expansion defect has been observed when non-separated BM cells of the NOD mouse were stimulated with cytokines other than GM-CSF [7], which points to alterations in the intracellular signaling pathways downstream from the cytokine receptors.

Concluding remarks

Taken together, analysis of the fresh isolated NOD BM cells did not reveal significant differences, with regard to the quantity and phenotypic quality of the DC progenitor pool. However when isolated and stimulated *in vitro*, the precursor cells deviated from the previously established maturation sequence defined in C57BL mice. When stimulated with GM-CSF *in vitro*, NOD BM precursors acquired faster the CD11c^{+} DC phenotype but failed to proliferate.

The focus of this small study was on the GM-SCF induced generation of myeloid DC. We did not investigate the potential of the same cells to generate other cell types, like Mϕ or PDC. It would be interesting to test whether the accelerated maturation without expansion also takes place when the NOD BM precursor cells are stimulated with other growth factors, like M-SCF or Flt3-L.

In conclusion, our observation of the seemingly normal DC progenitor pool in the NOD mouse BM that displays different activities when isolated from the natural surroundings, point to latent defects present in the earliest myeloid progenitors of the BM. They may appear mild in the broader context of all leukocytes *in vivo* but become quite exaggerated *in vitro*. Although latent and seemingly insignificant *in vivo*, these deficiencies might contribute to the faulty outcome

of the immune regulation and predispose the NOD mouse to autoimmunity.

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3.3

Bone marrow precursors of non-obese diabetic mice develop into defective macrophage-like dendritic cells *in vitro*

Tatjana Nikolic, Marjolijn Bunk, Hemmo A. Drexhage, Pieter J. M. Leenen

The non-obese diabetic (NOD) mouse spontaneously develops autoimmune diabetes. Dendritic cells (DC) play a crucial role in the autoimmune response. Previous studies have reported a defective DC generation *in vitro* from the NOD mouse bone marrow (BM), but a deviated development of myeloid precursors into non-DC in response to GM-CSF was not considered. In this study we demonstrate several abnormalities during myeloid differentiation of NOD BM precursors using GM-CSF *in vitro*. 1) We found reduced proliferation and increased cell death in NOD cultures, which explain the previously reported low yield of DC progeny in NOD. Cell yield in NOR cultures was normal. 2) In a detailed analysis GM-CSF-stimulated cultures, we observed in both NOD and NOR mice an increased frequency of macrophages, identified as $CD11c^+/MHCII^-$ cells with typical macrophage morphology, phenotype and acid phosphatase activity. This points to a preferential maturation of BM precursors into macrophages in mice with the NOD background. 3) The few $CD11c^+/MHCII^{high}$ cells that we obtained from NOD and NOR cultures, which resembled prototypic mature DC, appeared to be defective in stimulating allogeneic T cells. These DC had also strong acid phosphatase activity and a raised expression of monocyte/macrophage markers. In conclusion, in this study we describe a deviated development of myeloid BM precursors of NOD and NOR mice into macrophages and macrophage-like DC *in vitro*. Potentially, these anomalies contribute to the dysfunctional regulation of tolerance in NOD mice, yet are insufficient to induce autoimmune diabetes since they occurred partly in NOR mice.

Insulin-dependent diabetes mellitus (IDDM) or type I diabetes develops as a result of an autoimmune destruction of the β-cells in the islets of Langerhans [1]. To investigate the pathogenesis of IDDM, animal models such as the non-obese diabetic (NOD) mouse and the biobreeding (BB-DP) rat have proven to be valuable tools. These animals develop IDDM spontaneously. In NOD mice the sequence of events that occurs during this process closely resembles the disease development in humans [2].

Dendritic cells (DC) and macrophages play a pivotal role in the initiation and progression of the autoimmune response and the final destruction of the β-cells. In the pancreas of neonatal NOD mice elevated numbers of DC and macrophages are present [3]. Furthermore, an accumulation of DC and macrophages, around 4 weeks of age, marks the initiation of the autoimmune insulitis prior to the infiltration of lymphocytes [4, 5]. The final β-cell destruction is thought to occur by the activity of both lymphocytes and macrophages [4, 6]. Together, these findings underline the important roles of DC and macrophages in the different stages of the inflammatory process in the pancreas.

As antigen-presenting cells (APC), DC play a prominent role in both central and peripheral tolerance. Therefore, deficient induction and maintenance of tolerance by aberrant DC would be key to the initiation of autoimmunity [7]. According to such a view, a hampered negative selection by aberrant thymus DC leads to the incomplete deletion of autoreactive T cells. In addition, abnormal DC in the periphery might not stimulate regulatory T cells sufficiently, thus contributing to a failing control of the autodestructive process in the target organ. These notions have led to an extensive investigation of a putative deficient function of DC in type I diabetes. In both humans and BB-DP rats a functional deficiency of DC has been observed. Lower numbers of DC, an immature phenotype and an impaired stimulatory capacity have been found in both human and rat DC [8-10]. In contrast to these studies, *ex vivo* isolated NOD mouse DC from spleen and lymph nodes demonstrated only minor phenotypic and functional aberrations [11, 12].

In marked contrast to the reports on *ex vivo* DC isolated from pre-diabetic NOD mice, *in vitro* studies show significant abnormalities in DC yield and development when NOD DC are

generated from bone marrow (BM) precursors [12-16]. Studies where NOD BM precursors are stimulated with GM-CSF alone show generation of low numbers of DC that display an immature phenotype and a poor T cell stimulatory capacity [13, 14, 17]. However, in the latter reports the authors describe the phenotypic and functional anomalies of the *in vitro* generated NOD myeloid DC, without assessing a possibly deviated development of NOD myeloid precursors into non-DC. Since GM-CSF is an important inflammatory cytokine that influences the function and development of DC but also of other myeloid cell types [18], we decided to investigate the *in vitro* effects of GM-CSF on the generation of NOD BM cells in detail, with emphasis on the heterogeneity of the developing cell types.

Our study shows several anomalies in myeloid DC differentiation and maturation in NOD and NOR (as compared to C57BL and BALB/c) cultures that lead to a strongly decreased generation of prototypic and mature DC. Furthermore, phenotypically mature DC, when separated, appear to have various macrophage characteristics and a reduced T cell stimulatory capacity, despite normal expression levels of MHC and costimulatory molecules. The aberrant maturation of myeloid DC also occurs in Flt3-ligand (Flt3-L)-stimulated BM cultures, which points towards an intrinsic defect in myeloid DC development in mice with the NOD background.

Materials and Methods

Mice

Female NOD/Ltj (prediabetic) and C3Heb/Fej mice between 5 and 10 weeks of age were bred at the animal facility of the ErasmusMC, Rotterdam. Female C57BL, BALB/c female mice were purchased from Harlan (Horst, The Netherlands) and female NOR mice from Jacksons Laboratory (Bar Harbor ME, USA). Female C3HeB/FeJ mice were used as a source of allogeneic responder T cells. Animals were specific pathogen free and kept with free access to food and water in the animal care facility at the Erasmus MC, Rotterdam under the institutional guidelines for usage of experimental animals.

Monoclonal antibodies and conjugates

Monoclonal antibodies were used for cell sorting, flow cytometric analysis and evaluation of T cell activation in the MLR. Undiluted culture supernatants of the hybridomas 2.4G2 (anti- FcRγII/III - CD16/32), ER-MP21 (anti-transferrin R - CD71) as well as unlabeled Ab against the scavenger R type I (SR-AI/II - 2F8; kindly provided by Dr. S. Gordon) were detected by FITC- or R-phycoerythrin-(R-PE)- labeled goat-anti-rat IgG (mouse-absorbed; GaRa-FITC or GaRa-PE) purchased from Caltag Laboratories, San Francisco, CA. Directly labeled 53-6.7^{FITC} (anti-CD8α), RM4-5^{PE} (anti-CD4) and HL3^{PE} (anti-CD11c) were purchased from BD Biosciences (San Diego, CA). Biotinylated ER-TR3 (anti-MHC class II; I-A) was kindly provided by BMA Biomedicals AG (Augst Switzerland) and 10-2.16 (anti-MHC class II for NOD; I-A^{g7}) was produced, purified and biotynilated in our lab. These antibodies were detected by R-PE (Caltag) or allophycocyanin (-APC) (BD Biosciences) –conjugated streptavidin (SAV-PE or SAV-TC, respectively).

rGM-CSF- and Flt3-ligand- stimulated BM cultures

Single cell suspensions of BM, isolated from femora and tibiae, were prepared as described previously [19]. Total BM cells were cultured in RPMI-1640 (without Hepes; Biowhittaker) supplemented with 10% FCS (heat-inactivated; 0.2 mm filtered), 60mg/ml penicillin and 100mg/ml streptomycin (further referred to as culture medium). Growth factors were used according to published protocols for DC generation [20, 21]. In GM-CSF-stimulated cultures a final concentration of 20ng/ml recombinant GM-CSF (Biosource International, Camarillo, CA, USA) was used, while 100ng/ml of recombinant mouse Flt3-ligand (Flt3-L; R&D Systems Europe Ltd, Abingdon, UK) was used in Flt3-L-stimulated cultures. Cells were cultured at 37° C, 7% CO_2 for various periods of time, as indicated, up to 10 days.

Cell suspensions

Cultured BM-derived cells were isolated from culture dishes by vigorous pipeting to collect all cells. Cells were washed and kept until further use in culture medium containing no additional growth factors. When used for phenotypic analysis, cultured cells were washed with PBS supplemented with 0.5% (v/v) BSA (Organon Teknika, Boxtel, The Netherlands) and 20mM sodium azide. Single cell suspensions of spleen and lymph nodes were prepared and used for T cell isolation as described [11].

Cell sorting and flow cytometric analysis

For cell sorting, BM-derived cultured cells were labeled with two (anti-CD11c and anti-MHC class II) monoclonal antibodies as described previously [22]. Prior to sorting (FACS Vantage; Becton Dickinson), cell suspensions

were filtered over a 30mm sieve (Polymon PES, Kabel, Amsterdam, The Netherlands) to avoid clogging of the nozzle. After sorting, the purity of the cell suspensions was checked by re-running sorted samples and purity exceeded 95%. Suspensions were kept at 4°C throughout the staining and sorting procedure. Sorted cells were counted in a Bürker hemocytometer.

For phenotypic analysis by flow cytometry, samples of cultured cells (≥2x10^5 cells) were aliquotted into 96-microwell plates (round bottom, Nunc, Denmark) and labeled with antibodies as described before [22]. For dead cell exclusion labeling with 7-AAD (7-aminoactinomycin D; Molecular Probes Europe BV, Leiden, The Netherlands) was used prior to the measurement [23]. Events (3–5×10^4) were scored using a FACSCalibur flow cytometer and analyzed by CellQuest software (Becton Dickinson, Sunnyvale, CA).

Analysis of the cell cycle and apoptosis

Apoptosis and cell cycle characteristics were determined by using a propidium iodide (PI) staining as previously described [24, 25]. In short, 1x10^6 cells were fixed in ice-cold ethanol for >2h. Subsequently, cells were incubated at room temperature for 30 min in PBS containing 0.02 mg/ml PI, 0.1% v/v Triton X-100, and 0.2 mg/ml RNAse, left overnight at 4°C and analyzed with FACSCalibur. Doublet cells were excluded from the analysis by measuring peak area and width. The number of proliferating cells was determined by gating the region of the DNA histogram that contained cells in G1/G0, S and G2/M phases of the cell cycle. The percentage of apoptotic cells was determined by gating the sub-G1 peak in the same DNA histogram.

Cytochemistry

The acid phosphatase (AP) activity of the various culture-derived populations was determined using cytospins of sorted cells prepared on a Cytospin apparatus (Nordic Immunological Laboratories, The Netherlands). Cytospins were air-dried and used for the AP staining according to Katayama et al. [26], using naphtol AS-BI phosphate as a substrate and a hexazotized pararosanilin as a coupling agent (37ºC, 30 min). Slides were counterstained with hematoxylin. Preparations were mounted in DePex mounting medium (Gurr, BDH Ltd, Poole, UK).

Stained cytospins were quantified by counting a minimum of 300 cells per sample. For each mouse strain, four independent samples per experiment were counted using the following characterization. Acid phosphatase stains the lysosomal compartment [27] and based on the staining intensity and distribution we classified four different staining patterns: negative cells - granulocytes and myeloid precursors; weak cytoplasmic staining - cells with several small positive lysosomes, immature DC [28]; strong cytoplasmic staining - cells with a strong acid phosphatase activity in numerous lysosomes spread throughout the cytoplasm, typical for macrophages [29] and single dot staining - mature DC with a dot-like acid phosphatase active region located in the cell center [28].

Mixed leukocyte reaction assay

The capacity of *in vitro* generated BM-derived cells to activate allogeneic T cells was measured as previously described [19]. Briefly, stimulator cells (cultured DC) and responder T cells, isolated from lymph nodes, were resuspended in RPMI-1640 supplemented with 25mM Hepes, 10% FCS (heat inactivated; 0.2 mm filtered), 60mg/ml penicillin, 100mg/ml streptomycin, 20mg/l sodium pyruvate and 50 mM 2-mercaptoethanol (further referred to as MLR medium). T cells were resuspended at a concentration of 10^6/ml and the concentration of stimulator cells varied depending on the desired stimulator : responder cell ratio. The cells (100µl of each cell suspension) were incubated in round-bottom 96-well plates for 4 days at 37°C in 7% CO_2. Stimulator and responder cells, incubated separately in MLR medium, were used as negative controls. Mitogenic stimulation of T cells by concanavalin A (final concentration 1.25mg/ml) (Sigma Chemical Co., St. Louis, MO, USA) was used as a positive control.

We used induced expression of the transferrin receptor (TfR/CD71) as a measure for T cell proliferation in the MLR since the iron uptake through transferrin by stimulated cells has been shown to be a necessary requirement for their proliferation [30]. Control experiments demonstrated that results obtained by ^{3}H-thymidine incorporation method correlated directly with the results obtained with our method using the CD71/TfR expression detection ([19] and not published). In addition, we compared the kinetics of the CD71/TfR-expression with other activation markers (CD25, CD69) on allogeneic T cells stimulated by *in vitro* generated NOD DC. Expression pattern of CD71/TfR was similar to the expression of CD25 or CD69 and therefore we have chosen to use CD71/TfR expression analysis as a representative read-out of the T cell stimulation in the allogeneic MLR.

After 4 days of co-culture, cells were harvested and analyzed by flow cytometry. Triple labeling of cells with anti-CD4, anti-CD8 and anti-CD71/TfR antibodies was performed and 1.5 x 10^4 events were acquired within the live-cell gate. Data analysis was performed using Cell Quest analysis software. The percentage of CD71/TfR-positive cells was determined

within the $CD4^+$ and $CD8^+$ population.

Statistical analysis

Statistical analyses were done by paired two-tailed Student's *t*-test using the SPSS software package to determine differences within the means of the sample groups. Results are presented as the mean ±SEM, unless otherwise indicated.

Results

A low yield of DC in GM-CSF-stimulated NOD BM cultures is caused by reduced proliferation and increased apoptosis

The capacity of NOD BM precursors to generate DC *in vitro* has been studied previously and several groups reported a low yield of cells in NOD cultures when stimulated with GM-CSF alone [12-14, 17]. Similarly, we found that NOD cultures on average contained a significantly lower number of cells compared to the cultures from both C57BL and BALB/c mice ($p<0.01$). (Fig. 1A). This appeared not to be caused by a decreased frequency of GM-CSF - responsive precursors in NOD BM as determined by limiting dilution analysis (data not shown). Interestingly, the yield of cells in NOR cultures was similar to C57BL and BALB/c mice (Fig. 1A).

To study the events causing the low yield of cells in NOD cultures stimulated with GM-CSF, we followed kinetic changes in the number of proliferating versus apoptotic cells at different time points of the culture (days 2, 5 and 7) by labeling the nuclear DNA content with propidium-iodide (PI). Proliferation appeared to be quite constant during the culture, but the different mouse strains had different proliferation rates (Table 1). Of three mouse strains tested, BM cells from the C57BL mouse showed the highest proliferation rate, significantly higher than the NOD at days 5 and 7 ($p<0.01$). Proliferation rate in the BALB/c culture was on average also higher than in the NOD culture but this difference did not reach statistical significance, except for day 5 of the culture ($p<0.05$). Therefore, increased proliferation partly contributed to the higher cell number in the culture of the control strains.

Using the same DNA staining, we could determine apoptosis in the cultures (cells with the nuclear content <2n) and found the highest percentage of dead cells at the earliest time point (day 2) in all cultures (Table 1). This was due to apoptosis of many BM cells unable to respond and survive when stimulated by GM-CSF (mature granulocytes or cells that belong to the erythroid and lymphoid lineage). At later time points, the number of apoptotic cells decreased in the C57BL and BALB/c cultures, which was not the case in NOD cultures. Therefore, the observed higher cell death in NOD cultures additionally contributed to the lower yield at the end of the culture period. NOR mice were not tested in this assay.

A more sensitive method to quantify dead cells

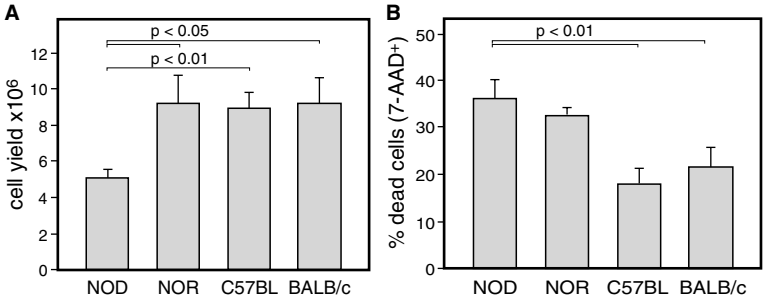

Figure 1. Low cell yield and increased cell death in NOD GM-CSF-stimulated BM cultures. Total BM was stimulated with GM-CSF for 7 days *in vitro*. A. Total yield of viable cells from NOD cultures was significantly lower than from NOR, C57BL or BALB/c cultures. Data represent average values ± SEM derived from 2 (of 10 performed) experiments in which the BM from all four strains was cultured simultaneously. $p<0.01$ NOD vs C57BL and $p<0.05$ NOD vs NOR and BALB/c B. The frequency of dead (7-AAD^+) cells at day 7 of culture in NOD is significantly higher than in C57BL and BALB/c but not higher than in NOR cultures. Average values (± SEM) derived from all 10 experiments are shown in the figure. $p<0.01$ NOD vs C57BL and BALB/c.

Table 1. Proliferation and apoptosis analysis of BM cultures at different time points

day of culture	proliferation[a]			apoptosis[b]		
	NOD	C57BL	BALB/c	NOD	C57BL	BALB/c
2	18.1 ± 3.8	26.5 ± 3.7	19.9 ± 4.5	25.8 ± 7	20.1 ± 4.9	23.1 ± 9.7
5	11.4 ± 0.5	23.2 ± 1.7[c]	19.0 ± 1.4[d]	11.4 ± 3.2	4.7 ± 1.6	10.5 ± 2.1
7	15.0 ± 1	24.6 ± 2.1[c]	19.7 ± 1.9	9.4 ± 2.2	3.1 ± 0.3	6.0 ± 1.9

[a] data represent average frequency (±SEM) of cells with nuclear content >2n
[b]data represent average frequency (±SEM) of cells with nuclear content <2n
[c] $p<0.01$ NOD vs C57BL; [d] $p<0.05$ NOD vs BALB/c

at the end of the culture was the uptake of 7-AAD shortly before the sample acquisition. Using this method we found an even more pronounced difference in the frequency of dead or dying cells in NOD cultures. Significantly higher percentages were found in NOD cultures as compared to both C57BL and BALB/c cultures ($p<0.01$) (Fig. 1.B). NOR mice were also tested and the percentage of dead cells was higher in NOR than in C57BL and BALB/c cultures. Therefore, NOR mice show aberrancy regarding cell apoptosis similar to NOD, yet cell yield is normal and comparable to C57BL and BALB/c mice (Fig. 1).

Diverged differentiation of BM cells to macrophages in NOD and NOR cultures

To investigate the possibility that NOD BM precursors demonstrated a reduced proliferation and an augmented apoptosis due to an aberrantly fast maturation, we analyzed BM cultures at different time points by double labeling for CD11c and MHC class II antigens. From the expression pattern of these two markers three DC developmental stages can be defined in normal BM cultures ([20] and morphological analysis below): granulocytes and myeloid precursors ($CD11c^-/MHCII^-$), immature DC ($CD11c^+/MHCII^{med}$) and mature DC ($CD11c^+/MHCII^{high}$).

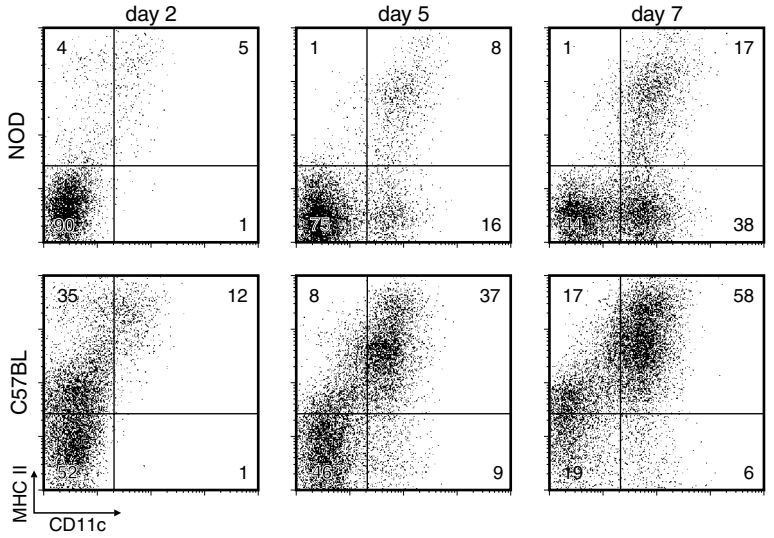

Figure 2. Deviated development of NOD DC in vitro. GM-CSF-stimulated cells from NOD and control (C57BL) were harvested at different time points and labeled with CD11c and MHC class II antibodies. After gating out dead cells ($7\text{-}AAD^+$), at days 2 and 5 of culture, a reduced percentage of $CD11c^+$ cells was present in NOD culture. Upregulation of MHC class II in NOD cultures was also diminished, leading to the development of fewer mature DC ($CD11c^+/MHCII^{hi}$) in culture. In addition, an increased percentage of $CD11c^+/MHCII^-$ cells developed. Numbers indicate the percentage of cells in the quadrants. Dot-plots are representative of five separate experiments with similar results.

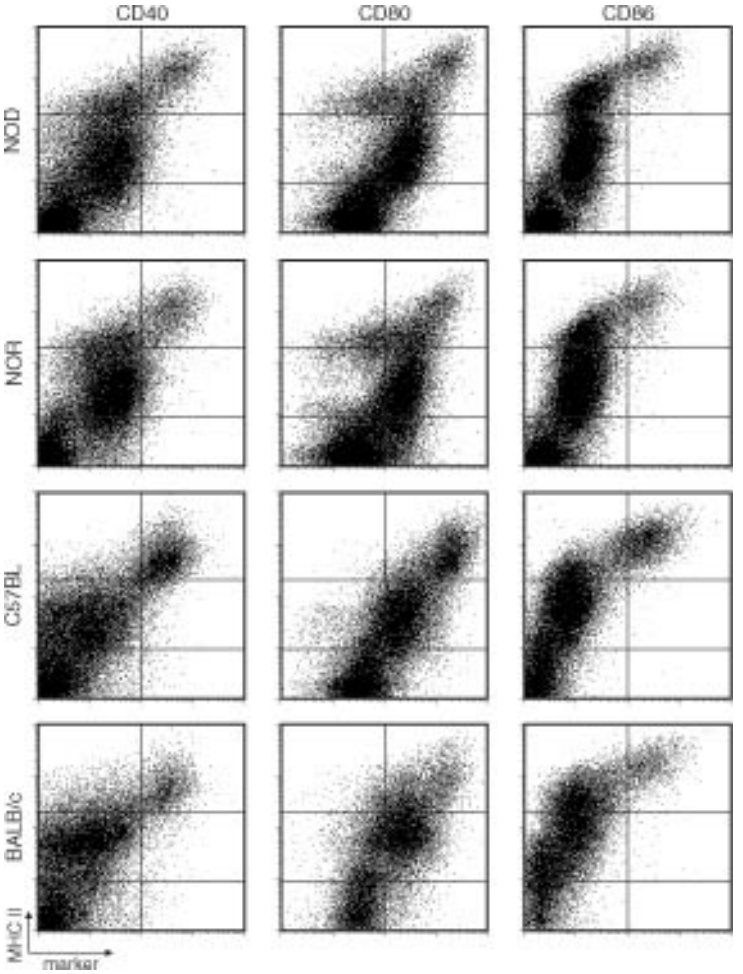

Figure 3.
Few mature DC (CD11c$^+$/MHCII^{hi}) develop in NOD and NOR cultures but they show normal expression of co-stimulatory molecules. NOD, NOR, C57BL and BALB/c cells obtained from 7day-GM-CSF-stimulated BM cultures were double labeled with MHCII and CD80, CD86 or CD40 monoclonal antibodies. Dot-plots show all culture-derived cells, after gating out dead cells (7-AAD$^+$). The figure shows representative dot-plots for each mouse strain from the same experiment. In total 3-7 independent cultures per mouse strain have been performed with similar results.

These three subsets were phenotypically easily discerned in cultures of C57BL mice (Fig. 2) as well as other control mouse strains (data not shown). In addition, a small population of CD11c$^+$/MHCII$^-$ cells was found in all BM cultures. These cells are considered to be monocytes/macrophages (from personal communication with Dr. M. Lutz and see below) and they measured less than 10% of viable cells in cultures of control mice.

In comparison to C57BL, the developing NOD BM cultures showed a strikingly different cellular composition over time. At days 2 and 5 of culture a reduced frequency of CD11c$^+$ cells was found in NOD cultures (6% in NOD vs. 13% in C57BL and 24% in NOD vs. 46% in C57BL, for days 2 and 5, respectively) (Fig. 2). This difference was less pronounced at day 7 of culture (55% vs. 64% CD11c$^+$ cells in NOD and C57BL, respectively). However, in marked contrast to the C57BL cultures, at day 7 the majority of CD11c$^+$ cells in NOD culture were MHC class II$^-$ and a significantly reduced proportion of cells expressed MHC class II molecules (17% in NOD cultures as compared to 58% CD11c$^+$/MHCII$^+$ cells in C57BL). From these data, we concluded that in response to GM-CSF NOD BM precursors do not show a fast maturation to myeloid DC but in contrast, a deviated maturation into CD11c$^+$/MHCII$^-$ cells.

Cells from BM cultures of NOR mice

displayed a CD11c/MHCII staining pattern similar to NOD cultures. At day 7 of culture, NOR cultures contained 27% myeloid precursors ($CD11c^-/MHCII^-$), 22% monocytes/macrophages ($CD11c^+/MHCII^-$), 29% immature DC ($CD11c^+/MHCII^{med}$), and 21% DC with mature phenotype ($CD11c^+/MHCII^{high}$).

Further, we analyzed the expression of the costimulatory molecules CD40, CD80 and CD86 by the different subsets, distinguished on the basis of MHC class II expression (Fig. 3). Expression of costimulatory molecules by cells derived from mice of the NOD background (NOD and NOR) was similar to the control cultures. Mature DC ($CD11c^+/MHCII^{high}$) from NOD and NOR cultures, although present at a reduced frequency, expressed similar levels of CD40, CD80 and CD86 compared to the C57BL and BALB/c. Immature, MHC class II^{med} DC in all mouse strains were $CD80^{low}$, $CD86^{low}$ and $CD40^-$, whereas $MHCII^-$ cells were negative for all three markers showing again that these cells in NOD and NOR cultures were not DC that merely failed to upregulate MHC class II molecules.

Taken together, subset analysis of BM cultures showed that NOD and NOR precursors have a decreased potential to develop into DC ($MHCII^+/CD11c^+$) when stimulated by GM-CSF. Yet, the relatively few immature ($MHCII^{low}/CD11c^+$) and mature ($MHCII^{high}/CD11c^+$) DC that develop in these cultures express normal levels of costimulatory molecules on their cell surface.

Poor stimulatory capacity of DC generated in GM-CSF cultures from NOD and NOR mice

In addition to the different frequency of mature DC, BM cultures showed different strain-specific capacities to stimulate T cells in the allogeneic MLR (Fig. 4). In two independent experiments, NOD as well as NOR DC failed to stimulate significant expression of CD71/TfR on the surface of allogeneic (C3H) T cells upon 4 days of co-culture (Fig. 4A and B). In control experiments, we excluded the low response of T cells due to the responder mouse strain as a possible cause for the low MLR outcome. In addition, poorer MLR capacity of stimulators from NOD and NOR cultures did not originate from the MHC diversity with the controls since DC of the congenic NOD^{H2b} mice with the MHC region derived from C57BL strain showed the same NOD-like low stimulation capacity (data not shown). Taken together, GM-CSF-generated DC from the mice that share the NOD background also share a poor stimulation capacity.

The poor stimulation capacity of NOD and NOR DC could not be overcome by an additional maturation stimulus. Unlike DC from C57BL and BALB/c cultures, NOD and NOR DC failed to increase their T cell activation potential when stimulated overnight with 100ng/mL LPS (Fig. 4C). However, the quality of T cells stimulated by any of the strains was similar with respect to the cytokine production: they produced significant amounts of IFNγ and little IL-4 or IL-10, therefore

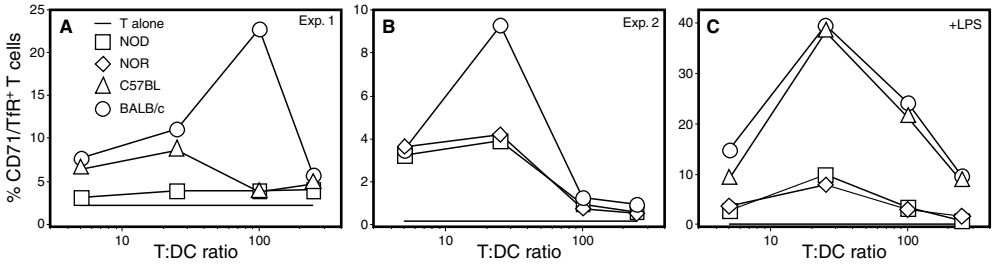

Figure 4. Poor stimulatory capacity of total culture-derived cells generated from NOD and NOR mice. A. and B. Total BM culture-derived cells were co-cultured for 4 days with allogeneic T cells from C3Heb/Fej mice in different APC: T cell ratios. The capacity of total culture-cells from NOD mice is compared with C57BL or BALB/c mice in Exp.1 and with NOR or BALB/c mice in Exp.2. In contrast to the other two mouse strains, NOD and NOR cells exhibited similar poor stimulatory capacity. C. LPS stimulation increased the stimulation potential of BM cells from all four strains. However, stimulated NOD and NOR cells exhibited again a much lower stimulatory capacity than C57BL or BALB/c cells. Data present the percentage of T cells ($CD4^+$ and $CD8^+$) that express CD71/TfR as indication of their productive stimulation by APC. A representative of at least 3 independent MLR cultures for each mouse strain is shown.

were Th1-like. When calculated per cell, all T cells produced similar levels of cytokines irrespective of the origin of DC by which they were stimulated (data not shown).

The very low frequency of mature DC in NOD cultures observed in phenotypic analysis might have been the reason for the poor performance of the whole population in the MLR. Therefore, BM subsets were purified by cell sorting and co-cultured with allogeneic T cells (Fig. 5). Although devoid of potential inhibitory cells and present in the same numbers as in the control, purified mature NOD DC ($CD11c^+/MHCII^{high}$) failed to stimulate T cells to a significant level when compared to C57BL mature DC (Fig. 5E). The percentage of T cells that had upregulated CD71/TfR when stimulated by NOD mature DC (Fig. 5E) was rather comparable to the proportion induced by the immature DC or monocytes/macrophages from C57BL culture (Fig. 5C or D). Other purified subpopulations from NOD cultures also exhibited lower stimulatory capacity than the corresponding C57BL subpopulations.

Morphology of mature DC in NOD cultures is similar to the immature DC from the C57BL

To characterize further the four cell subsets identified in flow cytometric analysis (see before), we investigated the morphology of cells separated by cell sorting from day 7 BM culture on the basis of their differential expression of CD11c and MHC class II molecules (Fig. 6A). As shown in figure 6B, the $CD11c^-/MHCII^-$ (R1) population contained myeloid precursors (slightly indented large nucleus with more basophilic cytoplasm) and neutrophillic granulocytes (typical segmented nucleus) in both NOD and C57BL cultures. The $CD11c^+/MHCII^-$ (R2) fractions, abundantly present only in NOD, contained morphologically distinguishable monocyte/macrophage-like cells in both cultures (asymmetrically positioned oval or kidney-shaped nucleus and a high cytoplasm/nucleus ratio). The $CD11c^+/MHCII^{med}$ (R3) cells derived from C57BL BM resembled immature DC (relatively large cells with an oval nucleus on one cell side and a large cytoplasm, more regular in shape) which formed relatively large clusters. In contrast, cells of the same $CD11c^+/MHCII^{med}$

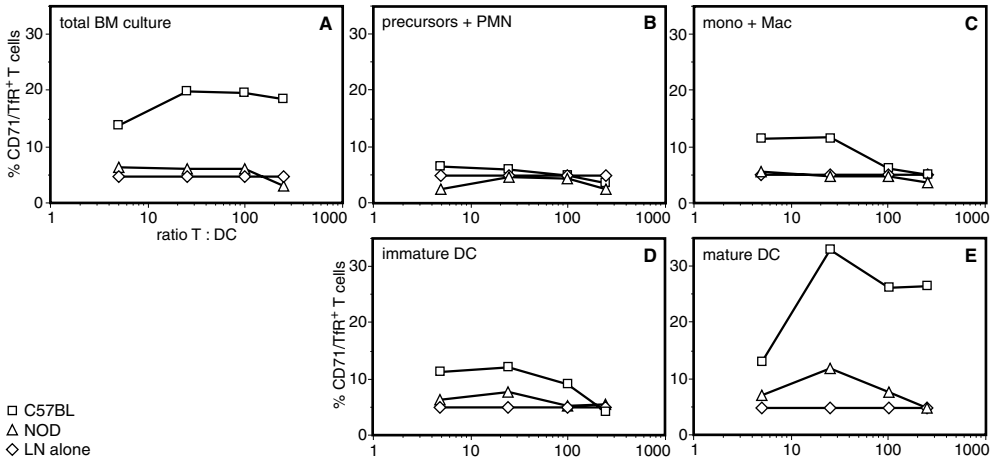

Figure 5. Poor capacity of sorted mature DC from NOD BM cultures to stimulate T-cells. Sorted cells from 7 day cultures were co-cultured with allogeneic T cells at different APC : T cell ratios. Data are presented as percentage of T cells that express CD71/TfR. Unseparated NOD culture-derived cells were virtually unable to stimulate T-cell proliferation, in contrast to their C57BL counterparts. As expected, purified mature DC ($CD11c^+/MHCII^{hi}$) from the C57BL culture displayed the highest stimulating capacity while NOD mature DC were poor stimulators even when purified. Data are a representative of two experiments performed independently, with similar results.

