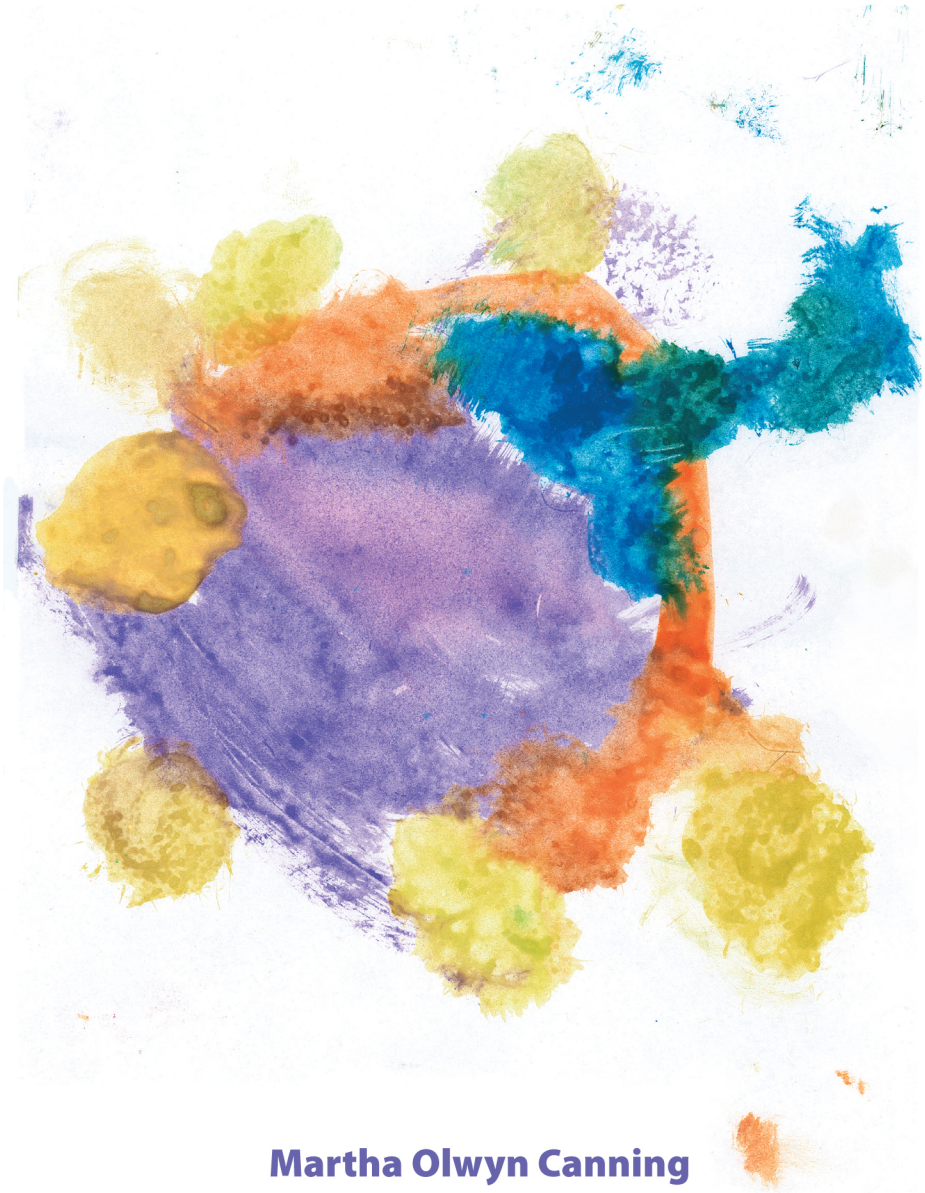
Dendritic cells and veiled accessory macrophages

Hormonal influences and autoimmune thyroid disease



Martha Olwyn Canning

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Front cover: dendritic cell by Julia Hunter Bonjer
Rear cover: dendritic cell by Emma Caroline Bonjer

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Dendritic Cells and Veiled Accessory Macrophages.
Hormonal Influences and Autoimmune Thyroid Disease.

Dendritische cellen en 'veiled accessory' macrofagen.
Hormonale invloeden en auto-immuunziekten van de schildklier.

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In memory of my mother

For Julia and Emma

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Chapter 1

Introduction

Adapted from M.O. Canning, C. Ruwhof, H.A. Drexhage. *Autoimmunity* 2003; 36: 429 – 42

I. THE SPECTRUM OF AUTOIMMUNE THYROID DISEASES IN THE HUMAN

Immune responses to thyroid specific autoantigens form the basis of autoimmune thyroid disease pathogenesis. Two polar forms of autoimmune reactivity of the thyroid gland exist in this disease spectrum: a catabolic form characterized by gradual inflammatory destruction of thyroid parenchyma leading to thyroid failure, and an anabolic form in which stimulation of the growth and metabolism of the thyroid parenchyma leads to goiter formation and hyperthyroidism. The catabolic form is best known as destructive autoimmune thyroiditis, whereas the anabolic form is generally referred to as Graves' disease.

Destructive Autoimmune Thyroiditis

Clinically the destructive autoimmune thyroiditis is sub-divided in Hashimoto goiter and atrophic thyroiditis. Both are characterized by infiltrates of immune cells, which destroy thyroid parenchyma, yet in Hashimoto thyroiditis there are re-growing thyroid follicles leading to goiter formation, while in atrophic thyroiditis re-growth is absent leading to a rapid atrophy.

Antibodies (Abs) involved in both subtypes are mainly directed toward the colloid and the thyroid cells, which themselves are players in the progression of the disease, expressing molecules of immunological interest such as HLA class I and II, CD40, adhesion molecules, cytokines and complement regulatory proteins (1). The most important target antigens of these autoantibodies are thyroperoxidase (TPO) and thyroglobulin (Tg) (Table I).

Table I.

Target Organ	Antigens	Indicative of (prevalence in disease)	Autoantibody Prevalence and Predictive Clinical Outcome
Thyroid	TPO/ TG	Hashimoto's Goiter (80-100%)	Healthy adults (5-15%) TPO antibody-positive
		Thyroid Atrophy (60-70%)	Females exhibit (per year) in 2-3% of cases a progression toward (subclinical) hypothyroidism
			TPO antibody-positive females with a serum TSH level > 6,0 mU/l* exhibit (per year) in 4-5% of cases a progression toward hypothyroidism
	TSH-R	Graves' disease (80-90%)	Healthy adults (0,3%)
		Thyroid Atrophy (30-40%)	The presence of antibodies always results in clinical manifestation of the disease. In pregnant females with (treated) Graves' disease or Thyroid Atrophy and positive TSH-R antibodies
			Congenital hyper- or hypothyroidism should be suspected in the neonate

Abs to TPO and Tg are found in high titre in the serum of 80-90% of patients with Hashimoto's disease, of 60-70% of atrophic thyroiditis patients, and of on average 10% of the normal healthy population. This latter prevalence is age and gender dependent, with females from

50 to 70 years of age displaying the highest prevalence of around 15% positivity of serum TPO-Abs. Since serum TPO-Abs are reliable markers of an existing thyroid autoimmune inflammation, it can be concluded that the incidence of autoimmune thyroiditis is considerable in the general population, particularly in "healthy" elderly women. This sub-clinical presence of autoimmune thyroiditis carries an increased risk of developing sub-clinical and overt thyroid failure (Table I).

TPO-Abs have recently been found to be significantly more prevalent in patients with bipolar disorder (28%) than population controls and psychiatric inpatients (3-18%) (2). The presence of TPO-Abs in bipolar patients was associated with thyroid failure, but not with age, gender, mood state, rapid cycling or lithium exposure.

Destructive autoimmune thyroiditis is also associated with neuro-endocrine disorders other than bipolar disorder: type 1 diabetes patients also exhibit a higher prevalence of this disease (3). Prevalences of up to 40% positivity for TPO-Abs have been found in type 1 diabetic patients and they develop thyroid failure in up to 10% of cases. This combination of type 1 diabetes and autoimmune thyroid failure is known as Polyglandular Syndrome (PGS) type 3a. Patients with atrophic gastritis/pernicious anemia have a high prevalence of TPO-Abs (30-50%) as well (3). Autoimmune thyroid failure occurs in 25% of patients with pernicious anemia; this combination is referred to as thyro-gastric disease or PGS type 3b.

Although TPO-Abs are a good marker of the destructive autoimmune thyroid process, they are not the actual effectors of the autoimmune destruction. Macrophages activated by auto-antigen specific T helper-1 (Th1) cells, cytotoxic CD8+ T cells, and apoptotic interactions between thyrocytes and these inflammatory cells are believed to play a more prominent role in the actual destruction of the thyroid parenchyma (4).

Receptor Antibodies

Although Tg and TPO are the best-known thyroidal antigens, others can also be the target of a thyroid autoimmune reaction. These other thyroidal antigens are f.i. the TSH-receptor and the IGF-1 receptor (5). These antibodies bind to these receptors and either mimic the binding of the actual ligand perfectly or imperfectly. The perfect mimicking receptor antibodies will lead to activation of the thyrocytes, i.e. the stimulation of thyroid hormone synthesis and growth (the so-called thyroid hormone stimulating antibodies, TSAbs, and the thyroid growth stimulating antibodies, TGAbs). The non-mimicking receptor antibodies will block the receptor and will lead to the blockade of hormone synthesis and growth (the so-called thyroid blocking antibodies (TBAbs)). The goiter in Hashimoto's disease results from the re-growth of destroyed thyroid follicles via a raised plasma TSH and the additional stimulation via stimulating antibodies, the TSAbs and TGAbs. Thyroid atrophy is due to the inability of the destroyed follicles to re-grow and respond to the raised TSH due to the presence of blocking antibodies, the TSBAbs and TGBAbs.

Graves' Disease

The clinical picture in Graves' disease is characterized by hyperthyroidism, a diffuse goiter and endocrine ophthalmopathy. This triad is referred to as the Merseberg triad.

The TSH-receptor (TSH-R) is the most important autoantigen in Graves' disease. Antibodies to this receptor are found in over 90% of patients with active disease. These Abs of IgG class are considered to be the direct cause of the disease, since monoclonal Abs to the TSH-R stimulate cAMP in cultured thyrocytes and since cAMP is involved in both hormone production and cellular proliferation of thyrocytes. Moreover, transplacentally transferred TSH-R Abs are able to induce Graves' hyperthyroidism in the neonate.

The antibody response to the TSH-R in Graves' patients is polyclonal and heterogeneous, and directed to various, partly overlapping, non-linear (conformational) epitopes in the TSH-binding as well as the non-TSH-binding domain of the TSH-R (5). In view of this heterogeneity of the TSH-R Abs and the coupling of the receptor to various 2nd messenger systems other than cAMP, it is not surprising that IgG fractions of Graves' sera are also capable of stimulating 2nd messenger systems belonging to the PLA2 and PLC pathways (6). There are even reports of Graves' IgG fractions that stimulate either the cAMP or the PLA2 pathway (6). Both varieties of TSH-R Abs are capable of inducing in vitro thyrocyte proliferation and when these varieties are found together in the serum of Graves' patients, these patients show the largest goiters and the most severe expression of hyperthyroidism (6).

There are also receptor antibodies that are not directed to the TSH-R but to another important receptor on thyrocytes, the IGF1-receptor (7,8,9). These antibodies are most likely synonymous with the previously described TGAbs in Graves' disease contributing to goiter formation in the virtual absence of stimulating hormone production.

The interactions of the various above described TSH-R and IGF1-R Abs may explain differences in symptom expression in Graves' patients, such as a strong hyperthyroidism with a small-to-absent goiter or the opposite, a hugh goiter with mild hyperthyroidism.

Ophthalmopathy is due to a retrobulbar autoimmune reaction, which has as its target the eye muscle and the retrobulbar pre-adipocytes (fibroblastic cells). Here again the main targets are considered to be the TSH-R and the IGF1-R on the retrobulbar pre-adipocytes (5).

II. ANIMAL MODELS OF AUTOIMMUNE THYROID DISEASE

Since the intensive and in depth study of patients has its obvious limitations, knowledge on the pathogenesis of autoimmune thyroid diseases has been gained also and often predominantly in studies on animal models of the disease. In the following paragraphs I would like to shortly introduce these animal models.

The Autoimmune Thyroiditis of the Obese Strain (OS) Chicken

One of the oldest models of endocrine organ-specific autoimmune disease is the Obese Strain (OS) Chicken, which suffers from a lymphocytic thyroiditis with a rapid onset of hypothyroidism (10). For the last 40 years chickens of the OS strain have been used to study the disease, which resembles severe destructive autoimmune thyroiditis of the human in many clinical, histopathological, serological and endocrinological aspects. Mononuclear cell infiltration of the thyroid gland commences in the second week after hatching and leads to an almost complete destruction of the thyroid architecture by 1-2 months of age. Limitations for extensive and up-to-date research in this model are the scarcity of immunological reagents for chickens and the absence of avian-cloned thyroid-specific genes.

The first genetic theory of endocrine organ-specific autoimmunity as a polygenic trait (1966) was proposed by Cole and based on breeding studies with this bird (11). The three-locus model of immune response MHC and non-MHC genes and genes coding for a hypothetical primary thyroid defect emerged from genetic analysis of OS families and from F2 crosses between OS and Cornell Strain (CS) chickens. Crossing experiments with another CS inbred line unrelated to OS revealed the existence of about 5 genes regulating the full development of the disease. Approximately three genes encode the susceptibility of the target organ to the attack by the immune system (one of them recessive) and the remaining one or two genes encode the hyper-reactivity of the immune system (12).

Iodine levels in food are an important environmental factor in the development of thyroiditis in the OS chicken, and the severity of the disease can be manipulated by iodine: Iodine deficiency attenuates, while iodine excess accelerates autoimmune thyroiditis (13). Iodine probably exerts these effects via inducing alterations in the metabolism of thyrocytes and even via toxic thyrocyte necrosis. Application of anti-oxidants delays the onset of the thyroiditis in the OS chicken, illustrating the importance of oxidative reactions in the toxicity of iodine (13). Iodine also has direct effects on the development and function of various immune cells (antigen-presenting cells, T cells and B cells) and the antigenicity of thyroglobulin (13,14).

The role of the stress system in the development of the disease in the chickens is illustrated by an altered immuno-endocrine communication via the HPA-axis in this strain of birds (15). The OS chicken shows a hypo-responsiveness to glucocorticoids and in particular to inhibitory factors released by this stress hormone in immune cells (15). Moreover, low levels of the central opioid peptide ß-endorphin have been shown in the hypothalamus of the OS chickens before onset of the disease, i.e. already at the embryonic stage. A further decrease in this brain peptide was observed in correspondence with the first signs of thyroid mononuclear infiltration (16).

The autoimmune thyroiditis of the BB-DP rat

The BB-DP rat is primarily a model for autoimmune diabetes (17). Inbred diabetes-prone BB (BB-DP) rats develop spontaneously a T cell-dependent, ketosis-prone diabetes and have a

profound T cell lymphopenia. BB-DP rats also suffer from a form of focal lymphocytic infiltrations in the thyroid that under normal conditions do not lead to hypothyroidism (18). These focal lymphocytic infiltrations show a high degree of architecture similar to that of secondary lymphoid organs (spleen and lymph nodes) with T cell zones, B cell follicles and high endothelial venules (HEV). These lymphocytic accumulations become more pronounced when the animals are fed a high iodine diet (19,20). Thyroid failure may become apparent after hemithyroidectomy of such animals.

There also exist sub-lines of the BB-DP rats, that are not lymphopenic and do not develop diabetes and thyroiditis. These lines are referred to as Diabetes Resistant or BB-DR. The lymphopenia of the BB-DP rat is primarily due to a lack of RT6+ T cells. RT6 is a marker for regulatory T cells. Transfers of RT6+ T cells from BB-DR rats to BB-DP rats prevent the development of diabetes and thyroiditis (17).

The autoimmune thyroiditis of the NOD mouse

The NOD mouse is – like the BB-DP rat - also predominantly studied for its autoimmune diabetes (21). NOD mice develop from an early age onwards (5 weeks) an initially non-destructive peri-insular accumulation of dendritic cells, accessory macrophages, T cells and B cells that persists for several weeks before it develops into a destructive form of insulitis (from 12 weeks of age onwards). Mild diabetes follows.

In the majority of the NOD strains there is only occasionally an association of diabetes with thyroid infiltrations (unlike in the BB-rat). In general the incidence of thyroiditis is very low in the NOD mouse, however it varies from colony to colony (22).

Certain dietary iodine regimens, however, have a triggering effect on thyroiditis development. When mice are made iodine deficient they develop a hyperplastic goiter. A single administration of a high dose of iodide to such mice has a necrotic effect on the hyperplastic iodine-deficient glands. In normal mice such dietary manipulation does not lead to thyroid autoimmunity. In NOD mice, however, it does lead to a Th1-mediated destructive autoimmune thyroiditis following the phase of early iodine-induced toxic thyrocyte necrosis (22). This again shows – as in the OS chicken - the importance of a local environmentally-induced factor (iodine-induced necrosis with a coinciding high antigen release and inflammation), which has to act in combination with a dys-regulated immune system (NOD mouse background) to start a full-blown autoimmune thyroiditis (22,23).

There are many genetic loci (over 15) on different chromosomes that associate with diabetes in the NOD mouse. The most important diabetic loci are linked to the MHC complex: NOD mice express an unique I-A locus, i.e. I-A g7 (histidine as residue number 56 and serine as residue 57, homologous to "diabetogenic" HLA-DQ ß non-aspartic acid 57 containing alleles in the human), but lack expression of I-Ea (homologous to DR α in humans) (24). There also exists a sub-line of NOD mice characterized by an alternative MHC haplotype, viz. the I-Ak allele instead of the I-Ag7 on the NOD background, and the mice are called NOD-$H2^{h4}$ mice.

These mice have under normal dietary conditions a prevalence of around 5% thyroiditis, but when kept on a continuously high iodine diet "spontaneously" develop autoimmune thyroiditis in virtually all animals (25).

Mouse thymectomy models of autoimmune thyroiditis

Thymectomy of Balb/C mice at day 3 results in a variety of organ-specific autoimmune diseases, including thyroiditis, gastritis, and oophoritis, but not insulitis (26). The inflammations are characterized by the presence of T cell infiltrates in the affected organs and the development of organ-specific antibodies in the serum. There is a strict temporal relationship between the development of the autoimmune syndrome and the day of thymectomy, which has to occur between the second and the fifth day after birth (27). Classically the model has been used to study oöphoritis and autoimmune gastritis (28). Autoimmune thyroiditis has hardly been studied in this model. The recent interest in CD4+CD25+ T cells as a specific subpopulation of thymus-derived suppressor or regulatory T cells has a clear historical association with the day 3 mouse thymectomy model (29,30). Day 3 neonatal thymectomy-induced autoimmune disease is due to a lack of CD4+CD25+ T cell migration into the periphery, since these regulatory cells typically migrate out of the thymus in this early period and since injection of purified CD4+CD25+ T cells into neonatally thymectomized mice prevents the development of autoimmunity, including autoimmune thyroiditis.

Animal models for "experimental allergic" Graves' disease

Classical models for the induction of organ-specific autoimmune disease are models that make use of immunizations with autoantigen in an adjuvant (the so-called "experimental allergic models"). A recent promising development in this area of experimental allergic diseases is the sensitization of mice with TSH-receptor (TSH-R) peptides, recombinant TSH-R preparations or with cDNA for the full-length human TSH-R cloned in an eukaryotic expression vector (genetic immunization) to create an animal model for Graves' disease (31). In these experiments it appeared easy to induce TSH-R antibodies (Abs) in all mice strains used with all the mentioned regimens. However the majority of the regimens were without any effect on the histology or function of the thyroid in most of the cases: Whereas H-2b and H-2k animals did not develop thyroiditis or thyroid function abnormalities, H-2d (Balb/C) mice did and some of these mice had hypo-thyroxinemia. Also NOD mice (H-2g7) developed thyroiditis and TSH-R Abs, and particularly in the NOD mouse model the thyroiditis was destructive and of Th1 character leading to clear hypothyroidism (32). This shows that the TSH-R is also able to induce destructive autoimmune thyroiditis, depending on the genetic background of an organism.

Hyper-thyroxinemia and orbital pathology (both clinical hallmarks of Graves' disease) were more difficult to induce using the above-described protocols. However two protocols showed some success:

1) immunizations of H-2k (AKR/N) mice intraperitoneally with MHC Class I identical fibroblasts double transfected with the TSH-R and MHC Class II led in 20% of cases to hyperthyroxinemia
2) immunizations of outbred Balb/C mice with TSH-R cDNA vectors led in 10% of cases to hyperthyroxinemia and TSH-R Abs that were able to stimulate c-AMP in cultured fibroblasts.

Even more interesting is the observation that transferring T cells from the latter mice to naïve mice (after an in vitro restimulation of the T cells with a recombinant TSH-R preparation) led to a Th1 thyroiditis with no signs of eye muscle infiltration when a NOD mouse model was used throughout. It led to a Th2 type thyroiditis with mild signs of eye muscle infiltration when Balb/C mice were used (31).

Recently it was shown that subcutaneous injections of dendritic cells, the most potent antigen-presenting cells (see below), infected with recombinant adenovirus expressing the TSH-R were able to induce a Graves'-like hyperthyroidism in female mice which was characterized by stimulating TSHR antibodies, elevated serum thyroxine levels and diffuse hyperplastic goiter (33). TSHR antibodies determined by ELISA were of both IgG1 (Th2-type) and IgG2a (Th1-type) subclasses, and splenocytes from immunized mice secreted interferon-gamma (a Th1 cytokine), not interleukin-4 (a Th2 cytokine), in response to TSHR antigen. Surprisingly, IFN-gamma secretion, and induction of antibodies and disease were almost completely suppressed by co-administration of alum/pertussis toxin, a Th2-dominant adjuvant. These data challenge the concept of a Th2 dominance in Graves' hyperthyroidism and provide support for the role of Th1 immune response in disease pathogenesis.

Clearly these models of experimentally-induced Graves' disease are promising and need further exploration, yet they can not be used to study the very early phases of "spontaneously developing" autoantigen presentation in "wild type-occuring" Graves' disease. The models may, however, be useful in the study of effector mechanisms playing a role in the signs and symptoms of Graves' disease.

III. RISK FACTORS FOR DEVELOPING THYROID AUTOIMMUNE DISEASE

Genotype

A genetic predisposition to autoimmune reactivity is associated with positivity for particular MHC class I and II haplotypes. Specifically, Graves' disease is associated in whites with HLA B8 and DR3, and in particular with DRB1*0304-DQB1*02-DQA1*0501 haplotype. MHC association studies in destructive autoimmune thyroiditis have revealed less consistent results. In whites, an association has been reported with various HLA alleles, including B8, DR3, DR4, DR5, DQA1*0201/*0301 and DQB1*03 (34).

Also non-MHC linked gene polymorphisms play a role in the development of autoimmune thyroiditis, such as polymorphisms in the CTLA-4 gene.

Effects of Age and Gender

Table I gave the prevalences of TPO-Ab positivity in the general population and the risk for developing overt and/or subclinical thyroid disease in case of antibody positivity. Figure 1 further illustrates the prevalence rates of TPO-Ab positivity in the general population as a function of age and gender. It is clear from this figure that thyroid autoimmunity has a predilection for the older aged and the female gender.

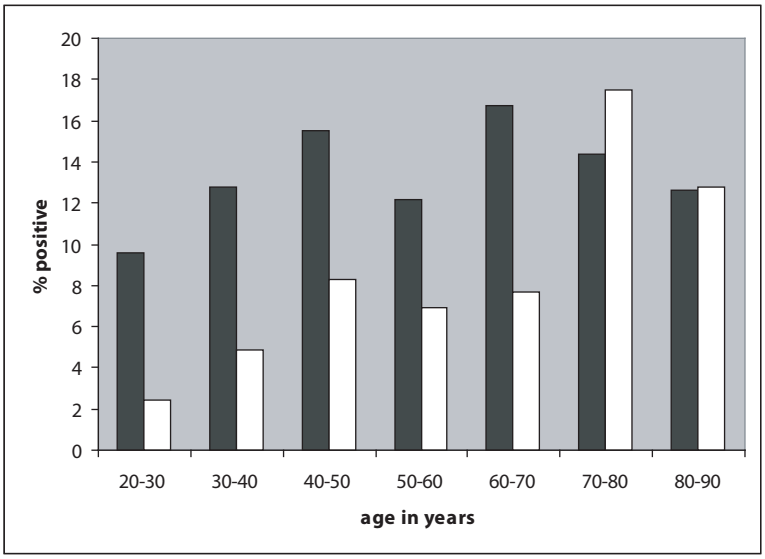

Figure 1. Prevalence of thyroperoxidase (TPO) antibody positivity in the normal population according to age and gender. TPO antibodies determined by Lumitest (Henning, Berlin, Germany). Black bars, females; white bars, males. n>150 in each age category.

The effect of ageing on the incidence of certain autoimmune diseases is generally ascribed to the decline in immune function in old age, so-called 'immuno-senescence'. Signs of immunosenescence are thymus involution, and the involution and fibrosis of secondary lymphoid tissues. Interestingly, serum levels of dehydroepiandrostenedione (DHEA), which is quantitatively the most abundant adrenal steroid hormone, also show a steady decline with ageing (35). There are reports showing that when DHEA is administered to aged individuals, whether animals or humans, their immune function is activated: they become more resistant to infections, their secretion of T-cell cytokines is enhanced, and monocyte numbers are increased. There are, however, also reports that refute such immune stimulating action of pharmacological doses of DHEA (36). In this respect, it is worth noting that DHEA administration does not lead to an attenuation of autoimmune thyroiditis in the ageing BB-DP rat.

With regard to the female preponderance in autoimmunity, the mode of action of sex steroids in the spontaneous autoimmune models still remains to be elucidated. Experimental studies show that the course of these autoimmune diseases can be modulated by procedures interfering with sex steroid levels, such as by castration or administration of sex steroids (37). In chicken and mouse models for autoimmune thyroid disease, oestrogen treatment of female or male animals, as well as castration of male animals, results in increased autoantibody levels. When castrated animals are treated with testosterone, autoantibody levels and autoimmunity decrease again.

However, results differ between distinct animal strains. Also, extrapolation of concepts derived from such animal studies to the human situation is problematic, because animal studies in general are performed in a genetically homogeneous population. To illustrate this, we have collaborated in a study on male to female transsexuals; castration followed by treatment with female hormones of these individuals did not lead to an increased prevalence of TPO antibodies up to the level found in the normal female population (38).

Effects of Environmental Factors

In addition to internal factors such as genotype, gender and age, environmental factors also play a role in the pathogenesis of thyroid autoimmune disease. The most important of these external factors are infectious agents, dietary intake, toxic agents and stress.

Infectious agents. Epidemiological studies have suggested a negative correlation between the pathogen weight in a population and the incidence of type 1-diabetes. In NOD mice and BB-DP rats, vaccination with *Mycobacterium bovis*, strain Bacillus Calmette–Guerin (BCG), or *Mycobacterium tuberculosis*- or QFA-containing preparations protect from developing diabetes in the animals, provided the treatment is initiated during the first 2 weeks of life (39). Staphylococcal enterotoxins have also been shown to prevent diabetes in NOD mice. Viruses can prevent diabetes too, and a plethora of viral strains such as EMCV-B, *Lymphochoriomeningitis virus* (LCMV) and others have been shown to interfere favourably with diabetes development in the rodent models of the disease (40). Although the mechanisms behind this protection are far from clear, it has been suggested that the viruses or bacteria act via antigenic competition, or via a direct superantigenic stimulation of T cells, releasing anti-inflammatory cytokines.

Independently of these potential protective roles, viruses and bacteria can also play a disease-promoting role. At least four mechanisms may contribute to autoimmune pathogenesis in this respect:

1. A virus may specifically infect a beta cell or a thyrocyte, leading to destruction of the cell. In this way, a non-specific inflammation of the target is induced, attracting APCs, which subsequently trigger an autoimmune response in susceptible (immune dysregulated) hosts (see above). Insulinotropic viruses include EMCV-D, reoviruses, rubella

and various enteroviruses, most notably *Coxsackie B virus* (41). Thyrotropic viruses are less well known, yet reoviruses may infect thyrocytes (42).

2. Viral or bacterial proteins sometimes share sequences with important organ-specific autoantigens. This has been suggested for *Coxsackie B virus* and glutamic acid decarboxylase (GAD), but has recently been disputed. Cross-reactivity (mimicry) has also been suggested between *Yersinia enterocolitica serotype 3* and the thyroid-stimulating hormone receptor (43). Cross-reactive epitopes could bypass existing T-cell tolerance to autoantigens to give rise to autoimmune responses.
3. A virus could induce the expression of neoantigens by future target cells. *Reovirus* type 1 induces an antibody-positive lymphocytic thyroiditis in mice (42). The infection is thought to introduce new epitopes next to, or as part of, thyroid autoantigens. This makes an immune reaction possible to these new epitopes, additionally eliciting an immune reaction to the coupled or adjacent thyroid autoantigen (bypass mechanism).
4. Finally, viruses and bacteria may directly influence the cells of the immune system, thus disturbing the delicate immune regulatory balance. *Avian leucosis virus* induces a lymphocytic thyroiditis with germinal centres in fetally infected chickens (44). The virus infects stem cells of the immune system, and has a direct effect on thymus and bursa development. Retroviruses and bacterial products might also disturb the immune balance by acting as superantigens, which cause expansion of subsets of T cells with T-cell receptors containing particular Vβ chains. It is conceivable that such T cells would include autoimmune reactive TH1 or TH2 cells.

Dietary factors. A second source of exogeneous factors contributing to autoimmune pathogenesis is constituted of specific food components. Both iodine excess and iodine deficiency are capable of disturbing the tolerance for thyroid autoantigens that exists in the healthy state (23,45). This sometimes leads to clinically overt thyroid autoimmune disease. An acute excessive iodine intake (e.g. the iodine treatment after the Chernobyl incident) in individuals with a predisposition for thyroid autoimmune disease induces a rise in the titre of TPO and thyroglobulin antibodies, and an outburst of Hashimoto-like lymphocytic thyroiditis in a proportion of such individuals with increased susceptibility.

Proposed pathogenic mechanisms are:

- An iodine-induced thyrocyte necrosis with a concomitant attraction of DCs and macrophages, and a release of autoantigens.
- A higher antigenicity of thyroglobulin due to a higher iodination grade.
- An enhanced maturation of accessory macrophages from monocytes due to a stimulating effect of iodinated compounds.
- A direct stimulation of B cells, T cells and macrophage peroxidase activity by iodine.

Any of these mechanisms may, by it self or in combination, break the existing tolerance for thyroid autoantigens and may cause disease development.

Iodine deficiency induces goitre formation and a diminished thyroid hormone production. In affected populations this leads to disease entities such as endemic goitre and endemic cretinism. In these environmentally induced thyroid disorders, local thyroid autoimmune phenomena have been described. These phenomena include a DC accumulation and clustering in the thyroid and a rise in the titre of anti-TPO and anti-thyroglobulin antibodies. In the BB-DP rat, a mild iodine deficiency leads to acceleration of the disease. Severe iodine deficiency leads, however, to a severe immunodeficiency in this animal, and hence to an ameloriation of the thyroid autoimmune response.

Toxic agents or drugs. Chemical toxins or drugs constitute a third source of pathogenic factors in the development of autoimmunity. Exposure to methylcholanthrene enhances the thyroid autoimmune response in Buffalo rats, a strain of rats genetically susceptible to experimentally induced autoimmune thyroid disease (46). Methyl-cholanthrene is thought to have a direct toxic action on the immune regulatory system rather than on the thyroid tissue of the rats.

Among other toxic factors, components from tobacco smoke have appeared to be most important in the development of Graves disease and autoimmune thyroiditis (45). In the UK, almost two-thirds of patients with Graves ophthalmopathy smoke cigarettes, in contrast to 10–20% of the normal healthy population. The mechanisms behind the association are not clear. Smoking might lead to immune dysregulations akin to the alterations seen in inherited forms of thyroid autoimmune disease. As such, smoking does lead to clear alterations in DC and macrophage function in the lung environment, and to an altered production of pro-inflammatory cytokines in the lung. Whether this is reflected in systemic monocyte, DC and macrophage dysfunction, or in a dysfunction of such cells in the thyroid, needs to be investigated. Alternatively, smoking might also lead to thyrocyte necrosis or thyroid metabolic abnormalities, processes that might also be driving forces behind a thyroid-specific autoimmunization.

Stress. A final putative external factor that modulates autoimmune pathogenesis is stress. BB rats exposed to daily stress, such as rotation, vibration or restraint stress, develop diabetes with a higher incidence than unaffected control animals. In contrast, in NOD mice, chronic stress introduced between 6 and 8 weeks of age, as well as repeated injections of saline, decreases the incidence of diabetes. Prenatal stress, however, accelerates the onset of diabetes (37). These examples illustrate the complex effects of stressors. It is suspected that stressors modulate the development of organ-specific autoimmunity by altering set points in the HPA axis and the IL-l system. This notion is supported by recent findings in patients with manic-depressive psychosis and severe melancholia. In these patients, the immune system is

severely dys-regulated, and the HPA axis and IL-1 system are grossly activated. Interestingly, the incidence of TPO antibodies is high in such patients.

Conclusion regarding the risk factors important for the development of autoimmune thyroid disease

Autoimmune diseases are complex, polygenic afflictions of which the penetrance is heavily dependent on various environmental influences. Only unfortunate combinations of genetic susceptibility and exogenous factors lead to full-blown disease.

IV. DENDRITIC CELLS (DC) AND THEIR HETEROGENEITY

In this thesis focus has mainly been on the role of antigen-presenting cells, such as the dendritic cells, in autoimmune thyroid diseases. The next section will therefore deal with a short introduction on the origin and function of dendritic cells.

Although almost all cells can act as antigen presenting cells to some degree, there are cells in the immune system that excel in this function, specifically B cells, accessory macrophages and above all, the DC. DC are critically involved in the initiation of primary T cell responses and the generation of T cell dependent auto-antibody formation (47).

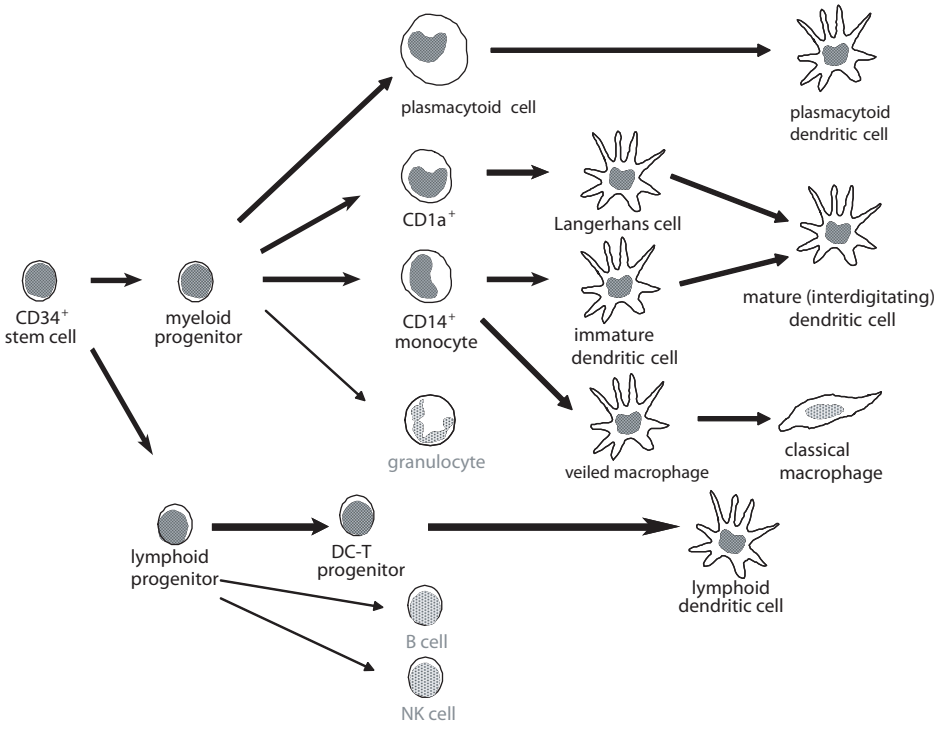

Figure 2. Developmental scheme of the various types of DC presently recognized

It is now well-accepted that the Langerhans cells (LC) of the skin pick up foreign antigens, process these antigens and travel with these - as socalled veiled cells (VC) - via the lymph to the draining lymph nodes to populate the T cell areas of these nodes as interdigitating cells (IDC) (47,48). The IDC act in presenting the transported and processed antigens to the surrounding T cells, and stimulate these lymphocytes to proliferate and to differentiate. LC, VC, IDC and similar antigen transporting and presenting cells (APC) from other sites are generally referred to as dendritic cells (DC), since the cells share many morphological and functional characteristics with the so-called dendritic cells of Steinman, originally isolated from the spleen of experimental animals, and playing a prime role in antigen presentation and T cell stimulation and skewing (47).

Cells belonging to the DC group are the most potent APC known, and have considerable potential for use in immunotherapy and tolerance induction. Therefore, there exists a great interest in generating sizeable numbers of such cells in vitro. Various methods have been devised for this, resulting in populations of accessory cells which differ in phenotype, functional capability and level of maturity, depending on the starting population and method of generation used. This has historically raised many questions about the lineage of DC, especially regarding possible precursors and routes of differentiation/maturation (49-55). Presently it is well accepted that apart from CD34+ precursor cells, also early not-commited thymocytes and monocytes may give rise to dendritic APC populations (56) and that the DC population is very heterogeneous.