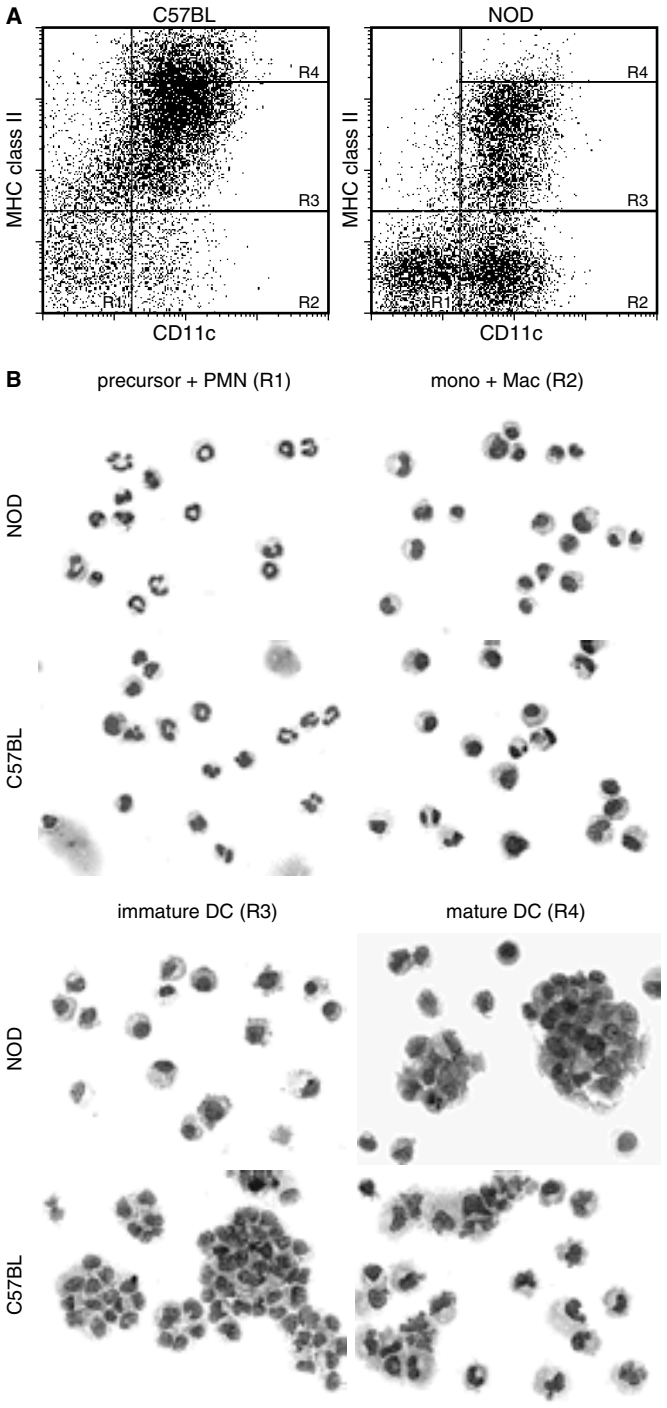

Figure 6.
Morphological analysis of cells in GM-CSF-stimulated culture shows similar subpopulations in NOD and control cultures. A. Four subpopulations of cultured cells can be defined with MHCII and CD11c: (R1) $CD11c^{-}/MHCII^{-}$; (R2) $CD11c^{+}/MHCII^{-}$; (R3) $CD11c^{+}/MHCII^{med}$ and (R4) $CD11c^{+}/MHCII^{hi}$. Represented gates are used for cell sorting. B. Sorted cells are morphologically identified as follows: R1-myeloid precursors and neutrophils; R2- monocytes/macrophages; R3- immature DC and R4- mature DC (more details in Results). Magnification x126.

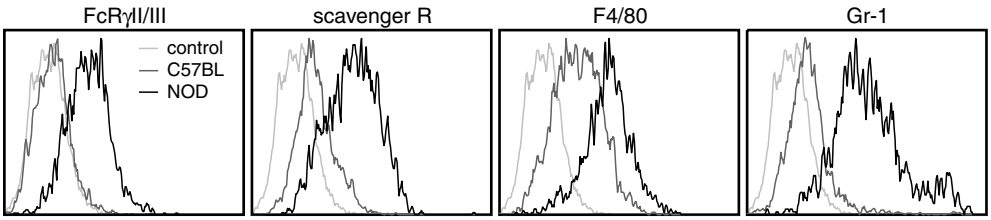

Figure 7. Elevated expression of monocyte/macrophage markers by mature NOD DC (CD11c^{+}/MHCII^{hi}) generated with GM-CSF. Cells were triple labeled with CD11c, MHCII and a marker of interest. The light grey line indicates a histogram of the isotype control; the histogram indicated with the black line represents the marker expression by mature DC from NOD and the dark grey line of C57BL cells. Histograms are derived from a representative staining of at least six independent experiments per mouse strain.

population in NOD cultures hardly formed any clusters. Finally, CD11c^{+}/MHCII^{high} (R4) cells from C57BL cultures had an irregular shape and a number of dendritic protrusions, thus resembling mature DC that mostly occurred as single cells or formed small clusters. The morphology of phenotypically mature DC separated from NOD cultures did not differ significantly from their C57BL counterparts but they formed large clusters comparable to immature C57BL DC.

Mature NOD and NOR myeloid DC express higher levels of markers typical for macrophages

The defective T cell stimulatory capacity and large cluster formation of separated mature DC from NOD BM cultures prompted us to investigate their phenotype further. Since NOD BM precursors seem to develop more readily into monocytes/macrophages (CD11c^{+}/MHCII^{-}) *in vitro*, we focused on macrophage markers in particular. As shown in Figure 7, mature DC (CD11c^{+}/MHCII^{high} cells) from C57BL cultures express very low-to-negative levels of FcRγ II/III, scavenger R, F4/80 and Gr-1. In contrast, much higher levels of these markers were found on mature NOD DC. When quantified from other mouse strains (Table 2), tested markers were higher on mature DC from NOD mice than on both C57BL and BALB/c mature DC. Interestingly, scavenger R and F4/80 were expressed on the NOR mature DC similarly like in the NOD while FcRγ II/III and Gr-1 values were intermediate between the NOD and the other two strains.

Mature DC from NOD cultures contain an abnormally high percentage of macrophage-like cells with a strong lysosomal activity

To assess the putative macrophage-like nature of mature DC generated in NOD cultures, we performed a cytochemical staining of acid phosphatase (AP) (for categorization/scoring see Material and Methods) in sorted cell populations (Fig. 8A). Initial examination revealed that the AP pattern of the sorted populations from the C57BL cultures overlapped to a great extent to their phenotype. This was not the case in sorted cells from NOD cultures. When quantified (Fig. 8B), the R3 population from NOD cultures with the phenotype of immature DC also included a significant percentage of cells with a lysosomal activity typical of macrophages (strong cytoplasmic staining) (49% for NOD vs. 8.3% for C57BL). An even more pronounced difference between NOD and C57BL existed in the sorted CD11c^{+}/MHCII^{hi} population (R4) of phenotypically mature DC. This population isolated from NOD cultures contained few cells with the staining pattern typical of mature DC (single dot staining) (3.3% for NOD vs. 83% for C57BL) and a remarkably high percentage of

Table 2. Intensity of monocyte/macrophage markers expression on CD11c^{+}/MHCII^{hi} DC from different mouse strains

marker	NOD (n=5)[a]	NOR (n=3)	C57BL (n=4)	BALB/c (n=1)
FcRγ II/III	118 ± 53[b]	59 ± 6	38 ± 22	44
scavenger R	162 ± 27	192 ± 6	11 ± 3	33
F4/80	148 ± 20	133 ± 8	28 ± 4	31
Gr-1	108 ± 26	70 ± 5	11 ± 4	7

[a] number of samples derived from a representative staining
[b] data represent an average MFI (± SEM)

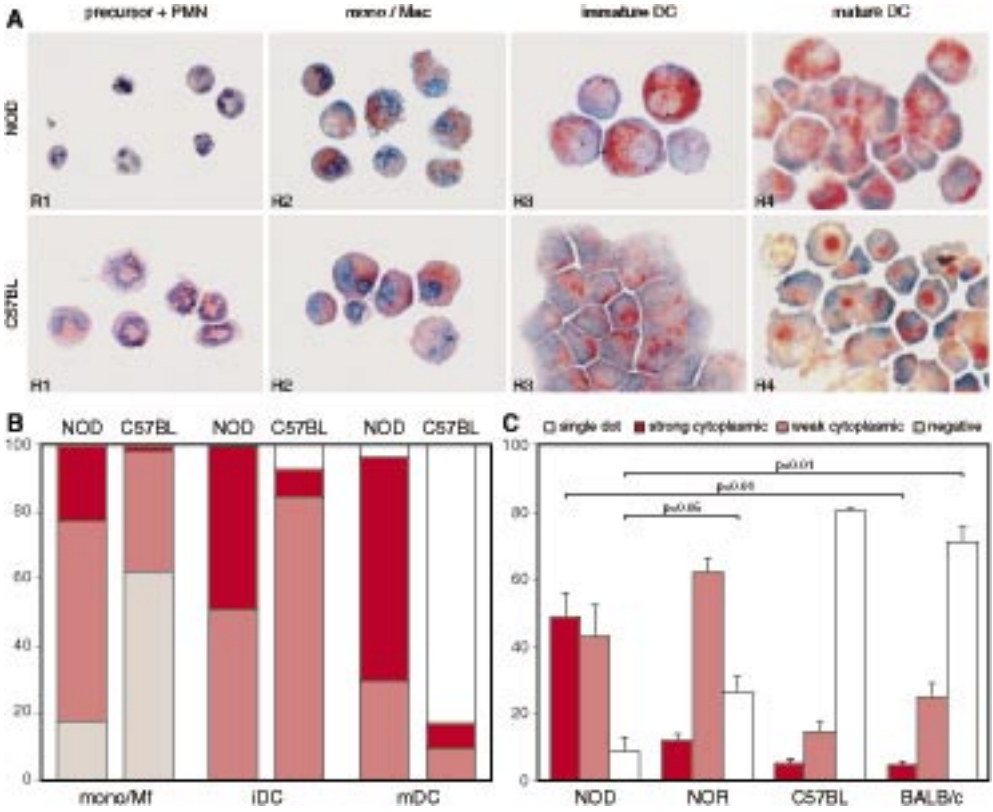

Figure 8. NOD mature DC have strongly increased acid phosphatase activity. A. Cytocentrifuge preparations of sorted cells were stained for AP and then counterstained with hematoxylin (magnification x285). B. Distribution of cells with different staining-pattern (see Material and Methods) in three indicated sorted populations from the NOD and C57BL cultures. Populations with a phenotype of immature and mature DC from NOD cultures contained a high number of cells with a strong AP activity (red area of the bar). C. Composition of the separated mature DC population ($CD11c^+/CD86^+$) from four different mouse strains (NOD, NOR, C57BL and BALB/c) with respect to the three cell types defined by the AP pattern (strong cytoplasmic staining – macrophages; weak cytoplasmic staining– immature DC; single dot pattern – mature DC). NOD and NOR cells contained more immature DC and fewer mature DC as judged by their AP activity. High frequency of cells with a high macrophage-like AP activity were found only in NOD $CD11c^+/CD86^+$ population. Data for A and B are representative of two independent experiments and C shows an average value ± SEM of four independent sortings for each mouse strain.

cells with a staining like macrophages (67.3% for NOD vs. 7.7% for C57BL).

To investigate whether this low percentage of real mature DC and a high percentage of macrophages in the alleged mature DC population is indeed NOD specific, we sorted mature $CD11c^+/CD86^+$ DC from 7 day GM-CSF stimulated cultures from NOD, NOR, C57BL and BALB/c cultures and quantified the AP staining. As shown in Figure 8C, NOD cells contained significantly lower number of cells with a single dot staining of mature DC than NOR, C57BL or BALB/c cells. However, both NOD and NOR contained more immature DC (weak cytoplasmic staining). Importantly, specifically $CD11c^+/CD86^+$ DC from NOD cultures contained significantly more cells with strong cytoplasmic (macrophage-like) AP staining than any other strain tested.

Aberrant myeloid DC development from NOD BM precursors stimulated with Flt3-L

It might be argued that the aberrant response we

observed mirrors the defect of NOD BM cells to respond to GM-CSF, rather than an inherent difficulty to develop into normal DC in culture. Flt3-L stimulates the development of both myeloid and plasmacytoid DC from mouse BM. Therefore, we cultured NOD, C57BL and BALB/c BM with Flt3-L according to the method of Gilliet et al. [21]

Similar to our findings for GM-CSF-stimulated cultures, the yield at day 10 of the Flt3-L-stimulated cultures was lower from NOD precursors compared to controls. In two independent experiments, the total cell yield in NOD cultures was 56% and 43% of the C57BL and 67% of the BALB/c cultures. When analyzed by flowcytometry, the proportion and the phenotype of plasmacytoid $CD11c^+/CD11b^-$ cells did not differ significantly among the strains (Fig.9). In contrast, the NOD cultures contained particularly fewer myeloid $CD11c^+/CD11b^+$ DC (16.2% in NOD vs 32.3% and 28.1% in C57BL and BALB/c, respectively) (Fig. 9A). Additionally, the phenotype of myeloid DC in NOD Flt3-L-stimulated cultures exhibited similar phenotypic abnormalities (Fig. 9B) as in GM-CSF-stimulated cultures. NOD myeloid DC failed to reach a mature phenotype, unlike the control, as they were primarily MHC class II^{low}, $CD80^-$ and $Gr\text{-}1^+$.

Therefore, the deviated development of myeloid DC in both Flt3-L and GM-CSF-stimulated cultures suggests an inherent abnormality in myeloid differentiation, rather than an aberrant response to a specific cytokine.

Discussion

The results presented in our study demonstrate several anomalies in the generation of myeloid DC from BM precursors *in vitro* in NOD and NOR mice. A) A low cell yield from BM precursors was specific for the NOD and not found in NOR mice. B) Both NOD and NOR showed an increased cell death of BM DC precursors, but this was more outspoken in the NOD mouse. C) An abnormally increased frequency of macrophages was found in GM-CSF-stimulated NOD and NOR BM cultures.

In addition, $CD11c^+/MHCII^{high}$ "DC" with an aberrant phenotype and macrophage-like AP activity were generated in NOD cultures. We conclude that myeloid precursors from NOD mice stimulated with GM-CSF predominantly mature into macrophage-like cells. Similar phenotypic abnormalities were evident in NOD myeloid DC generated with Flt3-L, pointing to an intrinsic deficiency of the precursors of mice with an NOD background to develop into real myeloid DC. The fact that only part of the NOD-associated anomalies in the generation of DC were present in the NOR underscores the multigenic complexity of the DC differentiation problems in mice of the NOD background.

Our first finding of the significantly lower yield of DC from NOD BM precursors *in vitro* is in agreement with previous reports [13, 14, 17]. Furthermore, we demonstrated that in NOD/Ltj mice a combined low proliferation and an increased apoptosis of GM-CSF-stimulated BM precursors lead to such observed low cell yield. Our data concur with recently reported findings of a lower proliferation and an increased apoptosis of DC precursors in the congenic NOD $H\text{-}2^k$ mouse [31]. Together, these data point to an intrinsic problem of proliferation and apoptosis of DC precursors under the control of NOD-specific genes independent of the $H\text{-}2^{g7}$ MHC region. NOR mice that share several gene segments with the NOD mouse (including the $H\text{-}2^{g7}$ MHC region) [32] also shared part of the high apoptosis in the BM culture with NOD, but had no problems to generate a normal cell yield.

Follow-up of DC maturation in NOD and NOR cultures revealed additional anomalies: BM derived cells ineffectively differentiated into $CD11c^+/MHCII^{hi}$ prototypic DC and a significant proportion of $CD11c^+$ cells did not express MHC class II and costimulatory molecules. The latter cells were not immature DC incapable to bring MHC class II molecules to the cell surface, as evaluated by immunocytochemistry. Additionally, 24-hour stimulation with LPS failed to induce maturation of these cells into typical mature DC. This, together with the morphology and phenotype of the cells supports the notion that the numerous $CD11c^+/MHCII^-$ cells in NOD and NOR cultures were monocytes and macrophages and not DC. The co-development of macrophages during *in*

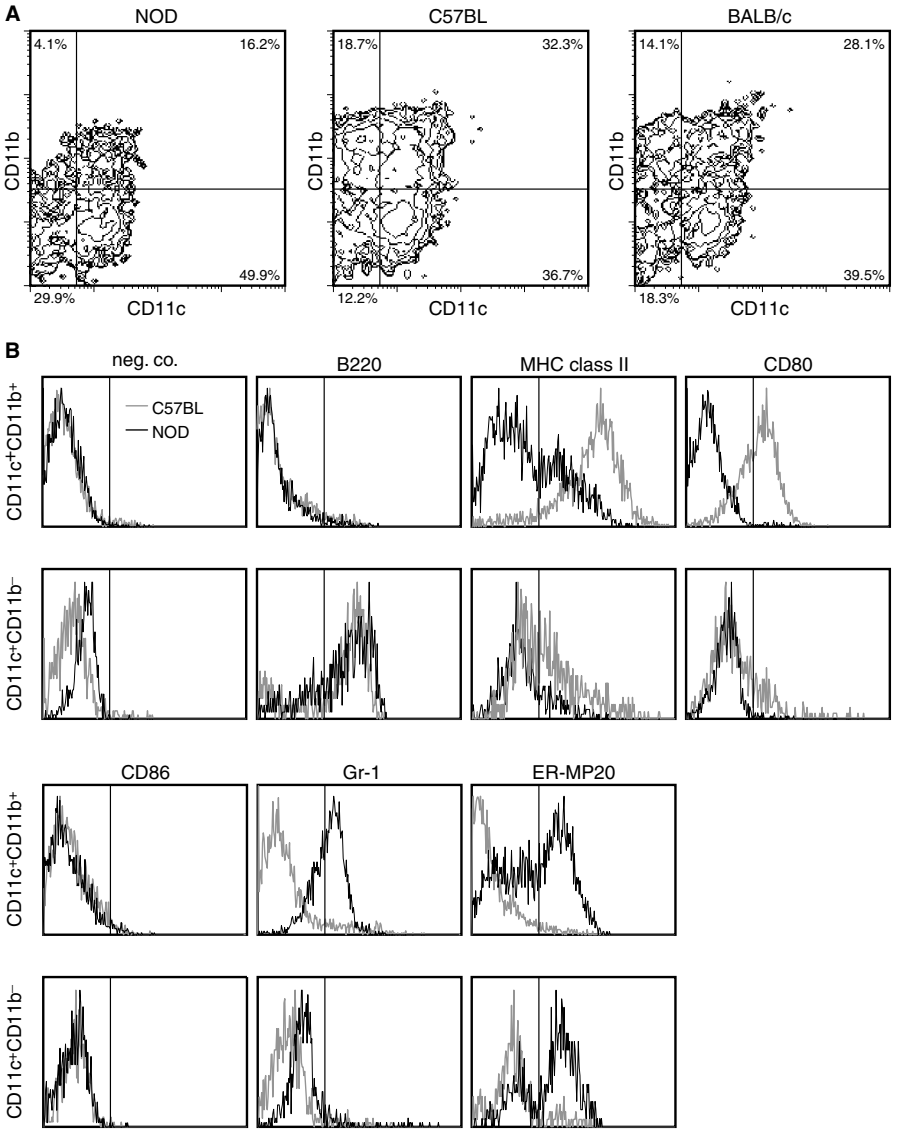

Figure 9. Aberrant generation of NOD myeloid DC in Flt3-L-stimulated cultures. BM cells were stimulated as reported by Gilliet et al.[21] A. Representative dot-plots of CD11c/CD11b staining indicates the development of different DC subsets under the influence of Flt3-L. Numbers express the frequency of plasmacytoid ($CD11c^+/CD11b^-$) and myeloid ($CD11c^+/CD11b^+$) DC in NOD and control (C57BL and BALB/c) cultures. B. Histograms show the phenotype of plasmacytoid and myeloid DC in NOD vs. control. Vertical line indicates the upper threshold of the negative control, the black line indicates the marker expression of gated plasmacytoid or myeloid DC in NOD culture and the grey line indicates the marker expression of the corresponding population in control culture. NOD myeloid DC displayed a similar deviant phenotype as observed in GM-CSF-stimulated culture. Plasmacytoid DC obtained from NOD and C57BL BM precursors had comparable phenotype. Similar data were obtained in two independent experiments.

vitro culture of BM precursors with GM-CSF has been reported previously in normal non-autoimmunity prone mice [33]. Likewise, we also found $CD11c^+/MHCII^-$ monocytes/macrophages in control cultures, but the frequency of these cells was very low in controls and noteworthy in NOD and NOR. Therefore, our data suggest that the decreased frequency of mature DC results from a deviated differentiation of myeloid precursors into macrophages (rather than DC) in BM cultures of mice with the NOD background.

A further abnormality we detected was that $CD11c^+/MHCII^{high}/CD86^+$ DC from NOD cultures that did express costimulatory molecules and approached the phenotype of prototypic mature DC the best, nevertheless had low T cell stimulation capacity, expressed increased levels of monocyte/macrophage markers and had an abnormally high percentage of cells with a strong AP activity. These by phenotype "mature" DC therefore seemed more related to macrophages (hence we called these cells macrophage-like DC). Interestingly, DC from NOR mice shared properties with the NOD DC like a similar low maturation rate and a poor T cell stimulatory capacity and some of the macrophage characteristics, such as the higher expression of scavenger R and of F4/80. However, NOD cultures exclusively contained a high percentage of macrophage-like DC as judged by acid phosphatase.

Our data once more indicate the complexity of the gene-background determinants that establish various deviations in the development of DC from BM precursors in mice with the NOD background. According to Prochazka et al , NOR mice possess C57BL/KsJ-derived genes on chromosomes 2, 4, 11, and 12. The remaining markers on 14 chromosomes, including the diabetogenic H-2g7 complex on chromosome 17, are of NOD origin. Interestingly, NOR mice, although being diabetes-resistant, share with NOD mice the T-lymphocyte accumulation in peripheral organs and a depressed IL-1 secretion by peritoneal macrophages. Yet, unlike NOD, NOR mice have a robust suppressor T-lymphocyte function.

The mechanisms that lead to the aberrant generation of DC in NOD cultures remain elusive. They might originate from intrinsic and genetically determined defects in BM precursors, or they might be induced by the numerous macrophages present and arising in the NOD cultures. Studies have been published that provide circumstantial support for each of the two possibilities [34-40].

With regard to the intrinsic defects in BM DC precursors, a deficient upregulation of the GM-CSF receptor or an abnormal downstream signaling from the receptor are good candidates for the abnormal cell maturation and increased cell death in culture, particularly in view of the defective response of NOD myeloid precursors to GM-CSF and IL-5, which share signaling-chain of the receptor [34]. However, we show that aberrant myeloid DC also develop when using Flt3-L as stimulator, which signals through a different receptor. Hence, we consider aberrations in a common signaling pathway more likely. Several studies so far have shown abnormalities in NF-κB degradation in NOD splenocytes and *in vitro*-derived NOD APC [35, 36]. It would be interesting to investigate whether similar defects can also be found in NOD and NOR DC precursors, thus at an earlier developmental stage.

Alternatively, the numerous macrophages present in the culture might inhibit a normal maturation of DC in the NOD and NOR cultures. Presence of macrophages in a culture is usually considered to be beneficial since they produce TNFα and thereby support spontaneous maturation of DC [37]. However, the abnormal cytokine profile that has been shown for NOD macrophages [38] might make them inhibitory instead of supportive for the growth and maturation of DC in culture. A potential inhibitory cytokine might be IL-6 as it has been shown that IL-6 shifts the differentiation of human monocytes from DC toward macrophages, while TNFα favors maturation into DC [39, 40]. Yet, in the supernatants collected from day 7 BM cultures we could not detect appreciable levels of TNFα, IL-6 or IL-1β and when stimulated with LPS, C57BL and NOD BM-derived cells produced similar levels of these cytokines (data not shown). Therefore, it is less likely that an increased production of inhibitory cytokines in NOD cultures is the decisive factor in the deviated DC generation. However, macrophages in the GM-CSF-stimulated NOD and NOR cultures could still inhibit DC maturation by direct cell-cell interactions or by soluble factors produced at

earlier stages in the culture.

An important difference between our current investigation and previous studies, in which an increased generation or enhanced stimulatory capacity of DC generated from NOD BM have been reported [15, 16], is that we did not use IL-4 in our culture system. A supportive effect of IL-4, added to GM-CSF, for the generation and maturation of NOD BM-derived DC was observed in several studies before [13, 14, 41, 42]. Also in our hands, NOD cultures showed an improved DC yield and stimulatory capacity in MLR when cultured with IL-4 added to GM-CSF (unpublished observation). Normally, IL-4 is not added to GM-CSF-driven DC culture in mice since GM-CSF alone is considered sufficient for the optimal generation of DC from the mouse BM [20]. However, unlike other mouse strains, BM precursors from NOD mice apparently critically depend on the presence of IL-4 for a normal and optimal development of DC. IL-4 is known to inhibit macrophage outgrowth in the culture [43] but it may also function by increasing the cells' sensitivity for DC-maturation factors such as TNF-α [44]. Whatever the mechanism may be, the beneficial effect of IL-4 in NOD cultures points to a subtle intrinsic deficiency in NOD BM precursors to develop into mature DC. This deficiency, magnified *in vitro*, might not be a decisive factor leading to obviously malfunctioning DC *in vivo* but probably contributes to the sensitivity of the DC system in NOD and NOR mice, which may be more prone to imbalance when challenged by external triggers in the NOD and protected by C57BL-inherited genes in the NOR mouse. In such way this subtle abnormality in DC differentiation might contribute to the autoimmune-prone genetics of NOD mice. However, since these anomalies are also largely present in NOR mice they are certainly not sufficient to precipitate autoimmune diabetes on their own.

It is unclear whether the correlate of a macrophage-like DC that we find in NOD BM culture exists *in vivo*. GM-CSF-stimulated culture might be considered a system in which inflammation-related DC are generated, and therefore one could imagine that NOD myeloid precursors could encounter similar problems to undergo normal maturation during inflammation *in vivo*. The immature $CD11c^{+}/MHCII^{low}$ DC produced under such circumstances would be the correlate of our macrophage-like DC *in vitro*. Findings from a recent study support this idea since NOD cells increasingly develop into $CD11c^{+}MHCII^{low}$ DC when reconstituting mixed BM chimeric mice [31]. Further studies on development and function of different DC subsets in the NOD mouse in steady state and inflammation would be necessary to confirm or reject this hypothesis.

In conclusion, in this study we describe an anomaly of myeloid BM precursors in NOD and NOR mice to develop preferentially into macrophages and abnormal macrophage-like DC when stimulated *in vitro*. Potentially, this defect contributes to the dysfunctional regulation of tolerance in NOD mice.

Acknowledgements

We thank Tar van Os for his support in preparation of the figures and Dr. Manfred Lutz for the productive discussion and advices, regarding *in vitro* cultures.

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3.4

A subfraction of B220$^+$ cells in murine bone marrow and spleen does not belong to the B cell lineage but has dendritic cell characteristics

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Although CD45R/B220 is commonly used as a pan-B cell marker in the mouse, not all B220$^+$ cells belong to the B cell lineage. Here we report the characterization of a subpopulation of B220$^+$CD19$^-$ cells in murine bone marrow, which failed to express markers that are present in early CD19$^-$ B cell precursors. Instead, these cells expressed low levels of MHC class II and CD11c, which are typically found on dendritic cells (DC). Moreover, these B220$^+$CD19$^-$CD11c$^+$ cells expressed Gr-1, indicating that they are related to the recently identified murine plasmacytoid DC or their progenitors. Therefore, we evaluated surface marker expression of the B220$^+$CD19$^-$CD11c$^+$ cells in lymphoid tissues of C57BL/6 mice, recombinase activating gene-1 deficient mice, lacking mature B and T lymphocytes, and mice with a targeted disruption of the Ig H chain μ membrane exon (μMT), lacking mature B lymphocytes. When comparing bone marrow and spleen, we found that the surface profiles of B220$^+$CD19$^-$CD11c$^+$ cells were remarkably similar, indicating that they are in a comparable maturation or activation stage in the two lymphoid compartments. In addition, the almost complete absence of peripheral B220$^+$ B$^-$lineage cells in μMT mice allowed the anatomical localization of the B220$^+$CD19$^-$CD11c$^+$ cells to the red pulp and the T cell areas in the spleen. Taken together, our findings indicate that the mouse bone marrow contains a recirculating population of B220$^+$CD19$^-$ CD11c$^+$ plasmacytoid DC, the development of which is largely independent of the presence of mature T and B cells.

In the mouse, B-lineage cells express the high molecular weight form of the common leukocyte antigen CD45, termed B220, on their cell surface [1]. B220 is already present on the earliest identified CD43$^+$ pro-B cells and expression is continued in pre-B, immature B and mature B cells, and is only downregulated at the late plasma cell stage [2, 3]. Although B220 is commonly used as a pan- B cell marker, it is also present on non-B lineage cells in the bone marrow (BM): a small subpopulation of B220$^+$ cells, which also expressed the NK1.1 marker, was shown to contain NK cell progenitors [4]. CD19 provides a more lineage-restricted marker *in vivo*, as it is a direct target gene of the transcription factor pax-5, which is critical for commitment to the B cell lineage [5]. Nevertheless, a subpopulation of CD19$^+$ cells can give rise to dendritic cells (DC) in culture [6].

The nature of the earliest B cell precursor populations in the mouse is not well defined and different, partially conflicting, models of early B lymphopoiesis have been devised [2, 3, 7]. In recent years, research has focused on the characterization of specific subpopulations of B220$^+$CD19$^-$ cells in the BM. In the model by Hardy et al. [2, 8], fraction A cells (CD43$^+$B220$^+$CD19$^-$HSA$^-$) are subdivided and only the fraction expressing the AA4.1 surface molecule was shown to include B lineage precursors, as identified by expression of Ig H chain m_o germline transcripts and the transcription factor pax-5. Tudor et al. [7] have identified a subfraction of DX5$^-$Ly-6C$^-$ primitive B220$^+$CD19$^-$ B cell precursors, which does not appear to overlap with the AA4.1$^+$ subpopulation of Hardy's fraction A cells.

Rolink et al. [4] have distinguished three subpopulations of CD43$^+$B220$^+$CD19$^-$ cells, including (1) NK1.1$^-$CD4$^-$ B cell precursors, (2) NK1.1$^+$ NK cell precursors, which are probably contained within the AA4.1$^-$ fraction A cells [8] and (3) NK1.1$^-$CD4$^+$ cells, which were not further studied. Potentially, these latter cells might represent DC, since recently B220$^+$ DC populations have been described that can be propagated from mouse liver [9] or are present in lymph node and spleen [10]. The latter population of B220$^+$ cells was shown to contain a significant proportion of CD4$^+$ cells and displayed characteristics of plasmacytoid

DC [10]. These cells have the capacity to stimulate T cell proliferation after activation with CpG and are major producers of IFNα after viral stimulation [11, 12]. They differ from most DC types as they are thought to enter lymph nodes

directly from the blood by transmigrating high endothelial venules. We hypothesized that if these plasmacytoid DC are also present in the BM, they may be contained within the NK1.1$^-$CD4$^+$ fraction of CD43$^+$B220$^+$CD19$^-$ cells.

In this report we have addressed this issue by phenotypical characterization of the NK1.1$^-$ subpopulation of CD43$^+$B220$^+$CD19$^-$ cells in the BM. These cells express surface markers that are typically present on DC, such as MHC class II and CD11c, albeit at low levels. We conclude that this cell population is similar to the CD11c$^+$B220$^+$Gr-1$^+$ plasmacytoid DC that were previously identified in lymph nodes and spleen [10]. Therefore, the B220$^+$CD19$^-$ CD11c$^+$ cells in the BM do not belong to the B cell lineage, but reflect a recirculating population of this specific subset of DC.

Materials and Methods

Mice

RAG-1$^{-/-}$ [13] and μMT [14] mice, which were on a 129/Sv and C57BL/6 background, respectively, were purchased from the Jackson Laboratory, Bar Harbor, ME. All mice were bred and maintained in the animal care facility at the Erasmus University Rotterdam.

Flow cytometric analysis

Preparations of single-cell suspensions and flow cytometry have been described previously [21, 22]. Events (1-3x10^5) were scored using a FACSCalibur flow cytometer and analyzed by CellQuest software (Becton Dickinson, Sunnyvale, CA). Low side scatter cells were gated to exclude contaminating Mϕ. The following mAb were obtained from Pharmingen (San Diego, CA): FITCconjugated anti-BP-1/6C3, anti-B220/RA3-6B2, anti- CD11c/HL3, anti-CD86, anti-κ/R5-240 and anti-IgM (II/41); PE-conjugated anti-CD11c/HL3, anti-CD19, anti-CD25, anti-CD43, NK1.1, anti-MHCII (M5/114), anti-CD69 and anti-CD80; Cy-Chrome-conjugated anti-B220/RA3-6B2; biotinylated anti-IgM (II/41), anti-CD19, anti-CD24/HSA (M1/69) and APC-conjugated anti-CD4. PE-conjugated IgD was obtained from SBA (Birmingham, AL). FITC-conjugated DEC205 monoclonal antibody was purchased from RDI (Flanders, NJ). The hybridomas PB493/AA4 [23, 24] and SLC/LM34 [17] were kindly provided by A. Rolink,(Basel Institute for Immunology, Switzerland), while F4/80 was provided by S. Gordon (University of Oxford, GB). PB493/AA4, ER-BMDM1, and 33D1 were used as purified hybridoma supernatants. F4/80 and SLC/LM34 were biotinylated according to standard procedures. Secondary antibodies were PE, Tri-Color or APC-conjugated streptavidin and FITC-conjugated goat anti-rat, purchased from Caltag Laboratories (Burlingame, CA). Intracellular flow cytometric detection of cytoplasmic Ig H or L chain (FITC-conjugated anti-Igκ or anti-IgM) was performed on cells that were first stained for cell surface markers and subsequently fixed in 2% paraformaldehyde and permeabilized using 0.5% saponin, as previously described [22].

Immunohistochemistry

Tissue samples were embedded in OCT compound and frozen 6-μm cryostate sections were acetone fixed and double labelings were performed as previously described [25]. The mAb antiB220/RA3-6B2 [1], anti-CD11c/N418, and MOMA-1 [26] were applied as purified hybridoma culture supernatants. Biotinylated anti-CD4 was purchased from BD Pharmingen.