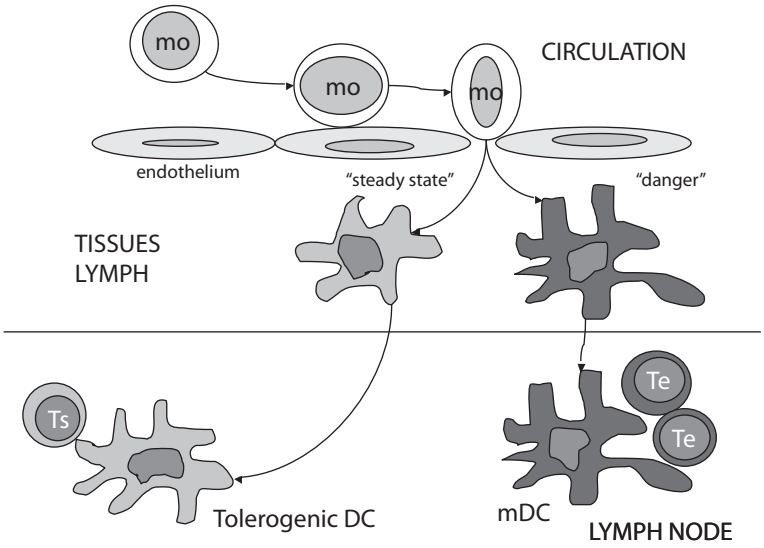

Figure 3. The traffic of monocyte-derived DC through tissues. Mo = monocyte, mDC = mature DC, Ts = suppressor T cell, Te = T effector cell

The "lymphoid" DC originate from pre T cells in the thymus and predominately populate the thymic cortico-medullary junction where the cell is instrumental in the deletion of erroneously created auto-reactive T cells (57).

"Plasmacytoid" DC originate from plasmacytoid precursor cells in the peripheral blood, characterized by a strong production of IFN-a and a positivity for CD123 (58).

The "myeloid" DC originate from a special CD34+ precursor in the peripheral blood (giving rise to epidermal S100+ Langerhans cells) and from CD14+ circulating monocytes (58). In the last decade culturing of monocytes for a week in the presence of GM-CSF and interleukin (IL)-4 (11) has become popular for generating large numbers of so-called monocyte-derived DC. The use of this methodology has resulted in the acceptance of blood monocytes as a major source of DC precursors.

The generally held current paradigm (47,59) is that the myeloid DC present in the interstitium of peripheral tissues are in a so-called "immature" state, suitable for their sentinel function. The immature cells express various molecules instrumental in the uptake of foreign and damaged material (mannose receptors, Toll receptors). They have a high capability for endocytosis enabling the cells to capture and process antigens, i.e. to place antigenic peptides in the grooves of the MHC-molecules in their endocytotic vesicles. Immature DC have a limited potency to stimulate T cells.

In response to a local inflammatory stimulus (the so-called "danger signals"), such as endotoxin (LPS) and bacteria, interstitial DC undergo maturation (Figure 3). The cells lose their antigen-capturing capacity and acquire a strong T cell stimulatory capacity by directing the antigen-loaden MHC molecules to the cell membrane and by up-regulating their co-stimulatory molecules CD80 and CD86. During maturation the cells migrate via the lymph to the T cell areas of the draining lymph nodes. For the latter process they change their make-up of chemokine receptors: immature DC are characterized by CCR1 and CCR5, while mature DC are positive for CCR7, enabling the latter cells to home to the lymph nodes since the lymphatics and the structures in the lymph node express the ligands for CCR7, i.e. CCL-15 and CCL-19. Fully mature lymph node DC are *the* initiators of immune responses in the draining lymph nodes (Figure 3). They are capable of giving strong proliferation signals to naïve antigen-specific T cells accumulated in the T cell areas of the lymph nodes due to the high expression of MHC class II molecules and co-stimulatory molecules on their cell membranes.

V. ABERRANT DENDRITIC CELLS IN THE ANIMAL MODELS OF SPONTANEOUS AUTOIMMUNE THYROIDITIS

The Early Accumulation and Clustering of DC and Macrophages in the Thyroids of the Animal Models of Spontaneously Developing Autoimmune Thyroiditis

A small increase in the number of DC and a homotypic clustering of the cells in the interstitium of the thyroid is one of the first signs of a developing thyroid autoimmune reaction in the Bio breeding diabetes prone (BB-DP) rat, the NOD mouse and the OS chicken (22,60,61). This local activation of DC precedes the T cell expansion and the production of auto-antibodies in the regional lymph nodes. At these early stages of the autoimmune reaction, thyrocytes are negative for MHC class II (22,61). Such MHC class II negative thyrocytes are very poor stimulators of T cell expansion (62). The thyroidal DC, on the other hand, are excellent in T cell stimulation and equal spleen T cells in this function (62). These arguments provide, at least in the animal models, sufficient proof to reject the idea that an aberrant expression of MHC class II molecules is an event initiating the thyroid autoimmune process.

In the BB-DP rat and OS chicken there are no signs of an early necrosis of thyrocytes attracting macrophages and DC to the necrosis-induced inflammatory reaction (as in the iodine-induced inflammation in the NOD model, see above). Interestingly, intrinsic disturbances in the growth and the differentiation of thyrocytes have been shown in both models, in the BB-DP rat leading to a high incidence of ultimo-brachial cysts and an altered production of IL-6 by thyrocytes (13,63). Whether such alterations are the cause of the early accumulation of DC is not known. In both models a high iodine diet does lead to acceleration of the autoimmune response and in the BB-DP rat to a higher influx of DC (13,20,64,65). With regard to relevant chemokines, MCP-1 does not seem to be involved in the early DC accumulation in the autoimmune thyroiditis of the BB-DP rat (63).

An abnormal differentiation of APC from precursors in the animal models of spontaneously developing autoimmune thyroiditis. A role in a defective tolerance induction?

Since the histology of thyroids to be affected by autoimmune disease clearly shows that DC are critically involved in the initiation of the autoimmune process and since normally steady state DC are involved in tolerance induction and not auto-sensitization (see above), it is important to note that there is accumulating evidence that the function and the differentiation of DC from precursors is aberrant in the BB-DP rat and in the NOD mouse.

Particularly in the NOD mouse studies have concentrated on the development of accessory macrophages and DC from bone-marrow precursors. This development has been found to be hampered in the majority of the studies, leading to the generation of DC in vitro with a lower grade of maturation and a lower capability to stimulate T cells (66,67). Contradictory findings have, however, also been made (68). This is probably due to the phenomenon that outcomes of DC differentiation studies from precursors are heavily dependent on the culture

conditions used, e.g. seeding concentrations of the cells, concentrations of growth factors and plastics used (Leenen, personal communication). Also in the BB-DP rat Sommandas et al have evidence that the generation of DC is hampered from bone-marrow precursors (to be published).

The differentiation abnormality from precursors leads to a deficit in APC with the appropriate grade of differentiation in the interstitium of peripheral tissues and in the draining lymphatic tissues, hence in the availability of the tolerogenic steady state DC at the appropriate places. In the BB-DP rat Simons et al found that in the very early thyroid infiltrates monocytic precursors are more abundant as compared to differentiated DC (62), and that lymph node and spleen DC of the BB-DP rat are still in a poorly differentiated state, showing a low expression of MHC class II and co-stimulatory molecules and a low capability of homotypic clustering (21,22). In the NOD mouse functional studies on thyroid interstitial DC are lacking. With regard to the spleen and lymph nodes of the NOD mouse, Piganelli et al (69) found that a mixture of spleen accessory macrophages and spleen DC were defective in stimulating T cells. Dahlen et al (70) confirmed these findings and in addition showed that such NOD macrophages and dendritic cells expressed lower basal levels of CD86. This low CD86 expression was not dependent on the MHC haplotype or on diabetes development since the NOD-related, diabetes-free mouse strains NON (H-2nb1) and NOR (H-2g7) exhibited similar low levels of CD86 expression. Radosevic et al have studied purified DC populations of the spleen and the lymph nodes of the NOD mouse and could not confirm that these professional APC were defective. In their hands the cells had reached a normal state of maturation and were perfectly capable of stimulating T cells (71). In fact there was an excessive proliferation response of the NOD T cells, when stimulated with such DC in vitro. This is most likely due to a defect other than the DC maturation defects in the immune system of this animal, namely a defect in the mechanisms of apoptosis of T cells leading to a hampered Activation Induced T Cell Death (AITCD, see below, 71).

Although it is not clear how precisely the above described differentiation defects of accessory macrophages and DC in the BB-DP rat and the NOD mouse play a role in the defective ability of the animals to mount tolerance to auto-antigens, there are a few indications on the mechanisms of the failure of tolerance induction. The less well-differentiated lymph node and spleen DC of the BB-DP rat are in particular less capable of expanding an important suppressor T cell population of the rat, the so-called RT6+ T cells (72). In the report of Dahlen et al (70) on the defective spleen APC of the NOD mouse the authors proposed that the low level of CD86 expression in the NOD mouse contributed to a defective regulation of autoreactive T cells by preventing a full activation of T cells and therefore the up-regulation of the CTLA-4 induced switch-off signal. In the BB-DP rat a similar mechanism may play a role: When this animal is treated with a stimulating anti-CD28 antibody (thus correcting the poor stimulating activity of the animals APC and activating the T cells to express CTLA-4), autoimmunity does not develop (Whalen and Rozing, personal communication). Also in the NOD mouse

interventions in the activation pathway between functionally active DC and T cells (by deleting CD80-CD28 interactions) disrupt tolerance induction by interfering in the generation of CD4+CD25+ T cells (73). Moreover transfers of in vitro fully differentiated and maturated DC prevent the development of type 1 diabetes in the NOD mouse (74,75).

Collectively these data show that the hampered differentiation of the DC in the animal models might lead to an inability to generate suppressor T cells as well as an inability to exert AITCD.

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Chapter 2

Experimental questions addressed in this thesis

HOW DO IN VITRO GENERATED VEILED CELLS RELATE TO CLASSICAL DENDRITIC CELLS?

Kabel et al and Mooi et al previously reported on a method for the generation of Antigen Presenting Cells (APC) from monocytes with a morphology, movement behaviour, marker pattern and function reminiscent of those of lymph-borne veiled cells (VC) (1,2). The VC generated using this culture procedure were found - but note more than a decade ago - to be functionally equal in T cell stimulation to DC populations obtained following well-accepted methods of that time, e.g. the method of Knight (1,2). We thus concluded at that time that such monocyte-derived VC were as typical DC as those yielded by other methods (1,2).

The hallmark of this method for the generation of VC from monocytes is that it results in the production of APC within twenty-four hours. It contrasts with other methods in that it employs strictly plastic non-adherent (polypropylene) culture conditions. To increase the yield of VC the monocytes are additionally exposed prior (and/or during) the culture period to iodinated compounds, such as metrizamide and thyroid hormones, and it was hypothesized that the active redox capacity of the iodonium atoms in these iodinated compounds may have affected the reactive-oxygen-species-generating system in the monocytes, and that this mechanism may have played a role in the enhanced generation of VC from monocytes (2). For the transition from monocytes to VC the presence of endogenous GM-CSF, TNF-α and IL-6 was also found to be essential in the culture system, although the extra addition of these cytokines was not needed (2).

Since then the field has progressed, showing the enormous heterogeneity of the DC populations including cells with different abilities in cytokine production, T cell subset stimulation and marker expression (3,4,5). I therefore have extended Kabel and Mooi's previous studies on the monocyte-derived VC and these are presented here in this thesis (Chapter 3). We firstly investigated the potential of two compounds (diphenyleneiodonium, DPI, and ascorbate with a known potent inhibitor activity for the reduced nicotinamide adeninedinucleotide phosphate (NADPH) oxidase, the major ROS producing membrane enzyme complex)(6,7,8) to enhance the transition of monocytes into VC. We also studied in greater detail the expression of recently-developed DC-specific markers and co-stimulating molecules on the generated VC, and their IL-12/IL-10 production and T cell-stimulating potential.

CAN DC AND VC GENERATION AND FUNCTION BE MODULATED BY HORMONES?

Dehydroepiandrosterone (DHEA) and glucocorticoids. Dehydroepiandrosterone (DHEA) is quantitatively the most abundant adrenal steroid hormone in humans and other mammals (9,10). The hormone is uniquely sulfated (DHEA-S) before entering the plasma, and the sul-

fated prohormone is converted to DHEA and its metabolites (11) in various peripheral tissues. No major endocrine functions have been ascribed to a direct action of DHEA-S and DHEA, although the hormones act as intermediaries in sex steroid synthesis (11). Both hormones, however, have been proposed as exerting important restoring effects on age-related processes, such as fat depot distribution and neurodegeneration. These effects also include major stimulation of cells of the (aging) immune system (12,13,14). However, these effects of DHEA have also been disputed (15,16).

There are no reports on the effects of DHEA on dendritic cell development. Mooi and Hoek previously reported that the exposure of monocytes to hormones (in particular to triiodothyronine) stimulated DC development from monocytes (2,17). We now have tested and report in this thesis (Chapter 4) the effects of exposure of monocytes to DHEA, prior to, and during, their differentiation into immature DC under the influence of GM-CSF and IL-4.

In biological systems, the effects of DHEA-S and DHEA are often opposed by the other important adrenal steroid cortisol (18). The ratio DHEA/cortisol is abnormal in various pathological conditions characterized by immune dysfunction, such as after thermal injury, in AIDS, in rheumatoid arthritis and in tuberculosis (19-23). The suppressive effects of glucocorticoids on T cells, B cells, monocytes and macrophages have extensively and in detail been studied (reviewed in 24, 25), but the reports on the effects of glucocorticoids on the function and differentiation of DC (26, 27) are relatively limited at the time and data in these reports are inconsistent regarding effects on marker expression, T cell-stimulatory capacity and cytokine production. We therefore contrasted our DHEA experiments with dexamethasone (DEX) experiments (Chapter 4) and tested the effect of this hormone on the process of transition from monocyte to immature DC also.

1α, 25-Dihydroxyvitamin D_3 (1,25(OH)$_2$D$_3$). 1α, 25-Dihydroxyvitamin D_3 (1,25(OH)$_2$D$_3$) is a steroid hormone known for its ability to regulate calcium metabolism. The presence of the vitamin D_3 receptor in almost all types of immune cells and the ability of 1,25(OH)$_2$D$_3$ to affect immune cell function in vitro is indicative of other actions of this hormone. The ability of 1,25(OH)$_2$D$_3$ to stimulate cell differentiation has been well characterized. For instance, this hormone can inhibit proliferation and induce differentiation of benign cells such as keratinocytes, malignant cells such as prostate, breast and colon adenocarcinoma cells and various leukemic cells.

1,25(OH)$_2$D$_3$ also plays a role in the differentiation of benign cells of the myeloid lineage. The differentiation of immature monocytes toward mature macrophages has been demonstrated to be fostered by this hormone in many reports (28,29,30). The hormone enhances macrophage-type activities such as phagocytosis and killing of bacteria, adherence and chemotaxis (31,32). 1,25(OH)$_2$D$_3$ is also known for its capability to induce TGF-beta production in monocytes and other cell types (33). Although TGF-beta is commonly considered as a tolerance-inducing or immunosuppressive cytokine, it has great plasticity and its action

on immune cells can be inhibiting or promoting, depending upon cell type, differentiation/activation status and environment. (34). Thus, its manner of regulating immune function is heavily context dependent.

Data on the effects of $1,25(OH)_2D_3$ on the accessory cell function of monocytes/macrophages demonstrate a decreased antigen presenting capability together with a reduced MHC class II antigen expression (11,12). In order to further investigate the effects of the immunoregulatory hormone $1,25(OH)_2D_3$ on myeloid DC differentiation, I also studied for this thesis the generation of iDC from blood monocytes in the presence of GM-CSF and IL-4, with the addition of $1,25(OH)_2D_3$ to the culture (Chapter 5).

ARE THERE ABERRATIONS IN DC AND VC IN PATIENTS WITH AUTOIMMUNE THYROID DISEASE?

In a previous report Jansen et al found aberrancies in the monocyte-derived veiled cells of type-1 diabetes: the yield of such cells was lower and their T cell stimulatory capacity reduced (35). At that time Jansen et al were of the opinion that veiled cells were as prototypic DC as generated by others via other methodologies. They thus took this observation as supporting a concept that DC generation might be disturbed in human endocrine autoimmune diseases in a similar fashion as it was in the animal models. To strengthen this concept we carried out experiments on monocyte derived veiled cells in autoimmune thyroiditis patients. In Chapter 6 the data of these studies are shown.

By now we know that there are considerable differences between monocyte-derived VC and DC (see Chapter 3). We have therefore also completed a study with classical monocyte-derived DC in autoimmune thyroiditis patients (addendum to Chapter 6).

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Chapter 3

Accessory cells with a veiled morphology and movement pattern generated from monocytes after avoidance of plastic adherence and of NADPH oxidase activation. A comparison with GM-CSF/ IL-4-induced monocyte-derived dendritic cells.

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ABSTRACT

Veiled cells (VC) present in afferent lymph transport antigen from the periphery to the draining lymph nodes. Although VC in lymph form a heterogenous population, some of the cells clearly belong on morphological grounds to the Langerhans cell (LC)/ dendritic cell (DC) series.

Here we show that culturing monocytes for 24 hrs while avoiding plastic adherence (polypropylene tubes) and avoiding the activation of NADPH oxidase (blocking agents) results in the generation of a population of veiled accessory cells. The generated VC were actively moving cells like lymph-borne VC in vivo. The monocyte (mo)-derived VC population consisted of $CD14^{dim/-}$ and $CD14^{bright}$ cells. Of these the $CD14^{dim/-}$ VC were as good in stimulating allogeneic T cell proliferation as immature DC (iDC) obtained after one week of adherent culture of monocytes in granulocyte-macrophage-colony stimulating factor (GM-CSF)/interleukin (IL)-4. This underscores the accessory cell function of the here-reported mo-derived $CD14^{dim/-}$ VC. Although the $CD14^{dim/-}$ VC had a modest expression of the DC specific marker CD83 and were positive for S100, expression of the DC specific markers CD1a, Langerin, DC-SIGN, and DC-LAMP were absent. This indicates that the here generated $CD14^{dim/-}$ VC can not be considered as classical LC/DC. It was also impossible to turn the $CD14^{dim/-}$ mo-derived VC population into typical DC by culture for one week in GM-CSF/IL-4 or LPS. In fact the cells died under such circumstances, gaining some macrophage characteristics before dying.

The IL-12 production from mo-derived $CD14^{dim/-}$ VC was lower, whereas the production of IL-10 was higher as compared to iDC. Consequently the T cells that were stimulated by these mo-derived VC produced less IFNγ as compared with T cells stimulated by iDC.