Results and Discussion

The identification of B220$^+$CD19$^-$CD11c$^+$ cells in BM

To investigate B220$^+$CD19$^-$ cells in detail, four-color flow cytometry experiments were performed on lymphoid tissues from C57BL/6 mice, recombinase activating gene-1 deficient (RAG-1$^{-/-}$) mice [13] and mice with a targeted disruption of the Ig H chain m membrane exon (μMT mice[14]). The RAG-1$^{-/-}$ and μMT mice were included to facilitate the analysis of non-B cell lineage B220$^+$ cells, as in these mice B-lineage cells are virtually absent in the peripheral lymphoid organs and limited to a small fraction of B220$^+$CD19$^+$ pro-B cells in the BM.

The B220-CD19 flow cytometry profile of BM cell suspensions from normal, RAG-1$^{-/-}$ and μMT mice revealed a population of CD19$^-$ cells in which the expression level of B220 was heterogeneous (Fig. 1). As early B cell progenitors were shown to express relatively low levels of B220 [15], we hypothesised that the putative non-B cell lineage cells would be present in the B220$^+$CD19$^-$ subfraction in which the expression level of B220 was higher than the level in B220^{low}CD19$^+$ pro-B cells. In all three mouse strains, ~3–5% of the BM cells with low side scatter characteristics, typical for lymphocytes or DC, were B220$^+$CD19$^-$.

On the basis of the expression profile of CD11c and NK1.1, the B220$^+$CD19$^-$ cell fraction was

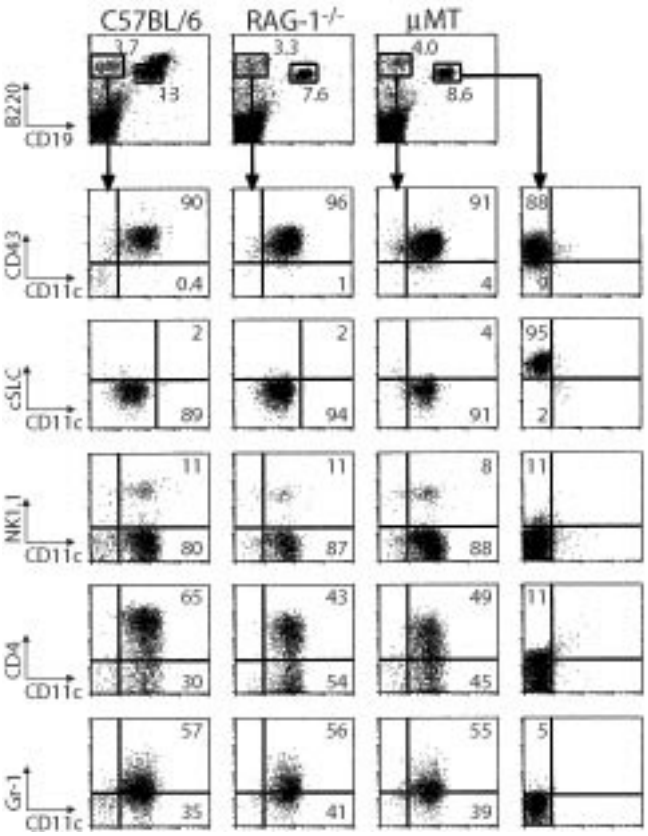

Figure 1.
Identification of CD11c$^+$ B220$^+$ CD19$^-$ cells in BM. Flow cytometric analyses of BM of wild-type, RAG-1$^{-/-}$ and μMT mice. The immunofluorescent profiles of B220$^+$CD19$^-$ cells are displayed as dot plots. Subsequently, the indicated gated B220$^+$CD19$^-$ cells were analyzed for the expression of CD11c, in combination with CD43, cSLC, NK1.1, CD4, or Gr-1 as indicated. A parallel analysis of these surface markers on B220$^+$CD19$^+$ cells from μMT BM is shown for comparison. Percentages within the indicated gates or quadrants are given. Data shown are representative for four to eight mice examined within each group.

divided into three separate subpopulations (Fig. 1). The small population of NK1.1$^+$ cells, which most likely reflected the previously characterized B220$^+$CD19$^-$NK1.1$^+$ population of NK cell precursors [4], was found to be CD11c$^+$. This is consistent with the finding that also mature NK cells in the spleen express low levels of CD11c (data not shown). Another small population consisted of NK1.1$^-$CD11c$^-$ cells. The largest population, containing 80–90% of the B220$^+$CD19$^-$ cells, consisted of NK1.1$^-$CD11c$^+$ cells. The CD11c molecule is the a_x chain that pairs with β2/CD18 to form the gp150,95 integrin, also known as CR4 and is present on the cell surface of DC [16]. We therefore hypothesized that the CD11c$^+$ B220$^+$CD19$^-$ cells may represent a unique DC subpopulation, probably related to the CD11c$^+$B220$^+$Gr-1$^+$ cells with characteristics of plasmacytoid DC in lymph nodes and spleen [10].

CD11c$^+$B220$^+$CD19$^-$ cells in the BM do not belong to the B cell lineage

As it has been demonstrated that early B cell precursors are positive for CD43, AA4.1 and cytoplasmic surrogate light chain and may express low levels of surface CD4 [2, 4, 7, 17], we analyzed the expression of these markers in combination with CD11c (Fig. 1). Like CD19$^+$ B cell precursors, the B220$^+$CD19$^-$CD11c$^+$ cells were CD43$^+$. However, in contrast with the CD19$^+$ B cell precursors, they did not express surrogate light chain in their cytoplasm and were surface AA4.1– (Fig. 1 and not shown). The expression of CD4 was heterogeneous (Fig. 1). In additional four-color analyses, the CD11c$^+$B220$^+$ CD19$^-$ cells in C57BL/6 mice were negative for surface IgM, intracellular Ig μH or κ L chain, or BP-1 (data not shown), which excluded the possibility that these cells (partially) reflected a more mature B-lineage

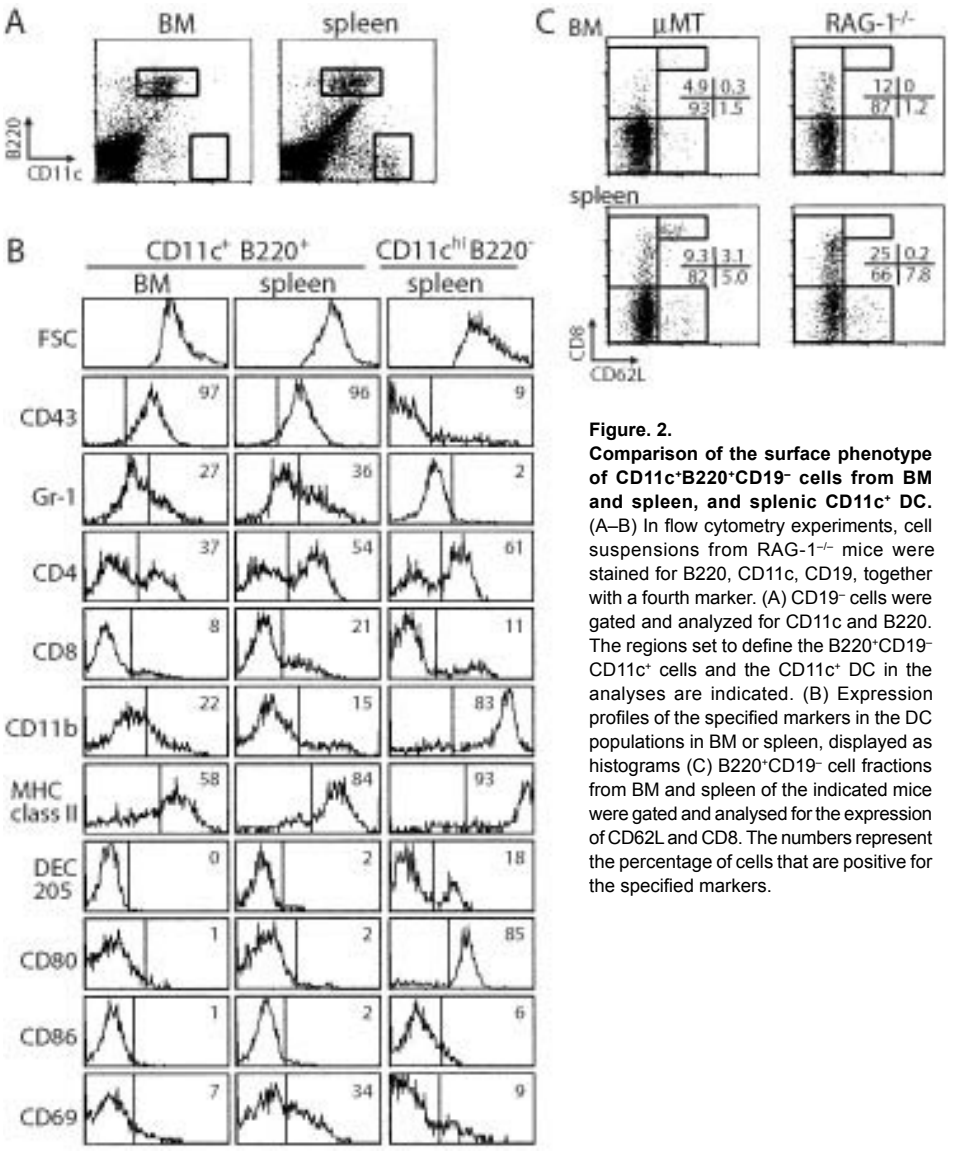

Figure. 2.
Comparison of the surface phenotype of CD11c$^+$B220$^+$CD19$^-$ cells from BM and spleen, and splenic CD11c$^+$ DC. (A–B) In flow cytometry experiments, cell suspensions from RAG-1$^{-/-}$ mice were stained for B220, CD11c, CD19, together with a fourth marker. (A) CD19$^-$ cells were gated and analyzed for CD11c and B220. The regions set to define the B220$^+$CD19$^-$ CD11c$^+$ cells and the CD11c$^+$ DC in the analyses are indicated. (B) Expression profiles of the specified markers in the DC populations in BM or spleen, displayed as histograms (C) B220$^+$CD19$^-$ cell fractions from BM and spleen of the indicated mice were gated and analysed for the expression of CD62L and CD8. The numbers represent the percentage of cells that are positive for the specified markers.

subpopulation that had lost CD19 expression. We therefore conclude that we have identified in the BM a novel population of CD43$^+$B220$^+$CD19$^-$ CD11c$^+$ cells, which apparently does not belong the B cell lineage, as these cells do not express markers that are typically present in early CD19$^-$ B cell precursors.

Surface characterization of B220$^+$CD19$^-$CD11c$^+$ cells in BM and spleen

To further investigate if the CD11c$^+$B220$^+$CD19$^-$ cells are related to the peripheral plasmacytoid DC, we analyzed the expression of Gr-1, a granulocyte marker that is also expressed on plasmacytoid DC in the mouse [10]. We found that the majority of

$B220^+CD19^-CD11c^+$ BM cells expressed moderate levels of Gr-1 (Fig. 1), supporting the notion that these cells in the BM are plasmacytoid DC or their progenitors. Indeed, we were able to identify similar $B220^+CD19^-CD11c^+Gr\text{-}1^+$ cells in spleen and lymph nodes. They were also found in the thymus, where they comprised ~ 50% of the small population of $B220^+$ cells (data not shown).

Next, we evaluated the surface profile of $B220^+CD19^-CD11c^+$ cells in the BM and the spleen of C57BL/ 6, RAG-$1^{-/-}$ and μMT mice (Fig. 2). We performed flow cytometric analyses, using CD11c, B220 and CD19 and a fourth marker. The marker expression profiles of $B220^+CD19^-CD11c^+$ cells in the two lymphoid tissues from the three groups of mice were similar, although some quantitative expression differences were noticed (see below). Also the $CD11c^+$ $B220^+CD19^-$ cell population in the spleen expressed CD43 and Gr-1. Consistent with the previous findings for $B220^+CD19^-CD11c^+$ plasmacytoid DC in lymph nodes and spleen [10], these cells expressed low levels of MHC class II were either or not positive for CD4 or CD8, but did not express the myeloid marker Mac-1/CD11b or co-stimulatory molecules, such as CD40, B7.1/ CD80 and B7.2/CD86 (Fig. 2 and data not shown). Since the CD4 or CD8 staining was present in RAG-$1^{-/-}$ mice that lack mature $CD4^+$ or $CD8^+$ T cells, it could not be attributed to pickup of marker protein from associated T cells, but rather reflected authentic CD4 or CD8 expression. As a control, we also analyzed the population of $CD11c^{high}B220^-$ DC. These cells displayed high levels of CD80, CD11b and MHC class II of which the expression level was ~10x higher, when compared with $CD11c^+B220^+$ DC (Fig. 2). When additional DC- and Mϕ-specific markers [16] were tested, we found that $CD11c^+B220^+CD19^-$ cells in BM or spleen did not express F4/80, ER-BMDM1/CD13, 33D1 or DEC-205 (Fig. 2 and data not shown). We noticed that the CD69 very early activation antigen was expressed on a higher proportion of $B220^+CD19^-CD11c^+$ cells in the spleen than in the BM (Fig. 2).

As plasmacytoid DC are thought to enter lymph nodes by L-selectin-mediated extravasation across high endothelial venules (HEV), we specifically analyzed $B220^+CD19^-$ cells for CD8 and CD62L expression in BM and spleen. We noticed that small fractions of the $CD8\alpha^-$ population and a significant portion of the $CD8\alpha^+$ population expressed CD62L, in particular in the spleen. Interestingly, RAG-$1^{-/-}$ mice specifically lacked the $CD62L^+CD8\alpha^+$ subpopulation of $B220^+CD19^-$ cells, when compared with C57BL/6 or μMT mice (Fig. 2 C). This finding indicates that the development of the $CD62L^+CD8\alpha^+$ subpopulation is dependent on the presence of T cells. This may relate to the observation that CD8α expression is induced in some DC by interaction with CD40L, expressed by activated T cells [18]. In addition, the finding of a fraction of CD62L-expressing cells in BM and spleen indicates that some of these plasmacytoid cells, once in the circulation, have the ability to enter the lymph nodes by extravasation across HEV.

Taken together, we conclude that the cells that we have identified in the BM are similar to the $CD11c^+B220^+$ $Gr\text{-}1^+$ cells with plasmacytoid DC characteristics in lymph nodes and spleen. The surface phenotype of $CD11c^+B220^+Gr1^+$ cells in BM and spleen is remarkably similar, suggesting that most of the $CD11c^+B220^+Gr\text{-}1^+$ cells within these organs are in a comparable stage of maturation or activation with equal functional abilities. This would imply that the $CD11c^+B220^+Gr\text{-}1^+$ cells in BM are not the precursors of the cells identified in spleen and lymph node, but rather that these cells reflect a recirculating population. However, we also observed that $CD11c^+B220^+CD19^-$ cells in the BM had lower forward scatter values and lower proportions of cells positive for CD4, CD8, MHC class II, L-selectin (CD62L) and the CD69 activation marker (Fig. 2). Therefore, the $CD11c^+B220^+CD19^-$ fraction in the BM may contain an additional subpopulation of more immature cells. The finding that the expression of particular markers varied between individual cells within the $CD11c^+B220^+Gr\text{-}1^+$ cell fractions, both in the BM and in the spleen, implies that these fractions may contain phenotypically and functionally distinct cell populations.

Localization of $CD11c^+B220^+CD19^-$ cells in the spleen

The absence of a unique marker for $CD11c^+B220^+CD19^-$ cells precluded the analysis of the localization of this cell population in

normal mice. However, we took advantage of the almost complete lack of $B220^{+}CD19^{+}$ cells in the spleen of B cell-deficient mice. As a result, the B220 marker almost exclusively identifies the $CD11c^{+}B220^{+}CD19^{-}$ DC in RAG-$1^{-/-}$ and μMT mice (Fig. 3A). By immunohistochemical analyses, using antisera specific for CD4, B220, CD11c and MOMA-1 (specific for metallophilic Mϕ), the spleens of 2-month-old C57BL/6 mice demonstrated a characteristic organisation in terms of segregation of red and white pulp (Fig. 3C and F). In the μMT mice, $B220^{+}$ DC were partly found as isolated cells in the T cell areas of the white pulp (identified by anti-CD4), and partly as isolated cells in the red pulp (Fig. 3B and E). The white pulp T cell areas also contained $CD11c^{low}$ interdigitating cells and were surrounded by a "marginal zone", which did not contain B cells, but had $CD11c^{high}$ DC, separated by a ring of MOMA-1^{+} metallophilic Mϕ (Fig. 3H). RAG-$1^{-/-}$ mice did not contain T

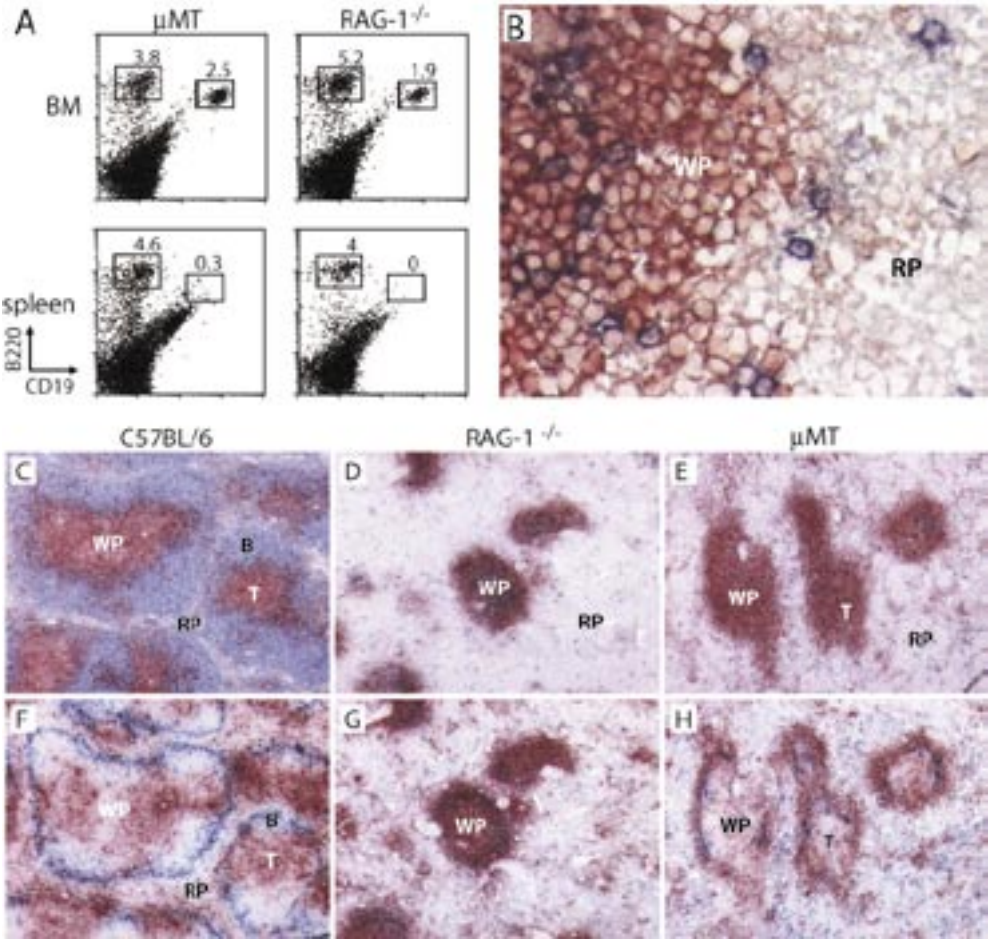

Figure 3. Localization of $CD11c^{+}B220^{+}CD19^{-}$ cells in the spleen. (A) The majority of splenic $B220^{+}$ cells in RAG-$1^{-/-}$ and μMT mice do not belong to the B cell lineage. Spleen and BM cells were analyzed for B220 and CD19 expression. Percentages of cells within the indicated gates are given. (B–H) Immunohistochemical analyses of 6- μm frozen spleen sections from the indicated mice. Sections were stained with anti-B220 (blue, B–E), anti-CD4 (brown, B–E), MOMA-1 for marginal zone metallophilic Mϕ (blue, F–H) and anti-CD11c for DC (brown F–H). (B) Detailed high magnification view of the spleen of a μMT mouse (original magnification: 400 x). (C–H) Original magnification: 50 x. RP = Red pulp; WP = White pulp; T = T cell area; B = B cell area.

cell areas, but nevertheless did show "white pulp" areas in which CD11c-expressing DC were present around central arterioles. These areas contained clusters of $B220^+$ DC, which were well separated from rings of MOMA-1^+ metallophilic Mϕ (Fig. 3D and 3G). Also in the RAG-$1^{-/-}$ mice, isolated $B220^+$ DC were present in the red pulp.

In summary, although the $B220^+$ DC showed a heterogeneous localization in the spleen, their major presence in T cell areas is consistent with the reported finding that $CD11c^+B220^+$ Gr-1^+ DC localize within T cell zones of the lymph nodes [10]. Because of the presence of the B220 marker on splenic B cells, we were unable to analyze the presence of $B220^+$ DC in the splenic B cell areas.

Concluding remarks

In this report, we have characterized a subpopulation of $B220^+CD19^-$ cells in murine BM, which express Gr-1 and low levels of MHC class II and CD11c on their cell surface. This unusual combination of markers was recently reported to be present on plasmacytoid DC [10]. In an evaluation of several cell surface markers, including CD4, CD8, CD43 and CD62L, we found that the surface profiles of $CD11c^+B220^+Gr\text{-}1^+$ cells in BM and spleen were remarkably similar. Therefore, we conclude that these cell populations are in a comparable maturation or activation stage in the two lymphoid compartments, suggesting that $B220^+CD19^-CD11c^+$ cells in the BM comprise a recirculating population of plasmacytoid DC.

The cells manifest an immature surface phenotype, characterized by low MHC class II expression and the absence of substantial co-stimulatory molecule expression. In line with these observations, we found that sorted $B220^+CD19^-CD11c^+$ cells from RAG-$1^{-/-}$ spleen did not activate naive allogeneic $CD4^+$ or $CD8^+$ T cells or induced cytokine production by T cells, consistent with the published data [10]. In our preliminary experiments, the isolated plasmacytoid DC did not proliferate in response to GM-CSF or IL-3, but produced IL-12 and IL-10 as a response to these cytokines. Furthermore, these cells were reported to produce high levels of IFNα after viral stimulation and to stimulate T cell proliferation after activation with CpG [10]. Therefore, the $B220^+CD19^-CD11c^+$ cells in the BM could also represent a major resource of IFNα -producing cells able to respond to viral infection in the BM. Upon activation, they may enter the circulation and migrate across HEV into lymph nodes [11]. Alternatively, they may stay in the BM, where local cytokine production may control hematopoiesis, as it has been shown that type I interferons inhibit growth and survival of B cell precursors [19] and negatively regulate multipotential, erythroid or granulocyte-macrophage progenitor cells [20]. In this context, it is very well possible that the observed phenotypical heterogeneity of $B220^+CD19^-CD11c^+$ DC, with respect to the CD4, CD8, CD62L and CD69 expression, may reflect their distinct *in vivo* functions in the BM. It is obvious that further experiments are required, not only to identify the possible functions, but also to investigate the origin of the $B220^+CD19^-CD11c^+$ DC in the BM.

Acknowledgements

These studies were partly supported by the Dutch Diabetes Research Foundation. We thank the people from the Erasmus University animal facility EDC for their assistance and C. Snoys (Dept. Cell Biology and Genetics, EUR) for cell sorting.

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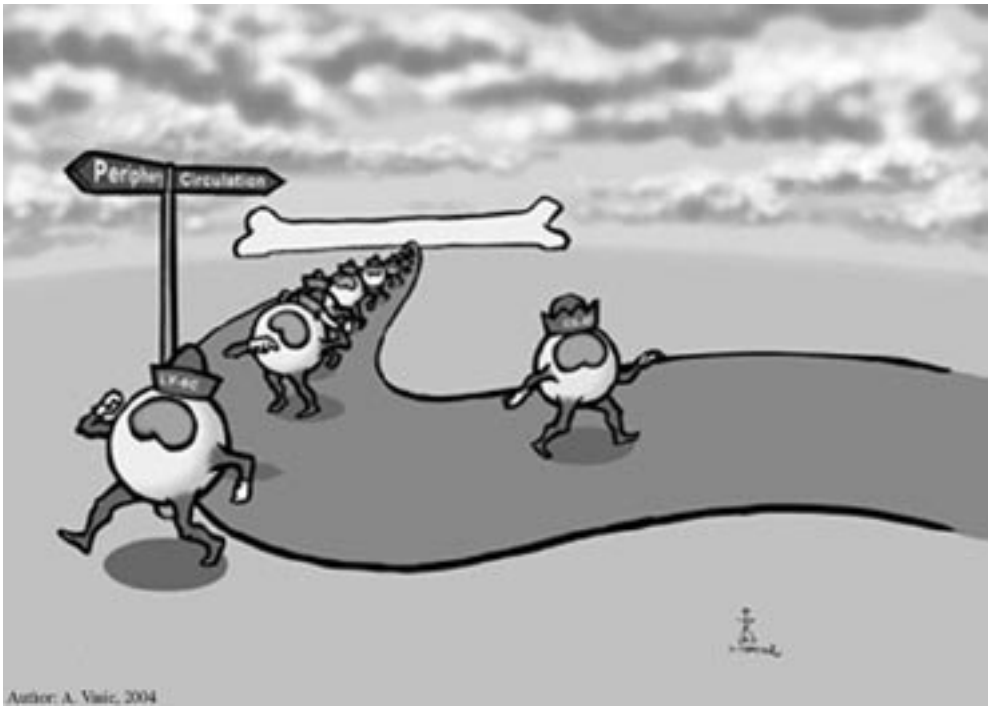
Chapter 4

Heterogeneity of the mouse circulating monocytes



4.1

Subpopulations of mouse blood monocytes differ in maturation stage and inflammatory response

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Blood monocytes are well-characterized precursors for macrophages and dendritic cells. Subsets of human monocytes with differential representation in various disease states are well known. In contrast, mouse monocyte subsets have been characterized minimally. In this study we identify three subpopulations of mouse monocytes that can be distinguished by differential expression of Ly-6C, CD43, CD11c, MBR, and CD62L. The subsets share the characteristics of extensive phagocytosis, similar expression of M-CSF receptor (CD115), and development into macrophages upon M-CSF stimulation. By eliminating blood monocytes with dichloromethylene-bisphosphonate-loaded liposomes and monitoring their repopulation, we showed a developmental relationship between the subsets. Monocytes were maximally depleted 18 h after liposome application and subsequently reappeared in the circulation. These cells were exclusively of the Ly-6C^{high} subset, resembling bone marrow monocytes. Serial flow cytometric analyses of newly released Ly-6C^{high} monocytes showed that Ly-6C expression on these cells was down-regulated while in circulation. Under inflammatory conditions elicited either by acute infection with *Listeria monocytogenes* or chronic infection with *Leishmania major*, there was a significant increase in immature Ly-6C^{high} monocytes, resembling the inflammatory left shift of granulocytes. In addition, acute peritoneal inflammation recruited preferentially Ly-6C$^{med-high}$ monocytes. Taken together, these data identify distinct subpopulations of mouse blood monocytes that differ in maturation stage and capacity to become recruited to inflammatory sites.

Circulating peripheral blood monocytes provide a mobile source of functionally competent cells of the innate immune system. They are constituents of the mononuclear phagocyte system, which encompasses various subtypes of dendritic cells (DC) and macrophages as well as their precursors [1, 2]. Different subtypes of monocytes in humans can be distinguished according to their expression of CD64 or CD14 and CD16 (see Ref. 3 for review). These subtypes both develop into macrophages or DC *in vitro*, but their developmental relationship is still unclear. Furthermore, differences in the occurrence of specific subsets in various disease states, such as systemic inflammation, suggest that monocyte subpopulations also are functionally specialized [3].

Compared with what is known about the human system, knowledge about mouse monocytes is much more limited. Bone marrow (BM) monocytes have been shown to develop into mature macrophages *in vitro* as well as *in vivo* [4, 5]. In addition, at least a fraction has the potential to develop into DC [6–8]. Monocytes can be identified in the BM on the basis of high level expression of Ly-6C (ER-MP20) and the absence of CD31 (ER-MP12), but an exclusive marker for mouse monocytes in the BM or the bloodstream is not yet available [4, 9, 10]. Characterizing peripheral blood monocytes, Lagasse and Weissman [11] and, more recently, Henderson et al. [12] showed peripheral blood monocytes to be a homogeneous population. Nevertheless, experiments conducted by Palframan et al. [13] and Geissman et al. [14] using CX3CR1$^{GFP/+}$ mice indicated that peripheral blood monocytes in these mice also encompass subtypes, differing in CX_3CR1, CCR2, and CD62L expression. Interestingly, the monocytes expressing CCR2, CD62L, and low levels of CX_3CR1 appeared to be preferentially recruited to inflamed peripheral sites by virtue of their recognition of CCL2/monocyte chemoattractant protein-1 and the CD62L-mediated interaction with high endothelial venules. Conversely, the CX3CR1^{high} monocytes migrated into noninflamed sites [14].

The goals of the present study were to identify and characterize the putative subpopulations of monocytes in normal mouse peripheral blood with respect to phenotype and function and to establish their hitherto unknown developmental

relationship. The initial criteria used to distinguish monocytes from other leukocyte types were their mononuclearity, which is read as low orthogonal (side)scatter in the flow cytometer, and their myeloid nature, as indicated by high level expression of CD11b/Mac-1. Additional required characteristics were high phagocytic capacity and the ability to develop into macrophages upon stimulation with M-CSF. Our results show that distinct subpopulations of monocytes can be identified in steady state peripheral blood of normal mice by differential expression of various surface markers, in particular Ly-6C (ER-MP20). Ly-6C^{high} monocytes are recent immigrants from the BM and have the capacity to migrate into sites of peripheral in- flammation. In contrast, Ly-6C^{low} monocytes have lost this potential. Moreover, we show that Ly-6C^{high} monocytes mature in the circulation and are the precursors for Ly-6C^{low} monocytes.

Materials and Methods

As the reported experiments comprise a collaborative effort among three different laboratories, materials and methods differed slightly between experiments performed at different locations, as indicated. However, results were validated by repetitions of experiments and extensive comparisons between our laboratories. All data shown are representative of at least three independently performed experiments.

Mice

Specific pathogen-free C57BL/6J female mice were purchased from The Jackson Laboratory (Bar Harbor, ME), Charles River (Sulzfeld, Germany), or Harlan (Horst, The Netherlands) and were housed in microisolator cages and given mouse chow and water ad libitum. Mice were 8–16 wk of age when used in experiments. All experiments with mice were performed with the approval of the animal care and use committees of Veterans Affairs Medical Center (Oklahoma City, OK), Erasmus Medical Center (Rotterdam, The Netherlands), or the State Review Board of Munster (Germany).

Antibodies

Specifications of mAbs and fluorescent conjugates against surface markers used in this study are listed in Table 1. Directly conjugated isotype control mAb were purchased from BD PharMingen (San Diego, CA). Anti-rat IgG conjugated with FITC was obtained from Dianova (Hamburg, Germany).

Table 1. Monoclonal Abs

marker	monoclonal antibody	used form (conjugate)	origin
CD3	145-2C11	FITC, APC	BD Pharmingen
CD11b / Mac-1	M1/70	FITC, PE, PerCP-Cy5, PE-CY5, APC	BD Pharmingen
CD11c	HL3	FITC, PE	BD Pharmingen,
CD16/32/ FcγRII/III	2.4G2	Ø[a]	ATCC, Rockville, MD, USA
CD19	1D3	PE	BD Pharmingen,
CD43	S7	FITC, PE	BD Pharmingen,
CD54 / ICAM-1	YN1/1.7	Ø	ATCC,Rockville, MD, USA
CD62L / L-selectin	MEL-14	PE	BD Pharmingen,
CD115 / M-CSF R	AFS98	Ø	Dr. Nishikawa, Kyoto, Japan
ER-MP58	ER-MP58	Ø	own laboratory
F4/80	F4/80	FITC	Caltag Laboratories
Ly-6C	ER-MP20	FITC	own laboratory
MHC class II	M5/114.15.2	PE	BD Pharmingen,
NK1.1	PK136	PE	BD Pharmingen,
scavengerR / SR-A	2F8	Ø	Dr. S. Gordon, Oxford, UK

[a] hybridoma supernatant

Mouse models of inflammation

Listeria monocytogenes strain EGD was stored in brain heart infusion broth (Difco, Detroit, MI) at 10^9 CFU/ml at 70°C. For experiments, 10 µl of stock culture were inoculated into 4 ml of broth and incubated overnight at 37°C with shaking. An aliquot of the overnight culture was diluted 1/10 into fresh broth and cultured an additional 4.5h. Bacteria were diluted to the desired concentration with sterile PBS before injection. *Leishmania major* (World Health Organization nomenclature: MHOM/IL/81/SE/BNI) were maintained by monthly passages in BALB/c mice. Promastigotes were grown in a 5% CO_2 atmosphere at 25°C in Schneider's *Drosophila* medium (Promocell, Heidelberg, Germany) supplemented with 10% FCS, 2% human urine, glutamine, and HEPES buffer, with penicillin and streptomycin as antibiotics. Mice were infected i.p. with 5.1 $\log_{10}$ CFU (1–2 LD50) of *L. monocytogenes* or by s.c. application of $2x10^7$ promastigotes (stationary phase) of *L. major* in 20µl of PBS into the left hind footpad. Animals were sacrificed, and blood was harvested 24, 48, and 72h after *L. monocytogenes* or 3 wk after *L. major* infection.

In other experiments, sterile inflammation was induced by injecting mice i.p. with 1.0 ml of sterile FCS (HyClone, Logan, UT). Inflammatory peritoneal

exudate cells were harvested 24h later. Mononuclear phagocytes were eliminated *in vivo* by i.v. injection of 0.2 ml of dichloromethylene- bisphosphonate (clodronate) liposomes into the lateral tail vein, as described previously [15]. Clodronate was a gift from Roche (Mannheim, Germany) and was incorporated into liposomes as previously described [16]. As an alternative approach, mononuclear phagocytes were labeled *in vivo* by i.v. injection of PBS-containing liposomes that were labeled with DiI or DiD (Molecular Probes, Leiden, The Netherlands).

Preparation of leukocytes

Mice were euthanized with an overdose of ketamine (2mg; Parke-Davis, Berlin, Germany; or Morris Plains, NJ) and xylazine (0.2 mg; CEVA Tiergesundheit, Dusseldorf, Germany; or Vedco, St. Joseph, MO) or by CO_2 exposure. Blood was obtained by axillary or femoral artery puncture or by heart puncture after exposing the organ and was collected in heparincoated tubes or in syringes containing 1.0 ml of PBS with 8mM EDTA. Control experiments showed that similar results were obtained by either method of exsanguination and anticoagulation. Erythrocytes were lysed using ACK lysis solution, and then leukocytes were washed twice by centrifugation at 250x*g* in DMEM (Life Technologies, Gaithersburg, MD) containing 5mM EDTA and 0.5% BSA. Peritoneal cells were collected 18–24h after induction of inflammation by lavage with 8ml of sterile iced PBS and were similarly prepared for further analysis.

Flow cytometry and cell sorting

Aliquots of ~10^6 cells in 1% BSA in HBSS (Biochrom, Berlin, Germany; or Life Technologies) were put into 96-well microtiter plates and then incubated with unlabeled mAb or directly conjugated primary mAb for 30min on ice. In experiments using PE-Cy5-conjugated mAb, the cells were first incubated with 3% normal mouse serum and anti-CD16/32 mAb (BD PharMingen) for 30min on ice before addition of directly conjugated mAb to block nonspecific binding. After the first incubation, the cells were washed three times and then incubated with fluorochrome-conjugated secondary Abs as needed. Controls included cells incubated with or without fluorochrome-conjugated control Abs and with unspecific isotype Ab, followed by fluorochrome-conjugated secondary Abs as needed. The cells were analyzed using a FACSCalibur equipped for four-color flow cytometry. Each measurement contained a defined number of 2x10^5 cells. Data were analyzed using CellQuest (BD Biosciences, Mountain View, CA) or WinMDI 2.8 software. Cell sorting was performed on a Moflo High Speed Cell Sorter and Analyzer (Cytomation, Ft. Collins, CO) with data acquisition using CyCLOPS version 2.1 software (Cytomation).

Morphological analysis

For morphological characterization of sorted cells, at least 2x10^4 cells from each population were sorted into FCS-coated plastic tubes filled with 2ml of HBSS. Suspensions of freshly sorted cells were cytocentrifuged onto slides, air-dried, and then fixed with equal parts ethanol/methanol. Differential counts, according to the morphological criteria established previously [10], were performed on May-Grünwald-Giemsa-stained cytospin preparations of peripheral blood leukocytes and blood smears by counting at least 200 cells/cytospin or blood smear at x400 magnification.

Culture of sorted cells

For analysis of *in vitro* development of sorted populations, 6–9x10^4 cells from each population were collected into 24-well glass tissue culture chambers (Nunc, Roskilde, Denmark). To test for macrophage development, cells were seeded at a density of 1x10^3 or 2x10^4 cells/ml in DMEM supplemented with 10% heat-inactivated FCS, 10% L cell-conditioned medium (as a source of M-CSF), L-glutamine, kanamycin, and nonessential amino acids. The cells were incubated at 37°C with 7% CO2 for 1–8 days, after which time they were fixed and stained for differential counting as described above.

Adherence and proliferation of cultured cells were evaluated daily with an inverted microscope using an ocular with eyepiece graticule. When several cells were found in close contact with each other and distinctly separated from neighboring cells, they were referred to as groups of 2–10 cells, clusters of 10–50 cells, or colonies when counting –50 cells.

Phagocytosis of latex particles and *L. major* promastigotes

Phagocytic activity was quantified by adding 2.5µl of a suspension of 5.4x10^9 latex particles/ml with an average diameter of 0.81µm (Difco) to adherent cells on 24-well plates for a final ratio of cells to latex particles of 1:14. Cells and latex particles were incubated for 4h at 37°C and then fixed with ethanol/acetone (50/50, v/v). In some experiments, phagocytosis was studied using *L. major* promastigotes. For this, freshly sorted cells were coincubated with promastigotes for 4h at a ratio of 1:5. Cells were washed and fixed in ethanol/acetone (50/50, v/v), and phagocytosis was quantified after staining with May-Grünwald-Giemsa. Cells were examined by light microscopy at x200 and x400 for the presence of intracellular parasites or ingested latex particles, respectively.

Results

Mouse blood monocytes are SSC^{low}CD11b^{high} cells heterogeneous for Ly-6C

To establish the flow cytometric identification of monocytes in normal mouse peripheral blood, we hypothesized that monocytes should be distinguishable from granulocytes and lymphocytes by their lower granular content, as reflected in low SSC, and their high level expression of Mac-1/CD11b [4, 10, 11]. To test this, we sorted different peripheral blood subsets based on SSC and Mac-1 expression (Fig. 1A) and determined which population contained monocytes. Morphological characterization by May-Grünwald-Giemsa staining showed that 95% of SSC^{low}CD11b^{high} cells were monocytic (see below). In addition, we observed that almost 95% of SSC^{low}M-CSF receptor (M-CSF R)$^{+}$ cells could be found within the CD11b^{high} window (Fig. 1A), suggesting that virtually all monocytes were contained in this population. When sorted, these SSC^{low}CD11b^{high} cells became adherent when cultured in M-CSF-containing medium and proliferated to some degree (Fig.1B). In contrast, almost all cells from the SSC^{high}CD11b^{high} population were granulocytes, whereas lymphocytes constituted the majority of SSC^{low}CD11b^{low} cells. Neither of these populations survived in the culture conditions for macrophages (Fig. 1B). More than 90% of the SSC^{low}CD11b^{high} cells were able to phagocytose L. major, whereas only SSC^{high} granulocytes, but not cells from other sorted populations, showed relevant phagocytic activity (Fig.1C). Further analysis of the SSC^{low}CD11b^{high} population with markers for NK cells (NK1.1), T cells (CD3), or B cells (CD19) showed that the SSC^{low}CD11b^{high} population contained no NK cells or lymphocytes (Fig. 2A). In contrast, SSC^{low}CD11b^{high} cells uniformly expressed significant levels of M-CSF R. Together, these findings indicate that the SSC^{low}CD11b^{high} population in peripheral blood encompasses almost exclusively monocytes on the basis of phenotype, morphology, and function.

After establishing parameters to discriminate monocytes from other leukocytes by flow cytometry, we tested whether this population displayed phenotypic heterogeneity. Initial analysis indicated that Ly-6C (ER-MP20) was variably expressed among SSC^{low}CD11b^{high} monocytes and allowed the distinction between subpopulations: Ly-6C$^{-/low}$, Ly-6C^{med}, and Ly-6C^{high} cells (Fig. 2A). The level of Ly-6C expression found on Ly-6C^{high} cells resembles that on BM monocytes [4]. Sorting and analysis of SSC^{low}CD11b^{high} cells according to the Ly-6C (ER-MP20) expression level showed that virtually all cells presented monocytic morphology (Fig. 2B) and extensive phagocytosis of latex beads and *L. major*.

Analysis of their proliferative activity in M-CSF-containing medium showed that cells from each subpopulation formed several groups of 2–10 cells in culture after a few days. Larger clusters (11–50 cells) and colonies (–50 cells) were most frequently seen in cultures of the Ly-6C^{high} population. This indicates that monocytes in all three subpopulations have the capacity to proliferate, but that cells with the highest proliferative potential reside in the Ly-6C^{high} population. Cells developing in these cultures were typically adherent and showed macrophage morphology (our unpublished observations). When we applied culture conditions suitable for the development of DC, using GM-CSF and IL-4, cells with morphological characteristics of DC and CD11c and MHC class II expression developed in a few days from all three different subsets (our unpublished observations), in accordance with the previously characterized development of DC from mouse monocytes [8, 17].

Further phenotypic characterization of the Ly-6C-defined monocyte subsets showed a uniform high level expression of ER-MP58, characteristic for cells of the myeloid lineage [18] (Fig. 2D). The ability to detect F4/80 varied between experiments, with all subsets expressing the Ag in some experiments, whereas in others no positive subsets were present. In the currently shown profile, most, but not all, monocytes in the different subsets express F4/80. The various subpopulations expressed only marginal amounts of mature macrophage markers such as FcγRII/RIII (CD16/CD32) or scavenger receptor type A (Fig. 2D). However, the subsets did show clear heterogeneity with regard to the expression of CD11c, CD43, and CD62L, with the latter being present primarily on Ly-6C^{high} monocytes, whereas CD11c and CD43 were found in particular on Ly-

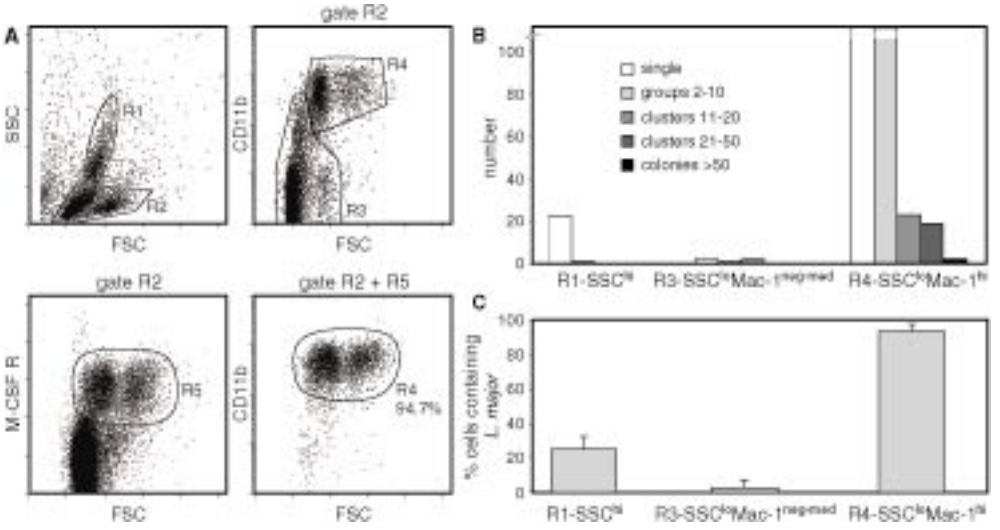

Figure 1. Phenotypic identification of mouse blood monocytes as SSC^{low}CD11b^{high} cells. A. Peripheral blood leukocytes were labeled with CD11b/Mac-1 Ab and analyzed on a flow cytometer. Gated cells (R1, R2+R3, and R2+R4) were separated by cell sorting. B. Cell survival and proliferation after 10 days of stimulation with M-CSF *in vitro* were analyzed under inverted microscope. C. Sorted cells were incubated for 6 h with *L. major* promastigotes (1:5 ratio) and then were fixed and stained. The frequency of phagocytic cells was quantified using light microscopy. A representative of three independent experiments is shown.

6C$^{-/low}$ cells.

Down-regulation of Ly-6C marks the maturation of mouse blood monocytes

Previous studies have indicated that BM monocytes are Ly-6C^{high} and that Ly-6C expression is rapidly lost during *in vitro* development into macrophages or DC [4, 8]. Using the same gatings as established for the peripheral blood monocytes, we confirmed that BM monocytes, identified as either SSC^{low}CD11b^{high} or SSC^{low}M-CSF R^{+} cells, were almost exclusively Ly-6C^{high} (Fig. 3A). Therefore, we investigated the possibility that Ly-6C^{high} peripheral blood monocytes might correspond to BM monocytes, which developed into Ly-6C$^{-/low}$ cells subsequently. *In vitr*o we found that sorted Ly-6C^{high} peripheral blood monocytes lost expression of Ly-6C within 3 days (our unpublished observations).

To analyze the subset relationship *in vivo* we injected mice i.v. with liposomes loaded with clodronate to deplete all phagocytic cells in the circulation and followed the kinetics of monocyte repopulation in the blood. Figure 3B shows that almost 90% of the SSC^{low}CD11b^{high} monocytes were eliminated by this procedure 18h after liposome injection. This result seemingly contrasts with the reported finding that blood monocytes could be depleted only partially by this method [19]. However, in that study monocyte depletion was evaluated only 48h after depletion, whereas we found that after 24h monocytes had started to repopulate and had reached normal numbers in 3–4 days (Fig. 3B). The monocytes reappearing at 2 and 4 days after depletion were almost exclusivelyof the Ly-6C^{high} BM phenotype (Fig. 3C). Ly-6C$^{-/low}$ monocytes were found in the circulation in significant numbers only from 7 days after depletion. These findings are consistent with the interpretation that Ly-6C^{high} monocytes give rise to Ly-6C^{med} and Ly-6C^{-} monocytes.

Attempting to approach this maturation sequence more directly, we sorted Ly-6C^{high} monocytes from green fluorescent protein (GFP)-transgenic mice and injected these into normal, unconditioned recipients. However, at o time point after transfer could GFP^{+} cells be retrieved from the circulation of recipient mice,suggesting

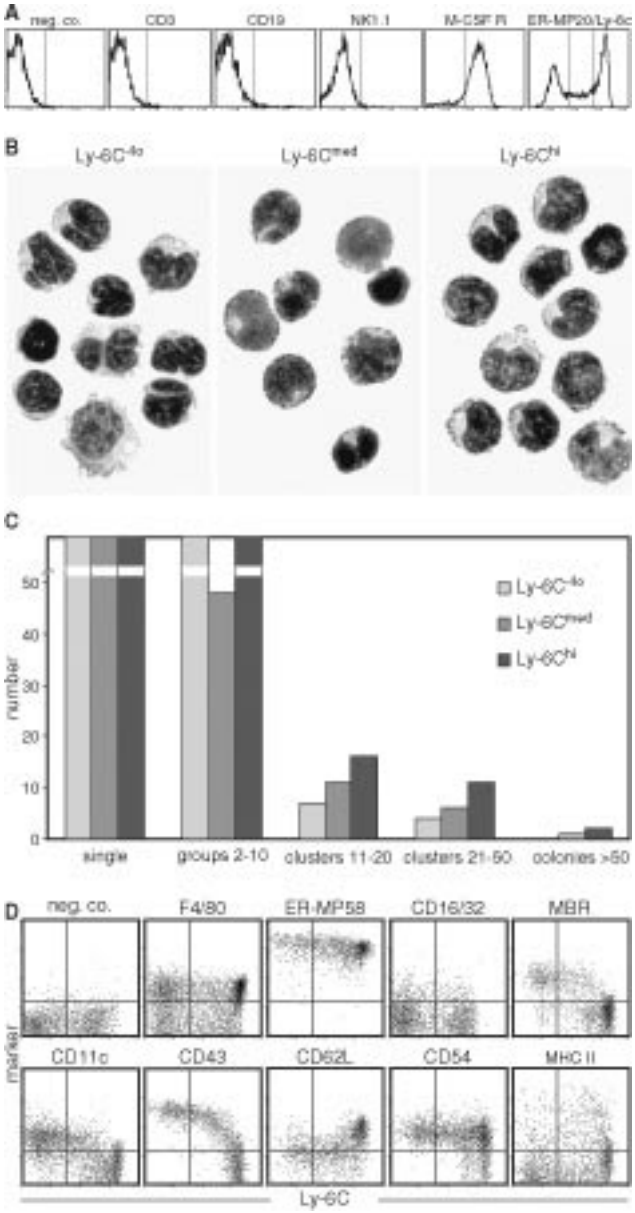

Figure 2.
Blood monocytes show heterogeneous expression of Ly-6C. A. SSC^{low}CD11b^{high} cells were negative for T- (CD3), B- (CD19), and NK-specific (NK1.1) markers, but uniformly positive for M-CSF R (CD115). They could be subdivided into three populations according to the expression of Ly-6C: Ly-6C^{high}, Ly-6C^{med}, and Ly-6C$^{-/low}$. These three subpopulations were separated by cell sorting and showed uniformly monocytic morphology (B) and differential proliferative capacity (C) when stimulated with M-CSF in vitro. D. Monocyte subsets differentially expressed various myeloid and activation markers relative to Ly-6C expression. In particular, CD62L was expressed by Ly-6C^{high} monocytes, whereas CD11c and CD43 were expressed by Ly-6C^{low} monocytes. Representative data are shown for at least four independent experiments.

that manipulation of these Ly-6C^{high} monocytes might induce aberrant behavior, leading to rapid margination *in vivo*. As an alternative approach, we synchronized monocyte development by clodronate liposome-mediated depletion and then labeled reappearing monocytes *in vivo* to follow their kinetic response. At 48h after depletion, virtually all circulating monocytes were Ly-6C^{high} (Fig. 3C). When we then injected fluorochrome (DiI)-labeled liposomes, we found that the vast majority of circulating monocytes became labeled and retained the Ly-6C^{high} phenotype for 2 days (Fig. 3D). From 3 days after fluorochrome application, DiI-labeled Ly-6C^{low} monocytes were observed

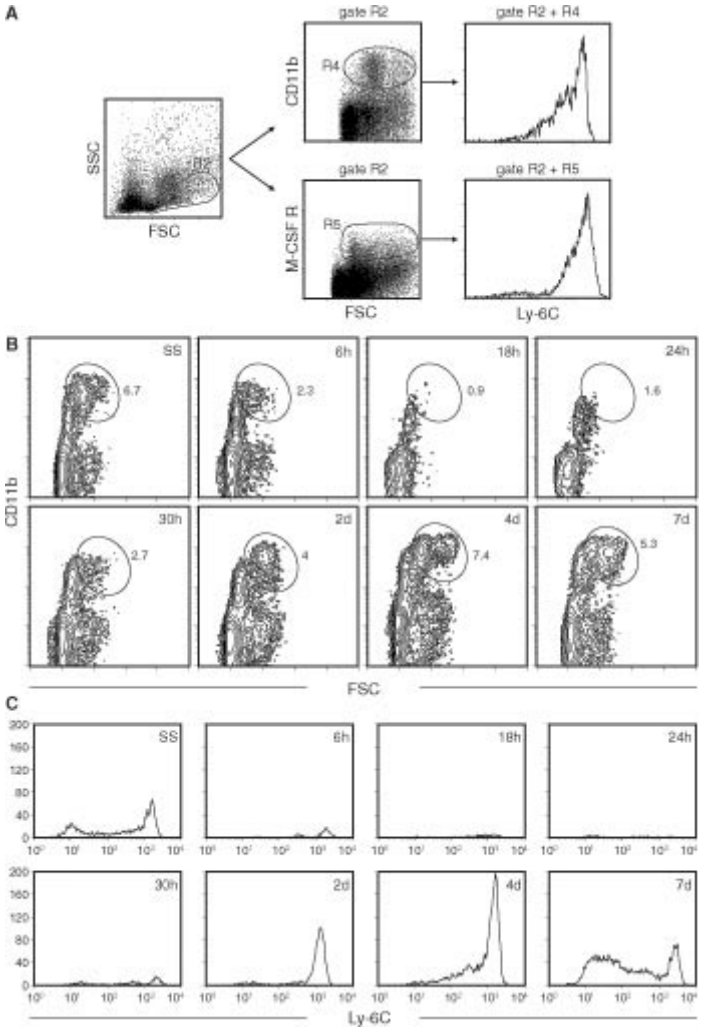

Figure 3.
Blood monocytes mature from Ly-6C^{hi} into Ly-6C^{low} cells. A. Bone marrow monocytes were identified by similar gating as performed on peripheral blood cells (see Fig. 1). They had uniformly high expression of Ly-6C, as reported previously. B. Upon i.v. injection of clodronate liposomes, virtually all peripheral blood monocytes were depleted within 24h. Recovery to normal numbers of monocytes (identified as SSC^{low}CD11b^{hi} cells) occurred by 4 days after depletion. C. Analysis of Ly-6C expression by monocytes, gated as SSC^{low}CD11b^{hi} cells, repopulating the blood after depletion. The first cells reappeared 2 days after clodronate liposome injection and were BM-like Ly-6C^{hi} monocytes, whereas Ly-6C^{low} monocytes were detected in the circulation from 7 days after depletion.

in the circulation, indicating that these cells had indeed developed from Ly-6C^{high} monocytes. In contrast, the vast majority of Ly-6C^{high} monocytes were not labeled at this point, demonstrating that they had been released into circulation after the fluorochrome pulse labeling. These experiments strongly support the concept that monocytes enter the circulation from the BM as Ly-6C^{high} cells and then develop into Ly-6C$^{-/low}$ monocytes before emigrating into peripheral tissues.

To follow the kinetics of the distinct monocyte populations in steady state, we attempted to label all monocytes by i.v. injection of fluorochrome (DiD)-labeled liposomes. Unexpectedly, we found that all monocytes, identified as SSC^{low}CD11b^{high} cells, disappeared from the circulation within 30 min after liposome injection (Fig. 3E) suggesting that phagocytosis of liposomes triggered monocyte margination. Monocytes started to reappear after 2–4h, but these cells were not DiD-labeled, indicating that they were not in direct contact with the circulation when the liposomes were injected. Presumably, these cells resided in the BM and entered the circulation after the liposomes were

injected. This was confirmed by the observation that these cells were of the Ly-6C^{high} BM monocyte phenotype. From 16h on, DiD-labeled monocytes, all of which were Ly-6C$^{-/low}$, were found in the peripheral blood. Because the previous experiments showed that Ly-6C^{high} and Ly-6C$^{-/low}$ monocytes had similar phagocytic activity of *L. major in vitro* and clodronate liposomes *in vivo*, it is unlikely that the Ly- 6C^{high} cells did not take up the DiD-labeled liposomes. Thus, these results suggest that the Ly-6C^{high} cells matured into Ly-6C$^{-/low}$ monocytes within the 16- to 48-h period. These experiments do not formally exclude, however, that only labeled Ly-6C$^{-/low}$ monocytes reappear in circulation, whereas Ly-6C^{high} monocytes have a different destination.

The immature Ly-6C$^{med/high}$ monocyte subset is selectively involved in inflammatory conditions

Previous studies have shown that monocytes recruited to peripheral inflammatory sites typically show a high level expression of Ly-6C [20, 21]. In view of our current findings, we asked whether this was due to a selective recruitment of immature BM-type monocytes. Therefore, sterile inflammation was elicited by i.p. injection of FCS. In the steady state peritoneal cavity, we observed that monocyte-like SSC^{low}CD11b^{high} cells were present, characterized by an intermediate expression of the macrophage marker BM8, well below that of resident peritoneal macrophages (Fig. 4A). These resident peritoneal monocytes were uniformly lacking Ly-6C (Fig. 4B). In contrast, the first inflammatory monocytes examined 18 h after FCS injection clearly showed significant

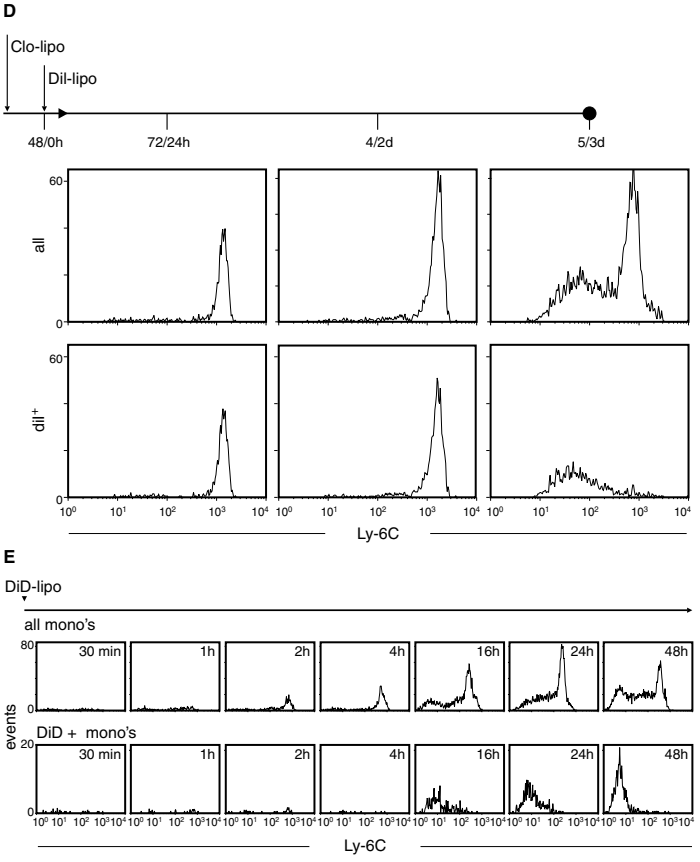

Figure 3. *(continued)*
Blood monocytes mature from Ly-6C^{hi} into Ly-6C^{low} cells. D. Ly-6C^{hi} monocytes that reappeared on day 2 after depletion were pulse-labeled *in vivo* by injecting DiI-loaded liposomes. By 3 days after the DiI labeling, Ly-6C^{hi} monocytes had developed into Ly-6C^{low} monocytes, whereas the vast majority of unlabeled monocytes at that time were of the Ly-6C^{hi} BM type. E. Injection of DiD-loaded liposomes into steady state mice caused all monocytes to disappear temporarily from the circulation within 30 min after injection. Measurable numbers of monocytes were found in circulation 2–4h later, but these were unlabeled Ly-6C^{hi} BM-type cells. DiD-labeled monocytes were again present 16h after application of DiD-labeled liposomes and expressed only low levels, if any, of Ly-6C reflecting their relatively mature status.

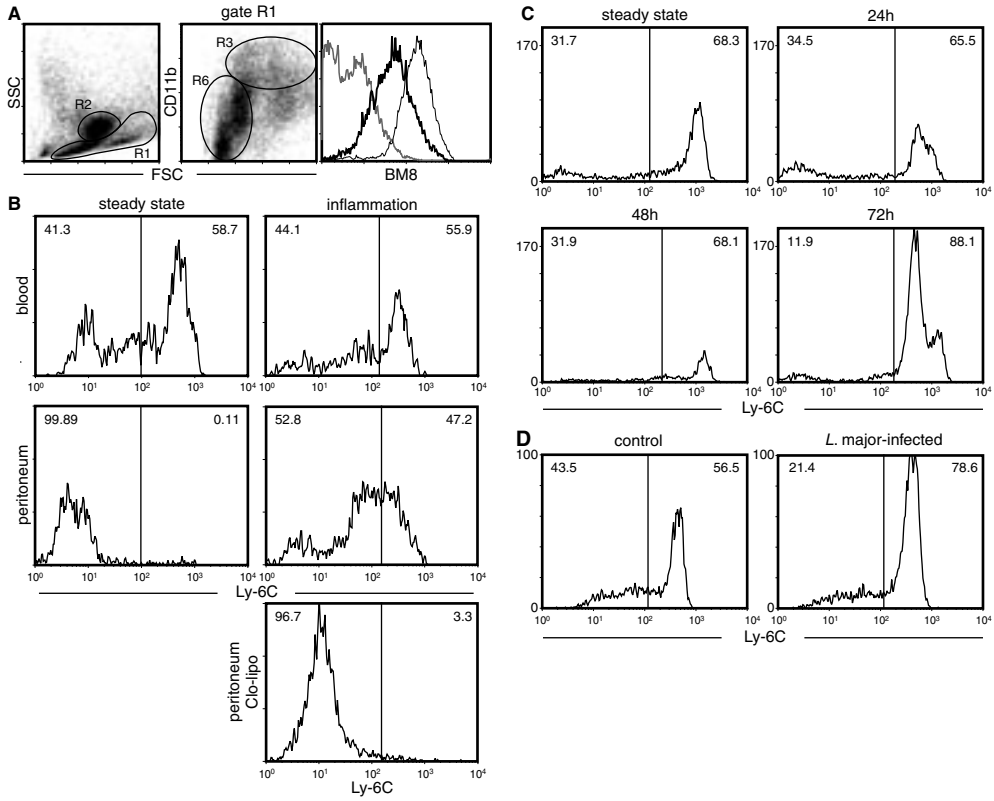

Figure 4. Selective contribution of Ly-6C$^{med/hi}$ monocytes to the inflammatory response. A. BM8 expression of monocyte-like SSC^{low}CD11b^{hi} cells (R1 + R3) in the peritoneal cavity at steady state is less than that of resident peritoneal macrophages (R2). The histogram showing the lowest BM8 expression is from SSC^{low} cells with low or no CD11b expression (R1 + R6). B. Sterile inflammation induced by i.p. injection of FCS 24h previously recruited specifically Ly-6C$^{med/hi}$ monocytes to the site of infection. This cellular influx is abolished by i.v. injection of clodronate liposomes at the same time as i.p. injection of FCS. C. Acute systemic infection with *L. monocytogenes* caused initial depletion of circulating monocytes during the first 48 h after infection, followed by specific accumulation of Ly-6C^{hi} monocytes, resulting in a noticeable monocytosis by 72h. D. Chronic parasitic infection with *L. major*, analyzed at 4 wk after infection, stimulated a shift toward Ly6C^{hi} monocytes in the blood.

Ly-6C expression (Fig. 4B). By comparison, the ratio between monocyte subsets in peripheral blood was unchanged. This suggested that Ly-6C^{high} immature monocytes were selectively recruited to the site of inflammation, in accordance with recent findings [14]. In contrast, immigration of Ly-6C^{high} monocytes was blocked completely when the inflammatory trigger was given to mice that were simultaneously depleted of circulating monocytes by i.v. injection of clodronate liposomes (Fig. 4B). The latter finding also indicates that FCS injection does not induce Ly-6C expression in resident peritoneal monocytes.

To investigate the monocyte subset kinetics in acute bacterial inflammation, we infected mice with *L. monocytogenes*. Fig. 4C shows that the ratio between Ly-6C^{high} and Ly-6C$^{med/low}$ monocytes did not change in the first 48 h after infection, but the absolute number of circulating monocytes decreased during this time. In contrast, there was a significant influx of Ly-6C^{high} monocytes 72h after infection, causing a notable monocytosis in the blood with a shift toward the immature Ly-6C^{high} subset. To test whether chronic infection caused a similar shift in the monocyte balance, C57BL/6 mice were infected s.c. with *L. major*.

After 4 wk, chronic infection was established as indicated by marked swelling of footpads and by dissemination of parasites into draining lymph nodes. At this stage also a significant shift was found toward the Ly-6C^{high} immature monocyte subset (Fig. 4D). Taken together, these findings confirm the idea that inflammatory monocytes are exclusively Ly-6C$^{med/high}$ cells. Furthermore, both acute and chronic infection are reflected in the blood by a shift in the balance between monocyte subsets toward the immature Ly-6C^{high} BM type.

Discussion

The circulating monocyte compartment in the mouse has been studied to only a limited extent due in part to the limited number of available cells and the lack of solid criteria to identify this cell type. In this study we characterized mouse peripheral blood monocytes in genetically nonmanipulated animals using an approach that is generally applicable in both steady state and experimental conditions. To identify monocytes among peripheral blood leukocytes, we used criteria that are universally recognized as characteristic of mononuclear phagocytes [22]. These are 1) mononuclearity, seen as low orthogonal light scatter in the flow cytometer; 2) myeloid nature, shown by high level expression of CD11b; 3) uniform phagocytic potential; and 4) the ability to develop into macrophages upon M-CSF stimulation. Application of these criteria indicated that monocytes in mouse peripheral blood were restricted to the SSC^{low}CD11b^{high} population and that this population included no other cells. These cells were uniformly positive for the M-CSF receptor (CD115), a proposed universal marker for mononuclear phagocytes (1). This way we identified ~6% of circulating leukocytes in the steady state as monocytes. With total leukocyte counts of ~10.10^6/ml, this amounts to 6 x 10^5 circulating monocytes/ml of mouse blood, which is consistent with earlier data [23].

In previous studies, Lagasse and Weissman used high level expression of CD11b, the absence or low level expression of Gr-1 and low SSC as discriminating criteria for monocytes and identified these cells as a single population [11]. Similarly, Henderson and colleagues [12] found mouse monocytes to be a homogeneous population using high level expression of the myeloid marker 7/4 and intermediate expression of Gr-1 as discriminating criteria. In contrast, Geissmann and colleagues [14] recently recognized distinct subsets among circulating monocytes in $CX_3CR1^{GFP/+}$ mice which differed in phenotypic and functional characteristics. In accordance with these results, we distinguished monocyte subsets in normal mice according to differential expression of Ly-6C (ER-MP20), CD62L, CD11c, and CD43. Cells of each subset showed extensive phagocytic activity, displayed typical monocytic morphology and developed into macrophages *in vitro* upon M-CSF stimulation. Thus, each population of SSC^{low}CD11b^{high} leukocytes, distinguished by variable Ly-6C expression, contains bonafide monocytes.

To date a possible relationship between these monocyte subsets had not been addressed [13, 14]. Using various approaches, we found that the monocyte subsets differing in Ly-6C expression represent different stages in a continuous maturation pathway. As such, recent immigrants from the BM enter circulation as Ly-6C^{high} monocytes, consistent with their BM-like phenotype [4], and gradually down-regulate Ly-6C expression while still in the bloodstream. *In vitro* studies indicate that this transition is performed within 24–48h [4, 8]. Under steady state conditions *in vivo* peripheral blood monocytes were estimated to have a similar average transit time in circulation of ~25h [24]. Our study provides evidence in support of such kinetics, because 16–24h after labeling monocytes with DiD *in vivo*, most monocytes were unlabeled and therefore recent immigrants, whereas labeled monocytes had developed into mature Ly-6C$^{-/low}$ cells within this time span. However, when we analyzed the kinetics of monocyte maturation in the circulation under certain inflammatory conditions, we found that it differs from steady state. When the forced release of monocytes from the BM during inflammation was simulated in a phagocyte depletion model, most Ly-6C^{high} cells remained in the circulation for –2 days before maturing into Ly-6C^{low} cells. This is probably due to the fact that monocytes are released in a less mature state under conditions of

peripheral phagocyte depletion or inflammation. These cells might need more time to acquire a minimal level of maturation, which is necessary before they can emigrate from the circulation and undergo further differentiation. Typically, only 4 days after depletion, the first repopulating macrophages and DC are observed in peripheral organs, in particular spleen and liver [25] (our unpublished observations). Do the different monocyte subsets have different developmental capabilities? Stimulation with M-CSF *in vitro* resulted in the development of macrophages from all subsets (Figs. 1 and 2). Similarly, all subsets acquired DC-like morphology and phenotype when stimulated *in vitro* with GM-CSF and IL-4. Therefore, the different monocytes seem to share a similar developmental potential. Recent *in vivo* transfer experiments show that Gr-1$^-$ monocytes, corresponding to mature Ly-6C$^{-/low}$ cells, reside longer in peripheral tissues of uninflamed recipient animals compared with Gr-1$^+$ monocytes, which correspond to Ly-6C^{high} cells [14]. The more mature Ly-6C$^{-/low}$ monocyte subset shows enhanced or induced expression of CD11c and CD43 Ags. Increased expression of CD43 and stimulation via CD43 have been shown to be associated with DC maturation in humans and rats [27, 28]. In addition, there was a clear phenotypic resemblance between the mature Ly-6C$^{-/low}$ monocytes and the recently identified CD11c^{int}CD45RA$^-$ pre-DC1, circulating DC precursors in the mouse [26]. Thus, the increased CD43 expression by the Ly-6C$^{-/low}$ monocytes together with induced CD11c expression probably reflect the maturation of these cells into a monocyte subtype with high potential to develop into DC.