Our data indicate that it is possible to rapidly generate a population of $CD14^{dim/-}$ veiled accessory cells from monocytes. The marker pattern and cytokine production of these VC indicate that this population is not a classical DC population. Rather, the cells might be related to the veiled macrophage-like cells also earlier described in afferent lymph.

INTRODUCTION

It is now well-accepted that the Langerhans cells (LC) of the skin pick up foreign antigens, process these antigens and travel with these - as veiled cells (VC) - via the lymph to the draining lymph nodes to populate the T cell areas of these nodes as interdigitating cells (IDC) (1, 2). The IDC act in presenting the transported and processed antigens to the surrounding T cells, and stimulate these lymphocytes to proliferate and to differentiate. LC, VC, IDC and similar antigen transporting and presenting cells (APC) from other sites are presently generally referred to as "dendritic" cells (DC), since the cells share many morphological and functional characteristics with the so-called dendritic cells of Steinman, originally isolated from the spleen of experimental animals, and playing a prime role in antigen presentation and T cell stimulation and skewing (2).

Cells belonging to the DC group are the most potent APC known, and have considerable potential for use in immunotherapy and tolerance induction. Therefore, there exists a great interest in generating sizeable numbers of such cells in vitro. Various methods have been devised for this, resulting in populations of accessory cells which differ in phenotype, functional capability and level of maturity, depending on the starting population and method of generation used. This has historically raised many questions about the lineage of DC, especially regarding possible precursors and routes of differentiation/maturation (2-9). Presently it is well accepted that apart from $CD34^+$ precursor cells, also early not-commited thymocytes and monocytes may give rise to dendritic APC populations (10).

In the last decade culturing of monocytes for a week in the presence of GM-CSF and interleukin (IL)-4 (11) has become popular for generating large numbers of so-called mo-derived DC. The use of this methodology has resulted in the acceptance of blood monocytes as a major source of DC precursors. These mo-derived DC express high levels of MHC-class-II and CD1a (11). The cells are considered 'immature', since these 'immature' DC can still take up and process antigens, and are able to increase their T cell accessory potential when cultured further ('mature') in the presence of lipopolysaccharide (LPS), IL-1, CD40 or TNFα (12-14). These then 'mature' DC show an increased T cell stimulating capacity (15). 'Mature' DC can be recognized by their increased expression of CD83 (13), while the costimulating molecules CD80 and CD86 are also markedly upregulated (12), as are the DC specific molecules DC-SIGN (16) and DC-LAMP (17). The 'immature' mo-derived DC (iDC) are considered to be the in vitro counterpart of the peripheral DC residing in the tissues, such as the LC (expressing the specific marker Langerin (18)) and the DC of the gut and bronchial mucosa, while the mature mo-derived DC (mDC) are considered to represent the DC residing in the T cell areas of the lymph nodes and spleen, the IDC (19).

We previously reported on an alternative method for the generation from monocytes of cells with the morphology, movement behaviour, marker pattern and functions reminiscent of those of lymph-borne veiled cells (VC) (4, 20). The hallmark of our method for the generation

of VC from monocytes is that it results in the production of APC within twenty-four hours. It contrasts with other methods in that it employs strictly plastic non-adherent (polypropylene) culture conditions. To increase the yield of VC the monocytes are additionally exposed prior (and/or during) the culture period to iodinated compounds, such as metrizamide and thyroid hormones, and it was hypothesized that the active redox capacity of the iodonium atoms in these iodinated compounds may have affected the reactive oxygen species generating system in the monocytes, and that this mechanism may have played a role in the enhanced generation of VC from monocytes (20). For the transition from monocytes to VC the presence of endogenous GM-CSF, TNF-α and IL-6 was also found to be essential in the culture system, although the extra addition of these cytokines was not needed (20).

The VC generated using this culture procedure were previously (more than a decade ago) found to be functionally equal in T cell stimulation to DC populations obtained following well-accepted methods of that time (e.g. the method of Knight) (4, 20). We thus concluded at that time that such mo-derived VC were as prototypic DC as those yielded by other methods (4, 21).

Since then the field has progressed, showing the enormous heterogeneity of the DC populations including cells with different abilities in cytokine production, T cell subset stimulation and marker expression (10, 22). We therefore have extended our previous studies on the mo-derived VC. We firstly investigated the potential of two compounds (diphenyleneiodonium, DPI, and ascorbic acid with a known potent inhibitor activity for the reduced nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, the major reactive oxygen species (ROS) producing membrane enzyme complex) (23-25) to enhance the transition of monocytes into VC. We also studied in greater detail the expression of DC-specific markers and costimulating molecules on the generated VC, their IL-12/IL-10 production and their T cell-stimulating and T cell-skewing potential.

Notable is the heterogeneity present in the expression of CD14 in the mo-derived VC population (4, 20). Since DC are generally regarded as CD14– cells, we also report here studies where we separated via MACS the $CD14^{bright}$ and $CD14^{dim/-}$ fractions to study these separately with respect to morphology, movement behaviour, marker expression, T cell stimulating and T cell-skewing capability and IL-10 and IL-12 production. The unseparated VC, as well as the $CD14^{bright}$ and $CD14^{dim/-}$ VC were compared to prototypic iDC obtained via the currently well-accepted method of culturing monocytes for one week in the presence of GM-CSF and IL-4. The $CD14^{bright}$ and $CD14^{dim/-}$ VC obtained after twenty-four hours and the iDC were also cultured for further periods under plastic adherent conditions with LPS or GM-CSF and IL-4 to study their capability to develop into 'mature' DC.

This report describes that although $CD14^{dim/-}$ mo-derived VC express S100 and higher levels of MHC class II and CD83 in comparison to their starting monocyte population and in addition are potent stimulators of allogeneic T cells (similar to iDC), that these cells lack important DC/ Langerhans cell markers like CD1a, Langerin, DC-LAMP and DC-SIGN. Also their survival

in GM-CSF/IL-4 was poor. We therefore presently consider this population as dissimilar from the classical DC. The cells are probably more related to the monocyte/macrophage series. Interestingly one of us (H.D.) reported almost two decades ago that the VC population in skin lymph of pigs is quite heterogenous, particularly after the application of a sensitizing agent. The population not only consisted of classical LC-like cells, but also of veiled macrophage-like cells reminiscent of those earlier described in lymphatics of sheep ().

MATERIALS AND METHODS

Isolation of monocytes from peripheral blood

Monocytes were isolated from the peripheral blood of healthy blood donors via well-accepted methods: Heparinized blood diluted with an equal volume of phosphate buffered saline (PBS) was distributed over Ficoll Isopaque (density 1.077 g/ml, Pharmacia, Uppsala, Sweden) and centrifuged for 15 minutes at 1000 g. Cells collected from the interface and washed were then suspended in RPMI 1640 with 25 mM HEPES and L-glutamine (GIBCO, USA), supplemented with 100 U/ml penicillin G, 0.1 mg/ml streptomycin (both Seromed, Biochrom, Berlin, Germany) and 10% FCS (Biowhitaker, Walkersville, MD, USA) (hereafter known as RPMI 1640^{+}) added. This cell suspension was distributed over Percoll (density 1.063 g/ml, Pharmacia), then centrifuged for 40 minutes at 400 g. Cells collected from the interface were washed and suspended in RPMI 1640^{+}. Monocyte purity was determined by non-specific esterase staining (NSE) (26). Cell suspensions containing 80% or more monocytes were frozen following standard procedures and stored in liquid nitrogen, providing a bank for experiments. Monocytes purified by elutriator centrifugation (4) were also used in a limited series of experiments (courtesy of CLB, Amsterdam, the Netherlands) in order to confirm results obtained via Ficoll/Percoll gradient separation.

Generation of VC

Monocytes were removed from liquid nitrogen and quickly thawed in a 37°C water bath. These cells were then washed twice in RPMI 1640 (without FCS) (5 minutes, 500 g) and suspended under plastic-nonadherent conditions (polypropylene tubes, Falcon, Becton Dickinson, San Jose, CA, USA) at a concentration of $2x10^{6}$/ml in RPMI 1640 (without FCS). Not only triiodothyronine (T3) and reversed T3 (rT3) were used, but also the known potent inhibitors of NADPH oxidase DPI (Sigma, St. Louis, USA) or L-ascorbic acid (Sigma) were added in dose-response to the cell suspension. The monocyte suspension was thereafter exposed to these compounds at 37°C, 5% CO_2, 100% humidity for 30 minutes. This was either followed by washing in RPMI 1640^{+} (5 minutes, 500 g) or not, and further culturing in RPMI 1640^{+} at 37°C, 5% CO_2, 100% humidity under plastic non-adherent conditions for 16 hours (overnight).

Following the 16 hour culture period, cells were centrifuged (5 minutes, 500 g) and resuspended in RPMI 1640^{+}. Cells were then examined under light microscopy at a magnification of 400x. VC were defined as large cells with actively moving cytoplasmic processes or veils. Viability was checked using trypan blue exclusion. Time-lapse cinematographic studies were also carried out. For this purpose cells were kept in a microincubator (37°C, 5% CO_2) on an inverted microscope (Axiovert, Zeiss, Germany) attached to a video camera (Sony, Japan) and a time lapse video recorder (Panasonic, Matsushita Electric Industrial CO., Osaka, Japan). The video recording interval was set at 0.18 sec and the tape running speed at 2.599 mm/sec. After recording, the images were analyzed using a computer (Acorn Computers Ltd., Cambridge, UK).

The cell suspension was then further purified and separated using large cell columns and a mini-MACS (Magnetic Cell Separation System, Miltenyi Biotec GmbH, Bergisch Gladbach, Germany) with mouse anti-human CD3 (Becton Dickinson, USA), CD19 (Coulter, Hialeah, Florida, USA), and CD56 (Becton Dickinson) monoclonal antibodies and goat anti-mouse magnetic beads (Miltenyi Biotec GmbH) to deplete contaminating T cells, B cells and NK cells. Separation into $CD14^{bright}$ and $CD14^{dim/-}$ cells was performed via mini-MACS using CD14 (Coulter) monoclonal antibody and goat anti-mouse magnetic beads (Miltenyi Biotec GmbH).

In a few experiments VC were further cultured (37°C, 5% CO_2, 100% humidity) for 2 days under plastic adherent conditions (polystyrene tubes, Falcon, Becton Dickinson) and GM-CSF and IL-4 added, as described below for the generation of DC to study the maturation potential of VC to become DC. Also VC were further cultured under adherent conditions with lipopolysaccharide (LPS 10 ng/ml) added.

Generation of DC by long-term adherent culture in GM-CSF and IL-4

Aside from generating VC, DC were obtained via the well-established method described by Ruppert et al. (27) and by Sallusto and Lanzavecchia (11). Briefly, monocytes were cultured for one week at 37°C, 5% CO_2, 100% humidity at a concentration of $3x10^5$ cells/ml in RPMI 1640^{+} with 800 U/ml GM-CSF and 1000 U/ml IL-4. Feeding of the cultures took place every two days, by removing 500 µl culture fluid and replacing this with 1 ml of fresh medium with cytokines.

Flow cytometry and immunocytochemistry

For analysis of marker expression by flow cytometry, all cell populations were stained by incubating for 10 minutes with mouse anti-human FITC- or PE-conjugated monoclonal antibodies, then washing three times. The monoclonal antibodies used were My4 (CD14, Coulter), CD1a (Coulter), B7.1 (CD80), B7.2 (CD86), Langerin (CD207), DC-LAMP (CD208) (all four from PharMingen, San Diego, CA, USA), and DC-SIGN (CD209) (R&D Systems, Minneapolis, USA). Immediately following the staining, cells were analyzed on a FACScan (Becton Dickinson).

The marker pattern of monocytes, VC and DC was also investigated by immunocyto-chemistry. Cytospins were prepared using a Cytospin apparatus (Nordic Immunological Laboratories, the Netherlands). These were air-dried for 1 hour, then fixed in 100% acetone (Merck, Darmstadt, Germany) for 10 minutes. Indirect immunoperoxidase staining was performed with the monoclonal antibodies My4 (CD14, Coulter), HLA-DR (Becton Dickinson), OKIa (Ortho Diagnostics, Raritan, NJ, USA), L25 (Dr. T. Takami, Gifu, Japan), Kim 6 (CD68, Behring, Marburg, Germany), RFD 7 and RFD 9 (gift of Dr. L.W. Poulter, London, UK). Application of rabbit-anti-mouse IgG peroxidase-labelled conjugate (1:50, Dako A/S, Glostrup, Denmark) in 1% normal human pool serum followed incubation with the monoclonal antibodies (RT, 1h). Cytospins were developed with a 0.05% 3,3'- diaminobenzidine (DAB, Sigma) solution containing 0.01% hydrogen peroxide for 3-5 minutes.

Cytospin preparations were also used to determine the reactivity of the various cell populations to acid phosphatase. The cytospins were air-dried, then incubated for acid phospatase staining according to Katayama *et al*. (28), using naphtol AS-BI phosphate as substrate and hexarotised pararosanilin as coupling agent (37 °C, 30 min). Slides were counterstained with hematoxylin.

Anti-S100 staining was performed on monocytes, VC and DC with a rabbit polyclonal antiserum against cow S100 (diluted 1:500; Dakopatts, Glostrup, Denmark). Cytospins were fixed overnight with Zamboni fixative at 4°C. Swine anti-rabbit serum (Dakopatts) was used as the secondary antibody, and was diluted 1:50 and supplemented with 4% normal rabbit serum. Non-specific adsorption of the antibodies was minimized by blocking with 4% normal swine serum (Dakopatts) prior to incubation with the secondary antibodies. Staining was ultimately achieved by incubation with rabbit peroxidase-anti-peroxidase-complex (diluted 1:50; Dakopatts) and diaminobenzidine HCI-nickel staining solution, essentially as described (21).

All cytospin preparations were mounted in DePeX mounting medium (Gurr, BDH Limited, Poole, England).

S100 enzyme immunoassay

The S100 protein concentrations in the media of monocyte- and VC cultures were measured using a double monoclonal antibody enzyme immunoassay (Sandwich EIA) (Inro/Medisera, Campro Scientific, Veenendaal, the Netherlands), essentially as described by Aurell *et al.* (29). Microtiter plates coated with a MoAb directed against S100 and an anti-S100 MoAb conjugated with β-galactosidase reacted with the S100 in the samples. O-nitrophenyl-β-galactoside was used as the staining substrate. Bovine S100 was used as the reference standard.

Mixed leukocyte reaction (MLR)

Allogeneic MLRs were performed in order to measure the accessory capability of the various APC populations generated. Responder T lymphocytes were obtained from healthy donors

and isolated following standard procedures with Ficoll-isopaque, Percoll density gradient centrifugation, and nylon wool adherence (Leuko-Pak, Fenwall Laboratories, IL, USA). Non-adhering cells recovered were greater than 90% CD3 positive. $1.5x10^5$ responder cells were cultured in 96-well, flat-bottom microtitre plates (Nalgene Nunc International, Rochester, NY, USA) with different numbers of irradiated (2000 rad) stimulator cells (monocytes, VC or DC) to achieve stimulator-to-T cell ratios of 1:5, 1:10, 1:20 and 1:40. The culture medium used was RPMI 1640 with 25 mM HEPES and L-glutamine, supplemented with 100 U/ml penicillin G, 0.1 mg/ml streptomycin and 10% human A^+ serum, in a total volume of 200 µl per well. Controls used were monocytes, VC or DC alone, and lymphocytes in the presence of 10-50 µg/ml phytoheamagglutinin (PHA) (Wellcome Diagnostics, UK). Cultures were performed in triplicate. On day 5, thymidine incorporation was measured by adding 0.5 µCi 3H-thymidine (specific activity = 185 GBq/mmol) to each well, then harvesting 16 hours later. Scintillation was counted on an LKB 1205 Betaplate liquid scintillation counter (Wallac, Turku, Finland).

T cell skewing capacities

The capacity of VC and DC to prime IFNγ-producing T cells (Th1 cells) was tested to demonstrate the T cell skewing capacities of these cell types. T cells were obtained out of the cell pellet generated after Percoll density gradient centrifugation of peripheral blood. This cell pellet was purified by removing contaminating B cells and NK cells, and monocytes via negative miniMACS selection using and anti-human CD-19, -56, and –14 magnetic beads (Miltenyi Biotec GmbH). 0.15 x 10^6 Irradiated (2000 rad) VC or DC (stimulator cells) were cultured in 24-well plates (Nalgene Nunc) with 1.5 x 10^6 T cells (1:10) (responder cells). The culture medium used was RPMI 1640 with 25 mM HEPES and L-glutamine, supplemented with 100 U/ml penincillin, 0.1 mg/ml streptomycin and 10% human A^+ serum. Controls used were T lymphocytes alone. On day 2, T cells were collected via miniMACS using MS^+ columns and anti-human CD3 (Miltenyi Biotec GmbH) to deplete VC and DC from the culture. The obtained T cells were placed in 24-well plates at a concentration of 1 x 10^6/ml and stimulated for 16-18 h by calcium ionophore (1000 ng/ml) and tetradecanoylphorbol acetate (TPA; 2 ng/ml) in Yssel's medium supplemented with 2.5% FCS. The next day, supernatants were collected and tested for the presence of IFN-γ by ELISA as indicated by the manufacturer (BioSource). The production of IFN-γ by T cells cocultured with VC or DC were corrected for the basal production of IFN-γ by T cells alone. The production of IFN-γ by T cells can be used as a measure of T cell skewing since Th1 cells predominantly produce IFN-γ, whereas Th2 cells produce IL-4, -5, -10, and –13 (30).

Phagocytosis

The ability of monocytes, VC and DC to phagocytose bacteria was tested using the Phagotest kit (Orpegen Pharma, Heidelberg, Germany). Cells were first cooled for fifteen minutes in an ice-water bath. *E. coli*-FITC was added, and the cells were then incubated for ten minutes in a

37°C water bath. The manufacturer's instructions were then followed regarding quenching, lysing and DNA staining. Analysis was performed on a FACScan (Becton Dickinson, USA).

IL-12/IL-10 production

Monocytes, VC and DC were placed in 24-well plates (Nalgene Nunc) at a concentration of $5x10^5$ cells/ml and cultured for 24 hours in $RPMI^+$ containing ultraglutamine (2 mM, Biowhitaker), penicillin/streptomycin (100 U/ml, 100 µg/ml, Biowhitaker) and serum free medium supplement (SF-1; Corning Costar Europe, Badhoevedorp, the Netherlands). To stimulate IL-10 production, the culture fluid contained staphylococcus aureus cowan 1 strain (SAC) (1:5000, Calbiochem, La Jolla, CA, USA). The IL-12 production was stimulated by SAC (1:5000) and γ-IFN (1000 U/ml, Biomedical Primate Research Centre, Rijswijk, the Netherlands). The production was measured by ELISA as indicated by the manufacturer (IL-10: ELISA Pelikine, CLB, Amsterdam, the Netherlands and the IL-12 (p 70): Eli-pair, Diaclone, Besançon, France).