The immature, Ly-6C^{high} subset of monocytes is characterized by a higher level of L-selectin (CD62L$^+$) expression compared with Ly-6C$^{-/low}$ monocytes. Given that CD62L mediates recruitment of leukocytes [29], we tested whether differential expression of this molecule was also manifested in differences in recruitment of the different subsets during an elicited peritoneal inflammation. We observed that the inflammatory Ly-6C^{high} monocytes [20, 21], but not the Ly-6C$^{-/low}$ monocytes, were recruited into acute inflammation. Recently, Palframan et al. [13] identified two populations of F4/80-positive mononuclear cells in peripheral blood of heterozygous CX$_3$CR1$^{GFP/+}$ mice, of which only one subset expressed CD62L and was recruited to lymph nodes draining inflamed skin. In accordance, Geissmann et al. [14] showed preferential migration of the CD62L$^+$Gr-1$^+$CCR2$^+$ monocyte subset, which corresponds to our Ly-6C^{high} population, into the peritoneal cavity during thioglycolate-induced inflammation in CX$_3$CR1$^{GFP/+}$ mice. We found in preliminary studies that Ly-6C^{high} monocytes express elevated levels of CCR5 and CXCR4 compared with the mature Ly-6$^{-/low}$ monocytes. Together, these findings clearly show that particularly the immature Ly-6C^{high} monocytes, but not the mature Ly-6C$^{-/low}$ monocytes have a high potential to migrate to inflammatory sites. Peripheral inflammation stimulates the production of monocytes in the BM and their subsequent release into circulation. When this is substantial, there is a skewing of the monocyte population toward a higher frequency of immature Ly-6C^{high} cells. This can be considered a so-called left shift in the monocyte compartment comparable to that seen in the neutrophil compartment, when more immature bandforms are found during acute inflammation. Such a monocytic left shift is typically observed after 72h during primary infection with *L. monocytogenes*. It results in greatly increased numbers of monocytes in circulation, whereas the BM is fully occupied with myelopoiesis [9]. We observed a significant skewing toward immature cells also in chronic infection caused by *L. major*. This probably reflects the increased myelopoiesis that is long recognized in chronic infection with *L. major* [30].

The identification of distinct monocyte subsets in the mouse raises the question of how these relate to monocyte subsets present in humans. A recent comparison between human and mouse monocytes indicated that CX3CR1^{low} (our Ly-6C^{high}) monocytes correspond to the classical CD14^{high}CD16$^-$ monocytes, whereas CX$_3$CR1^{high} (our Ly-6C$^{-/low}$) monocytes resemble CD14^{low}CD16^{high} cells [14]. Interestingly, in human patients with sepsis an increase has been observed of CD64$^+$ monocytes, which primarily correspond to the major subset of CD14$^+$16$^-$ monocytes [31]. In analogy to our findings in infected mice, this might represent a so-called left shift toward immature monocytes also in severe inflammations in humans. Taken together, in

this study we identify different subsets of mouse monocytes in peripheral blood, which behave differently depending on host conditions. Our data indicate that the cells enter the circulation from the BM as Ly-6C^{high} cells. Under steady state conditions, monocytes mature in circulation, which is reflected in strong down-regulation of Ly-6C and up-regulation of CD43 expression. In peripheral inflammation, however, only Ly-6C$^{med/high}$ monocytes are recruited to the affected sites to become inflammatory exudate macrophages. Increased monocytopoiesis under these circumstances is apparent from the increased frequency of immature Ly-6C^{high} monocytes, which may be considered a left shift in the monocyte compartment. The destiny of the Ly-6C$^{-/low}$ monocytes remains more speculative at present. Their phenotypic change suggests maturation toward DC with increased CD43 expression facilitating stimulation through this receptor to become more mature DC. Furthermore, the anti- and proadhesive properties of CD43, depending on the presence of the appropriate ligands [32], suggest a more selective homing pattern for the mature Ly-6C$^{-/low}$ monocytes compared with the immature Ly-6C^{high} monocytes.

Acknowledgments

Many people were important in the genesis of this study. We thank Tar van Os for preparation of the figures; Eva Nattkemper, Ruth Goez, Berlinda den Broeder, Karin Fischer, and Meike Steinert for technical support; and Jeremy Gill for his enthusiastic input. We are grateful to Ellen van Lochem for advice on human monocyte development, and to Jim Henthorn (William K. Warren Medical Research Institute) for expert assistance with flow cytometry and cell sorting. Dr. Nishikawa is thankfully acknowledged for making available the anti-M-CSF R Ab.

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4.2

Diabetes-prone NOD mice show an expanded subpopulation of mature circulating monocytes, which preferentially develop into macrophage-like cells in vitro

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In the non-obese diabetic (NOD) mouse, a model of autoimmune diabetes, dendritic cells (DC) and macrophages are important for the initiation and progression of autoimmunity and the final destruction of β-cells. Monocytes are direct precursors of DC and macrophages. The recently described phenotypic discrimination between immature (Ly-6C^{high}) and mature (Ly-6C^{low}) monocytes in the mouse circulation enabled us to investigate the apportioning between blood monocytes in the NOD mouse. NOD mice had an abnormal high number of mature monocytes in circulation and this phenomenon appeared to be intrinsic to the NOD background, since NOR and NOD-H2b mice also showed this altered balance. After depletion, immature Ly-6C^{high} monocytes returned more rapidly to the circulation in NOD mice, while the transition of immature to mature monocytes had normal re-appearance kinetics. This suggests that the bone marrow in NOD mice is able to release more monocytes to ensure the restoration of a larger mature circulating compartment. In addition, while monocytes from C57BL mice down regulate their capability to adhere to fibronectin and ICAM-1 upon maturation, the mature NOD monocytes retained the high adhesion capacity. Furthermore, both monocytes groups of NOD mice differentiated preferentially into macrophage-like F4/80^{high} cells during *in vitro* overnight culture.

In conclusion, mice with the NOD background show an enhanced monocyte-production capacity, raised numbers of mature monocytes in the circulation and a pro-inflammatory macrophage-directed set point of these monocytes.

Type I diabetes is an autoimmune disease in which a self-destructive immune process against the pancreatic β-cells leads to insulin deficiency. In the non-obese diabetic (NOD) mouse, a widely used animal model for autoimmune diabetes, dendritic cells (DC) and macrophages (Mϕ) are important for the initiation and progression of autoimmune diabetes and the final destruction of β-cells. Morphological studies show raised numbers of DC and Mϕ already in the pancreas of neonatal NOD mice [1]. A further accumulation of DC and Mϕ around islets at 4-5 weeks of age precedes the peri-insular infiltration of lymphocytes. An infiltration of lymphocytes and Mϕ into the islets characterizes the initiation of the final β-cell destruction [2, 3].

Monocytes are direct precursors of DC and Mϕ [4-6]. It has been generally accepted that the majority of DC and Mϕ originate from blood monocytes, especially in inflammation [7, 8]. Mouse blood monocytes can phenotypically and functionally be separated into two subsets [9, 10]. Monocytes that have recently emigrated from the bone marrow (BM) and express high levels of the Ly-6C molecule belong to the first subset. These Ly-6C^{high}, immature monocytes, are readily attracted to sites of inflammation, have the potential to develop into DC or F4/80^{+} inflammatory Mϕ [6, 10] and co-express CCR2, which enables the cells to be attracted towards inflammation-induced MCP-1 signals. These immature monocytes also express CD62L molecules and are therefore able to enter lymph nodes in non-inflammatory conditions through high endothelial venules (HEVs) [11]. The second subset of blood monocytes is generated through a maturation step from the immature monocytes, marked by a reduction of the surface expression of the Ly-6C molecule and an increase in expression of CD43 [10]. These Ly-6C^{low} monocytes that express CD11c and CX_3CR1 (fractalkine receptor), migrate to peripheral organs, such as liver and lung, in the absence of inflammation and have been proposed to be the precursors for the steady state pool of DC and Mϕ [9].

Geissmann et al have suggested that the mouse monocyte subsets correspond to the previously discriminated subtypes of circulating monocytes in humans. According to this view, CX_3CR1^{lo} (Ly-6C^{high}) mouse monocytes correspond to CD14^{+}CD16^{-} human monocytes, while the CX_3CR1^{hi} (Ly-6C^{low}) monocytes correspond to

the $CD14^+CD16^+$ human monocyte population. Changes in the composition of the monocyte pool due to different pathological conditions have been demonstrated in humans. The $CD14^+CD16^+$ monocytes expand greatly in acute and chronic infections, systemic inflammatory syndromes, AIDS and renal failure [12-17]. However, we found normal numbers of both monocyte populations in type-1 diabetic (T1D) patients and the same has been observed in multiple sclerosis (MS) patients [18, 19]. Other subdivisions in circulating human monocytes have been made, such as the subdivision based on the ability to adhere to fibronectin [20]. The fibronectin-adhering, so-called pro-inflammatory "P-monocytes" constitute 20-30% of the circulating monocytes, express high levels of adhesion molecules, have an enhanced chemotactic responsiveness, phagocytosis and pro-inflammatory cytokine production capability [21, 22]. Interestingly, we found that fibronectin-adhesive monocytes are present in equal numbers in both the $CD14^+CD16^+$ and the $CD14^+CD16^-$ population, indicating that the division based on the fibronectin-adherence and the division based on the CD16 expression are probably not identical. This is also indicated by our finding of raised number of fibronectin-adhesive monocytes in T1D patients as compared to healthy controls, in the presence of normal numbers of $CD14^+CD16^+$ cells [18].

The phenotypic discrimination between the Ly-$6C^{high}$ immature and Ly-$6C^{low}$ mature monocytes enabled us to investigate the apportioning between immature and mature blood monocytes in the mouse variant of autoimmune diabetes, i.e. the NOD mouse model. Here we describe an excess of mature Ly-$6C^{low}$ monocytes in the blood of mice with the NOD background, which had abnormal high fibronectin adhesive properties. The kinetic studies on the re-appearance of the monocyte subpopulations after depletion with clodronate-loaded liposomes (lip-CL_2MDP) showed that in NOD mice more monocytes rapidly leave the BM to fill the larger mature blood monocyte compartment.

Previously we reported a hampered capability of monocytes of T1D patients to develop during an overnight culture into DC-like cells [23]. We therefore also tested the capability of both immature and mature monocytes of NOD mice to differentiate into cells with a DC- or Mϕ-like phenotype during an *in vitro* overnight culture and found an enhanced capability of NOD monocytes to create more progeny with a Mϕ-like phenotype.

Taken together we here give evidence in mice with the NOD background for an enhanced production of monocytes from BM precursors, raised numbers of mature monocytes in the circulation with an abnormal adhesion capability and a preferential development of Mϕ-like cells from both the immature and mature forms of circulating NOD monocytes.

Material and methods

Animals

Mice used in this study were all female and between 5-16 weeks old when used in experiments. Diabetic NOD mice were 25 weeks old. C57BL/6j, BALB/c mice were purchased from Harlan (Horst, The Netherlands), NOR mice were purchased from Jacksons Laboratory (Bar Harbor ME, USA) and NOD/Ltj, NOD.B10H2^b (NOD-H2b) and C3Heb/Fej mice were bred in the animal care facility at the Erasmus MC, Rotterdam. All mice were specific pathogen-free and kept with free access to food and water in the animal care facility at the Erasmus MC Rotterdam, under the institutional guidelines for usage of experimental animals approved by Erasmus University Animal Welfare Committee. Glycosuria in NOD mice was tested with the Gluketur test (Roche Diagnostics GmbH, Mannheim, Germany).

Antibodies

Specifications of monoclonal antibodies and fluorescent conjugates against surface markers used in this study are listed in Table 1. Directly conjugated isotype control mAb were purchased from BD Pharmingen (San Diego, CA). Anti-rat IgG conjugated with FITC- or R-phycoerythrin-(R-PE)- (mouse-absorbed; GaRa-FITC or GaRa-PE) were purchased from Caltag Laboratories, San Francisco, CA. Biotinylated Ab were detected by allophycocyanin-(APC) conjugated streptavidin (SAV-APC) purchased from BD Pharmingen.

***In vivo* elimination of mononuclear phagocytes**

Multilamellar liposomes containing clodronate (dichloromethylene bisphosphonate, a gift from Roche Diagnostics) (lip-CL_2MDP) in the aqueous phase were prepared as described previously [32, 33]. Liposomes

Table 1. Monoclonal antibodies used in this study

marker	monoclonal antibody	used form (conjugate)	origin
CD3	145-2C11	FITC, APC	BD Pharmingen
CD11b	M1/70	PerCP-Cy5.5, APC	BD Pharmingen
CD11c	HL3	FITC, PE	BD Pharmingen
CD16/32	2.4G2	Ø[a]	ATCC, Rockville, MD, USA
CD19	1D3	PE	BD Pharmingen
CD43	S7	FITC, PE	BD Pharmingen
CD54	YN1/1.7	Ø	ATCC, Rockville, MD, USA
CD62L	MEL-14	PE	BD Pharmingen
CD115	AFS98	Ø	Dr. Nishikawa, Kyoto, Japan
ER-MP58	ER-MP58	Ø	own laboratory
F4/80	F4/80	FITC	Caltag
Ly-6C	ER-MP20	FITC	own laboratory
MBR	MIV 38	Ø	Dr. Falkenberg, Bochum, Germany
NK1.1	PK136	PE	BD Pharmingen

[a] hybridoma supernatant

consisted of phosphatidyl choline and cholesterol in 6:1 molar ratio. After washing, the liposomes were resuspended in PBS. Mononuclear phagocytes were eliminated *in vivo* by intravenous (i.v.) injection of 0.2 ml clodronate liposomes into the lateral tail vein, as described before [34].

Preparation of leukocytes

Mice were euthanized by CO_2 exposure. Blood was obtained by heart puncture and collected in heparin-coated tubes. Erythrocytes were eliminated using BD lysing buffer (San Diego, CA) and leukocytes were washed by centrifugation at 1500rpm for 5 min in phosphate buffered saline (PBS) pH 7.8 containing 0.5% BSA (Biowhittaker, Verviers, Belgium) (further referred to as FACS buffer). Cells were finally resuspended, counted and used for further analysis.

Flow cytometry and cell sorting

For phenotypic analysis aliquots of 0.5-1x10^6 cells were pipetted into 96-microwell plates (round bottom, Nunc, Denmark) and incubated with the prepared mix of monoclonal antibodies. Each incubation step was performed at a room temperature for 10min. Cells were analyzed using a FACSCalibur equipped for 4-color flow cytometry. Depending on the size of the sample up to 5x10^5 events were obtained. Data were analyzed using CellQuest software (Becton Dickinson, Sunnyvale, CA).

Cell sorting was performed on a FACSDiva by applying a previously described protocol [4]. Lysed blood samples (pooled from 5 or 10 mice) were first washed in a sterile PBS supplemented with 5% heat inactivated FCS (Biowhittaker) (further referred to as a sorting buffer) and incubated for 30min on ice with the mix of CD11b, Ly-6C (clone ER-MP20) and Ly-6G antibodies. Subsequently, cells were washed with a sorting buffer and filtered over a 30mm sieve (Polymon PES, Kabel, Amsterdam, The Netherlands) to avoid clogging of the nozzle. After sorting, the purity of the cell suspensions was checked by re-running sorted samples and purity exceeded 95% (unless stated othervise). Cells were kept at 4°C throughout the staining and sorting procedure. Sorted cells were counted in a Bürker hemocytometer.

Adhesion test

For adhesion capacity test, sorted monocytes were suspended in RPMI-1640 culture medium (Biowhittaker) containing antibiotics (60µg/ml penicillin and 100 µg/ml streptomycin) supplemented with 1% heat-inactivated FCS. Monocytes were then plated on the coated chambertek glass slides (Nalge Nunc International, Naperville, USA) at a density of 0.2 x 10^5 cells per chamber. Slides were coated with 10 µg/ml fibronectin or 10g/ml ICAM-1 (Sigma, Steinheim, Germany). After 60min incubation at 37°C, cells were washed with PBS and fixed with 4% paraformaldehyde (PFA; Sigma) supplemented with 3% glucose. Subsequently, cells were permeabilized using 0.5% Triton X-100 (Sigma Chemical, Saint Louis, USA) and stained with 0.1 mg/ml FITC-labeled phalloidin (Sigma Chemical) for 30-45 min. After washing and mounting the slides, the cells were counted using a fluorescence microscope. Adhesion was expressed as the number of cells in 10 high power fields (hpf) at 200x magnification. Two individuals performed the counting, independently.

Culture of sorted cells

For analysis of *in vitro* differentiation capacity of monocytes, sorted cells were seeded into 96-well culture plate at concentration of 1x10^6/ml in the RPMI-1640 medium supplemented with antibiotics and 10% FCS. The cells were incubated at 37°C with 5% CO_2 for 24h, alone or stimulated with 100ng/ml LPS (Sigma). After 24h incubation, cells were used for phenotypic analysis.

Statistical analysis

To determine differences between the groups, data were compared by a two-tailed Student's T-test using the SPSS software package. Results are presented as the mean ± SEM, unless indicated differently.

Results

Both Ly-6C^{high} (immature) and Ly-6C^{low} (mature) are found in the blood of NOD mice

In the NOD mouse blood, an identical definition of the two subsets of monocytes could be applied as the previously reported [10] (Fig. 1A). Similarly to the C57BL mouse, more than 97% of SSC^{lo}CD11b^{hi} cells in the NOD mouse blood were M-CSF-receptor positive (CD115 in the Fig. 1C) and could be separated into two populations defined by Ly-6C expression (Fig. 1A). The markers CD11c, CD43 and CD62L also had a similar distribution in NOD as in C57BL mice (Fig. 1B). Further phenotyping of Ly-6C^{high} and Ly-6C^{low} monocytes did not reveal reproducible differences between the two mouse strains (Fig. 1C), apart from the macrophage marker, F4/80, which had a reproducibly higher surface expression on both subsets of NOD monocytes.

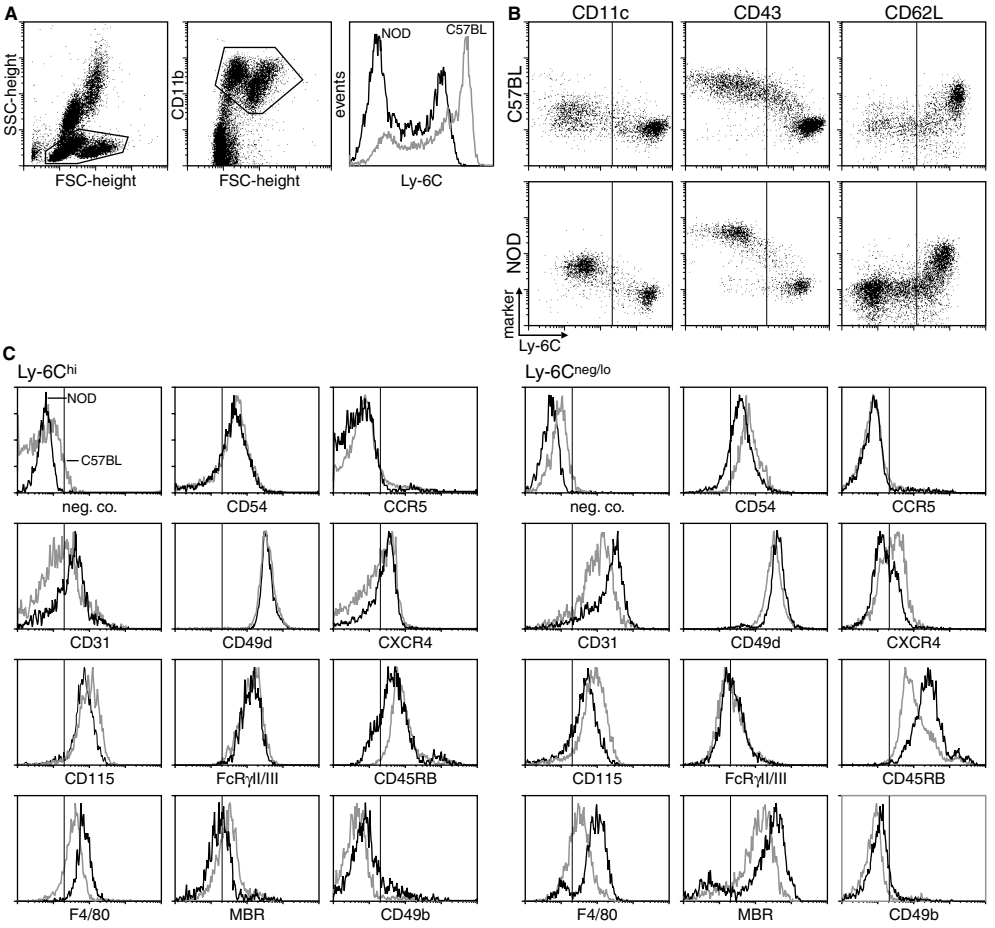

Figure 1. Definition and phenotypic analysis of blood monocytes in the NOD mouse. A. Application of the same gating of SSC^{lo} and CD11b^{hi} cells separates two monocyte populations as seen from the bi-phasic profile of the Ly-6C histogram. B. Although lower on NOD monocytes, Ly-6C separation discriminates identical two monocytes populations as in the C57BL mice. C. Additional markers tested show a differential or similar expression between two monocyte subsets, but almost identical expression between NOD and C57BL mice within a particular population. Exceptionally, F4/80 molecule is higher on both Ly-6C^{hi} and Ly-6C^{lo} monocytes in the NOD mouse. Dot-plots and histograms show a representative staining of a minimum of 3 independent stainings for each marker.

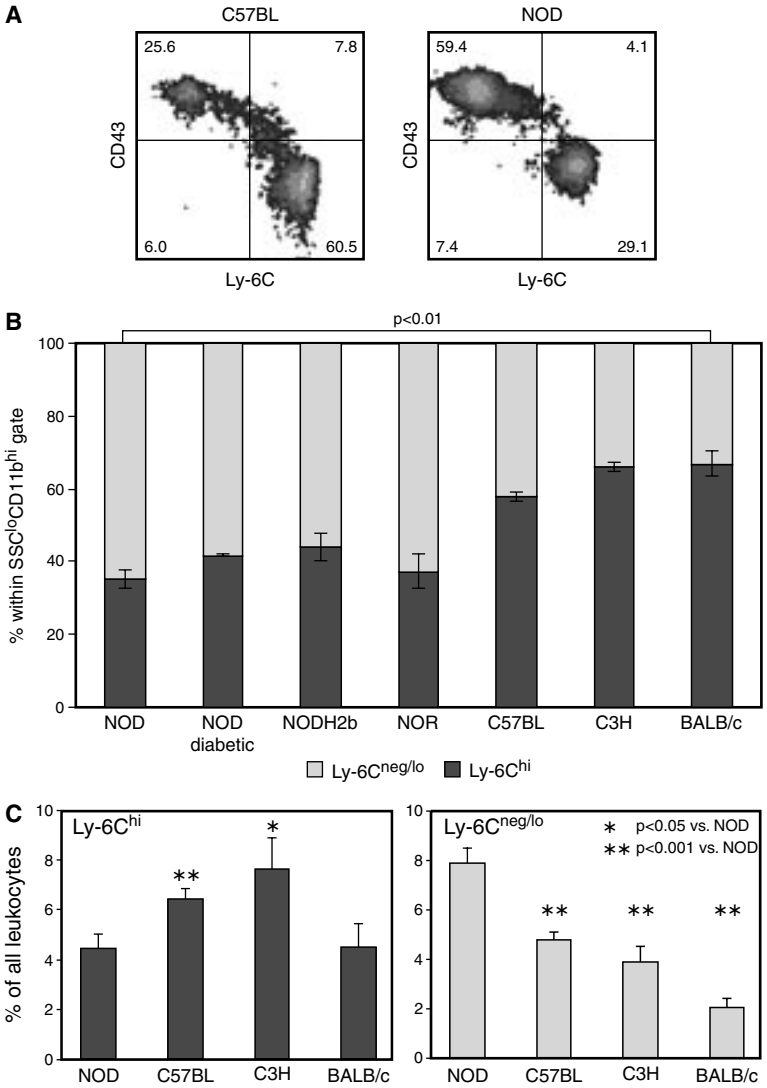

Figure 2. Different balance between Ly-6C^{hi} and Ly-6C^{lo} monocytes in the blood of mice with a NOD genetic background. A. Representative dot-plot of the Ly-6C/CD43 profile of NOD and C57BL monocytes shows a higher frequency of Ly-6C^{lo} monocytes in the NOD mice. B. Analysis of the frequency of blood monocytes in different mouse strains revealed a specific shift towards Ly-6C^{lo} monocytes in all mouse strains with the NOD genetic background. C. Analysis of the frequency within all nucleated cells. Frequency of Ly-6C^{hi} monocytes is significantly lower in the NOD than in the C57BL and the C3H mice but not different from the BALB/s mice. In contrast, percent of Ly-6C^{lo} monocytes in the NOD mouse blood is significantly higher than in all mouse strains tested. Graphs represent average value ±SEM calculated from 26 C57BL mice, 20 NOD mice and 6-9 mice of other mouse strains.

Mice with the NOD background have more mature Ly-6C^{low} monocytes in the blood.
Although the phenotype of the NOD monocyte subsets did not differ from that of C57BL mice, we noticed that the frequency of the Ly-6C^{high} monocytes was repeatedly lower in NOD mouse blood when compared to C57BL blood, as demonstrated in a representative dot-plot (Fig. 2A). Correspondingly, Ly-6C^{low} monocytes in the NOD blood formed the major monocyte population. This overrepresentation of mature monocytes in the blood was specific to the NOD mouse. When we determined the frequency of monocyte subsets in several unrelated mouse strains (Fig. 2B), between 58-67% monocytes were of the Ly-6C^{high} type in the strains of mice unrelated to the NOD. In the strains with the NOD background, Ly-6C^{high} monocytes never exceeded 44%. In addition, both healthy and diabetic NOD/Ltj mice, as well as NOR and NOD-H2b mice, had the higher percentage of the Ly-6C^{low} of monocyte (Fig. 2B). Hence, all mice with the NOD background had a reversed ratio of monocyte subsets in favor of the Ly-6C^{low} cells.

In addition we calculated the absolute numbers of the two subsets in the peripheral blood. All tested mouse strains had similar absolute numbers of leukocytes and similar frequency of monocytes (SSC^{lo}CD11b^{hi}) within all leukocytes per ml blood (not shown). Absolute number of Ly-6C^{high} monocytes in the NOD mouse blood was significantly lower as compared to the C57BL and C3H mice ($p<0.001$ and $p<0.05$, respectively) (Fig. 2C), but BALB/c mice had similar numbers of circulating Ly-6C^{high} monocytes like NOD mice. The number of Ly-6C^{low} monocytes was more discriminative and remarkably higher in NOD mice when compared to all other tested control strains ($p<0.001$ for all three control strains) (Fig. 2C). Taken together, a mild shortage in the Ly-6C^{high} monocytes and a clear excess of Ly-6C^{low} monocytes, typify the disturbed monocyte-ratio in the blood of mice with the NOD background.

Ly-6C^{low} monocytes from NOD mice display unusually high adhesion to fibronectin and ICAM-1, typical for Ly-6C^{high} monocytes
To check the ability of the two subsets of NOD monocytes to adhere to fibronectin and ICAM-1, we sorted Ly-6C^{high} and Ly-6C^{low} monocytes from the mouse blood and tested their adhesiveness to these compounds *in vitro*.

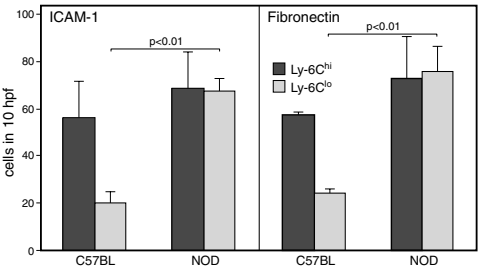

Figure 3. High in vitro adherence of sorted Ly-6C^{lo} monocytes from the NOD mouse. Blood monocytes were sorted on the FACSDiva and put to adhere to (A) ICAM-1 or (B) fibronectin for 60min. Subsequently cells were washed, stained and quantified on the fluorescent microscope. A high number of Ly-6C^{hi} monocytes from both mouse strains adhered to both substrates. In contrast, while Ly-6C^{lo} monocytes from the C57BL downregulate the adhesion capacity upon maturation, the Ly-6C^{lo} monocytes from the NOD mice failed to do so. Graphs represent an average±SEM from three independent pools of a minimum of 5 mice per pool, obtained in two independent experiments.

As shown in Figure 3, majority of Ly-6C^{high} monocytes isolated from both NOD and C57BL adhered to fibronectin and ICAM-1. In contrast, much lower percentage Ly6C^{low} monocytes from C57BL mice adhered to these compounds; hence the maturation of monocytes into resident cells in the circulation leads to a decline in the ability to attach. Interestingly, Ly-6C^{low} monocytes in NOD mice preserved the high adhesion capacity to both fibronectin and ICAM-1, comparable to that of Ly-6C^{high} monocytes (Fig. 3).

NOD monocytes show an enhanced spontaneous differentiation *in vitro,* predominantly in the direction of Mϕ-like cells.
To check for the capacity of the two subpopulations of monocytes to spontaneously differentiate *in vitro*, we again sorted and incubated them in culture fluid for 24h ,without adding additional cytokines.

In the case of C57BL cells, overnight culture of sorted immature Ly-6C^{high} monocytes induced a spontaneous up regulation of the CD43 molecule – characteristic of mature monocytes - on the majority of the cells (Fig. 4A). In addition, the

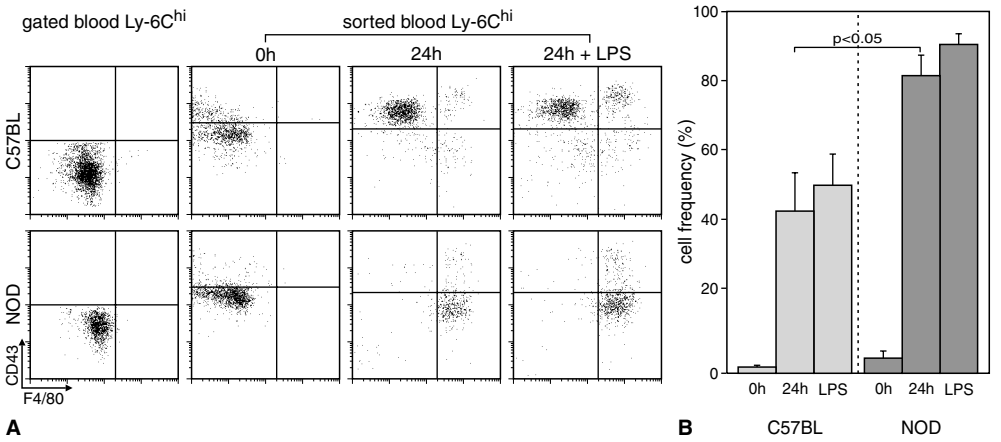

Figure 4. Different in vitro maturation of Ly-6C^{hi} monocytes from NOD mice. A. Maturation of Ly-6C^{hi} monocytes upon in vitro cultivation for 24h with or without LPS goes through different phenotypic stages in NOD mice when compared to the C57BL. Dot-plots show a representative staining of Ly-6C^{hi} monocytes prior and after the sorting as well as after 24h incubation alone or with LPS. B. Frequency (±SEM) of Ly-6C^{hi} monocytes that become F4/80^{hi} cells, in vitro. Data are derived from 4 independent samples per mouse strain obtained from a pooled blood of 5-6 mice per strain, in three independent experiments.

Mϕ marker F4/80 became also higly expressed on some of the cells. In NOD mice, however, Ly-6C^{high} cultured immature monocytes did not acquire CD43. Instead, the majority had strongly upregulated the F4/80 molecule (note that we already mentioned the higher F4/80 expression on the fresh blood monocytes from the NOD mouse). Quantitative analysis demonstrated that significantly more Ly-6C^{high} monocytes of NOD mice spontaneously became F4/80^{high} Mϕ-like cells ($p<0.05$) (Fig. 4B). LPS was an additional stimulus for C57BL immature monocytes to increase F4/80, after which the frequency of F4/80^{high} cells from C57BL and NOD samples was not statistically different anymore.

Overnight culture of sorted mature Ly-6C^{low} C57BL and NOD monocytes spontaneously yielded three cell groups: undifferentiated mature monocytes (CD43^{+}F4/80^{low}), cells with a DC-like phenotype (CD43^{low}F4/80^{med}CD11c^{+}MHCII^{hi}) and cells with a Mϕ-like phenotype (CD43^{high}F4/80^{high}CD11c^{low}MHCII^{low}). Although these three groups were present in both NOD and C57BL samples, they had a different frequency in these mice (Fig. 5A). In overnight cultures, more than half of C57BL Ly-6C^{low} monocytes remained unchanged, while in NOD cultures there were few remaining Ly-6C^{low} monocytes ($p<0.001$). The majority of the NOD cells had differentiated into Mϕ-like cells and their frequency was significantly higher in samples of the NOD mouse ($p<0.05$). These effects of an enhanced and preferential differentiation into Mϕ-like cells were more pronounced with LPS as an additional stimulus (Fig. 5B). The percentage of DC-like cells was similar in the 24-hour NOD and C57BL cultures.

Taken together, in NOD both Ly-6C^{high} immature monocytes and Ly-6C^{low} mature monocytes show an enhanced spontaneous differentiation, and predominantly in the direction of cells with a Mϕ-like phenotype.

An enhanced release of monocytes from the bone marrow in the blood of NOD mice.

To follow the monocyte maturation *in vivo*, we made use of clodronate-loaded liposomes (lip-CL_2MDP). As shown in Table 2, a single injection of lip-CL_2MDP caused an almost complete depletion of monocytes (SSC^{lo}CD11b^{hi}) from the blood within the first 18h in both NOD and C57BL mice. Apparently the NOD monocytes were equally able to phagocytose and fragment liposomes as the C57BL monocytes.