RESULTS

Accessory cells with a movement pattern of lymph-borne VC are generated by overnight plastic non-adherent culture of monocytes after exposure to NADPH oxidase blocking compounds.

We confirmed our previously-described observations that the procedure using monocytes cultured overnight under non-adherent conditions (polypropylene-tubes) after exposure and in the presence of the iodinated compounds T3 or rT3 yielded 40 – 60% ($n \geq 30$) of cells with a veiled morphology, and a movement pattern in time-lapse cinematography characteristic of lymph-borne veiled cells (Fig. 1).

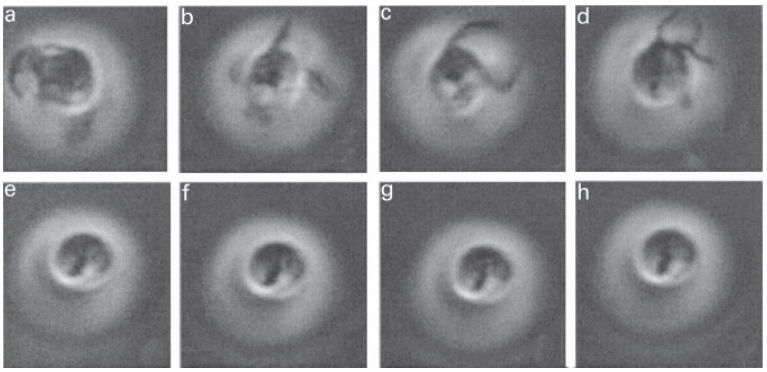

Figure 1. Time-lapse cinematographic pictures of a living monocyte-derived veiled cell (a-d) and an original monocyte (e-h). Pictures are taken 20-30 seconds apart. Magn.x1000. Note the constant changes in shape of the veiled cell extending and withdrawing long, thin cytosplasmic extensions (a-d), this in contrast to the rigid round shape of the monocyte (e-h).

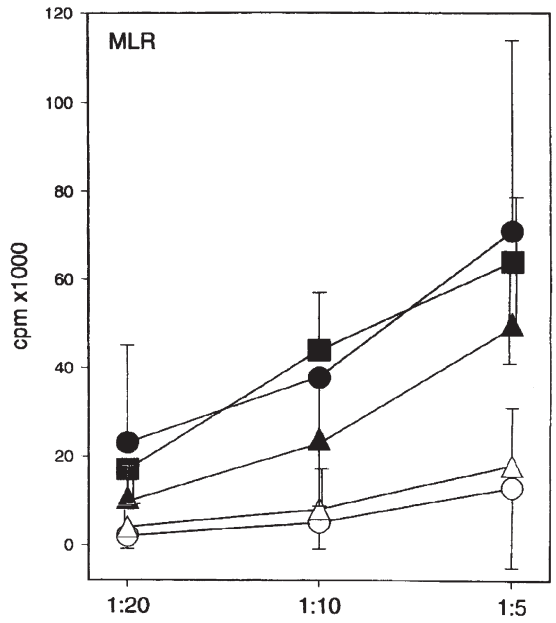

Figure 2. The T cell stimulatory capacity in alloMLR of the various antigen presenting cell (APC) populations studied. Mean cpm x 1000 (± s.d.) are given for various APC:T cell ratios (1:20; 1:10; 1:5). △: original monocytes (n=13); ▲: thyroid hormone-activated monocytes cultured overnight under nonadherent conditions (n=13); ●: as ▲, but MACS-separated into $CD14^{dim/-}$ VC (VC) (n=22); ○: as ▲, but MACS-separated into $CD14^{bright}$ VC (n=12) and ■: monocytes cultured for 7 days under plastic-adherent conditions in GM-CSF and IL-4 (DC) (n=6).

The VC suspension also showed a marked improved stimulatory capacity in the MLR as compared to the original monocytes (Fig. 2) (e.g. T3 as iodinated compound 14,218 ± 4307 cpm, DC: T cell ratio 1:20), and a reduced reactivity with the enzyme acid phosphatase (9 ± 4%).

Markers (as measured by immunocytochemistry on cytospins) that were upregulated in the VC population as compared to the original monocytes were HLA-DR (OK1a, 44 ± 17% vs 8 ± 5%), and the HLA-DQ related molecules RFD1 (61 ± 23% vs 6 ± 5%) and L25 (32 ± 14% vs 9 ± 8%). The DC specific marker CD83 was also upregulated, but to a lesser extent (9 ± 1% vs 3 ± 2%). Importantly there was almost no expression of DC-SIGN (a specific DC marker (16)), DC-LAMP (a specific IDC marker (17)) and Langerin (a specific Langerhans cell marker (18)) in the mo-derived VC population. There were no differences in results acquired from monocytes obtained by Percoll gradient separation or by elutriation in a limited series of experiments (n=3). For reasons of convenience, only Percoll-purified monocytes were used in further experiments.

With regard to DPI and ascorbate, Table I gives the data (plus total yield of cells). As can be seen DPI and ascorbate were equally as good as T3 in accelerating the transition of monocytes into VC. When exposed to these compounds and further cultured under plastic non-adherent

conditions monocytes turned for more than 40% into VC when optimal concentrations were used. In the presence of ascorbate the total yield of cells was slightly higher (45%, see Table I) as compared to DPI and T3, these latter conditions yielding in total around one third of the cells.

Table I. The generation of veiled cells (percentages ± standard deviation (upper part)) and total cell numbers (yield in percentages ± standard deviation (lower part) (n>5) from monocytes after exposure of the monocytes to agents which block NADPH-oxidases and further culture under non-plastic adherent conditions.

	Concentration			
	Nil	**10^{-12} M**	**10^{-9} M**	**10^{-6} M**
Triiodothyronine	24 ± 9	40 ± 12 [a]	30 ± 10	-
Diphenylene iodonium	24 ± 9	42 ± 8 [a]	36 ± 4 [a]	35 ± 6 [a]
Ascorbate	24 ± 9	42 ± 13 [a]	68 ± 10 [a]	52 ± 3 [a]

	Cell Yield			
	Nil	**10^{-12} M**	**10^{-9} M**	**10^{-6} M**
Triiodothyronine	29 ± 9	32 ± 9	-	-
Diphenylene iodonium	29 ± 9	32 ± 10	24 ± 9	35 ± 11
Ascorbate	29 ± 9	34 ± 7	42 ± 8	45 ± 5 [a]

[a] Significantly different from nil value, $p<0.05$. T test EXCEL computer program.

To see whether the monocytes cultured in the presence of GM-CSF/IL-4 and under plastic-adherent conditions also yielded any VC after one day, we examined monocytes exposed to these conditions and compared them to VC generated under above described conditions. After one day of culture, only few of the monocytes plastic adherent-cultured in GM-CSF and IL-4 displayed a veiled morphology (20 ± 5%) and the cells lacked movement patterns of lymph-borne VC. They also hardly stimulated T cells in the mixed leukocyte reaction (e.g., at a stimulator-to-T cell ratio of 1:20 1,000 ± 100 cpm).

$CD14^{dim/-}$ and $CD14^{bright}$ monocyte-derived VC

Since the VC obtained were clearly heterogeneous with respect to negative, dull and bright CD14 expression, and since classical DC are CD14–, we further purified the population on this basis. For the outcomes of the following experiments it did not matter which was the blocking compound used to generate the $CD14^{dim/-}$ and $CD14^{bright}$ VC, since marker expression, capabilities to stimulate T cells, to produce cytokines or to phagocytose were found predominantly linked to the separated populations itself.

Two different VC populations could be obtained using the mini-MACS with a mouse anti-human CD14 monoclonal antibody: $CD14^{dim/-}$ and $CD14^{bright}$ VC. The morphology and cinematography of the $CD14^{dim/-}$ and $CD14^{bright}$ VC was markedly similar, with 73 ± 8% and 82 ± 8% of the cells, respectively, displaying actively moving, long veils. In cytospin analysis HLA-DR was expressed by high numbers of both $CD14^{dim/-}$ and $CD14^{bright}$VC, i.e. over 80%. RFD1 expression

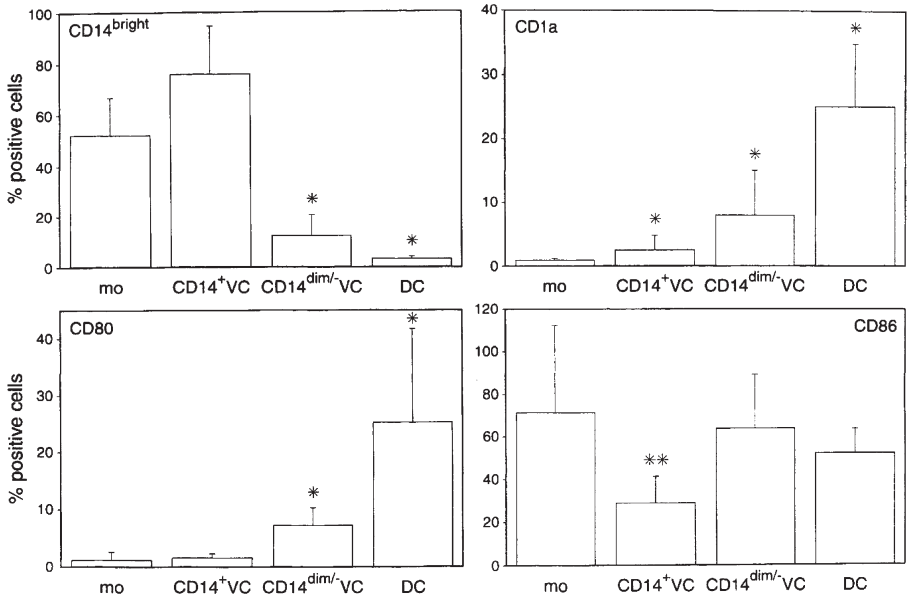

Figure 3. The CD14^{bright}, the CD1a, the CD80 and CD86 expression as determined by FACS analysis of original monocytes (mo, n=5), CD14^{bright} VC (VC, n=4), CD14$^{dim/-}$ VC (n=4) and 7 day cultured DC (DC) (n=5). Mean (± s.d.) of percentage positive cells are given; only large cells - the fraction containing veiled/DC - were included. For abbreviations see legend Fig. 1.

was 74 ± 13% and 66 ± 19%, respectively. Noteworthy the CD14$^{dim/-}$ VC and CD14^{bright}VC were both acid phosphatase negative (monocytes were largely positive).

Further flow cytometric analysis of surface markers (Fig. 3 and Table II) of these two MACS-separated populations revealed that the majority of the CD14$^{dim/-}$ fraction (Fig. 3) indeed expressed no CD14: there was a bright expression of CD14 on only 13 ± 8%, and a dim expression of CD14 by 38 ± 21%. The CD14^{bright} VC brightly expressed CD14 in 76.1 ± 18.5% of the cells. The MFI data on CD14 (Table II) corroborated these numerical findings.

The DC specific marker CD83 was expressed by 21 ± 4% of the CD14$^{dim/-}$ VC, and the MFI level of CD83 expression was clearly above that of monocytes and CD14^{bright} VC (Table II and Fig. 4) and comparable to that of iDC. Langerin, DC-SIGN and DC-LAMP did not come to expression in CD14$^{dim/-}$ VC (Table II). Only a small percentage of the CD14$^{dim/-}$ VC expressed CD1a (8 ± 7%) and CD80 (7 ± 3%) with a weak expression (Fig. 3 and Table II); 64 ± 25% of the CD14$^{dim/-}$ VC expressed CD86 (Fig. 3), and the MFI of CD86 expression was clearly above that of monocytes and CD14^{bright} VC, but lower than that of iDC (Table II). CD40 also came to a higher expression on CD14$^{dim/-}$ VC than on monocytes and CD14^{bright} VC (Table II).

CD14^{bright} VC expressed almost no CD1a and CD80. CD83 was expressed by only 3 ± 1% of these cells, and at a low level (Table II and Fig. 4). The cells lacked expression of DC-SIGN, DC-LAMP, and Langerin. Of note is that fewer CD14^{bright} VC expressed CD86 as compared to monocytes, but the positive cells had a higher level of expression (yet lower as compared to

Table II. FACS analysis of monocytes, CD14^{bright} VC, CD14$^{dim/-}$ VC and iDC (n=6 populations of 6 different donors). The mean fluorescence intensity and standard deviation are given.

Marker	Monocytes	CD14^{bright} VC	CD14$^{dim/-}$ VC	iDC
CD14	2925 ± 1095	2295 ± 743	674 ± 350[a]	280 ± 230[a]
HLA-DR	212 ± 113	403 ± 96[a]	910 ± 359[a]	870 ± 570[a]
CD83	12 ± 6	24 ± 9	101 ± 32[a]	150 ± 63[a]
CD1a	9 ± 2	19 ± 7	16 ± 4	270 ± 350[a]
CD80	7 ± 3	27 ± 10	37 ± 31	290 ± 130[a]
CD86	55 ± 13	154 ± 42[a]	291 ± 73[a]	400 ± 270[a]
CD40	89 ± 40	124 ± 63	215 ± 81[a]	710 ± 297[a]
DC-LAMP	19 ± 2	28 ± 3	23 ± 4	276 ± 189[a]
DC-SIGN	36 ± 9	7 ± 20	44 ± 12	7775 ± 809[a]
Langerin	7 ± 3	10 ± 2	9 ± 7	59 ± 37

[a] Significantly different from monocytic value, $p<0.05$. T test EXCEL computer program.

CD14$^{dim/-}$ VC, Table II). There was an equal expression of CD40 by the CD14^{bright} VC as compared to the original monocytes (Table II).

Classical mo-derived iDC after seven days in plastic adherent culture with GM-CSF and IL-4 formed a population of cells that contained large numbers of cells with a typical dendritic morphology. Of note is that CD14^{bright} expression was clearly reduced (Fig. 3 and Table II) as compared to the original monocytes, while at the same time CD1a and CD80 expression were markedly increased, both being higher than in the CD14$^{dim/-}$ and CD14^{bright} VC (Fig. 3 and Table II). Also the intensities (MFI) of CD86 and CD83 expression were clearly upregulated in comparison to monocytes, but not statistically significantly higher than in the CD14$^{dim/-}$ VC population (Table II). CD 40, DC-LAMP and DC-SIGN expression was markedly upregulated as compared to monocytes, CD14$^{dim/-}$ VC and CD14^{bright} VC (Table II).

The generated VC and DC were also tested for immunoreactivity and production of the calcium-binding protein S100 by immunocytochemistry and EIA. Using immuno-cytochemistry, 71 ± 11% of the CD14$^{dim/-}$ VC were positive for S100, as compared to 25 ± 9% of the CD14^{bright} VC and 22 ± 6% of the original monocytes (n=2). The percentage of the unseparated VC positive for S100 was 62 ± 5% (n=2). Immature DC were S100^{+} in <1%, although many of the cells were faintly stained. These immunocytochemical data correlated well with S100 protein concentrations in the media of the various cell populations in culture, resulting in the highest concentration of S100 in the CD14$^{dim/-}$ VC (1.64 ng/ml, n=1) followed by the unseparated VC (1.34 ng/ml, n=1), CD14^{bright} VC (1.11 ng/ml, n=1) and the starting monocyte population (0.97 ng/ml, n=1). The immature DC were not tested in this respect.

Functional capabilities (accessory/skewing function, IL-12/IL-10 production, phagocytosis) of VC generated by 24 hr non-adherent culture of monocytes as compared to GM-CSF/IL-4-induced monocyte-derived DC

Accessory function. Firstly, the two VC populations, the original blood monocytes and the iDC generated by culturing monocytes under adherent conditions in GM-CSF and IL-4 for seven

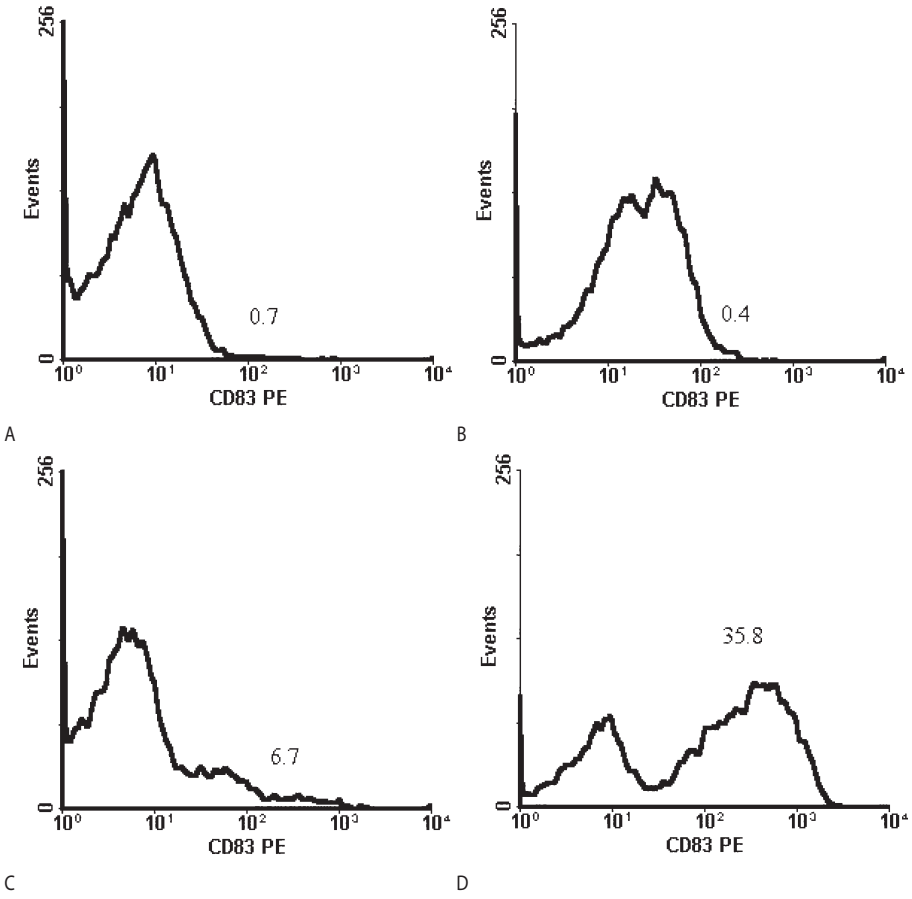

Figure 4. A representative example of the CD83 expression as determined by FACS analysis of monocytes and their descendent $CD14^{bright}$ VC, $CD14^{dim/-}$ VC, and iDC. Histogram profiles of the expression of CD83 of monocytes (a), of thyroid hormone-activated monocytes cultured overnight under nonadherent conditions and MACS-separated into $CD14^{+}$ VC (b), of thyroid hormone-activated monocytes cultured overnight under nonadherent conditions and MACS-separated into $CD14^{dim/-}$ VC (c), and of monocytes cultured for 7 days in GM-CSF/IL-4 (iDC) (d). Percentages of cells positive for CD83 are indicated in the plots.

days were cocultured with allogeneic T cells in the MLR to study their accessory capability. Different numbers of stimulatory cells were cultured with a fixed number of allogeneic T cells, resulting in stimulatory-to-T cell ratios of 1:5, 1:10, and 1:20 (Fig. 2). At all ratios tested the $CD14^{dim/-}$ VC were as good stimulators of T cell proliferation as iDC: at a ratio of 1:5 71,380 ± 43,730 vs 64,183 ± 22,844 cpm, respectively; at a ratio of 1:10 39,143 ± 27,175 and 44,849 ± 14,421 cpm, respectively, and at a ratio of 1:20 23,094 ± 22,644 and 17,678 ± 8,491 cpm, respectively. In contrast, $CD14^{bright}$ VC were relatively poor stimulators of T cell proliferation: 13,656 ± 18,559 cpm at a stimulatory-to-T cell ratio of 1:5, comparable to monocytes (18,118 ± 13,440 cpm), and almost non-stimulatory at a ratio of 1:20: 1,993 ± 2,265 cpm.