Monocytes started to appear in the blood around 48h post-injection in both NOD and C57BL mice. From this moment till the end of the

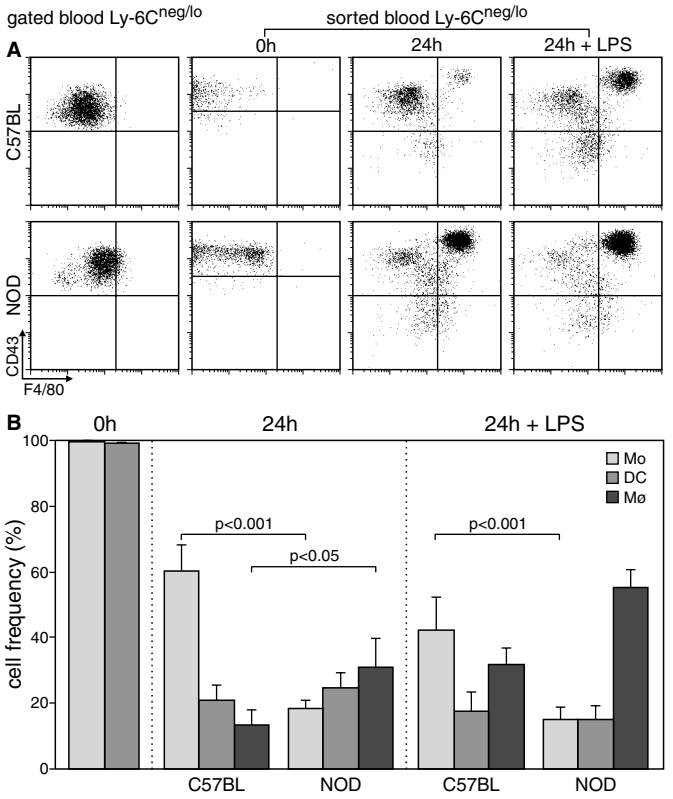

Figure 5.
Predominant maturation of Ly-6C^{lo} monocytes from NOD mice into Mϕ. A. Phenotype of monocytes (CD43^{med}F4/80^{lo}), DC (CD43^{lo}F4/80^{med}) and Mϕ (CD43^{hi}F4/80^{hi}) from NOD and C57BL mice after 24h culture with or without LPS is similar, as seen on representateive dot-plots. B. Graph represents the average frequency of monocytes (light grey bar), DC (dark grey bar) and Mϕ (black bar) ±SEM calculated from the 4 samples obtained in the same experiments as in the figure 6. NOD mouse shows as high spontaneous maturation of Ly-6C^{lo} monocytes into F4/80^{hi} Mϕ, in vitro.

observation period, the percentage of monocytes (SSC^{lo}CD11b^{hi}) was considerably higher in NOD mice (Table 2). However, due to the high standard deviation, this difference did not reach statistical significance.

Like we previously established for the C57BL mice, the first monocytes found in the blood of NOD mice at 48h were exclusively immature Ly-6C^{high} cells (Fig. 6). After 96h, the frequency of Ly-6C^{high} monocytes started to decline in both mouse strains (Fig. 6A). Interestingly, the release from the BM of immature Ly-6C^{high} cells was larger in NOD mice and at 48h the NOD contained significantly more Ly-6C^{high} monocytes in the blood as compared to C57BL mice (Fig. 6A). Accordingly, the kinetics of the return of Ly-6C^{high} monocytes in the circulation was noticeably different between NOD and C57BL mice (Fig. 6B). In C57BL mice the frequency of Ly-6C^{high} monocytes rose gradually to the starting point (at 96h) and never exceeded the steady state range. In contrast, Ly-6C^{high} monocytes of the NOD mouse reached the steady state level as soon as 48h and continued to rise to the level 2x higher than the steady state, at 96h. Thereafter the number of Ly-6C^{high} monocytes in the NOD mouse rapidly declined to the steady state level at

Table 2. Return of blood monocytes after the i.v. depletion with lip-CL_2MDP

	Time after depletion with lip-CL_2MDP (hours)					
	0	18	24	48	96	192
NOD	13.5 ± 2.9[a]	1.4 ± 0.9	1.3 ± 0.5	7.6 ± 4.9	13.5 ± 6.2	22.1 ± 13.2
C57BL	13.1 ± 8.4	2.2 ± 1.7	2.3 ± 1.5	3.4 ± 1.5	9.7 ± 5.9	15.9 ± 12.2

[a] percent within leukocytes/ml blood ±SD

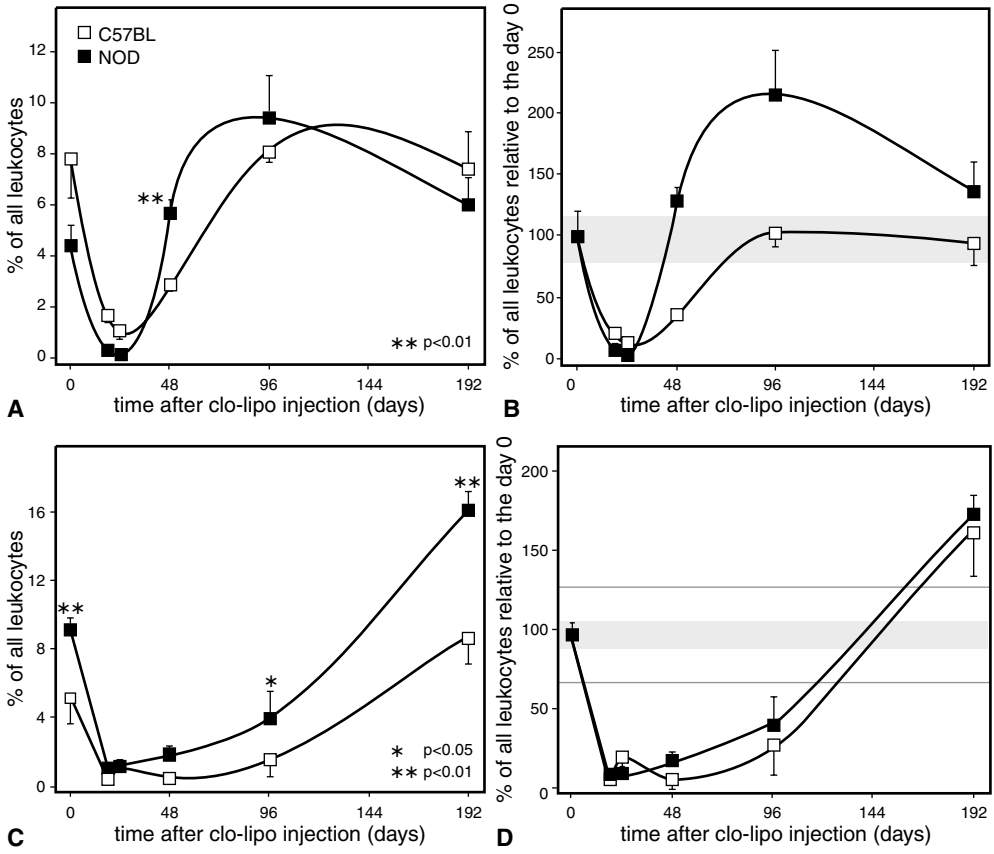

Figure 6. Normal restoration but different kinetics of the monocyte-return after a depletion with lip-CL_2MDP (i.v.) Changes in frequency of (A) Ly-6C^{hi} and (C) Ly-6C^{lo} monocytes in the NOD (black squares) and C57BL mice (empty squares) at different time points after injection of lip-CL_2MDP. Kinetic of the return of (B) Ly-6C^{hi} and (D) Ly-6C^{lo} monocytes in the NOD and C57BL mice when calculated relative to their own steady state. Data represent average frequency±SD of a minimum of 3 mice for each time point and each mouse strain.

192h (Fig. 6B).

With regard to the return of the Ly-6C^{low} monocytes in the circulation, higher levels of Ly-6C^{low} monocytes - similar to the situation before depletion - were found in the blood of NOD mice starting from 96h till the end of the observation period (Fig. 6C). Importantly, the return of Ly-6C^{low} monocytes had identical kinetics in both NOD and C57BL mice (Fig. 6D). Therefore, an enhanced release of Ly-6C^{high} monocytes from the BM might be the decisive determinant in the finally larger number of mature monocytes in the NOD circulation.

Efflux of Ly-6C^{low} monocytes from the blood of the NOD mice is normal.

Besides the enhanced monocyte influx from the BM, a reduced efflux of the mature monocytes to the periphery could also cause the elevated number of Ly-6C^{low} monocytes in the NOD mouse blood. We therefore determined the quantities of Ly-6C^{low} monocytes in the peritoneal cavity and the spleen in the steady state and after the application of lip-CL_2MDP. The Ly-6C^{high} monocytes are virtually absent from these compartments (not shown).

In the steady state, the frequency of Ly-6C^{low} monocytes in the spleen and peritoneal cavity of NOD and several different mouse strains was

Table 3. Frequency of Ly-6C^{lo} monocytes in different compartments

	blood		spleen		peritoneum	
	NOD	C57BL	NOD	C57BL	NOD	C57BL
untreated [a]	8.56 ± 1.05*	5.55 ± 0.65	0.59 ± 0.05**	0.96 ± 0.08	6.81 ± 1.24	6.32 ± 1.50
treated (d7)	4.90 ± 0.74	3.57 ± 0.43	0.71 ± 0.05*	0.97 ± 0.07	6.07 ± 0.77	3.35 ± 1.56

[a] percent of all leukocytes/ml blood ± SEM
** p<0.01 NOD vs. C57BL; * p<0.05 NOD vs. C57BL

similar, across the group of different mouse strains (Fig. 7). After depletion, Ly-6C^{low} monocytes returned to the steady state point in both the spleen and peritoneal cavity within a week (Table 3); importantly with similar kinetics in NOD and C57BL mice (not shown). Hence we found no sign of a reduced efflux of monocytes from the circulation to the periphery in NOD mice.

While performing these experiments we also noticed that Ly-6C^{low} monocytes were clearly underrepresented in the NOD mouse spleen (in favour of Mϕ and DC), when compared to the C57BL strain. We therefore calculated the monocyte: Mϕ+DC ratio in the spleen of different mouse strains and found a significantly lower ratio (more mature cells than monocytes) in the NOD compared to all mouse strains tested including NOR mice (Fig. 7-insert). This indicated that the balance between monocytes and their progeny in the spleen is also tipped towards the more differentiated/mature cells in mice with the NOD background.

Discussion

The recent advances in the phenotypic definition of two monocyte subsets in the normal mouse blood, i.e. the Ly-6C^{high} immature monocytes and the Ly-6C^{low} mature monocytes, enabled us to analyze blood monocytes in diabetes-prone NOD mice and compare with our previously reported data on monocyte subsets in human T1D.

In this study we found that NOD mice display an altered balance between Ly-6C^{high} immature circulating monocytes and Ly-6C^{low} mature monocytes, i.e. the mice displayed an abnormal high number of circulating mature monocytes. This

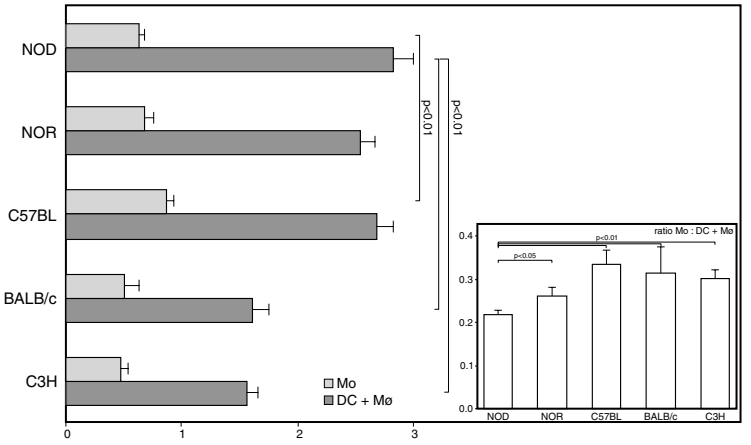

Figure 7. Different balance between mature (Ly-6C^{lo}) monocytes and DC and Mϕ in the spleen of NOD mice. Data are calculated as a frequency of all nucleated cells and an average±SEM of monocytes (light gray bars) and DC+Mϕ (dark grey bars) from different mouse strains are given in the graph. Insert-graph represent an average±SEM of a calculated ratio monocyte:mature cell (DC+Mϕ). P values are derived from a double-sided Student's t-test. Data are calculated from 13 C57BL mice, 19 NOD mice and 5-8 mice of other mouse strains.

phenomenon appeared to be intrinsic to the NOD background, since NOR and NOD-H2b mice also showed this altered balance. In addition we found in the NOD spleen more DC and Mϕ relative to the monocytes as compared to control strains. These observations support a view that mice with a NOD background show a skewing of cells of the monocyte lineage towards more differentiated and mature forms in both the circulation and the periphery.

After depletion with lip-CL_2MDP we found an overshoot of immature Ly-6C^{high} monocytes released in the first two days in the blood of NOD mice, while the transition of immature to mature NOD monocytes had normal kinetics. The re-appearance kinetics are compatible with a view that in NOD mice more monocytes are able to leave the BM after depletion (perhaps also in the steady state) to finally fill a larger compartment of more differentiated and mature forms of the monocyte lineage.

What could be the mechanisms behind the raised release of NOD monocytes from the BM compartment? Theoretically more Ly-6C^{high} monocytes are able to leave the BM when there is a larger production of monocytes per unit time or when there is an enhanced release of monocytes from stored pools of immature monocytes in the BM. For the latter mechanism we did not obtain evidence in a set of preliminary experiments: i.e. we found a similar composition of the myeloid BM cells in NOD and C57BL mice before and after depletion (unpublished results). Hence we favor the view that mice with the NOD background have a capacity for a larger and faster production of monocytes from monoblasts in the BM, when stimulated to do so.

The here described mature Ly-6C^{low} monocyte population corresponds to the Gr1^{-} CCR2^{-}CX$_3$CR1^{hi} mouse monocyte as defined by Geissmann and colleagues [9]. But does it absolutely relate to the CD14^{+}CD16^{+} monocytes described in the human?

The CD14^{+}CD16^{+} monocyte population is generally considered to act as an important pro-inflammatory effector subset based on their inflammation-related amplification in the blood. According to Geissmann et al the human CD16^{+} monocytes share many features with mouse CX$_3$CR1^{hi} monocytes. They are not recruited to inflammations but "resident" cells and the precursors for the steady state Mϕ and DC. Both mouse (CX$_3$CR1^{hi}) and human CD16^{+} "resident" monocytes are negative for the pro-inflammatory chemokine receptor CCR2 [9], suggestive of their exclusion from inflamed tissues. Hence, a parallel can be drawn between the enlarged CD16^{+} pool during acute and chronic inflammations in the human [12-17] and the enlarged pool of Ly-6C^{low} monocytes in the NOD mouse that suffers from various (autoimmune) chronic inflammations. However, our data showing the larger pool of mature monocytes in the inflammation-free mice with the NOD background, suggest that this expansion in the NOD mouse is most likely due to an enhanced generation of monocytes in the BM determined by the genetic background. This further implies that the imbalance towards mature forms of circulating monocytes relates to the proneness to develop (autoimmune) chronic inflammations rather than to the presence of such inflammations per se.

Our data point to the possibility that the Ly-6C^{high}/Ly-6C^{low} monocyte subdivision in the mouse might not completely correspond with the CD14/CD16 division in the human. Quantitatively, there are clearly more Ly-6C^{low} monocytes in normal mouse blood than CD16^{+} monocytes in normal human blood (40% vs. 10%, respectively). Also, while we here describe that Ly-6C^{high} monocytes have a selective capability to adhere to fibronectin and ICAM-1, we previously reported that both CD16^{-} and CD16^{+} monocytes are capable of such adherence [18]. Finally there is the discrepancy between the populations in autoimmune diabetes: the frequency of CD16^{+} circulating mature monocytes in human cases was not raised, while we here show more mature Ly-6C^{low} monocytes in the NOD mouse blood.

In fact, a better overlap might be between the Ly-6C^{high}/Ly-6C^{low} subdivision and the distinction on the basis of fibronectin-adherence, since Ly-6C^{high} monocytes in normal mice have a clearly raised ability to adhere to fibronectin like the "P-monocytes" in humans [20]. However the overlap between the Ly-6C^{high} and the "P-monocytes" is also not absolute. In patients with T1D, we found a clearly raised adherence of

monocytes to fibronectin (i.e. a raised number of "P-monocytes"). We also found raised numbers of NOD monocytes that adhered to fibronectin, if taken irrespectively of whether they expressed the Ly-6C. Yet, the number of Ly-6C^{high} monocytes (normally the fibronectin-adhering population in the mouse blood) was not raised (on the contrary). Taken together, the functional flexibility of monocytes might make a definition of monocyte subpopulations troublesome.

As mentioned, the mature NOD monocyte population had the same high adhesive properties to fibronectin and ICAM-1 as that of NOD immature monocytes. This subset of in principal "non-inflammatory" monocytes hence with characteristics of inflammation-related monocytes, implies that the normal down regulation of this characteristic during monocyte maturation in the blood had not taken place in the NOD mouse.
Perhaps the abnormal Ly-6C molecule in myeloid cells of the NOD mouse plays a role in this aberrancy [24]. Cross-linking of Ly-6C molecules induces integrin expression on the cell surface and has been associated with cell adhesion [25]. It is unclear whether the recombination in the Ly-6C gene changed the function of this molecule in the NOD mice and will aberrantly influence the expression and avidity of integrins involved in fibronectin and ICAM-1 binding (It is here also important to note that the somewhat lower Ly-6C expression on the immature Ly-6C^{high} NOD monocytes did not prevent the accurate separation of the NOD monocytes in our experiments).

With regard to the capability of blood monocytes of the NOD mouse to differentiate into cells with a DC- or Mϕ-like phenotype, we here describe a tendency of both immature and mature monocytes to preferentially differentiate *in vitro* into cells with a Mϕ-like phenotype, i.e. F4/80^{high}CD11c^{low}MHCII^{low} cells. This strengthens our previously expressed view – using BM precursors - that a differentiation into the DC direction is hampered in NOD mice and skewed into the Mϕ direction [26]. This abnormally skewed production of Mϕ-like cells could be due to dysfunctional signaling pathways. This brings to mind the high NF-κB activity, that has been found previously in NOD mouse DC and Mϕ [27, 28].

In conclusion, mice with the NOD background show an enhanced production of monocytes when stimulated, larger numbers of mature monocytes in the periphery and a preferential development of Mϕ-like cells from both immature and mature forms of monocytes. We can only speculate on the contribution of the described phenomena to the proneness of NOD mice to develop various autoimmune conditions.

We excluded the possibility that they are the consequence of the autoimmune inflammations, since they were also present in pre-diabetic NOD mice and inflammation-protected NOD-H2b and NOR mice. Interestingly, in autoimmune prone SJL mice we also found a shifted ratio in circulating monocyte subpopulations, similar to the NOD (not shown).

So, if the here-described phenomena are causally related to autoimmunity, which could be the mechanisms? Firstly the larger number of mature forms of monocytes and Mϕ might support the destructive character of the autoimmune inflammations. In addition, the imbalance between the generation of DC and Mϕ from monocytes in favor of the Mϕ lineage may play a role. DC are essential not only in the induction of immunity, but also - and perhaps more importantly - in the tolerance maintenance. Indeed transfers with optimally functioning DC have proven to prevent the development of autoimmune diabetes in the NOD mouse [29-31]. Therefore, a deficient induction and maintenance of tolerance by insufficiently produced high-quality DC could form a key to the initiation of autoimmunity.

Acknowledgements

The investigation presented here was financially supported by the Dutch Diabetes Research Foundation (Grant 96.606). We would like to acknowledge the contribution of Samorah Pigot for the technical assistance. In addition, we thank Tar van Os for his important contribution in preparing the figures.

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Chapter 5

Conclusions and Discussion

Background of the study

Conclusions from the study

A state of the art view on mouse monocyte-derived dendritic cells and macrophages

Monocytes and monocyte-derived dendritic cells and macrophages in autoimmune-prone NOD mice

Future directions

Background of the study

The role of dendritic cells (DC) and macrophages (Mϕ) in the regulation of the immune homeostasis has been widely recognized. An important aspect of such regulation is the maintenance of immune tolerance. An inability of DC and Mϕ to adequately regulate immune responses, in either unengaged (steady state) or inflammatory situations leads to a potential break of tolerance and autoimmunity.

In the non-obese diabetic (NOD) mouse, autoimmune destruction of pancreatic β-cells leads to the insulin deficiency and diabetes. Antigen-presenting cells - DC, Mϕ and B cells - are involved in the diabetogenic process. B cells play an important part in the conversion of the benign peri-insulitis into a destructive insulitis process [1]. DC and Mϕ are involved in both the initiation and the progression of the autoimmune process, both locally (in the target organ) and in the lymphoid tissues (reviewed in [2]). NOD mice benefit from therapeutic regimens that target the antigen-presenting system.

An aberrant function of Mϕ and DC has been suggested to underlie the T1D not only in the NOD mouse, but also in the BB-DP rat and the human. Functional defects of Mϕ have been described in the NOD and other autoimmunity-prone mouse strains and include an abnormal cytokine production (an elevated interleukin IL-12 and an imbalance in TNF-α/IL-10), a low phagocytosis of apoptotic cells (that fails to induce an anti-inflammatory cytokine pattern) and a poor antigen-specific T cell stimulation by APC from the NOD spleen [3-8]. Investigations of the DC function, which employed a generation of these cells in vitro, have led to a conclusion that DC show an abnormal differentiation [9-14]. However, various observations from the in vitro system were not accompanied with similar findings in vivo, especially for DC. Aberrancies of purified spleen and lymph node DC have been found in NOD mice, yet they are relatively mild or disputed [15, 16]. Whether such DC defects affect T cell tolerance is largely unknown. The proposed abnormal selection and apoptosis of autoreactive T cells or the defects in the regulatory T cell pool might be caused by intrinsic defects in NOD mouse lymphocytes, induced by the aberrant DC or may be mediated by other cells (thymic epithelial cells, B cells, Mϕ).

This unclear contribution of DC to the tolerance defects was partially caused by the incompletely understood ontogeny of the DC system and the functional/developmental relations with other cells of the immune system. The approach described in this thesis mainly involved the application of monoclonal antibodies in the quest for a precise definition of the various differentiation stages of Mϕ and DC and their precursors in the normal mouse and in the diabetes-prone NOD mouse. Apart from that, depletion studies (using clodronate-loaded liposomes; lip-CL_2MDP) have been carried to study the kinetics of return of the Mϕ, DC and their precursors both in the circulation and in the pancreas of NOD mice.

Conclusions from the study

The following conclusions can be drawn from our studies:

1. DC and Mϕ return slowly to the islets after their depletion in NOD mice. In addition to the role in the induction of islet autoimmunity, they appear to be essential in the maintenance of T- and B-cells in the peri-insular infiltrate in later stages of the disease (Chapter 2).

2. DC and Mϕ share progenitors in the mouse BM. Three subpopulations are able to give rise to DC when cultured in the presence of GM-CSF:

   $$CD31^{high}Ly\text{-}6C^{-} \rightarrow CD31^{+}Ly\text{-}6C^{+} \rightarrow CD31^{-}Ly\text{-}6C^{high}$$

   The kinetics of DC differentiation indicates that the three populations are successive maturation stages of myeloid DC precursors. All precursor stages are also able to differentiate into Mϕ when stimulated with M-CSF (Chapter 3.1). In addition, the $CD31^{+}Ly\text{-}6C^{+}$ population also harbors the precursors for a new DC type, the $CD11b^{-}CD11c^{low}B220^{+}$ plasmacytoid DC (Chapter 3.4 and not shown)

3. Bone marrow of NOD mice contains normal numbers of DC progenitors with comparable phenotype. However, when stimulated *in vitro* with GM-CSF, the NOD DC progenitors exhibit a reduced proliferation capacity together with an accelerated differentiation (Chapter 3.2). Also *in vivo*, when circulating monocytes need to be rapidly replenished (e.g. after depletion), the NOD mouse is able to produce more monocytes per unit time in the first phases of this replenishment than control mouse strains (Chapter 4.2).

4. An *in vitro* stimulated generation of myeloid DC from NOD bone marrow results in aberrant DC with a macrophage-like phenotype. These aberrant DC are functionally defective and have a lower capacity to stimulate T cells as compared to normal myeloid DC (Chapter 3.3).

5. Mouse blood monocytes, an important precursor-reservoir for mouse DC and Mϕ, undergo maturation in the circulation. This results in two pools of blood monocytes, i.e. the $Ly\text{-}6C^{high}CD43^{low}$ immature monocytes and the $Ly\text{-}6C^{low}CD43^{high}$ mature monocytes (Chapter 4.1). The immature, inflammatory monocytes are predominantly recruited to sites of inflammation to become exudate Mϕ, in accord with their pro-inflammatory chemokine-receptor make-up and integrin-mediated adherence capability (Chapters 4.1 and 4.2). The resident, mature population is thought to be the precursor population for the steady state DC and Mf in the non-inflamed tissues. Resident mature monocytes have down regulated their inflammatory chemokine receptors and up regulated the receptor for fractalkine [10]; they have also reduced their integrin-mediated adherence capability (Chapter 4.2).

6. In the NOD mouse, and also in other strains with the NOD background, the mature ($Ly\text{-}6C^{low}CD43^{high}$) circulating pool of monocytes is expanded, but has abnormally retained a high integrin-mediated adherence capability. Futhermore, in the NOD mouse, not only bone marrow precursors (see before), but also immature and mature blood monocytes differentiate more readily into Mϕ-like cells than into DC-like cells (Chapter 4.2).

A state of the art view on mouse monocyte-derived dendritic cells and macrophages

The various differentiation/maturation stages of mouse monocytes as common dendritic cell and macrophage precursors

DC and Mϕ are widely spread throughout the body. All lymphoid and non-lymphoid organs and tissues contain at least one type of Mϕ and/or DC. Due to the enormous diversity and plasticity of the DC and Mϕ system and due the lack of appropriate methodology, the development of DC and Mϕ from their precursors was not known in detail. However, in the last few years, significant information has been collected by us and others about the developmental stages of the monocyte-derived DC and Mϕ in the mouse. Figure 1 represents a schematic overview of the development of mouse monocyte-derived DC and Mϕ through the three compartments/ phases, starting in the BM through the circulation into the tissues.

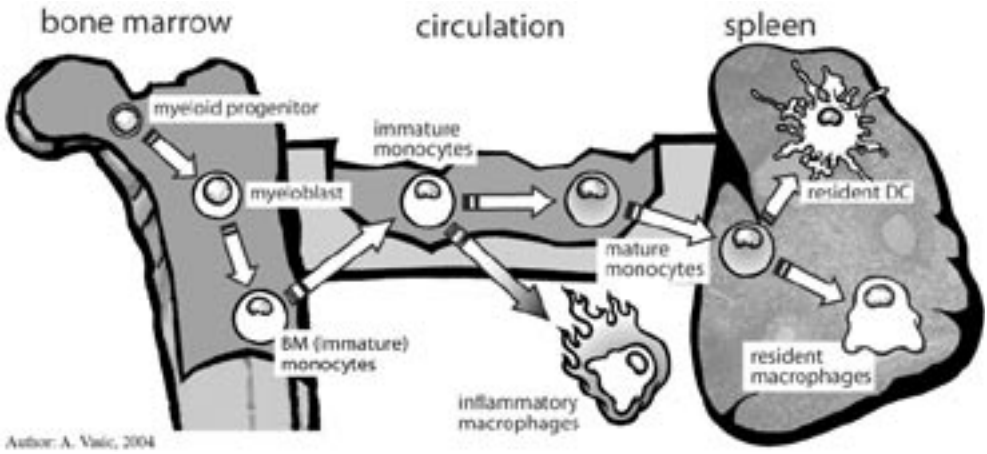

Figure 1. Maturation sequence of myeloid cells in the mouse. Three developmental stages can be defined in the bone marrow (BM). The last BM stage are immature monocytes, which mature in the circulation into monocytes able to give resident DC and Mϕ in the periphery. Immature monocytes can also develop into inflammatory macrophages, if they encounter inflammation-related signals

Phase I – Maturation in the BM to immature monocytes.

Three successive stages of the precursors for Mϕ have been defined previously in the BM of normal mice, using the markers Ly-6C and CD31 [18]. In the study described in Chapter 3.1, we show that myeloid DC also derive from these precursor populations and share their precursors with Mϕ throughout the development in the BM [19]. Furthermore, we observed that an initial stimulation of the common DC/Mϕ progenitors with M-CSF did not deprive the cells from the capacity to develop into DC upon switching to GM-CSF. This demonstrates that over a prolonged period of development, progenitors of the mononuclear phagocyte system are capable to either developing into Mϕ or DC, depending on local conditions. Therefore, a maturation sequence of the Mϕ/DC progenitors in the BM, defined by Ly-6C and CD31

molecules goes as follows:

$$CD31^{high}Ly\text{-}6C^{-} \rightarrow CD31^{+}Ly\text{-}6C^{+} \rightarrow CD31^{-}Ly\text{-}6C^{high}$$

With regard to the plasmacytoid DC, Bruno et al demonstrated that the $CD31^{+}Ly\text{-}6C^{+}$ population also contains the precursors for IFNα producing cells [20], which were the same as the $B220^{+}$ PDC (not shown). In addition, it has recently been demonstrated that early precursors for all DC subtypes reside within the $Flt3^{+}$ precursor population in the BM, regardless of their lymphoid or myeloid lineage orientation [21].

Phase II – Maturation in the blood to mature monocytes.

The last stage of the DC/ Mϕ precursor development in the mouse BM represents monocytes. The cells are characterized histo-morphologically and have a high expression of the Ly-6C molecule. In the circulation, the name "inflammatory" monocytes was designated to the same cells, [17]. These monocytes have the capacity to rapidly extravasate and migrate to the inflammation site, where they develop into inflammatory exudate Mϕ [17, 22]. In addition, the expression of CD62L enables these monocytes to enter the lymph node through the HEV [23, 24].

As reported in Chapter 4.1, we demonstrated that the monocytes undergo a maturation step in the circulation, through which they loose Ly-6C, CCR2 and CD62L molecules (and therefore the ability to enter inflammations and lymph nodes) and gain CD43, CD11c, fractalkine receptor and several other markers [22]. Therefore we identified the $Ly\text{-}6C^{low}$ monocytes as mature, and others as resident monocytes [17]. Apart from the change in phenotype, the maturation of monocytes also included the reduction of the ability to adhere to ICAM-1 and fibronectin when compared to the immature monocytes (Chapter 4.2). Mature, resident monocytes probably still have a high endocytic capacity as judged by their capacity to engulf and break up the lip-CL_2MDP (Chapter 4.1).

Peripheral non-inflamed compartments, such as the spleen and the peritoneal cavity contain almost solely mature monocytes (Chapter 2 and 4.1). In addition, transferred $Ly\text{-}6C^{low}$ monocytes preferentially migrate to peripheral tissues, such as lungs and liver, independently of whether the transfer was performed in healthy mice or in an inflammatory setting [17]. Therefore, the fate of mature monocytes is to develop into resident DC and Mϕ of non-inflamed tissues. To enter non-inflamed tissues via blood endothelium, mature monocytes probably utilize adhesion molecules different than those employed by the immature monocytes, such as CD11c and CD31 chemo-attracted via the fractalkine receptor. With regard to the entry into the spleen, mature $Ly\text{-}6C^{low}$ monocytes are able to reach this lymphoid organ directly without a migration through endothelial cell layers, i.e. via the sinuses in the red pulp.

It is not clear which interactions or stimuli are needed to promote the maturation of $Ly\text{-}6C^{high}$ into $Ly\text{-}6C^{low}$ monocytes. Since $Ly\text{-}6C^{high}$ monocytes are integrin-adherent cells, a (transient) adhesion to endothelium might form a key stimulus. Soluble factors like hormones, cytokines or chemokines might play a role as well. Alternatively, monocytes might already be programmed in the BM to undergo a maturation sequence in a certain time-window. In this time window the cells are able to react to inflammation-related stimuli. If such stimulus were absent, cells would spontaneously undergo maturation to become $Ly\text{-}6C^{low}$ monocytes with a different destiny.

Phase III – Maturation in the periphery to DC and Mϕ.

In vitro, various growth factors and stimuli have been defined that regulate the transition of monocytes to DC or Mϕ (GM-CSF, IL-4, M-CSF, Flt3-L, TLR, etc), yet the factors that determine the differentiation step of mature monocytes in the tissues *in vivo* towards Mϕ or DC are largely unknown. *In vivo*, this step is probably regulated by a combination of intracellular events induced through intimate contacts with other cells (e.g. endothelial cells in reversed transmigration) and/or with soluble factors or factors bound to the extracellular matrix, that monocytes meet upon arrival in the tissues. In such view local factors of inflammatory and non-inflammatory character determine which particular morphological, phenotypic and most importantly functional properties monocyte-derived cells will have. Alternatively, it might also be possible that pre-programming steps in the BM and/or the blood have prepared the cells to differentiate into distinct directions.

The potential of monocytes to differentiate into both DC and Mϕ *in vitro* or *in vivo*, depending on the used growth factor, has been shown by us and others before [18, 19, 25]. Here we show that mature monocytes (Chapter 4.1) are capable to spontaneously differentiate into both DC-like ($CD11c^{+}MHCII^{hi}$) and Mϕ-like ($F4/80^{high}MHCII^{low}$) cells. Due to the practical inability to purify high monocyte numbers for functional studies, we cannot firmly state that these cells are indeed functional DC and Mϕ. The obtained cells are reminiscent of the immature DC and Mϕ that need additional stimuli to mature into functionally competent cells. An investigation on whether the here described "spontaneously" generated immature DC and Mϕ could be diverged into only DC or only Mϕ would shed more light on the question until which point these two cell types blend together.

The terminal differentiation of the immature steady state DC and Mϕ into potent cytokine-producing professional APC requires an additional stimulus. Depending on the strength as well as the quality of the activating stimulus, DC and Mϕ will become either strong immune inducers or strong immune suppressors. The current dogma dictates that antigens met in a non-inflammatory context will lead to APC that regulate/suppress immunity (so-called tolerogenic DC) (rev in [26]). Interestingly, the maturation level at which DC have a tolerogenic function, acquired through the uptake of the antigen under "non-danger" conditions, does not represent an end-stage, since these tolerogenic DC can be turned into immune stimulators; e.g. upon CD40 ligation [27].

Relation of the Ly-$6C^{high}$ and Ly-$6C^{low}$ monocytes to other monocyte-like cells reported in the mouse

In the last few years, other groups have described monocytes and monocyte-like cells, which in our view overlap or are closely related to either Ly-$6C^{high}$ or Ly-$6C^{low}$ monocytes.

1. A precursor population for monocyte-derived DC1 cells has recently been reported in the mouse blood [28]. When stimulated with GM-CSF and TNFα *in vitro*, these $CD11c^{low}CD45RA^{-}$ mouse pre-DC developed into mature $MHCII^{hi}$ DC that stimulated T cell proliferation. Based on a closer analysis of the used cell purification from the blood (PBMC separation; the separation of the low-density fraction on the Nycodenz; depletion of CD3, CD19 and Gr-1 positive cells and thereafter of auto-fluorescent and $DX5^{+}$ cells)

and the phenotype of the pre-DC ($CD11b^+CD11c^{low}CD45RA^-$), it is highly likely that this population overlaps with our mature monocyte ($Ly\text{-}6C^{low}$) population.

2. Cells involved in the innate immunity and tumor-mediated suppression are also closely related or even overlap with immature blood monocytes. Serbina et al recently described a TNF/iNOS- producing DC population in the spleen that mediates an innate immune defense against infectious agents, for which immature ($Ly\text{-}6C^{high}$) monocytes are likely precursors [29, 30]. Interestingly, cells with similar phenotype, $CD11b^+Gr\text{-}1^+CD31^+$, so-called iMacs, were also found to produce iNOS and to mediate an immune-suppression in the tumor bearing and in the immuno-suppressed mice [31, 32]. However, the iMacs population contains probably both granulocytes and $Ly\text{-}6C^{high}$ monocytes. We therefore doubt whether the claimed suppressor-function can solely be attributed to the monocytes in this cell fraction.