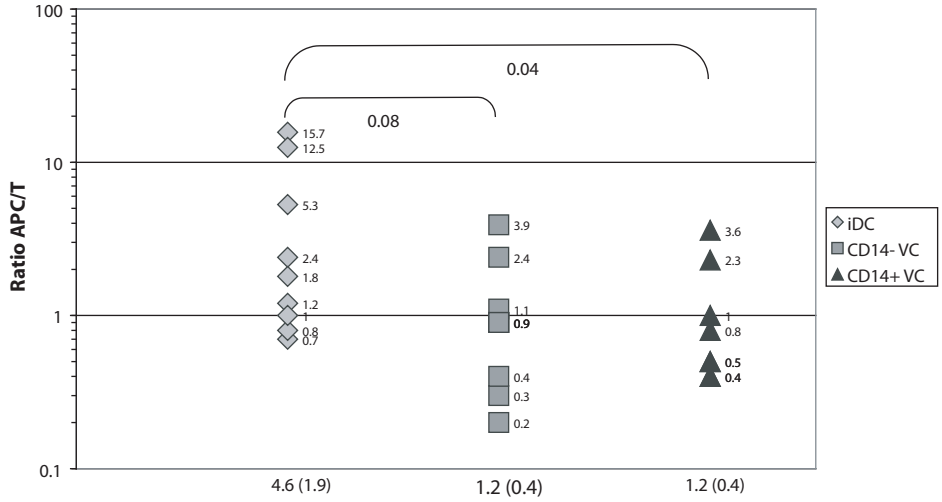

Figure 5. T cell skewing capacities of the various APC populations studied. The induction of IFN-γ-producing T cells (Th1 cells) by VC and DC populations cocultured for 2 days with T cells at a concentration of 1:10. Mean (± s.e.) of IFN-γ production by T cells was calculated as the ratio of IFNγ production by T cells cocultured with VC or DC corrected for the basal production of T cells. ◇: monocytes cultured for 7 days under plastic-adherent conditions in GM-CSF and IL-4 (iDC); □: thyroid hormone-activated monocytes cultured overnight under nonadherent conditions MACS-separated into $CD14^{dim/-}$ VC; ▲, thyroid hormone-activated monocytes cultured overnight under nonadherent conditions MACS-separated into $CD14^{bright}$ VC.

IL-12/IL-10 production. The $CD14^{bright}$ and $CD14^{dim/-}$ VC populations were also tested for the production of IL-10 and IL-12, and compared to the original monocyte population and the GM-CSF/IL-4- induced iDC (Fig. 6). Monocytes and the $CD14^{bright}$ VC produced the greatest amounts of IL-10 (3106 ± 503 pg/ml, n=21 and 3472 ± 1046 pg/ml, n=10, respectively) (measurements are for 0.5 x 10^4 cells). The production by the $CD14^{dim/-}$ VC was similar at 2347 ± 754 pg/ml (n=10). In contrast, the iDC exhibited a very low IL-10 production (326 ± 82 pg/ml, n=8), which had clearly decreased from the original monocyte population (875 ± 316 pg/mL, n = 8 vs. 333 ± 76, n = 17, respectively, p = 0.02).

With regard to IL-12, a shut-down in production occurred in the $CD14^{bright}$ VC population (74 ± 29 pg/ml, n=5) when compared to IL-12 production by monocytes (333 ± 76 pg/ml, n=17, p=0.02). $CD14^{dim/-}$ VC had a production of IL-12 equal to that of the original monocytes. The level of IL-12 production had increased in the DC as compared to their original monocyte populations (875 ± 316 pg/ml, n=8 vs. 333 ± 76, n=17, respectively, p=0.02).

Skewing function. Furthermore, the VC and DC populations were tested for their T cell skewing capacities by their capability to prime T cells that produce IFNγ (a Th1 cytokine). As shown in Figure 5 DC stimulated the production of IFNγ by T cells by 4.6-fold (s.e. 1.9), whereas both $CD14^{dim/-}$ VC and $CD14^{+}$ VC stimulated the production of IFNγ by T cells just 1.2-fold (s.e. 0.4).

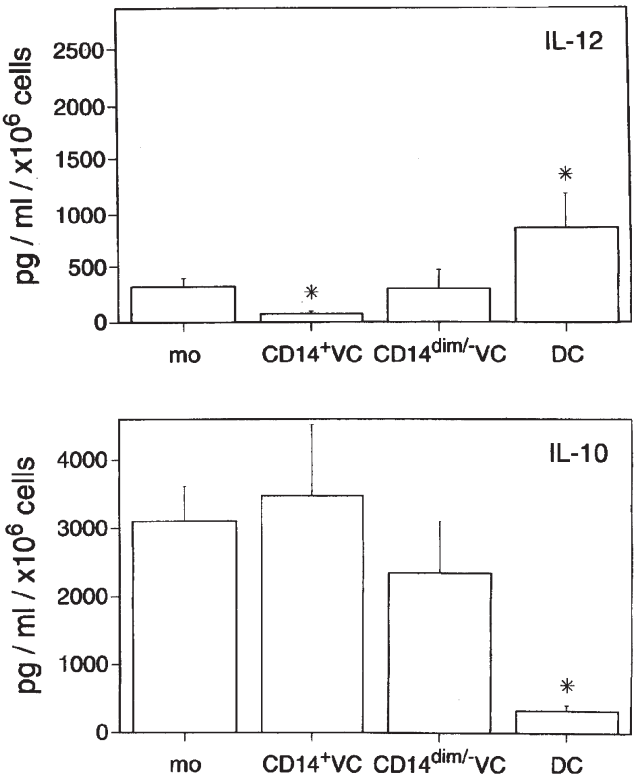

Figure 6. The IL-12 and IL-10 production of original monocytes (mo, n=17), $CD14^{bright}$ VC (VC, n=10), $CD14^{dim/-}$ VC (n=10) and 7 day cultured DC (n=8). Mean (± s.d.) of production (pg/ml/10^6 cells) are given. [a] $p<0.01$ vs. orginal monocytes.

Taken both VC populations together, the VC were significantly (p=0.05) less potent in generating Th1 cells as compared to iDC.

Phagocytosis. The APC populations generated under non-adherent and plastic-adherent conditions were finally tested and compared to monocytes for their ability to perform phagocytosis. During a ten minute incubation with *E.coli*, 54 ± 23% of the monocytes phagocytosed these bacteria. Both the $CD14^{dim/-}$ and $CD14^{bright}$ VC (24 ± 12%, p=0.06 and 33 ± 11%, p=0.03, respectively) were less capable of phagocytosing *E. coli* than were monocytes. The iDC only had a minor capability to phagocytose *E. coli* (0.8 ± 0.7% immature DC phagocytosed E.coli).

Phenotype of the $CD14^{dim/-}$ and $CD14^{bright}$ VC obtained from non-adherent-cultured monocytes after further long-term culture under plastic adherent conditions in the presence of GM-CSF/IL-4 or LPS

To see if our VC could become iDC or, like iDC, become mature DC we cultured the $CD14^{dim/-}$ and $CD14^{bright}$ VC under plastic adherent conditions in the presence of GM-CSF and IL-4, or LPS (Table III). After culturing under these conditions, however, the viability of the two VC populations greatly decreased.

Of the $CD14^{dim/-}$ VC, only 50-65% (GM-CSF/IL-4) or 25-50% (LPS) were viable after two days. When cultured with GM-CSF/IL-4, the $CD14^{dim/-}$ VC exhibited a decrease in CD14 expression before dying (12 ± 7% positive cells) as compared to the original population (26 ± 14% positive cells). There was however no increase in CD1a expression (3 ± 2% positive cells on day 3, as compared to 4 ± 3% positive cells on day 1) and even a decrease in CD80 expression (6 ± 6% positive cells on day 3, as compared to 18 ± 7% positive cells on day 1). Expression of CD86 was reduced from 43 ± 28% positive cells on day 1 to 16 ± 15% positive cells on day 3. Hence there were absolutely no signs of further maturation into the direction of DC-like cells. These signs were also absent during culture with LPS. Under these conditions the cells in fact changed to rounded-up macrophage-like cells before dying (Table III): the cells lost their veils and became acid phosphatase positive (dotted macrophage-like patterns) while losing CD14 expression.

When the $CD14^{+}$ VC were cultured in GM-CSF/IL-4 or LPS there was also a considerable death of cells (though slightly less than with the $CD14^{dim/-}$ DC), and viability results of 50-55% (GM/CSF/IL-4) and 60-70% (LPS) were found after two days of culture. When cultured with GM-CSF/IL-4, the $CD14^{bright}$ VC clearly lost their CD14 expression: 30 ± 23% positive on day 3 of culture as compared to 81 ± 8% initially. This however was not combined with noteworthy increases in CD1a expression (6 ± 3% positive cells on day 3, as compared to 3 ± 1% positive cells on day 1) and CD80 expression (9 ± 5% positive cells on day 3, as compared to 4 ± 1% positive cells on day 1). CD86 expression was even slightly decreased (33 ± 16% positive cells on day 3, as compared to 46 ± 13% positive cells on day 1). Hence there were again no signs of "maturation". Also in the presence of LPS the $CD14^{bright}$ VC did not acquire a typical mature DC phenotype.

DISCUSSION

This study shows that based upon the avoidance of plastic adherence (culture in polypropylene tubes) and avoidance of NADPH oxidase activation (via exposure to blocking agents) a heterogeneous population of accessory cells with long actively moving protrusions could be generated from blood monocytes within 24 hours.

These rapidly in vitro-generated mo-derived veiled cells resembled in a number of aspects the veiled cells described in lymph: the cells displayed a movement behaviour known of lymph-borne VC, they were MHC class II and S100 positive and they were acid phosphatase negative.

Table III. The viability and phenotypic characteristics of $CD14^{bright}$ and $CD14^{dim/-}$ VC before and after culture in cytokines (GM-CSF and IL-4) or LPS

Cell population	Viability	Veils	Acid Phosphatase	$CD14^{bright/dim}$*	CD1a *	CD80*	CD86*
$CD14^{dim/-}$ VC (MACS-separated)	92 - 99%	70 - 80%	neg.	26 ± 14%	4 ± 3%	18 ± 7%	43 ± 28%
* same, 2 days additional culture with cytokines	50 - 65%	60 - 70%	neg.	12 ± 7%	3 ± 2%	6 ± 6%	16 ± 15%
* same, 2 days additional culture with LPS	25 - 50%	10 - 20%	pos.	11 ± 10%	5 - 6%	-	50 - 60%
$CD14^{bright}$ VC (MACS-separated)	90 - 97%	75 - 95%	neg.	81 ± 8%	3 ± 1%	4 ± 1%	46 ± 13%
* same, 2 days additional culture with cytokines	50 - 55%	85 - 90%	neg.	30 ± 23%	6 ± 3%	9 ± 5%	33 ± 16%
* same, 2 days additional culture with LPS	60 - 70%	80 - 85%	neg.	15 - 20%	5 - 7%	-	20 - 30%

• by FACS analysis (all cells are included), neg. = negative or weak juxtanuclear staining, pos.= strongly staining dots, - = not done, mean ± s.d. or range.

Two major populations of in vitro-generated mo-derived veiled accessory cells could be distinguished based upon the expression of CD14: a $CD14^{bright}$ fraction and a $CD14^{dim/-}$ fraction. These populations differed from each other and from classical DC in functional capacity, marker expression and survival capability. The $CD14^{dim/-}$ fraction had a clearly increased T cell stimulatory capacity as compared to monocytes and were the far better stimulators of allogeneic T cell proliferation as compared to the $CD14^{bright}$ fraction. In fact, they were comparable in allogeneic MLR to iDC which also formed a more or less $CD14^{negative}$ homogeneous population, as is also known from literature. However, the $CD14^{dim/-}$ VC did not or hardly express CD1a, Langerin, DC-SIGN and DC-LAMP, and CD80 only to a limited extent. They were however clearly positive for S100 and a considerable proportion for CD83. Although the strong S-100 positivity of our in vitro generated VC is reminiscent of that of various peripheral subsets of DC-like cells such as those of the skin (31), lung (32), gut (33) and endocrine glands (21, 34, 35), we nevertheless do not consider our cells as classical DC. The absent to lower level of expression of CD1a, Langerin, DC-SIGN and DC-LAMP and CD80 by our in vitro generated $CD14^{dim/-}$ mo-derived veiled accessory cells and the lower level of production of IL-12 and greater production of IL-10 in comparison to mo-derived DC indicate that the $CD14^{dim/-}$ veiled accessory cells must be accessory cells separate from classical DC. To investigate the possible potential of our $CD14^{dim/-}$ VC to mature into classical DC, we tried to culture them for an additional week using the same culture conditions as has been reported for the generation of immature and mature DC, and added GM-CSF, IL-4 or LPS to the cultures. We found that the $CD14^{dim/-}$ VC do not remain viable under these culture conditions where, also in our hands, iDC do remain viable and gain in MHC class II, CD83 and CD80 expression. In fact, the $CD14^{dim/-}$ VC did not gain in CD1a or CD80 expression at all, and even became macrophage-like cells before dying in LPS culture conditions. This underscores the separate character of the $CD14^{dim/-}$ VC from classical DC, and indicates that the cell is mainly related to the monocyte-macrophage series. It is thus likely that our $CD14^{dim/-}$ mo-derived VC require other factors than GM-CSF/IL-4 and LPS for their sustenance, and we recently found that IL-3 is such a factor (to be published).

Interestingly Randolph et al (36) created a population of veiled/dendritic cells from monocytes somewhat reminiscent of ours using a system of reverse transmigration. When monocytes were cultured with endothelium on a collagen matrix the reversed transmigrated cells differentiated within 2 days into a subset of $CD14^{-}CD1a^{-}CD83^{-}$ veiled/dendritic cells. These veiled/dendritic cells generated from monocytes during the process of reversed transmigration reverted in part to macrophages when exposed to LPS or particles in the subendothelial collagen (36), the remaining part was however able to transform -in contrast to our cells- into mature DC expressing CD83.

Since the $CD14^{dim/-}$ veiled macrophage-like cells were equally good T cell stimulators as the iDC (as measured in the MLR) and good producers of IL-10, while their CD80 expression and IL-12 production were low, it was tempting to speculate that these $CD14^{dim/-}$ VC do not

stimulate Th1 cells. Indeed we found that our CD14$^{dim/-}$ VC induced T cells that produced less IFNγ as compared to iDC. It is further worthy to note that S100-expressing accessory cells with a similar costimulatory molecule expression and cytokine production profile as our CD14$^{dim/-}$ VC population have been described in vivo in lung digests (38) and in the bronchoalveolar lavage fluid (39). These human airway-associated APC have also been suggested to be predominantly Th2-stimulatory cells (38, 39), since airway APC of the rat with a similar immature marker pattern were proven to stimulate Th2 pathways (40).

Apart from CD14$^{dim/-}$ VC, there was the CD14^{bright} population. Although veiled and actively moving, these VC hardly expressed DC related markers and costimulatory molecules, and had the same low T cell stimulatory capacity as monocytes. We therefore do not consider this population as specialized in expanding T cell populations. This poses the question of what the function of these (still largely monocytic) VC might be. Our studies show that their prime role does not lie in phagocytosis, as is also indicated by their low acid phosphatase content. CD14^{bright} veiled monocytic cells were, however, very actively moving cells and formed large homotypic clusters as well as clusters with T cells (cinematographic studies, not shown here), suggesting a role for this population in immunoregulation. Noteworthy in this respect are its very low IL-12 production, combined with a high IL-10 production and moderate MHC-class-II expression. This profile suggests that the CD14^{bright} VC population might provide signals for anergy induction, but this assumption clearly needs further investigation. It is also worthy to note that the CD14^{bright} cells did not change into more typical DC-like cells after a further culture in GM-CSF/IL-4 or LPS. Again the viability of the cells was a problem.

In conclusion, what is evident from this body of work, is that within 24 hours a heterogenous population of veiled cells can be generated from monocytes when avoiding plastic adherence and avoiding the activation of NADPH oxidase. Of this population particularly the CD14$^{dim/-}$ fraction has acessory capabilities in T cell stimulation. However, the marker pattern and cytokine production of these VC indicate that this population is not a classical DC population. The cells might earlier be related to veiled macrophage-like cells also described in afferent lymph in the 1970-1980s; however detailed studies on such lymph-borne cells using markers developed in the last decades are required to strengthen or refute such view.

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Chapter 4

Opposing effects of dehydroepiandrosterone and dexamethasone on the generation of monocyte-derived dendritic cells

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ABSTRACT

Background: Dehydroepiandrosterone (DHEA) has been suggested as an immunostimulating steroid hormone, of which the effects on the development of dendritic cells (DC) are unknown. The effects of DHEA often oppose those of the other adrenal glucocorticoid, cortisol. Glucocorticoids (GC) are known to suppress the immune response at different levels and have recently been shown to modulate the development of DC, thereby influencing the initiation of the immune response. Variations in the duration of exposure to, and doses of GC (particularly dexamethasone (DEX)) however, have resulted in conflicting effects on DC development.

Aim: In this study, we describe the effects of a continuous high level of exposure to the adrenal steroid DHEA (10^{-6} M) on the generation of immature DC from monocytes, as well as the effects of the opposing steroid DEX on this development.

Results: The continuous presence of DHEA (10^{-6} M) in GM-CSF/IL-4-induced monocyte-derived DC cultures resulted in immature DC with a morphology and functional capabilities similar to those of typical immature DC (T cell stimulation, IL-12/IL-10 production), but with a slightly altered phenotype of increased CD80 and decreased CD43 expression (markers of maturity).

The continuous presence of DEX at a concentration of 10^{-6} M in the monocyte/DC cultures resulted in the generation of plastic-adherent macrophage-like cells in place of typical immature DC, with increased CD14 expression, but decreased expression of the typical DC markers CD1a, CD40 and CD80. These cells were strongly reactive to acid phosphatase, but equally capable of stimulating T cell proliferation as immature DC. The production of IL-12 by these macrophage-like cells was virtually shut down, whereas the production of IL-10 was significantly higher than that of control immature DC.

Conclusion: The continuous presence of a high level of GC during the generation of immature DC from monocytes can modulate this development away from DC towards a macrophage-like cell. The combination of a low CD80 expression and a shutdown of IL-12 production suggests the possibility of DEX-generated cells initiating a Th2-biased response. These effects by DEX on DC development contrast with those by DHEA, which resulted in a more typical DC although possessing a phenotype possibly indicating a more mature state of the cell.

INTRODUCTION

Dehydroepiandrosterone (DHEA) is quantitatively the most abundant adrenal steroid hormone in humans and other mammals (1, 2). The hormone is uniquely sulfated (DHEA-S) before entering the plasma, and the sulfated prohormone is converted to DHEA and its metabolites (3) in various peripheral tissues. No major endocrine functions have been ascribed to a direct action of DHEA-S and DHEA, although the hormones act as intermediaries in sex steroid synthesis (3). Both hormones, however, have been proposed as exerting important restoring effects on age-related processes, such as fat depot distribution and neurodegeneration.

These effects also include major stimulation of cells of the (aging) immune system (4-6). However, these effects of DHEA have also been disputed (7, 8).

Dendritic cells (DC) are antigen-presenting cells *par excellence*, and the only cells capable of stimulating naïve T cells and thus capable of initiating primary immune responses (9). A well-accepted method of generating DC from monocytes is the culture of monocytes in the presence of the cytokines GM-CSF and IL-4 for 7 days (10). This procedure yields the socalled

`immature' DC, with a retained capability for uptake and processing of antigens but with a relatively low capability to stimulate T cells. A further exposure of `immature' DC to pro-inflammatory stimuli (e.g. IL-1, lipopolysaccharide (LPS)) generates mature forms of

the cell with an enhanced capability to stimulate T cells, but an almost lost capability for antigen uptake and processing (11).

There are no reports on the effects of DHEA on dendritic cell development. We previously reported that the exposure of monocytes to hormones (in particular to triiodothyronine) stimulated DC development from monocytes (12, 13). We now have tested and report here the effects of exposure of monocytes to DHEA, prior to, and during, their differentiation into immature DC under the influence of GM-CSF and IL-4.

In biological systems, the effects of DHEA-S and DHEA are often opposed by the other important adrenal steroid cortisol (14). The ratio DHEA/cortisol is abnormal in various pathological conditions characterized by immune dysfunction, such as after thermal injury, in AIDS, in rheumatoid arthritis and in tuberculosis (15-19). Although the suppressive effects of glucocorticoids on T cells, B cells, monocytes and macrophages have extensively been studied (reviewed in 20, 21), there is a growing, but still limited number of reports on the effects of glucocorticoids on the function and differentiation of DC (22-26). Data in these reports are inconsistent regarding effects on marker expression, T cell-stimulatory capacity and cytokine production. We therefore contrasted our DHEA experiments with dexamethasone (DEX) and tested the effect of this hormone on the process of the transition from monocyte to immature DC also.

MATERIALS AND METHODS

Isolation of monocytes from peripheral blood

Monocytes were isolated from the peripheral blood of healthy blood donors via well-accepted methods. Heparinized blood diluted with an equal volume of phosphate buffered saline (PBS) was distributed over Ficoll Isopaque (density 1.077 g/ml, Pharmacia, Uppsala, Sweden) and centrifuged for 15 min at 1000 g. Cells were collected from the interface and washed and then suspended in RPMI1640with 25 mMHEPES and L-glutamine (GIBCO, LifeTechnologies, Breda, the Netherlands), supplemented with 100U/ml penicillinG, 0.1 mg/ml streptomycin (both Seromed, Biochrom, Berlin, Germany) and 10% FCS (Bio Whitaker, USA) (hereafter knownas RPMI 1640^{+}) added. This cell suspension was distributed over Percoll (density 1.063 g/ml, Pharmacia, Uppsala, Sweden), then centrifuged for 40 min at 400 g. Cells collected from the interface were washed and suspended in RPMI 1640+. Monocyte purity was determined by non-specific esterase staining (NSE) (27). Cell suspensions containing 80% or more monocytes were frozen following standard procedures and stored in liquid nitrogen, providing a bank for experiments. Monocytes purified by elutriator centrifugation were also used (courtesy of CLB, Amsterdam), in order to confirm results obtained via Ficoll/Percoll gradient separation.

Culture of DC from peripheral blood monocytes

DC were obtained via the well-established method first described by Sallusto and Lanzavecchia (10). Briefly, monocytes were cultured for 1 week at 37 ˚C, in 5% CO2 and 100% humidity at a concentration of 3×10^5 cells=ml in RPMI 1640^{+} with 800 U/ml GM-CSFand 1000 U/ml IL-4. The cultures were fed every 2 days, by removing 500 µl culture fluid and replacing this with 1 ml of fresh medium containing cytokines. In order to test the effects of exposure to DHEA and DEX on the monocyte-to-DC transition, these hormones were added at an optimal concentration of 10^{-6} M to monocytes in RPMI 1640 culture medium (without FCS) and incubated for 30 min at 37 ˚C, 5% CO2, 100% humidity after which FCS (10%), GM-CSF (800 U/ml) and IL-4 (1000 U/ml) were added to the culture. DHEA and DEX were also fed to the cultures every 2 days along with fresh medium and cytokines. All culture medium used was tested and found to be free of endotoxin. Both hormones were purchased free from endotoxin contamination.

Flow cytometry and immunocytochemistry

For analysis of marker expression by flow cytometry, all cell populations were stained by incubating for 10 min with mouse anti-human FITC- or PE-conjugated monoclonal antibodies, followed by three washes. The monoclonal antibodies used were My4 (CD14, Beckman Coulter, Hialeah, FL, USA), CD1a (Beckman Coulter, Hialeah, FL, USA), B7.1 (CD80, Becton Dickinson, San Jose, CA, USA), B7.2 (CD86, PharMingen, Los Angeles, CA, USA), CD 40 (Serotec, Oxford, UK), CD43 (Biosource, Camarillo, CA, USA) and CD83 (Immunotech, Marseilles, France). Imme-

diately following the staining, cells were analyzed on a FACScan (Becton Dickinson, San Jose, CA, USA).

The reactivity of the various monocyte/DC populations to acid phosphatase was determined using cytospins prepared on a Cytospin apparatus (Nordic Immunological Laboratories, the Netherlands) Cytospins were air-dried, then incubated for acid phosphatase staining according to Katayama *et al*. (28), using naphtol AS-BI phosphate as substrate and hexarotised pararosanilin as coupling agent (37 ˚C, 30 min). Slides were counterstained with hematoxylin. Preparations were mounted in DePex mounting medium (Gurr, BDH Ltd, Poole, UK).

Mixed leukocyte reaction

Allogeneic mixed leukocyte reactions (MLRs) were performed in order to measure the accessory capability of the various DC populations generated. Responder T lymphocytes were obtained from a healthy donor and isolated following standard procedures with Ficollisopaque,

Percoll density gradient centrifugation, and nylon wool adherence (Leuko-Pak, Fenwall Laboratories, IL, USA). Non-adhering cells recovered were greater than 90% CD3 positive. A total of 1.5 x 10^5 responder cells were cultured in 96-well, flat-bottom microtitre plates (NUNC A/S International, Denmark) with different numbers of irradiated (2000 rad) stimulator cells (monocytes or DC) to achieve stimulator-to-T cell ratios of 1:5, 1:10, 1:20 and 1:40. The culture medium used was RPMI 1640 with 25 mM HEPES and L-glutamine, supplemented with 100 U/ml penicillin G, 0.1 mg/ml streptomycin and 10% human A^+ serum, in a total volume of 200 µl per well. The controls used were monocytes or DC alone, and lymphocytes in the presence of 10-50 µg/ml phytohemagglutinin (PHA) (Wellcome Diagnostics, Zeist, the Netherlands). Cultures were performed in triplicate. On day 5, thymidine incorporation was assayed by adding 0.5 µCi [^{3}H]-thymidine to each well. Cells were harvested 16 h later and the radioactivity counted on an LKB 1205 Betaplate liquid scintillation counter (Wallac, Turku, Finland).

IL-12/IL-10 production

The DC were placed in 24-well plates (NUNC) at aconcentration of 5 x 10^5 cells/ml and cultured for 24 h in $RPMI^+$ containing ultraglutamine (2 mM, BioWhitaker), penicillin/streptomycin (100 U/ml, 100 µg/ml, BioWhitaker) and serum free medium supplement (Pepro Tech, Rocky Hill, NJ, USA). To stimulate IL-10 production, the culture fluid contained *Staphylococcus aureus* cowan 1 strain (SAC) (1:5000, Calbiochem, La Jolla, CA, USA). The IL-12 production was stimulated by SAC (1:5000) and γ-IFN (1000 U/ml, Biomedical Primate Research Centre, Rijswijk, the Netherlands). The production was measured by ELISA as indicated by the manufacturer (for IL-10, ELISA Pelikine, CLB, Amsterdam, the Netherlands; IL-12, Eli-pair, Diaclone, Besançon, France).

RESULTS

DHEA and DEX influence the phenotype of DC in culture

Dose-response curves were carried out to determine the optimal concentration of DHEA to add to the monocytes in culture. On the basis of our previous experience using various hormones to generate veiled cells (12, 13), the optimal concentration was determined to be that which resulted in the greatest number of cells displaying a veiled morphology. The optimal concentration for DHEA was found to be 10^{-6} M. In the case of DEX, we had previously carried out dose ± response curves and have shown that although concentrations of 10^{-9} M were already effective, a concentration of 10^{-6} M added to DC obtained from bronchial alveolar lavage was optimal for obtaining a decreased function as well as a decrease in costimulatory molecule expression (29). We have therefore used 10^{-6} M DHEA and 10^{-6} M DEX in our subsequent experiments.

The addition of DHEA and DEX to the cultures resulted in the generation of DC with significantly different phenotypes than those generated with cytokines alone (Fig. 1), while not affecting cell viability or cell survival, as determined by cell recovery numbers and trypan blue exclusion.

Adding DHEA to the cultures resulted in a DC population with a marked upregulation of the costimulatory molecule CD 80 (45 ± 9% vs 22 ± 8% positive cells in the control culture, P = 0.05). This was contrasted by a sharp downregulation of CD80when DEX was added (1 ± 0% vs. 22 ± 8% positive cells in the control culture, P = 0.05). Moreover, in the presence of DEX the morphology of the cells had changed, appearing as largely adherent macrophage-like cells with blunted cytoplasmic processes. The expression of the co-stimulatory molecule CD86 remained constant, regardless of exposure to either DHEA or DEX.

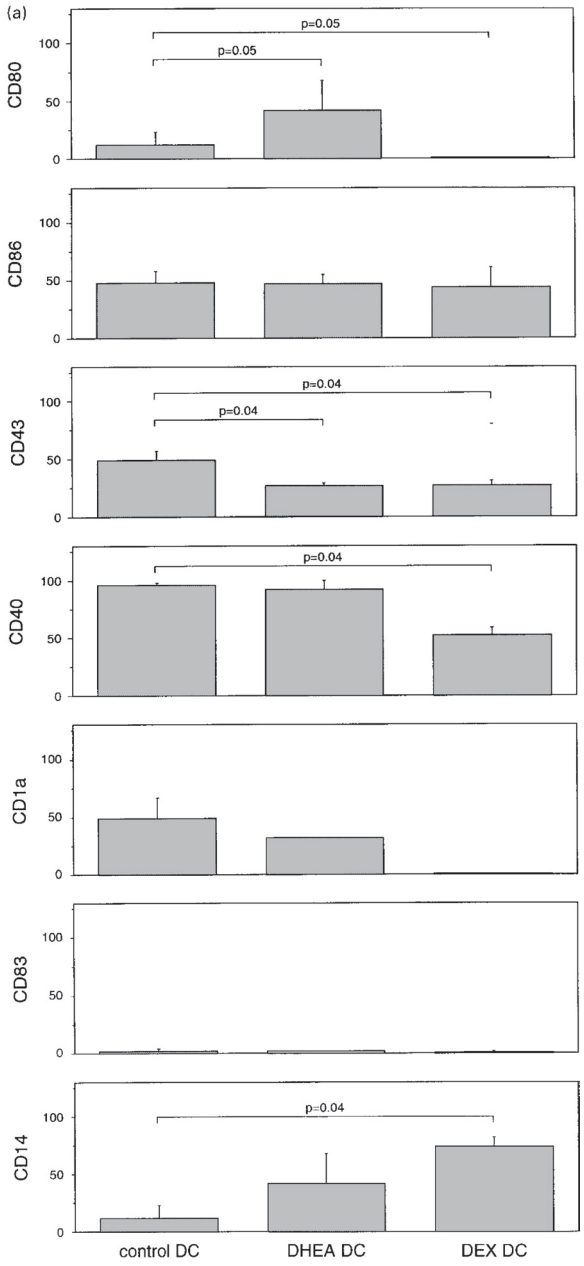

Figure 1a. The CD80, CD86, CD43, CD40, CD1a, CD83 and CD14 expression as determined by FACS analysis of control monocyte-derived DC and DC derived from monocytes in the continuous presence of 10^{-6} M DHEA (DHEA DC) or 10^{-6} M dexamethasone (DEX DC).
Means (±s.d.) of percentage positive cells are given; only large cells were included. *P* values are as indicated, n = at least five separate experiments.

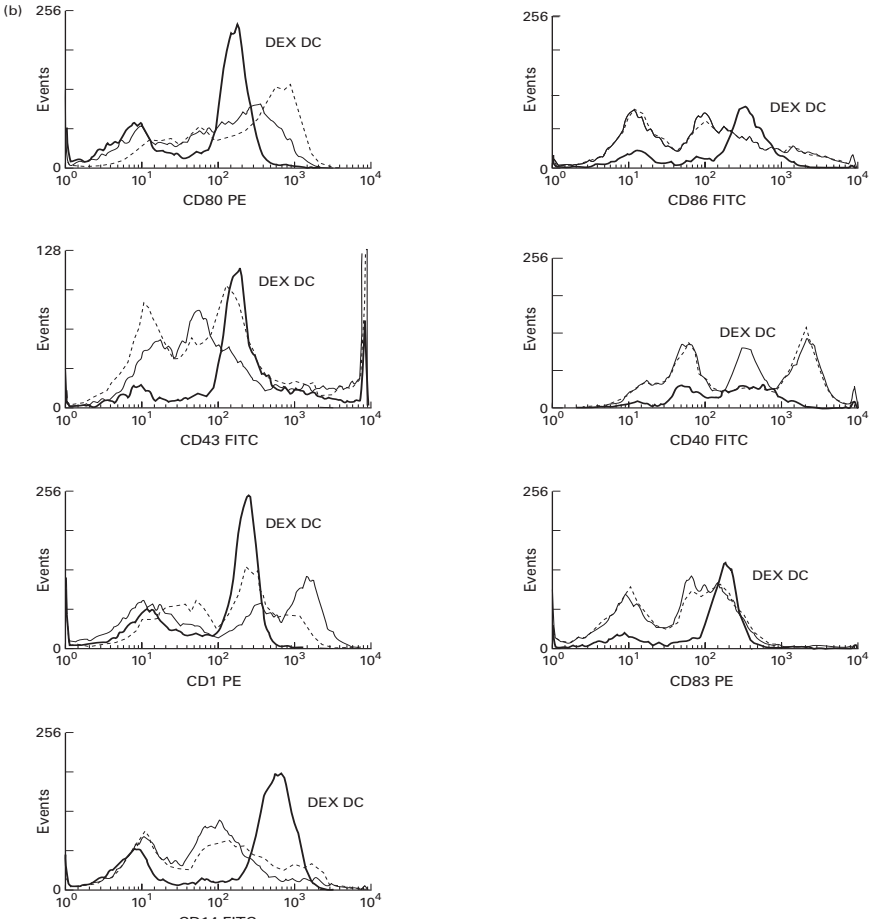

Figure 1b. The CD80, CD86, CD43, CD40, CD1a, CD83 and CD14 expression as determined by FACS analysis of control monocyte-derived DC and DC derived from monocytes in the continuous presence of 10^{-6} M DHEA (DHEA DC) or 10^{-6} M dexamethasone (DEX DC).

Overlapping histogram profiles of the expression of each marker in a representative experiment. Control DC are represented by fine line, DHEA DC are represented by a dotted line and DEX DC are represented by a thick line.

Both hormones decreased the expression of the antiadhesion molecule CD43 (a marker of immature DC, see Discussion) from 49 ± 8% positive cells in the control culture to 27 ± 2% positive cells in the DHEA culture (P = 0.04) and 27 ± 4% positive cells in the DEX culture (P = 0.04). Whereas DHEA had no effect on CD40 expression, the addition of DEX to the culture significantly downregulated its expression (96 ± 2% positive cells in the control culture vs 52 ± 7% positive cells in the dexamethasone culture, P = 0.04). Both CD43 and CD40 play a role in the further maturation of immature DCs by inducing homotypic interactions (see Discussion) or when ligated by CD40 ligand on T cells respectively.

The expression of CD1a was also downregulated by DEX from 49 ± 18% positive cells in the control culture to 1 ± 1% positive cells in the DEX culture (Fig. 1). The presence of DHEA in the culture also resulted in a slight decrease in CD1a expression, from 49 ± 18% positive cells in the control culture to 32 ± 0% in the DHEA culture (Fig. 1).

The expression of the mature DC marker CD83 was extremely low in all cultures, underscoring the immature status of all DC populations under study.

As expected, most dendritic cells from the control cultures were CD14 negative. A significant increase in CD14 marker expression was seen in cells from cultures containing DEX (74 ± 8% positive cells vs 12 ± 11% positive cells in control cultures, P = 0.04). Cells from the cultures containing DHEA did not express CD14 in significantly higher amounts than those from the control cultures. Moreover, staining of cytospin preparations revealed that cells from the DEX cultures were strongly reactive with acid phosphatase in contrast to those from the DHEA- and control cultures which reacted weakly with acid phosphatase.

Thus, in summary, the addition of DHEA enforced the DC-character of the cells with a high CD80 and low CD43 expression, whereas DEX in fact changed the phenotype of the cells into more macrophage-like $CD14^+$ cells, lacking CD1a CD80 and CD40 expression.