3. Apart from being the precursors for mature APC, or involved in the innate immunity, as proposed by the groups of Geismann and Shortman, the mature monocytes might also have another function. In a recent study, cells with a potential to stimulate the generation of antigen specific regulatory T cells could be generated *in vitro* using as growth factors GM-CSF in combination with IL-10. These tolerogenic DC could also be isolated from the spleen as $B220^-CD11c^{low}CD11b^+CD45RB^+$ cells [33] and are thus clearly of myeloid origin. We were able to find the same $CD11c^{low}CD11b^+CD45RB^+$ cells in fresh mouse spleen suspensions, but found these cells to overlap with the $CD11b^+CD43^{hi}CD11c^{low}Ly\text{-}6C^{low}$ mature monocytes (Chapter 2). Therefore the Th/Tr stimulating profile of spleen mature monocytes needs to be tested to make a decesive link between the two populations.

Comparison of $Ly\text{-}6C^{high}$ /$Ly\text{-}6C^{low}$ monocytes to plasmacytoid dendritic cells

We were able to identify three pre-DC populations in the mouse blood: $CD11b^+CD11c^-Ly\text{-}6C^{high}$ - immature monocytes, $CD11b^+CD11c^{low}Ly\text{-}6C^{low}$ - mature monocytes and $CD11b^-CD11c^{low}B220^+$ - plasmacytoid DC. These three cell groups share some functional properties, and differ in others. The overview of their comparison is presented in the Table 1.
All three types of cells are **precursors for the mouse APC**. They can all differentiate into DC of different sort (Chapter 4.1, [25, 34]), but only the myeloid-related monocytes are able to differentiate into Mϕ (Chapter 4.1,[25]).
Functionally, a direct **contribution to innate immunity** is clear for immature monocytes and PDC. Yet, the two groups of cells produce different mediators to exert this function (TNFa/MCP-1 and IFNα, respectively) (unpublished observation and [34]). In addition, PDC play a part in the **manipulation of the adaptive immune response**, which is the task for monocytes as well. However, PDC exert this function in both the steady state and in inflammation; monocytes have divided these tasks between the two subsets [17, 35, 36]. PDC mediate the stimulation of both Th1 and Th2 immune response [36]. In addition, an **immune regulatory role** of the PDC has been suggested in the steady state [35, 37]. Immature monocytes are under inflammatory conditions engaged in innate immunity and in the steady state will proceed toward the mature circulating monocyte-stage, which will finally contribute to adaptive immunity.

Monocytes and PDC also differ in **migration patterns**. Immature monocytes enter both lymph nodes and site of inflammation; mature monocytes exclusively go to non-inflamed tissues, while PDC home only to the T cell areas in spleen and lymph nodes (Chapter 4.1, [17, 37]).

Taken together, the three circulating DC-precursor populations differently contribute to pro- and the anti- inflammatory immune forces. Their traffic in the steady state or in response to danger signals is dynamic and controlled by cytokines and chemokines. They are responsive to different signals and able to stimulate different aspects of the immune response. Further studies are needed to provide detailed knowledge on the importance of the balance between these three populations of DC precursors in the circulation for the maintenance of immune homeostasis.

Table 1. Specific and shared properties of the two subsets of monocytes and PDC

	Ly-6C^{hi} Mo[1]	Ly-6C^{lo} Mo	PDC
Differentiation			
Macrophage	yes	yes	no
Dendritic cell	yes	yes	yes
Function			
Activation of innate immunity	yes	no	yes
Activation of adaptive immunity	no	yes	yes
Regulation of immune response	no	yes	yes
Migration			
Lymphoid organs			
Steady-state	no	yes[?]	yes
Inflammation	yes	no	yes
Non-lymphoid tissues			
Steady-state	no	yes	no
Inflammation	yes	no	no

[1] monocytes; [?] not clear

Monocytes and monocyte-derived dendritic cells and macrophages in autoimmune-prone NOD mice

Monocytopoiesis in the NOD mouse bone marrow – an accelerated maturation at the expense of cell proliferation under extreme conditions?

In BM and blood DC and Mϕ precursors went through the same developmental stages in the NOD mouse as in the C57BL mouse, i.e. through the three stages in the BM and the two maturation stages of monocytes in the blood.

However, when isolated from the natural surroundings (bone marrow) and stimulated with GM-CSF *in vitro*, the earliest progenitors ($CD31^{hi}Ly\text{-}6C^{-}$) and the myeloblasts ($CD31^{+}Ly\text{-}6C^{+}$) isolated from the NOD mouse BM exhibited an aberrant differentiation pace. The cells rapidly differentiated into $CD11c^{+}$ cells, without going through several proliferation cycles necessary for renewal and high output such as C57BL cells did. We found a similar reduction in precursor renewal when undivided NOD BM was cultured in the presence of GM-CSF (Chapter 3.3.). When stimulated with Flt3-L, the expansion rate was any way small in the cultures of all mice strains (not shown). However, the number of obtained myeloid DC was in particular low in the NOD cultures, again pointing to a potential problem of the NOD mouse to continuously produce sufficient numbers of myeloid DC due to a diminution of precursor pools.
In contrast to these *in vitro* stimulation assays, we did not observe any quantitative deficiencies of DC or precursor monocytes *in vivo* in the NOD mouse. After a complete depletion of phagocytic cells by lip-CL_2MDP in the spleen, a normal functional DC pool was replaced in both the NOD and the C57BL mouse within one week (Chapter 2). Furthermore the experiments in which we depleted blood monocytes by lip-CL_2MDP (Chapter 4.2) demonstrated that also blood monocytes reached original levels after 8 days in both the NOD and C57BL. This excludes the *in vivo* existence of a deficient continuous renewal of precursors for DC in the NOD mouse.

However, it must also be noted that after lip-CL_2MDP depletion of blood monocytes in the NOD mouse there was an even higher release of immature monocytes from the BM noticeable 2 days after depletion in comparison to C57BL mice. Is this faster release in the first 48 hrs related to the potency for an accelerated differentiation of NOD bone marrow DC precursors *in vitro* under the above-described cytokine-stimulated conditions?

The larger mature pool of circulating monocytes in NOD mice.

NOD mice had an abnormal high number of mature $Ly\text{-}6C^{low}$ monocytes in circulation and this phenomenon appeared to be intrinsic to the NOD background, since NOR and NOD-H2b mice also showed this altered balance. After depletion, the re-appearance kinetics showed that immature $Ly\text{-}6C^{high}$ monocytes returned more rapidly to the circulation in NOD mice, while the transition of immature to mature monocytes had normal kinetics. This suggests that the BM in NOD mice is able to release more monocytes to ensure the restoration of a larger mature circulating compartment in the same period. However, this transition to mature monocytes in the NOD mouse is not ordinary. While normally $Ly\text{-}6C^{low}$ mature monocytes down regulate their capability to adhere to fibronectin and ICAM-1, mature NOD monocytes retained this

high adhesive pro-inflammatory property.

These data in autoimmunity-prone mice, when interpreted in light of findings in human diabetic patients [38], point to the possibility that the Ly-6C^{high}/Ly-6C^{low} monocyte subdivision in the mouse does not correspond with the CD14/CD16 division in the human, as was previously suggested [17]: while we here show more mature Ly-6C^{low} monocytes in NOD mouse blood, the frequency of CD16^{+} circulating mature monocytes in human type 1 diabetes (T1D) is not raised. Moreover, there are quantitatively clearly more Ly-6C^{low} monocytes in normal mouse blood than CD16^{+} monocytes in normal human blood. Also, while we here describe that Ly-6C^{low} monocytes have a low capability to adhere to fibronectin and ICAM-1 as compared to Ly-6C^{high} cells, we previously reported that CD16^{-} and CD16^{+} monocytes have the same capability for such adherence [38]. Hence, a word of caution is necessary in trying to define monocyte subpopulations. The functional flexibility of the cells might make such definition troublesome. Monocyte compartment is flexible and dynamic and it is probably more worthwhile to characterize monocytes functionally and on the basis of complex patterns of CD molecule expression in different situations. Subdivision of the cells suggests a fixation in states, which might not be compatible with the inherent property of monocytes to change in order to adapt to their surroundings.

The preferential *in vitro* maturation of NOD myeloid precursors into Mϕ and Mϕ-like DC with a reduced T cell stimulatory capacity

Others have previously reported that NOD BM cells require the presence of IL-4 in addition to GM-CSF and IFNγ in addition to M-CSF to induce a complete maturation to DC and Mϕ respectively [11, 14, 39-41]. Also in our experience GM-CSF alone was insufficient in the NOD mouse system to induce the *in vitro* maturation of normally competent DC: the cells had a reduced capability to stimulate T cells. Moreover we found a clear skewing of the cells into a Mϕ direction. In Chapter 3.3 we report that the DC in the NOD GM-CSF-stimulated BM culture had an expression pattern of surface molecules typical of Mϕ: FcRg II/III, SR-A, F4/80 and Gr-1. Interestingly, up regulation of the same surface molecules, characterized as the induction to differentiate into Mϕ, occurred when myeloid precursor (M1) cells were transfected with the human GM-CSF receptor (GMR) and stimulated with hGM-CSF *in vitro* [42].

A common feature of NOD cells is to develop into Mϕ or Mϕ-like cells. Besides BM precursors, also sorted monocytes from the NOD mouse blood demonstrated a spontaneous maturation into cells with a Mϕ phenotype (Chapter 4.2). Whether these cells are indeed real functional Mϕ, and which typical Mϕ functions they would be able to perform, or more related to the Mϕ-like DC as we found in GM-CSF stimulated cultures, remains to be investigated.

Increased adherence capacity might be related to an unusual behavior of NOD cells *in vitro*. In all studies in NOD mice in which we found Mϕ-like cells or the preferential development into Mϕ, increased adherence was also evident. Mature monocytes, which overnight promptly acquired a Mϕ-like phenotype, had an unusual capacity to adhere to fibronectin and ICAM-1 (Chapter 4.2). The Mϕ-like cells from the GM-CSF-stimulated NOD BM (Chapter 3.3) were also strongly adherent and stretched on the bottom of the culture dish. Finally, when the sorted BM progenitors were cultured in the Teflon bags (a culture condition that does not promote adherence) (Chapter 3.2), the majority of the NOD cells were stretched and adherent on the bottom of the bag. In the latter study, we did not check for the Mϕ markers, but the preferential

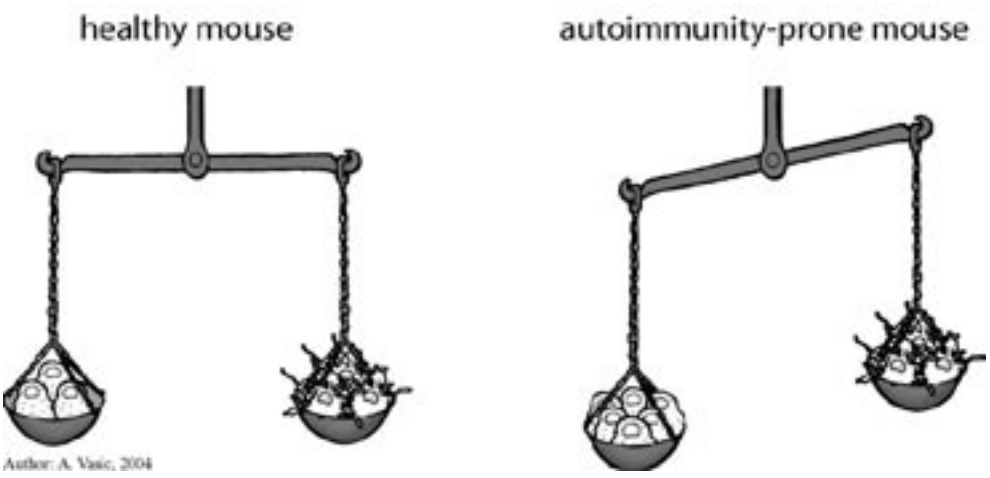

Figure 2. Skewed maturation of myeloid progenitor cells towards Mϕ in the NOD mouse might underlie pronness to autoimmunity. In the normal, healthy situation, a proper balance between the anti-inflammatory and pro-inflammatory forces (in this case viewed as the balance between DC and Mϕ) secures the proper function of the immune system. In the autoimmunity-prone NOD mouse, the pro-inflammatory forces tip the balance and disturb a proper regulation of the immune system.

maturation without expansion pointed to an atypical behavior of these cells as well.

It is generally assumed that Mϕ adhere more and stronger to surfaces than DC. However, DC stimulated overnight with LPS also adhere to the bottom of the culture dish. Since we observed a rapid maturation in all above-mentioned studies, in our system we cannot discriminate between attachment related to an increased maturation or to Mϕ-like characteristics of the cells.

The putative mechanisms that might underlie the aberrant maturation pathways in the NOD mouse

In the process of cell development, progenitors proliferate and differentiate, yet en route on the developmental pathway, the proliferative and lineage potentials are progressively lost while specific differentiated functions are acquired [43]. The factors that are required for the proliferation and differentiation of specific cell types have largely been identified, but the mechanisms for the coordination of proliferation and differentiation and the mechanisms that ultimately ensure a permanent exit from the cell cycle in terminally differentiated cells are poorly understood. Differentiation and proliferation are often viewed as antagonistic, however mechanisms that control these different processes work concomitantly and probably influence each other. There are signaling pathways that play a role in both of the processes, but also those that are specific for either proliferation or differentiation. For example, mechanisms that inhibit proliferation and support Mϕ differentiation are becoming more clear [44]. They include CREB proteins that indirectly stimulate growth-arrest and promote Mϕ differentiation. Interestingly, the same factors were found to regulate ICAM-1 expression, adhesion and cell morphology in U937 leukemia cell-line [45]. In contrast, Notch signaling inhibits myeloid differentiation and promotes expansion of hematopoietic stem/progenitor cells [46].

Our data suggest that the proliferation and differentiation capacity of NOD BM cells are not disturbed in the natural surroundings. All studies, in which we found a poor development

of competent DC from precursors and a preferential differentiation into Mϕ-like cells, were performed with cells that had been isolated and cultured *in vitro* (Chapter 3.3 and Chapter 4.2). We did not find the same aberrancies *in vivo*. Hence, support cells or the non-disrupted architecture in the tissues must play a prominent part in correcting the aberrancies we found *in vitro*. Direct contacts between the cells that support haematopoiesis and myeloid progenitors, as well as locally provided cytokines and growth factors, play an important role in the fate of the hematopoietic progenitors [47]. The NOD BM cells might be more dependent on the support from such stromal cells for proliferation and cell renewal than BM cells of other mouse strains. Isolation from this support and exposition to the differentiation-stimulating cytokine (e.g. GM-CSF) led NOD cells into a precocious maturation neglecting cell renewal.
Irrespective of their relevance *in vivo*, the poor renewal capacity of NOD myeloid precursors and the generation of NOD Mϕ-like DC found *in vitro* point in the direction of an aberrant function of signaling molecules down stream from the GM-CSF-R and other cytokine receptors.

The GM-CSF receptor consists of an α-chain (a low affinity receptor) and a β-chain; together they form a high affinity complex, capable of transducing a proliferative signal [48-50]. In mouse, the β-subunit of the GM-CSF receptor is shared with the IL-5 and in humans also with the IL-3 receptor. It is thus of interest that not only defective *in vitro* responses of NOD myeloid precursors to GM-CSF and Flt3-L have been found (this thesis), but also to IL-5 and IL-3 [51]. Interestingly, similar cell renewal defects as found here for the NOD myeloid precursors have been found when the signaling from the human GM-CSF receptor was inhibited in $CD34^+$ cells and cells subsequently stimulated with cytokines that signal through the b-subunit of this receptor [52]. However, the signal inhibition did not influence the lineage composition of the progeny, indicating that only part of the NOD differentiation problem is related to the might be related to β-chain of the GM-CSF receptor.

Problems in three major signaling pathways have been found in the NOD mouse. BM cells from the NOD mouse have a reduced ability to stimulate PKC-coupled second messenger pathways [41]. A mutation in the Stat5b gene within the Idd4 region has recently been found in the NOD mouse [53]. Interestingly, similar intrinsic defects to expand myeloid cells *in vitro*, like in NOD mice, have been found in STAT5 deficient mice [54]. Furthermore, stimulated NOD mouse BM cells (DC, Mϕ and B cells) exhibit a hyper-activation of the NF-κB pathway [3, 55, 56]. A faster activation of the ERK1/2 pathway after LPS stimulation of NOD peritoneal Mϕ, leading to a faulty regulation of the CD49d expression[57].

The three pathways found aberrant in NOD have been linked to cell functions and behavior. The NF-κB pathway is the major regulating mechanism for cytokine production and an abnormal cytokine production pattern found in NOD mice is caused by a hyper-activation of NF-κB [4, 58]. PI3K and ERK1/2 pathways are differentially involved in the stimulation of cell survival, cell proliferation, and cell death after stimulation with the LPS [59]. The importance of LPS-activated (TLR4) pathways is further illustrated by the observation that activation through LPS leads to a block conversion of inflammatory monocytes into DC and directs it into Mϕ [60]. This is precisely one of the aberrancies characteristic of the NOD found in our studies. It would be interesting to investigate this conversion *in vivo* in the NOD mouse, having in mind the skewed maturation of monocytes into Mϕ that we found *in vitro* and the fact that also other molecules like extracellular matrix proteins can be ligands for TLR4 [61] (Note that we also found an enhanced adhesion of mature circulating monocytes to fibronectin).

Can we relate the here-described aberrancies in NOD monocytes, DC and Mϕ to the proneness for autoimmunity in the NOD mouse?

The role of DC and Mϕ in the process of the peri-insular accumulation of lymphocytes in the NOD mouse has been suggested previously [62]. In chapter 2 of this thesis, we investigated the effect of the depletion of phagocytic cells (monocytes, DC and Mϕ) in NOD mice that have already progressed to an advanced stage of peri-insulitis. Both Mϕ and DC appeared to be important for the organization of the lymphocyte accumulations in the pancreas of NOD mice.

The transient depletion of the DC and Mϕ in the target organ also significantly delayed the progression to destructive insulitis. A beneficial role of phagocytic cell depletion to prevent diabetes development has been reported previously [63, 64]. The focus of our studies were the alterations in the (peri-)insular infiltrate as a result of the DC and Mϕ depletion and the new message from our study is that DC and Mϕ in the endocrine pancreas are not only important for the initiation of the inflammation and attraction of new lymphocytes to the site, but that they are also essentially required to keep lymphocytes at the spot. There are also other reports in non-autoimmune conditions that show that elimination of phagocytic cells reduce lymphocytic infiltration in the pancreas, e.g. lymphocyte infiltration is reduced by phagocytic cell depletion in transplanted fetal pig pancreas xenografts in NOD mice [65]. A similar vision of DC being important for the maintenance of autoimmune lesions has been proposed in the RIP-LCMV mouse model. [66]. Similarly, the loss of marginal zone Mph as the result of chronic Leishmania infection severely abrogates normal trafficking of lymphocytes in the white pulp of the spleen [67].

Interestingly, the DC and Mϕ came back to the NOD pancreas and were again capable of organizing the inflammatory infiltrate. This points to a persistent signal that comes from the pancreas to initiate a local inflammation. It is therefore unlikely that age-related defects in the pancreas or age-related defects in the function of APC and T cells cause the initiating signal for the lymphocyte accumulation.

How can we relate the in this thesis described enhanced production of NOD monocytes from NOD monoblasts, the raised numbers of mature NOD monocytes in the periphery and an *in vitro* aberrant development of Mϕ-like and less competent DC from NOD precursors to the proneness of NOD mice to develop various autoimmune conditions?

To begin with, we can exclude the possibility that the above described phenomena are the consequence of the autoimmune inflammations, since they were also present in pre-diabetic NOD mice and inflammation-protected NOD-H2b and NOR mice. So, if the here-described phenomena are causally related to autoimmunity, the question of the mechanisms causing them remains.

Mϕ are generally considered as pro-inflammatory cells. In such view, the larger number of mature forms of monocytes and Mϕ might support the destructive character of the autoimmune inflammations in the tissues. However an opposite point of view can also be taken by considering Mϕ as anti-inflammatory. In the steady state, Mϕ together with DC mediate the uptake of apoptotic cells as a physiological mechanism of the disposal of old and non-functioning cells. Disposal of apoptotic cells and maintenance of T cell tolerance are linked by the fact that phagocytes that take up apoptotic cells produce anti-inflammatory mediators such as IL-10 or TGFβ [68-70] and uptake of apoptotic cells by Mϕ actively modulates the

cells and prevents autoimmunity [59, 71]. It is therefore noteworthy that a decreased uptake of apoptotic cells by NOD Mϕ has been found [7], which might deprive them from an anti-inflammatory potential and render most NOD Mϕ pro-inflammatory. Indeed, a imbalanced cytokine production has been found in NOD and SJL mice [6]. In addition, mice with genetic defects in the disposal of apoptotic cells or in which Mϕ had a reduced ability to take up apoptotic cells, had constitutive activated APC and were prone to develop autoimmunity [72-74]. However, these mice developed a lupus-like systemic autoimmunity. Therefore, we cannot directly translate these findings to the diabetes in NOD mice. However, in view of the shared and similar cytokine imbalance with SJL mice and in view of the fact that antinuclear antibodies have also been found in NOD mice [75], a Mϕ defect as exemplified in a deficient uptake of apoptotic cells might contribute to the proneness to autoimmunity in general.

In addition, the imbalance between the generation of DC and Mϕ from NOD bone marrow precursors and from NOD monocytes may play a role. DC were produced in lower quantities and had a reduced T cell stimulatory capacity. DC are essential not only in the induction of immunity, but also - and perhaps more importantly - in both central and peripheral tolerance. Furthermore, self-antigens that originate from dying cells, molecule shedding or direct capture from live cells, are presented by DC in draining lymph nodes, in which the highly autoreactive cells that encounter the congnate self either die or rapidly expand and acquire the activated T_{reg} phenotype [76, 77]. Therefore, a deficient induction and maintenance of tolerance by insufficiently produced high-quality DC could form a key to the initiation of autoimmunity in the NOD mouse. Compensation of such a defect could be accomplished by inducing optimally functioning DC. Indeed, transfers of competent DC have proven to prevent the development of autoimmune diabetes in the NOD mouse [39, 78-80].

Last but not least it must be noted that we did not find indications that the NOD mouse possesses relatively more Mϕ than DC *in vivo* and that the spleen and lymph node DC were functionally defective *in vivo*, although the *in vitro* aberrancies were clear. Hence, our *in vitr*o data may just reflect the aberrancies found in intracellular signalling in the NOD mouse, i.e. the reduced ability to stimulate PKC-coupled second messenger pathways [41], the mutation in the Stat5b gene within the Idd4 region [53], the hyper-activation of the NF-κB pathway [3, 55, 56] and the faster activation of the ERK1/2 pathway after LPS stimulation [57]. In such view these signaling aberrancies might also play a role *in vivo* in the function of cells other than the DC and Mϕ or in the function of monocytes, DC and Mϕ in the pancreas, cell populations which we have not tested.

Future directions

Studies presented in this thesis contribute to a better understanding of the normal development and the heterogeneity of the monocyte-macrophage-dendritic cell system. Studies in which the development of this system was addressed in the NOD mouse resulted in the discovery of a plethora of (sometimes) discrete aberrancies of which the relevance is not yet clear. In fact more questions can now be raised than answers given regarding the role of this system in autoimmune diabetes. Several directions for follow-up investigations can be proposed; whether all of them are equally appealing to pursue remains to be seen.

The various members of the monocyte-macrophage-dendritic cell system contribute in different ways to immune homeostasis. Since we are now familiar with the differentiation pathways and means to define different stages of cell development in the monocyte-macrophage-dendritic cell system and since imaging systems have become available to study labeled cells *in vivo*, studies become feasible in which precursors would be labeled to follow the development into the three cell groups (Mϕ, DC and PDC) and possibly their behavior in the NOD mouse pancreas and immune system *in vivo*. This would significantly contribute to our understanding of the development and function of the system in these mice. It must be noted in this respect that it is difficult to separate precursor and monocyte subpopulations without functionally influencing the cells. More sensitive techniques of cell labeling or applying transgenic mice might be helpful to unravel above posed questions. Such approach would be particularly valuable since it would provide not only means to investigate the role of the system in the pathophysiology of autoimmune diabetes but would also provide the technology to (exogenously) manipulate cells of the monocyte-macrophage-dendritic cell system for therapeutic applications.

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Immune system protects us from harmful microbes and tumor development. At the same time, the immune system makes sure that the unnecessary immune reaction against harmless foreign substances (known as antigens) or self-originating structures (self-antigens) either does not occur or is stopped before it induces irreparable damage to a healthy organ. Therefore, the immune system is able to make a distinction between the "dangerous" and the "harmless" irrespective of its origin. If a "dangerous" is encountered, defense mechanisms are activated that generate an inflammation. Elimination of the inflammation inducers leads to a deceleration of inflammation and a wound healing, which is actively regulated. The response against harmless antigens (either foreign or self) is also actively suppressed and is called tolerance. The mechanisms utilized for the controlled activation and inhibition of the inflammation and for the tolerance acquisition enable the balanced function of the immune system, called immune regulation.

In some situations the immune regulation can be disturbed and the immune system starts to destroy healthy cells leading to an irreparable damage. This action of the immune system is called an autoimmune reaction and as a consequence an autoimmune disease develops. Such a process directed against β-cells in the islets of Langerhans in the pancreas leads to the autoimmune disease termed type 1 diabetes (also known as a sugar disease).

Macrophages (Mϕ) and dendritic cells (DC) importantly contribute to the proper function of the immune system. These two cell types comprise a heterogeneous group of cells called mononuclear phagocytes, which differ in the phenotype, function or the origin. They are sentinels that reside in all organs and are the first that encounter the infectious agents, transformed cells, or some other harmful substances. As an immediate reaction, they activate the inborn immunity and start an inflammatory reaction that subsequently leads to the activation of other immune forces. Later, they also mediate the reduction of the inflammation and help the wound healing. In addition, they are also important for the activation of the specific immune forces (including tolerance): DC take up the antigen, present it to other cells of the immune system and transduce a signal whether that antigen should be destroyed or ignored. Taken together, mononuclear phagocytes can perform several different function and enable a balanced function of the immune system, called homeostasis.

Besides heterogeneity in the function, the origin of DC and Mϕ can be diverse. Most of them are generated from the bone marrow (BM). The origin of DC can be myeloid and lymphoid while most Mϕ are of myeloid origin. Myeloid DC have a close developmental link with Mϕ, but can perform quite distinct functions. It has not been clear at which point in the development they start to diverge.

Taken together, immune tolerance/regulation prevents the development of autoimunity. DC and Mϕ are actively involved in these processes. Therefore we have hypothesized that a proper immune regulation/tolerance depends on a correct function of DC and Mϕ; abnormal function of these cells will lead to the break of tolerance en autoimmunity. Furthermore, abnormal development could precede an abnormal cell function. Therefore, in the mouse that spontaneously develops autoimmune diabetes (Non-Obese Diabetic - NOD mouse), abnormal development of DC and Mϕ leads to the failure of immune regulation and the autoimmunity. Therefore, we have focused our investigation on the developmental pathway of DC and Mϕ from the BM of healthy and autoimmune NOD mice.

The experimental approach described in this thesis mainly involves application of monoclonal antibodies, which are generated to recognize the specific proteins present on a

cell surface. In this way, we could label the cells in quest for the precise definition of various differentiation stages of Mf and DC and their precursors in a normal mouse and in the diabetes-prone NOD mouse.

Previous investigation has shown that the NOD mouse pancreas contains higher number of DC and Mϕ from the earliest diabetes stages on, even before any other sign of autoimmunity is present. However, it is hard to evaluate from the histological studies whether the contribution of these cells in the pancreas is beneficial or harmful for the organ. Experimental methods applicable in a NOD mouse would not allow the isolation of substantial numbers of viable DC and/or Mϕ from the pancreas. Therefore no substantial information existed about the functionality of these cells in the pancreas, once the autoimmune process has already started. Therefore, we performed study (presented in the **Chapter 2**) in which we interrupted the autoimmune process in the pancreas by depleting phagocytic cells (DC and Mϕ) with clodronate-loaded liposomes (lip-CL_2MDP). In this study we observed that the disappearance of DC and Mϕ from the pancreas did not follow the same time-line as in the spleen or the circulation. They started to disappear from the pancreas at the time that both the blood and the spleen were almost completely reloaded with new DC and Mϕ. Interestingly, the disappearance of DC and Mϕ from the pancreas resulted in the complete clearance of other inflammatory cells (which are not sensitive to lip-CL_2MDP), leading to the significant delay of destructive autoimmunity in the pancreas. However, this protection was not absolute and several weeks after the treatment with lip-CL_2MDP, DC and Mϕ returned to the islets and the autoimmune inflammation developed again in the NOD pancreas.
Taken together, data from this study pointed to a possibility of a beneficial treatment of the ongoing destruction in the pancreas by modifying the cells of the mononuclear phagocyte system.

In the **Chapter 3** of this book, four studies encompass the investigation of the system of DC and Mϕ precursors in the BM. In the first study (Chapter 3.1), we investigated whether the DC and Mϕ share progenitors in the mouse bone marrow. We found that three subpopulations were able to give rise to DC, when cultured in the presence of GM-CSF:

myeloid progenitors → myeloblasts → BM monocytes

The kinetics of DC differentiation from these cells indicated that the three populations were successive maturation stages of the myeloid DC precursors. Cell at all three stages were also able to differentiate into Mϕ when stimulated with M-CSF.
In the Chapter 3.2, we investigated the BM of NOD mice and found no significant differences when compared to the healthy (C57BL) mice. The NOD BM also had normal numbers of DC progenitors with comparable phenotype to the C57BL mouse BM. However, when stimulated *in vitro* with GM-CSF, the NOD DC progenitors exhibited a reduced proliferation capacity and an accelerated differentiation.
Similar abnormal behavior of the NOD BM cells *in vitro* was apparent in the following study, in which a GM-CSF stimulated generation of myeloid DC from NOD bone marrow resulted in aberrant DC with a macrophage-like phenotype. These aberrant DC were functionally defective and had a lower capacity to stimulate T cells as compared to normal myeloid DC (Chapter 3.3).
In the fourth study of the chapter 3, we identified an additional DC population. Those were the $CD11b^{-}CD11c^{low}B220^{+}$ plasmacytoid DC, which were the recirculating cells present in the

BM, but also all other lymphoid organs that we investigated. These cells were the representatives of the DC of lymphoid origin in the mouse.

Taken together, the three circulating DC-precursor populations differently contribute to pro- and the anti- inflammatory immune forces. Their traffic in the steady state or in response to danger signals is dynamic and controlled by cytokines and chemokines. They are responsive to different signals and able to stimulate different aspects of the immune response. Further studies are needed to provide detailed knowledge on the importance of the balance between these three populations of DC precursors in the circulation for the maintenance of immune homeostasis.

The last developmental form of DC and Mϕ precursors in the BM is the monocyte. In the **Chapter 4** we investigated further transformation of the monocytes in the blood on their way to become DC and Mϕ in the peripheral tissues. In the chapter 4.1, we identified this conversion through the differential expression of three molecules (CD11c, CD43 and Ly-6C) on the monocyte surface. The starting monocyte population had a high expression of Ly-6C and was CD11c and CD43 negative. The conversion included the gradual loss of the Ly-6C and acquisition of CD43 and CD11c. Although the gradual change resulted in a "smear" of different phenotypes (as presented in the Figure 1 of this chapter), majority of cells were localized in two easily definable groups, which we named immature (Ly-6C^{high}) and mature (Ly-6C^{low}) monocytes, referring to their development stage.
This change of the cell phenotype also affected their differentiation capacity. While the immature monocytes were able to rapidly migrate to the inflammation site and develop into exudates Mf, the mature monocytes failed to do so. The latter cells were ready to respond to the different kind of stimuli and move to the periphery to develop into Mϕ and DC that function in a non-compromised situation.

When we analyzed the same cells in the NOD mouse blood (Chapter 4.2), we found higher number than normal of mature monocytes and these cells had combined mature and immature properties. This pointed to a different maturation of monocytes in the NOD blood, which may influence their capacity to develop into Mϕ or DC. Indeed, when we isolated the NOD monocytes and let them mature overnight, like the BM precursors in the Chapter 3.3, they differentiated more readily into Mϕ-like cells than into DC-like cells. The indication of the abnormally fast maturation *in vivo*, when circulating monocytes needed to be rapidly replenished (e.i. after depletion with lip-CL_2MDP), the NOD mouse was able to produce more monocytes per unit time in the first phases of this replenishment than control mouse strains (Chapter 4.2).

In conclusion, the maturation of DC and Mϕ precursors in the NOD mouse follows the normal pathway when the phenotypic changes are taken in account. However, the maturation of these cells occurs much faster in the NOD mouse and this influences the functional properties of generated cells. This accelerated maturation of common DC and Mϕ precursors also affects their balanced maturation into these two cell types since they preferentially mature into Mϕ and NOD DC. Shifting the developmental balance between the DC and Mϕ potentially disturbs the balance between the pro- and anti-inflammatory forces, which unlocks the key to the initiation of autoimmunity in the NOD mouse.

Het afweer (immuun) systeem is een systeem van cellen die ons verdedigt tegen gevaarlijke microben maar ook tegen tumoren die kunnen ontstaan. De eiwitten die het immuunsysteem kan herkennen en bestrijden, noemen we antigenen. Ons immuunsysteem is in staat om onderscheid te maken tussen vreemde antigenen en antigenen afkomstig uit het eigen lichaam (zelf-antigenen). Echter, de verdedigingsmechanismen kunnen worden aangezet tegen schadelijke antigenen ongeacht of ze van oorsprong vreemd of eigen zijn. Dit noemen we de ontstekingsreactie. Als de schadelijke prikkels geëlimineerd zijn, wordt de ontstekingsreactie geremd en het beschadigde weefsel geneest. Als de prikkels voor een langere periode aanwezig zijn ontwikkelt zich een chronische ontsteking, die grote schade in een weefsel kan veroorzaken. Als er een ontstekingsreactie tegen onschadelijke prikkels ontstaat, wordt dit door het immuun systeem actief afgeremd en dit proces heet tolerantie. Er zijn dus mechanismen in ons lichaam die zorgen voor de gebalanceerde functie van het immuun systeem ook wel immuun regulatie genoemd.
Helaas functioneert de immuun regulatie in sommige situaties niet zoals het hoort. De ontstekingsreactie kan zich tegen eigen cellen keren en dit kan tot onherstelbare schade in het lichaam leiden. Zo'n reactie noemt men "auto-immuun reactie" (immuniteit tegen eigen) en de ziekten die ontwikkelen als gevolg van deze immuniteit worden "auto-immuunziekten" genoemd. Een van de auto-immuunziekten is diabetes of suikerziekte. Diabetes ontwikkelt zich als gevolg van een immuunreactie tegen β-cellen in de eilandjes van Langerhans in de pancreas (alvleesklier). De β-cellen maken het hormoon insuline. Insuline zorgt voor de opname van suiker (glucose) die kan worden gebruikt als energie bron. Als er meer dan 80% van de β-cellen vernietigd worden ontstaat er een tekort aan insuline en zo ontstaat suikerziekte.

Macrofagen (Mϕ) en dendritische cellen (DC) behoren tot de cellen van het immuunsysteem en ze zijn heel belangrijk voor het goede functioneren ervan. Deze twee typen cellen vormen een heterogene groep cellen (mononucleaire fagocyten) die zich onderscheiden door hun vorm (fenotype), door de functies die ze uitvoeren en door hun afkomst. De Mϕ en DC bevinden zich overwegend in het bindweefsel van alle organen. In deze positie, komen ze als eerste in aanraking met infectieverwekkers, getransformeerde cellen en andere gevaarlijke stoffen. Op deze manier functioneren ze als wachters van het immuunsysteem. Samen met andere mechanismen van het aangeboren (niet-specifiek) immuunsysteem, nemen Mϕ en DC dus plaats in de eerste verdedigingslijn. Zij fagocyteren ook de onbekende stoffen en vertonen stukjes ervan aan andere cellen van het immuunsysteem (T cellen). Hierbij stimuleren ze T cellen om op een bepaalde manier tegen een bepaald antigen te reageren. Mϕ en DC kunnen dus ook stimuleren en activeren de cellen van het verworven (specifiek) immuunsysteem. Mononucleaire fagocyten zorgen in latere fase ook voor het verzachten van een ontsteking en/of helpen bij het helen van wonden. Bovendien zijn er een aantal soorten mononucleaire fagocyten die belangrijk zijn voor immuun tolerantie. Kortom, DC en Mϕ zijn belangrijk voor het evenwichtig functioneren van het immuunsysteem (immuun homeostase).
Naast de verschillen in vorm en functie, variëren de mononucleaire fagocyten ook in hun afkomst. Cellen met dezelfde functie kunnen een verschillende afkomst hebben en cellen van dezelfde afkomst kunnen verschillende functies hebben. DC en Mϕ staan tijdens hun ontwikkeling onder invloed van factoren die ze tegenkomen in de omgeving waar ze verblijven. Zo kunnen onrijpe DC met dezelfde afkomst zich ontwikkelen in rijpe DC met

verschillende eigenschappen, afhankelijk van het weefsel waar ze uiteindelijk terechtkomen. Dit bevordert hun heterogeniteit.
De meeste DC en Mϕ zijn afkomstig uit het beenmerg. De onrijpe beenmergcellen van waaruit ze zich ontwikkelen heten precursoren (voorlopercellen). DC precursoren kunnen van afkomst lymphoid (verwant met lymfocyten - specifiek afweer) of myeloid (verwant met granulocyten - aspecifiek afweer) zijn. Mϕ precursoren zijn voornamelijk myeloid. Daardoor zijn Mϕ en myeloide DC vaak beschouwd als soortgelijke cellen die andere functies uitvoeren. Het was alleen niet bekend op welke punt de vertakking in hun ontwikkeling plaatsvindt.
Hoewel DC en Mϕ uit dezelfde precursoren komen, voeren ze als rijpe cellen verschillende (soms tegenovergestelde) functies uit. Wij stellen in onze hypothese dat het goede verloop van de immuun regulatie gedeeltelijk afhankelijk is van het goede verloop van DC en Mϕ ontwikkeling. Verder, stellen wij in dat een abnormale ontwikkeling van DC en Mϕ in ons proefdiermodel, Non-Obese Diabetic (NOD) muis, een basis vormt voor de abnormale functie van de immuun regulatie en leidt tot auto-immuniteit en diabetes. De NOD muis is en proefdier die, net als de mens, spontaan diabetes kan ontwikkelen.

Als experimentele aanpak in dit project hebben wij voornamelijk gebruik gemaakt van monoklonale antilichamen (eiwitten die aan specifieke antigenen binden) die gemaakt zijn om bepaalde antigenen op de oppervlakte van Mϕ, DC en hun voorlopercellen te herkennen. Hiermee waren wij in staat om onderscheid te maken tussen verschillende ontwikkelingsstadia van de DC en Mϕ zowel in de gezonde als in de NOD muizen.

Eerder onderzoek in de NOD muis heeft aangetoond dat DC en Mϕ aanwezig zijn in de pancreas van de NOD muis vanaf de vroegste stadia van diabetes ontwikkeling. Zelfs op het moment dat er geen aanwijzing is van een actieve auto-immuniteit, zijn er verhoogde aantallen van DC en Mϕ gevonden in de pancreas van een jonge NOD muis, vergeleken met de gezonde controle muizen. Dit aantal bleef hoog in de NOD muis tijdens het hele auto-immuun proces. Toch, was onduidelijk welke functie deze cellen in de pancreas uitvoeren.
In hoofdstuk 2 van dit proefschrift hebben we de studie beschreven waarin we met behulp van liposomen (lip-CL_2MDP) de pancreas van de NOD muis hebben vrij gemaakt van DC en Mϕ op het moment dat de auto-immuun reactie al vol aan de gang was. Deze liposomen waren alleen giftig voor DC en Mϕ en niet voor andere cellen die in de pancreas aanwezig waren. Toch, was het hele ontstekingsproces platgelegd vanaf het moment dat DC en Mϕ weg waren. Dit resultaat heeft duidelijk aangetoond dat DC en Mϕ van essentieel belang zijn voor het activeren en onderhouden van de ontstekingsreactie in de NOD pancreas.

Aangezien onze hypothese was dat de afwijkende ontwikkeling van DC een bijdrage levert aan het ontstaan van auto-immuniteit, hebben we in het vervolg studies gekeken naar de ontwikkeling van dit celtype uit het beenmerg.
In hoofdstuk 3 hebben wij gekeken naar de *in vitro* ontwikkeling van de DC voorlopers. Uit de analyse van het beenmerg van een gezonde muis is gebleken dat DC en Mϕ tot de laatste stap van hun ontwikkeling uit dezelfde voorlopers gekweekt kunnen worden (Hoofdstuk 3.1). Wij hebben dezelfde ontwikkelingsfasen in het beenmerg van de NOD muis gevonden. Alleen ontwikkelden gingen deze cellen sneller dan normaal (Hoofdstuk 3.2). De snelle ontwikkeling van de NOD beenmerg voorlopers tijdens de kweek leidde tot de aanmaak van

verkeerde DC die de eigenschappen van zowel DC als Mϕ hadden. Deze cellen waren niet goed in staat om T cellen te stimuleren (Hoofdstuk 3.3).
Tenslotte, hoofdstuk 3.4. beschrijft een studie waarin wij een nieuwe cel type in het beenmerg van een muis gevonden hebben. Deze cellen zijn ook DC die een lymphoide afkomst hebben.

Hoofdstuk 4 bestaat uit twee studies. Hierin hebben wij gekeken naar de verdere ontwikkeling van DC en Mϕ in de muis en met behulp van antilichamen tegen de eiwitten CD11c, CD43 en Ly-6C, hebben wij een manier gevonden om twee ontwikkelingsstadia van monocyten (directe voorgangers van DC en Mϕ) in de bloedbaan te onderscheiden (Hoofdstuk 4.1). Als eerste zijn er onrijpe monocyten, die in staat zijn om deel te nemen in een ontstekingsreactie (als zij de juiste prikkel krijgen) en die zich ontwikkelen tot ontstekingsmacrofagen. Als deze monocyten geen ontstekingprikkel tegenkomen, worden het rijpe monocyten. Deze rijpe monocyten zijn voorlopers van de Dc en Mf die in het gezonde bindweefsel zitten.
In hoofdstuk 4.2 laten we zien dat deze indeling van monocyten ook voor de NOD muis toegepast kan worden. We vinden wel dat de NOD muis meer rijpe monocyten in het bloed had dan normaal. De rijping van monocyten van de NOD muis was ook abnormaal snel en deze cellen werden sneller Mϕ dan DC, vergeleken met de normale muizen.

Samengevat kunnen we zeggen dat de ontwikkeling van DC en Mϕ uit de NOD muis beenmerg afwijkend is. De ontwikkeling van DC volgt alle normale stadia, maar wanneer het gestimuleerd wordt, verloopt het sneller in de NOD muis dan in een normale muis. Tijdens deze versnelde ontwikkeling, zijn NOD cellen gevoeliger dan normaal en wijken snel af richting macrofagen. Dit leidt tot de afwijkende balans tussen de activatie en de remming van het immuunsysteem een levert daardoor een bijdrage aan de verstoorde regulatie en ontwikkeling van auto-immuniteit in de NOD muis.

Абнормално развиће дендритичних ћелија код аутоимунитет-осјетљивог NOD миша

скраћени садржај

Имунски систем нам омогућава заштиту (имунитет) од опасних микроба и тумора. Протеини које имунски систем препознаје, и против којих усмјерава своје дјеловање, се називају антигени. Имунски систем је способан да разлучи да ли антигени потичу од "опасних" структура, које су у стању да нанесу штету организму (инфективни микроби или трансформисане туморске ћелије), или су "безопасни" мада могу бити сопственог или страног (полен биљака, прашина ...) поријекла. Уколико је примијећени антиген опасан по здравље, долази до активирања механизама урођеног имунитета и до запаљења. Поред тога, механизми специфичног имунитета се активирају и најчешће воде до елиминације извора опасности. Уколико се ово не деси, запаљење постаје хронично. Ако је страни агенс елиминисан, запаљење се смирује и оштећено ткиво зараста. Иницирање запаљења, као и његово заустављање су високо регулисани.

Поред иницијације и регулације запаљења, имунски систем је такође у стању да спријечи активацију имунског одговора према сопственим антигенима; ово је имунска толеранција. У појединим ситуацијама долази до нарушавања имунске регулације/толеранције те тако активност имунитета против сопствених ткива доводи до непоправљиве штете у организму. Оваква реакција је означена као аутоимунска реакција (имунитет према сопственом), а болести, које се развијају као посљедица овакве реакције, се називају аутоимунске болести. Једна од аутоимунских болести је и дијабетес или шећерна болест, која се развија као посљедица реакције према β-ћелијама у панкреасу (гуштерачи). β-ћелије производе хормон инсулин који је веома битан за уношење енергије у облику шећера (глукозе) у ћелије. Када више од 80% β-ћелија у панкреасу буде разорено, долази до недостатка инсулина, који организам не може компензовати и појављују се први знаци болести.

Макрофаги и дендритичне ћелије припадају ћелијама имунског система и врло су битне за његово правилно функционисање. У ствари, ова два типа ћелија сачињавају хетерогену групу, које се разликују по изгледу (фенотипу), функцијама које извршавају, или по поријеклу. Све различите врсте макрофага и дендритичних ћелија се једним именом називају мононуклеарни фагоцити. Они представљају чуваре који се налазе у везивном ткиву свих органа. Овако постављени, они први долазе у контакт са инфективним агенсима, трансформисаним ћелијама или другим опасним материјама. Оне фагоцитирају ("поједу") тај непознати агенс и његове елементе презентују другим ћелијама (тзв. специфичног имунског система, Т ћелијама) те им тако дају сигнале да ли да на одређени антиген реагују, или не, и на који начин. Ова посљедња способност се назива презентација антигена.

Функција мононуклеарних фагоцита може да буде различита, један тип ових ћелија је задужен за подстицање запаљења, други мононуклеарни фагоцити воде рачуна да се непотребно запаљење стиша или помазу зарашћивање рана. Коначно, одређене врсте мононуклеарних фагоцита су јако битне за имунску толеранцију. Укратко, дендритичне ћелије и макрофаги су битни за балансирану функцију имунског система која се назива имунска хомеостаза.

Поред разлика у изгледу и функцији, мононуклеарни фагоцити могу бити различитог поријекла. Већина дендритичних ћелија и макрофага се развија у коштној сржи, и по

поријеклу припадају ћелијама бијеле крвне лозе. Све бијеле крвне ћелије се развијају из ћелија које се називају прогенитори. Прогенитори за дендритичне ћелије и макрофаге могу да буду мијелоидни (сродни гранулоцитима) или лимфоидни (сродни лимфоцитима), па према томе дендритичне ћелије и макрофаги могу бити лимфоидног или мијелоидног поријекла. Сви макрофаги су углавном мијелоидног поријекла, док дендритичне ћелије могу бити и мијелоидне и лимфоидне. Мијелоидне дендритичне ћелије и макрофаги су, према томе, веома блиски по поријеклу, али могу извршавати врло различите функције и до недавно се није сасвим сигурно знало у ком тренутку њиховог развића долази до раздвајања ова два типа ћелија.

Правилна функција имунског система је условљена исправном функцијом ћелија припадника мононуклеарно-фагоцитног система. Да ли ће нека од ових ћелија исправно функционисати зависи и од њиховог развића. Због тога смо ми поставили хипотезу, да код експерименталних мишева који спонтано развијају аутоимуни дијабетес (NOD мишеви), абнормално развиће дендритичних ћелија и макрофага доприноси погрешној имунској регулацији. Фокус нашег истраживања у овом пројекту је био дефинисање развојних облика дендритичних ћелија и макрофага код здравих и NOD мишева.

Експериментални приступ описан у овој тези је углавном подразумијевао кориштење моноклоналних антитијела, која су направљена да препознају специфичне протеине на површини ћелија. На овај начин је било могуће "обиљежавати" ћелије у циљу њихове диференцијалне анализе и дефиниције специфичних фаза развића дендритичних ћелија и макрофага и њихових прекурсора, код здравих и дијабетичних NOD мишева.

Ранија истраживања су показала да су дендритичне ћелије и макрофаги присутни у увећаном броју у панкреасу младих NOD мишева (у моменту када још нема знакова аутоимунске реакције). Ове ћелије остају присутне у панкреасу током читавог деструктивног процеса у панкреасу. Ипак, из ранијих хистолошких студија је било тешко донијети закључак о стварном доприносу дендритичних ћелија и макрофага током деструктивног процеса, и питање да ли оне убрзавају или успоравају аутоимунски процес у панкреасу је остало неодговорено. Експерименталне методе које је могуће примијенити у моделу NOD миша нису омогућавале изоловање значајних количина вијабилних (живих) дендритичних ћелија и макрофага из панкреаса. Због тога није постојало довољно информација о функционалности ових ћелија у самом панкреасу у тренутку када је аутоимуни процес већ започео. У студији која је приказана у 2. поглављу у овој књизи, ми смо пореметили аутоимунски процес у панкреасу тако што смо елиминисали дендритичне ћелије и већину макрофага из панкреаса користећи липозоме у којима се налази клодронат (токсична супстанца). Пошто само дендритичне ћелије и макрофаги имају способност да "поједу" (фагоцитирају) липозоме, они су специфично елиминисани док на остале ћелије у панкреасу нисмо утицали. Липозоме смо инјектирали у стомак миша, чиме је постигнута њихова системска дистрибуција.

Занимљиво запажање проистекло из ове студије је да елиминација дендритичних ћелија и макрофага није пратила исту кинетику у панкреасу и осталим органима (у слезини или у крвотоку). Они су почели да нестају из панкреаса много касније него из осталих органа, и то у моменту када је слезина већ у потпуности била попуњена ново-развијеним дендритичним

ћелијама и макрофагима. Поред тога, иако смо липозомима директно елиминисали само дендритичне ћелије и макрофаге из панкреаса, у моменту када су ове ћелије почеле да нестају, и све остале ћелије имунског система (Т ћелије, В ћелије итд) су га напустиле. Као резултат, панкреас је био потпуно очишћен од запаљења, 4 недјеље након третмана липозомима. Ипак, ова промјена није била апсолутна. Послије 4 недјеље, дендритичне ћелије, макрофаги и све остале имунске ћелије су се полако вратиле у панкреас. Ипак, третирани мишеви су били значајно заштићени и већина њих није више развила дијабетес до краја периода праћења (25 недјеља касније).

Из ове студије смо закључили да је могуће утицати на развој дијабетеса и у моменту када је аутоимунски процес увелико започео, и то манипулацијом ћелија фагоцитног система, што отвара нове могућности у дизајнирању терапија за спречавање или успоравање развоја аутоимунског дијабетеса.

Поглавље 3 у овој књизи је сачињено од 4 студије чији је фокус био развиће дендритичних ћелија у костној сржи мишева. У првом и у другом раду (поглавља 3.1 и 3.2) смо се бавили дефинисањем развојних ступњева дендритичних ћелија код здравих и код NOD мишева. Уз помоћ моноклоналних антитијела, дефинисали смо три фазе развића дендритичних ћелија:

мијелоидне прогениторе → мијелобласте → моноците костне сржи

Ове три групе ћелија су биле познате и раније и знало се да су оне у стању да се развију у макрофаге, али се није знало да ли ове исте ћелије имају способност да дају и дендритичне ћелије. Такође смо показали да и код NOD мишева, све три популације имају исти потенцијал да се развију у дендритичне ћелије, а и да не постоје разлике у броју или фенотипу ових ћелија између здравих и болесних мишева. Ипак, када смо изоловали ове ћелије из костне сржи NOD миша и стимулисали их са GM-CSF-ом, фактором раста за дендритичне ћелије, примијетили смо да NOD ћелије почињу да сазријевају у дендритичне ћелије много брже него нормално, што им није давало времена да се умноже. Због тога је број дендритичних ћелија који би се развио из костне сржи NOD миша био значајно мањи него код контролних животиња. Ова разлика је била нарочито уочљива када су ћелије култивисане 7 дана (по класичном протоколу) (поглавље 3.3). Поред значајно мањег броја добијених ћелија, у овом случају ни њихов фенотип (изглед) није било нормалан нити су имале способност да активирају наивне Т ћелије (што је њихова типична функција). У ствари, добијене ћелије из костне сржи NOD миша су по изгледу и функцији више личиле на макрофаге него на дендритичне ћелије, указујући на њихово абнормално развиће.

Четврта студија у овом поглављу дефинише нову популацију ћелија, које су назване плазмацитоидне дендритичне ћелије. Ово је нова врста дендритичних ћелија које су по поријеклу лимфоидне и које смо пронашли не само у костној сржи него и у свим осталим органима које смо испитали. Функција ових ћелија није у потпуности расвијетљена. Оне највјероватније играју улогу у активирању имунског система као одговор на вирусне инфекције.

У четвртом поглављу смо пратили даљи развој моноцита након што напусте костну срж и уђу у крвоток/циркулацију. Пронашли смо да моноцити у крви доживљавају трансформацију на путу према периферним ткивима, гдје постају дендритичне ћелије и макрофаги. Ову трансформацију смо детектовали помоћу моноколналних антитијела која

препознају молекуле CD11c, CD43 и Ly-6C (тзв. маркере за моноците). Моноцити, када тек напусте костну срж, су само позитивни за маркер Ly-6C, да би касније постепено губили овај маркер, а добијали све више CD43 и CD11c молекула на својој површини. На овај начин смо дефинисали читаву скалу моноцита који су имале различите количине (експресију) ова три маркера на својој површини (фенотип). Ипак, већина моноцита је могла бити подијељена између двије фазе, које смо назвали Ly-6C^{high} (незрели) и Ly-6C^{low} (зрели) моноцити, именом алудирајући на њихов степен развоја. Промјена фенотипа моноцита је такође праћена промјеном њиховог развојног капацитета. Док су незрели моноцити у стању да убрзано мигрирају на мјесто запаљења и тамо се развију у макрофаге, зрели моноцити то не раде него реагују на сигнале друге врсте и имају способност да се развију у дендритичне ћелије или макрофаге у некомпромитованој ситуацији.

Анализа је, наравно, извршена и код NOD миша (у поглављу 4.2). Код NOD миша смо нашли вишак зрелих моноцита у крви и уз то су ове ћелије имале комбиноване особине зрелих и незрелих моноцита. Ово је указало на различито сазријевање моноцита код NOD миша, што је потенцијално указивало на њихово абнормално развиће. И стварно, када смо изоловали NOD моноците и оставили их да сазрију у култури преко ноћи, слично као и прекурсори из костне сржи, и NOD моноцити су убрзано сазрели у макрофаге а не у дендритичне ћелије. Индикација да се ово дешава и у стварности, у мишу, је произишла из експеримента у коме смо моноците из крви елиминисали употребом липозома и пратили којом се брзином поново развијају из костне сржи. Костна срж NOD миша је била у стању да производи много више моноцита у јединици времена него нормално.

У закључку, развој и сазријевање дендритичних ћелија и макрофага из прекурсора у NOD миша прати нормалан пут када се узме у обзир промјена фенотипа. Ипак, сазријевање се дешава много брже, што утиче на функцију насталих ћелија. Ово убрзано и абнормално сазријевање заједничких прекурсора за дендритичне ћелије и макрофаге тако утиче на балансиран развој ова два типа ћелија, пошто код NOD миша прекурсори постају прије макрофаги него дендритичне ћелије. Ово помијерање развојне равнотеже према макрофагима потенцијално мијења и равнотежу између активације и заустављања про- и анти- запаљењских процеса, чиме се откључава брава за активирање аутоимунитета код NOD мишева.

7-AAD	7 aminoactinomycin
Ag	antigen
AICD	activation induced cell death
APC	antigen-presenting cell
-APC	allophycocyanin
AP	acid phosphatase
BB-DP	Biobreeding diabetes prone
BSA	bovine serum albumin
BM	bone marrow
CD	cluster of differentiation
DC	dendritic cells
ELISA	enzyme-linked immuno sorbent assay
ER	Erasmus
FACS	fluorescence activated cell sorter
FITC	fluorescein isothiocyanate
Flt3-L	Flt-3 ligand
FCS	fetal calf serum
GM-CSF	granulocyte-macrophage colony stimulating factor
ICAM –	intracellular adhesion molecule
IDDM	insulin independent diabetes mellitus
IFN	interferon
IL	interleukin
IP-10	interferon inducivle protein-10
Lip-CL_2MDP	clodronate loaded liposomes
LN	lymph node
LPS	lipopolysaccharide
MLR	mixed leukocyte reaction
Mf	macrophages
M-CSF	macrophage-colony stimulating factor
MACS	magnet activated cell sorting
mAb	monoclonal antibody
MHC	major histocompatibility complex
MPS	mononuclear phagocyte system
NK	natural killer cell
NKT	natural killer T cell
NF-κB	nuclear factor kB
NOD	non-obese diabetic

PE	phycoerythrin
PI3K	phosphoinositide-3 kinase
PerCP	peridin chlorophyll protein
PBS	phosphate-buffered saline
RIP-LCMV	rat insulin promoter-lymphocytic choriomeningitis virus
RPMI	Roswell Park Memorial Institute medium
SAV	streptavidine
STAT	Signal Transducers and Activators of Transcription
TNF	tumor necrosis factor
Th	helper T cell
Treg	regulatory T cell

I have moved a lot in my life, changed towns, changed countries... I changed country even without moving, on several occasions ;o). This seems to have created a kind of pattern, reflected in other aspects of my life; I have changed labs quite a lot as well ;o)) In the last couple of years, since I'm The Netherlands, I have changed the lab three times (strangely though, I still work with some people I originally started with ;o)). This constant moving has one delightful consequence; I have met many people and made many friends. Therefore, there will be a lot of thanking and a lot of names in the coming page or two. I'm writing this text mostly in English because I want everyone to understand and know how much all of you mean to me.

It would be unfair to start from anyone else but my lab-mates from 853. Three groups in one lab under the hat of Willem van Ewijk, who encouraged the multinational nature of his group and made me feel like home in his lab (although we could not have chewing gums in the lab - rules and regulations!!!). People in 853 were not only my colleagues but also my friends. Many of them/you still are.

Katarina, hvala ti za moje prve uspiješne korake u labu. Imati nekog ko govori moj rođeni srpski (pardon hrvatski ;o)) jezik je bilo od nemjerivog značaja u momentu kada sam tek došla, a i kasnije. Jane, Judith, Rogier, Rita, Annabrita, Femke, it was (and still is) wonderful to know you and to work with you. We had much fun, with you I learned a lot, you helped me go through the rough times. Thanks. I'm looking forward to coming years of our friendship.

Rudi, thanks for my first published paper in this book. You taught me how to plan an experiment, but also many other things in research. Sabine, Gemma, Jan-Piet, Martijn, Rogier (weer! ;o), Kam-Wing, Petra, Ester, although we did not spend much time working together, it was very nice to share the lab with you.

Pieter, there are only few words that can describe how much I'm thankful for everything you have done for me. You introduced me to a real science and gave me a chance to develop. You have had the time and patience to help me organize my arrival here, when the situation in my country was far from normal. Many brainstorms with you, your hesitation to accept new views unless I provide enough experimental evidence for them (and unless they fit in the large scheme ;o) and an almost limitless freedom to plan experiments that would lead me to the truth, created a healthy and inspirational playground for me. You were also my friend and family when the real family was far and have always had "an ear" for my personal problems, boyfriends, looking for an apartment, driving exam…thank you.

Marleen, thanks for my first steps in the lab, teaching me to culture DC, to isolate and label antibodies. You were also my friend and I hope we will enjoy many more "ladiesnights" with the rest of the ladies.

My dearest paranimf, Berlinda, it was great to work with you but also to go out, chat in the coffee/tea breaks, do all the other things we did together and I'm sure we will keep doing.

Thanks for help and good times to my lab-colleagues from "Pieter's group", Adri, Marcel, and all other former and current members of the Autoimmunity Unit: MarjanV, Harm, Wai-Kwan, Vinod, Pieter S., Dariusz, Ester, Patricia, and also to Marjan V. and her group: Gerben, Jojaneke, Loneke, Corine, Hui, Rebecca, and all others I forgot to mention (not intentionally).

Prof. Drexhage, beste Hemmo, I always admired your firmness. The last few months were the most intensive in our communication ;o), I hope you did not find me too stubborn on some occasions. I always enjoyed our discussions, but also the nice new-years borrels at your place. Thank you very much for all the help during creation of this thesis.

My students, one for each year, Marjolijn, Anouk, Bernice, Milouda and Samorah, and of course Jeremy, it was challenging to take the responsibility to scientifically educate you. I hope you have had as much fun working with me as I have with you.

My AIO life would not be the same without the AIO weekends, which I enjoyed a lot, especially with my AIO club from '99 (Sabine, Lizette, Wai-Kwan) but also Gerben, Rogier (third time!!! ;o)), Patricia, Ester, Floor and the "older" AIO's Saskia, Wim, Martijn, Judith.

Nice memories from my AIO years at the Immunology department also include many other people. It seems unfair to miss any name but if I would start naming you all and everything each of you ment to me, I would need to write an extra book. So, you won't mind if I simply say thanks to you all, for all the help, nice lab-days, borrels and for making me feel like home on the 8th floor. I still need to name FC-DJ– Berlinda, Edwin, Marcia, Anja, Wouter with whom I improved my organizing skills ;o)). Also Rene, Harm en Edwin, who assisted me in the long sorting experiments, without which this thesis would be less interesting. A special thanks to Tar, for all the hours you have spend making the figures for me, and not only that, for being a friend, for teaching me stuff about graphic design.

I can't finish the part related to Erasmus MC without mentioning my new department, Longziekten, Prof. Hoogsteden en Dr. Bart Lambrecht, who helped me solve the "verblijfsvergunning" problem on time, and gave me the opportunity to spend unlimited amount of working hours working on my thesis. Also all the people from Longziekten, Annabrita, Danielle, Femke, Harmjan, Hermelijn, Ivette, Joost, Nanda, Monique, Karolina, Alex, Leonie, Hamida, Thomas, Hendrik-Jan, Bianca, Mirjam, Brigit and Lous, thanks for these wonderful months of laughter and joy that gave me the energy and patience to finish this book.

I also want to mention two special collaborators, Dr. Doug Drevets and Dr. Cord Suderkotter, who helped me open the door to the mononuclear phagocyte world.

Dearest Sacha, my "twin-AIO"; I put you in the middle between the colleagues' dear to me and my dear friends outside the EUR. You belong to both. Every time you would arrive from Paris was an intellectual holiday for me. You could challenge my thoughts in a productive way. Together, we created pretty nice studies. I hope we will work together one day. In the mean time, do not forget Rotterdam ;o))

In the remaining few sentences I would like to thank many friends here in The Netherlands, my old friends in Belgrade, Bijeljina and those scattered around the Europe or Canada, for being my friends ;o) Sometimes is that enough for help. Sanja, Bojana, hvala vam na vašem prijateljstvu, za sve što ste uradile za mene i pomogle mi da riješim probleme kojim sam obasipana prošle godine. Aleksandra i Bojane, moji drugari sa najduzim stažom ;o)) žao mi je što ne možemo češce da budemo zajedno ali vaša energija je prelazila duge kilometere i pomagala mi da istrajem. Mom ličnom sponzoru, teta Agneši hvala za potporu i finansijsku i moralnu, a naročito putem masaža koje čovjeka ožive i daju mu novu snagu. Aleksandre, riječi su izlišne; ipak, hvala ti za najljepše ilustracije na svijetu, za pomoć kad god je bila potrebna, za lijepu riječ a najviše za ljubav i strpljenje koje imaš za mene.

Mojoj široj porodici iz Crvene Crkve i užoj porodici iz Bijeljine ;o) puno hvala za bezgraničnu ljubav koja mi daje krila da letim. Mama i tata, vi ste ipak šampioni. Na kraju, Dragana, moja najdraža sisterko na svijetu, i moj saborcu u mnogim bitkama pa i ovoj .... drži mi fige …. idemo….

Tatjana Nikolic was born on October 1, 1973 in Zenica, Yugoslavia. After she had graduated from Gymnasium in Bijeljina, (currently in Republika Srpska, BiH) in 1992, she continued her education in Serbia at the University of Belgrade School of Biology. In 1997, Tanja completed her internship at the Institute of Medical Research at the Military Medical Academy in Belgrade and with honors obtained her Bachelor of Science degree.

This was her first encounter with the research in the field of Immunology. During the Master of Science studies (1997-1999) that she performed at the Toxopasmosis Laboratory of the Institute for Medical Research in Belgrade, her interest in the mechanisms of the innate immunity had evolved. During this period she was awarded two congress-awards from the European society for Microbiology and infectious diseases, as a young investigator, first in Hamburg, and a year later in Berlin, Germany.

In 2000 she moved to the Netherlands to pursue the search for the new knowledge during her PhD studies at the Department of Immunology of the Erasmus University in Rotterdam. The focus of her research was the development of dendritic cells in the diabetic mouse, which was performed under supervision of Prof Hemmo Drexhage and Dr. Pieter Leenen. She has been involved in collaborative projects at the Erasmus MC and with several groups in Europe and United States. From jan 2004, she is is employed at the Department of Pulmonary and Critical Care Medicine, Erasmus MC, Rotterdam, where she continued the work on the development and function of the cells of the innate immune system in the group of Dr. Bart Lambrecht.

Tatjana Nikolić, autor ove knjige, rođena je 1. oktobra 1973. godine u Zenici. Nakon završene srednje škole u Bijeljini, svoje školovanje nastavila je u Beogradu, gdje je studirala Molekularnu biologiju na Prirodno-matematičkom fakultetu Univeziteta u Beogradu. Diplomski rad pripremala je na Institutu za medicinska istraživanja Vojno-medicinske akademije u Beogradu i tada se po prvi put susrela sa istraživanjima na polju imunologije. U aprilu 1997. godine diplomirala je sa ocjenom 10.

U periodu do 1997. do 1999. godine radila je u Laboratoriji za toksoplazmozu u sklopu Instituta za medicinska istraživanja u Beogradu, gdje se ozbiljnije zainteresovala za istraživanje mehanizama urođenog imuniteta. Uporedo sa ovim istraživačkim radom, upisala je poslediplomske studije na Biološkom fakultetu u Beogradu. U toku ovog perioda, kao mladi istraživač, dobila je dvije međunarodne nagrade od Evropskog društva za mikrobiologiju i infektivne bolesti, prvo na kongresu u Hamburgu a godinu dana kasnije i u Berlinu.

Da bi nastavila sa naučno istraživačkim radom 2000. godine preselila se u Nizozemsku (Holandiju) gdje je nastavila sa doktorskim studijama na Odjeljenju za imunologiju Erasmus univerziteta u Roterdamu. Predmet njenih istraživanja, koja su se odvijala pod nadzorom Prof. Hemmo Drexhage-a i Dr. Pieter Leenen-a, bio je razvoj dendritičnih ćelija kod miševa sa dijabetesom. Učestvovala je u udruženim(zajedničkim) projektima u okviru samog Erasmus medicinskog centra, kao i sa nekoliko istraživačkih timova u Evropi i SAD-u.

Od januara 2004. godine radi u Odjeljenju za plućne bolesti u Erasmus medicinskom centru u Roterdamu, gdje je nastavila da istražuje razvoj i funkcije ćelija urođenog imunskog sistema, u grupi Dr. Bart Lambrecht-a.

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[#]shared author contribution

Stellingen behorende bij het proefschrift

Quo vadis

Aberrations in the Development of Dendritic Cells in the Autoimmunity-prone Non-Obese Diabetic Mouse

Rotterdam, 3 november 2004

Tatjana Nikolic

Stellingen

1. Prototypical macrophages and dendritic cells are the extremes in a continuum of functionally and/or developmentally related cells recognized as mononuclear phagocytes. *(this thesis)*

2. The terminal differentiation of monocytes into dendritic cells or macrophages is determined by signals in the connective tissue of the organ they populate. *(this thesis)*

3. Subdivision of monocytes suggests a fixation in states, which is not compatible with the inherent property of monocytes to change to adapt to their surroundings. *(this thesis)*

4. Problems in three major intracellular signaling pathways exist in the NOD mouse. *(this thesis)*

5. A deficient induction and maintenance of tolerance by insufficiently produced high-quality DC forms a key to the initiation of autoimmunity in the NOD mouse. *(this thesis)*

6. Initiation of the autoimmune response is entirely non-specific and depends only on the presence of misplaced peripherally activated pro-inflammatory monocytes/macrophages. *N. S. Stoy Medical Hypotheses (2002) 58(4), 312-326*

7. The protective and destructive activities of the immune system are comparable to the ambiguous acts of Brutus and his conspirators who assassinated Julius Caesar (15 March 44 B.C.), the leader they had sworn to protect.

8. Flow cytometry for the purpose of quantitative analysis of cellular properties is an invaluable research tool.

9. In an atomic system there exist a number of so-called *stationary states,* which possess a peculiar, mechanically unexplainable stability, of such a sort that every permanent change in the motion of the system must consist in a complete transition from one stationary state to another. *Niels Bohr*

10. All essential knowledge relates to existence; only such knowledge as has an essential relationship to existence is essential knowledge. *Soren Kierkegaard*

11. A researcher that has enough supplies of Cup-a-Soup sachets and Senseo coffee-pads does not have to worry about his future.