Functional differences induced by DHEA and DEX in DC culture

Dendritic cells were co-cultured with allogeneic T cells in mixed leukocyte reactions in order to measure their capacity for stimulating T cell proliferation (Fig. 2). Dendritic cells gener-

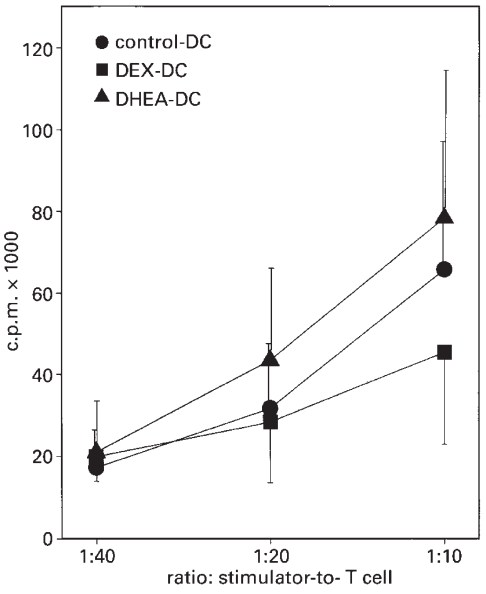

Figure 2. The T cell stimulatory capacity in allo-MLR of the various DC populations studied. Mean c.p.m. x 1000 (± s.d.) are given for various DC: T cell ratios (1:40; 1:20; 1:10)

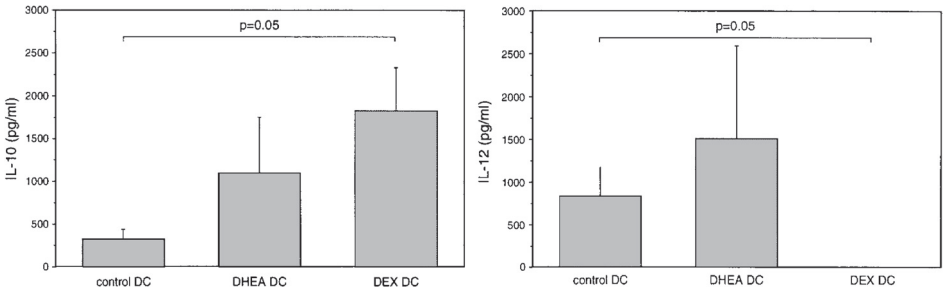

Figure 3. The Il-10 and IL-12 production of control DC, DHEA DC and DEX DC (see Figure 1 for abbreviations). Means (± s.d.) of production (pg/mL/10^6 cells) are given. *P* values are as indicated, *n* = at least five separate experiments.

ated under the influence of DHEA stimulated T cell proliferation as successfully as the control immature dendritic cells. Dendritic/macrophage-like cells generated under the influence of DEX were also able to stimulate T cell proliferation at a comparable level to the control dendritic cells; however, a non-significant difference at a stimulator-to-T cell ratio of 1:10 may suggest that these DCmay be slightly inferior at higher ratios. As the number of mixed leukocyte reactions performed was limited (*n* = 3); further experimentation should elucidate-whether or not DC generated in the presence of DEX are truly as capable of stimulating Tcell proliferation (at all ratios) as control immature DC. The ability of all DC populations generated to stimulate T cell proliferation at a stimulator-to-T cell ratio of 1:40 was comparable to the control PHA response. At a stimulator-to-T cell ratio of 1:10, this response was three times that of the PHA control.

Dendritic cells generated in the presence of DHEA produced IL-10 and IL-12 in not significantly higher amounts than the control DC (Fig. 3). Dendritic/ macrophage-like cells generated under the influence of DEX, however, produced significantly more IL-10 than the control DC (1825 ± 503 pg/ml vs. 323 ± 114 pg/ml respectively; mean ± S.E.M.; P = 0.05) and virtually shut down their production of IL-12, in contrast to the control DC (0 ± 0 pg/ml vs. 831 ± 490 pg/ml respectively; mean ± S.E.M.; P = 0.05).

DISCUSSION

Glucocorticoids are well known for their potent immunosuppressive effects when given in pharmacological doses. There are numerous reports of such suppressive effects on T cells, B cells and macrophages (reviewed in 20, 21). With regard to DC, various studies indicate that exposure to glucocorticoids decreases the function and number of DC *in vivo* (30-32). Literature on the effects of glucocorticoids on the *in vitro* generation of immature and mature DC from their precursors, or on the T cell stimulatory capability of glucocorticoidexposed DC is increasing (22-26). These reports until now collectively show that the function of DC and

the generation of immature and mature DC from monocytes is negatively influenced by the *in vitro* addition of glucocorticoids. Nevertheless, the precise data on the effects of glucocorticoids on DC marker expression, function and maturation are inconsistent. The reasons for the differences among observations are largely unclear, yet it is likely that they include the different maturation states of the DC used and the different exposure times to different doses of glucocorticoids in these experiments. When Van den Heuvel *et al*. (24) exposed GM-CSF/IL-4-cultured human monocytes not continuously, as in this report, but only for brief periods to DEX (early or late in the culture period), a population of immature DC was generated with a decreased accessory capability. This difference in function occurred in these experiments in the absence of changes in the expression of co-stimulatory molecules. Piemonte *et al.* (23) exposed GM-CSF/IL-4-cultured human monocytes not for brief periods, but continuously to DEX; they however used a lower concentration of DEX than in this report (i.e. 10^{-8} M). These authors again induced cells with a DC morphology, but with a lower expression of CD86, CD40 and CD1a, a higher expression of MHC-class II, adhesion molecules and CD14, as well as an enhanced antigen uptake via the mannose receptor. The cells again had a poor T cell stimulatory capability. Vieira *et al.* (22) reported that when they similarly produced immature DC in relatively low concentrations of glucocorticoids, they were poor in the production of IL-12 p-70, tumor necrosis factor (TNF)-α and IL-6 (when stimulated with LPS); however, these authors found a normal expression of CD80 and CD86, a normal antigen uptake, and a normal T cell stimulatory capability of such cells.

Vanderheyde *et al.* (25) used already-generated immature monocyte-derived DC and exposed these cells to methyl-prednisolone GM-CSF/IL-4 (100 µg/ml) for 24 h. The cells enhanced their antigen uptake and downregulated their basal expression of CD86 and their T cell stimulation potential. The treatment also prevented LPS-induced maturation, but had limited effects on CD40-induced further maturation. A recent study by Matasic *et al.* (26) also exposed alreadygenerated immature monocyte-derived DC to DEX. The authors confirmed that 500 nM DEX prevented further DC maturation. In fact, the treatment redirected the differentiation of the DC to a more monocyte-macrophage type of cell (high CD14, high CD68, low T cell stimulatory potential).

Here we describe that a continuous exposure of monocytes to a high concentration of DEX (10^{-6} M) completely abolished the generation of cells with an immature phenotype and morphology of DC from monocytes, and induced a set of largely plastic-adherent macrophage-like cells, expressing increased levels of CD14 and decreased levels of `typical' DC markers such as CD1a, CD40 and CD80. These cells were also strongly reactive to acid phosphatase, yet had a T cell stimulating potential in allo-MLR similar to that of 'immature' DC. They did not, however, produce IL-12 but did produce high quantities of IL-10. The CD80 expression of these macrophage-like cells was very low. In combination with the virtual shutdown of IL-12 production, this suggests that these cells could possibly initiate a Th2-biased response. *In vivo* studies also indicate that exposure to glucocorticoids is able to increase the numbers of

(suppressor) macrophages (31, 33). Our data are in line with such an effect, suggesting that the MØ/DC balance is affected by glucocorticoids, which skew this dynamic equilibrium in a macrophage direction when monocytes are continuously exposed to DEX during DC maturation. During this exposure, DEX passively diffuses through the cell membrane, binding to the resting glucocorticoid receptor (GR). The resulting activation of the GR causes it to bind to glucocoticoid-responsive elements (GRE) in the nucleus, influencing gene transcription.

Taken together, a picture is emerging in which glucocorticoids have multiple effects on DC biology. Besides the previously reported negative effects on DC trafficking (34), function (25, 30) and increases in apoptosis of DC (35), glucocorticoids also have shown in many studies variable `suppressive' and `altering' effects on the maturation of DC. This probably depends on the dose and time schedule of exposure, and the maturation state of the target DC population.

Serum DHEA-S levels show a steady decline with aging, coinciding with the decline in immune function in old age (`immunosenescence'). When aged individuals, being it experimental animals (36-38) or humans (39) are administered DHEA, their immune function has been claimed to be restored; they become more resistant to infections, their secretion of T cell cytokines (e.g. IL-2) is enhanced, whereas monocyte numbers are increased. Hence, DHEA is viewed by some as an immunostimulating hormone. However, data are also accumulating that such effects of DHEA are minimal or absent (7, 8).

Our data on the effects of DHEA on DC development are in line with the view that DHEA has a modest potentiating effect on immune function. DHEA synergized in our hands with GM-CSF and IL-4 to generate DC with a higher expression of the co-stimulating molecule CD80 (whereas the expression of CD86 remained equal to that of the control immature DC) and a lower expression of CD43. The sialoglycoprotein CD43 (sialophorin/leukosialin) is a negatively-charged anti-adhesion molecule. When human DC are treated with an antibody to CD43, their clustering capability is enhanced and their phenotypic and functional maturation is mediated (40, 41). Thus, the downregulation of CD 43 expression by DHEA-induced DC reported here may indicate a somewhat greater level of maturation than that of the control DC, which would be in accordance with the higher CD80 expression of these cells. However, CD83 expression was still low after DHEA exposure. An argument against their higher state of maturation is that the DC generated in the continuous presence of DHEA produced IL-10 and IL-12 in similar amounts to the control DC. Their T cell stimulatory capacity was, however, somewhat higher, although this was not statistically significant. Despite this seeming lack of significant functional differences, the phenotypic profile of the DHEA-induced DC (high CD80 expression) could signal the potential of these DC to direct the development of naý»ve Th cells toward a Th1 phenotype (42). DHEA would then be the first hormone known to possess this capability, which until now has only been demonstrated by pro-inflammatory cytokines such as IL-1 and TNF, and substances like LPS (43).

In saying so, we must be aware that it is still a matter of debate whether immune cells (or any other cells) possess specific receptors for DHEA. The effects of DHEA might not be exerted via DHEA-specific receptors, but rather via receptors for active androgenic metabolites of DHEA-S and DHEA generated in immune cells (44). Specifically leukocytes, including macrophages, possess the sulphatases and other enzymes important in this conversion (45, 46).

In conclusion, the data reported here show that the adrenal hormones DHEA and glucocorticoids both have effects on monocyte-to-immature DC maturation, though in a largely opposite manner. The DC maturation data as presented here are suggestive of DHEA inducing somewhat more mature DC with a possible Th1-skewing potential, whereas glucocorticoids induce macrophage-like APC with a possible Th2-skewing potential. Similar opposing effects of DHEA and cortisol on the Th1-Th2 balance *in vivo* have been suggested (47, 48) and refuted (49, 50) before.

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Chapter 4A

The effects of dexamethasone exposure on the generation of veiled accessory macrophages from monocytes

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INTRODUCTION

In chapter 3 of this thesis we described that apart from classical DC, a population of veiled accessory cells could also be generated from monocytes.

Culturing monocytes for 24 hrs while avoiding plastic adherence (polypropylene tubes) and avoiding the activation of NADPH oxidase (blocking agents) resulted in the generation of a population of veiled accessory cells (VC). The generated VC were actively moving cells like lymph-borne VC in vivo. The monocyte (mo)-derived VC population existed of $CD14^{dim/-}$ and $CD14^{bright}$ cells. Of these the $CD14^{dim/-}$ VC were as good in stimulating allogeneic T cell proliferation as immature DC (iDC) obtained after one week of adherent culture of monocytes in granulocyte-macrophage-colony stimulating factor (GM-CSF)/interleukin (IL)-4. This underscores the superb accessory cell function of the mo-derived $CD14^{dim/-}$ VC.

Although the $CD14^{dim/-}$ VC had a modest expression of the DC specific marker CD83 and were positive for S100, expression of the DC specific markers CD1a, Langerin, DC-SIGN, and DC-LAMP were absent. This indicates that the here generated $CD14^{dim/-}$ VC cannot be considered as classical LC/DC. The IL-12 production from mo-derived $CD14^{dim/-}$ VC was also lower, whereas the production of IL-10 was higher as compared to iDC. Consequently the T cells that were stimulated by these mo-derived VC produced less IFNγ as compared with T cells stimulated by iDC.

It was impossible to turn the $CD14^{dim/-}$ mo-derived VC population into typical DC by culture for one week in GM-CSF/IL-4 or LPS. In fact the cells died under such circumstances, gaining some macrophage characteristics before dying. We also found that culturing the cells in IL-3 let them survive while the cells now gained clear macrophage characteristics as an adhesive capacity and acid phosphatase activity, underscoring the actual macrophage character of the cells.

We concluded that the veiled macrophage-like cells might form part of the veiled cell population in afferent lymph draining to peripheral lymph nodes and described in the 1970-1980s, since similar cells formed part of this heterogeneous population. However detailed studies on such lymph-borne cells using markers developed in the last decades are required to strengthen or refute such view.

Since dexamethasone had clear suppressing effects on the generation of classical DC from monocytes (see chapter 4) we studied here whether exposure of monocytes to dexamethasone would influence their capability to turn into veiled accessory macrophages.

MATERIALS AND METHODS

Isolation of monocytes from peripheral blood

Monocytes were isolated from the peripheral blood of healthy blood donors via well-accepted methods: Heparinized blood diluted with an equal volume of phosphate buffered saline (PBS) was distributed over Ficoll Isopaque (density 1.077 g/ml, Pharmacia, Uppsala, Sweden) and centrifuged for 15 minutes at 1000g. Cells collected from the interface and washed were then suspended in RPMI 1640 with 25 mM HEPES and L-glutamine (GIBCO, USA), supplemented with 100 U/ml penicillin G, 0.1 mg/ml streptomycin (both Seromed, Biochrom, Berlin, Germany) and 10% FCS (Biowhitaker, Walkersville, MD, USA) (hereafter known as RPMI 1640^{+}) added. This cell suspension was distributed over Percoll (density 1.063 g/ml, Pharmacia), then centrifuged for 40 minutes at 400g. Cells collected from the interface were washed and suspended in RPMI 1640^{+}. Monocyte purity was determined by non-specific esterase staining (NSE) (26). Cell suspensions containing 80 % or more monocytes were frozen following standard procedures and stored in liquid nitrogen, providing a bank for experiments. Monocytes purified by elutriator centrifugation (4) were also used in a limited series of experiments (courtesy of CLB, Amsterdam, the Netherlands) in order to confirm results obtained via Ficoll/Percoll gradient separation.

Generation of VC

Monocytes were removed from liquid nitrogen and quickly thawed in a 37°C water bath. These cells were then washed twice in RPMI 1640 (without FCS) (5 minutes, 500g) and suspended under plastic-nonadherent conditions (polypropylene tubes, Falcon, Becton Dickinson, San Jose, CA, USA) at a concentration of $2x10^{6}$/ml in RPMI 1640 (without FCS). The monocyte suspension was thereafter exposed to dexamethasone at a concentration of 10^{-6}M at 37°C, 5% CO_2, 100% humidity for 30 minutes. This was followed by washing in RPMI 1640^{+} (5 minutes, 500 g) and further culturing in RPMI 1640^{+} at 37° C, 5 % CO_2, 100% humidity under plastic non-adherent conditions for 16 hours (overnight).

Following the 16 hour culture period, cells were centrifuged (5 minutes, 500g) and resuspended in RPMI 1640^{+}. Cells were then examined under light microscopy at a magnification of 400x. VC were defined as large cells with actively moving cytoplasmic processes or veils. Viability was checked using trypan blue exclusion. Time-lapse cinematographic studies were also carried out. For this purpose cells were kept in a microincubator (37°C, 5% CO_2) on an inverted microscope (Axiovert, Zeiss, Germany) attached to a video camera (Sony, Japan) and a time lapse video recorder (Panasonic, Matsushita Electric Industrial Co., Osaka, Japan). The video recording interval was set at 0.18 sec and the tape running speed at 2.599 mm/sec. After recording, the images were analyzed using a computer (Acorn Computers Ltd., Cambridge, UK).

The cell suspension was then further purified and separated using large cell columns and a mini-MACS (Magnetic Cell Separation System, Miltenyi Biotec GmbH, Bergisch Gladbach, Germany) with mouse anti-human CD3 (Becton Dickinson, USA), CD19 (Coulter, Hialeah, Florida, USA), and CD56 (Becton Dickinson) monoclonal antibodies and goat anti-mouse magnetic beads (Miltenyi Biotec GmbH) to deplete contaminating T cells, B cells and NK cells. Separation into $CD14^{bright}$ and $CD14^{dim/-}$ cells was performed via mini-MACS using CD14 (Coulter) monoclonal antibody and goat anti-mouse magnetic beads (Miltenyi Biotec GmbH).

Flow cytometry and immunocytochemistry

For analysis of marker expression by flow cytometry, all cell populations were stained by incubating for 10 minutes with mouse anti-human FITC- or PE-conjugated monoclonal antibodies, then washing three times. The monoclonal antibodies used were My4 (CD14, Coulter), CD1a (Coulter), B7.1 (CD80), B7.2 (CD86), (both from PharMingen, San Diego, CA, USA). Immediately following the staining, cells were analyzed on a FACScan (Becton Dickinson).

The marker pattern of monocytes, VC and DC was also investigated by immunocyto-chemistry. Cytospins were prepared using a Cytospin apparatus (Nordic Immunological Laboratories, the Netherlands). These were air-dried for 1 hour, then fixed in 100% acetone (Merck, Darmstadt, Germany) for 10 minutes. Indirect immunoperoxidase staining was performed with the monoclonal antibodies My4 (CD14, Coulter) and HLA-DR (Becton Dickinson). Application of rabbit-anti-mouse IgG peroxidase-labelled conjugate (1:50, Dako A/S, Glostrup, Denmark) in 1% normal human pool serum followed incubation with the monoclonal antibodies (RT, 1h). Cytospins were developed with a 0.05% 3,3'- diaminobenzidine (DAB, Sigma) solution containing 0.01% hydrogen peroxide for 3-5 minutes.

All cytospin preparations were mounted in DePeX mounting medium (Gurr, BDH Limited, Poole, England).

Mixed leukocyte reaction (MLR)

Allogeneic MLRs were performed in order to measure the accessory capability of the various APC populations generated. Responder T lymphocytes were obtained from healthy donors and isolated following standard procedures with Ficoll-isopaque, Percoll density gradient centrifugation, and nylon wool adherence (Leuko-Pak, Fenwall Laboratories, IL, USA). Non-adhering cells recovered were greater than 90% CD3 positive. $1.5x10^5$ responder cells were cultured in 96-well, flat-bottom microtitre plates (Nalgene Nunc International, Rochester, NY, USA) with different numbers of irradiated (2000 rad) stimulator cells (monocytes, VC or DC) to achieve stimulator-to-T cell ratios of 1:5, 1:10, 1:20 and 1:40. The culture medium used was RPMI 1640 with 25 mM HEPES and L-glutamine, supplemented with 100 U/ml penicillin G, 0.1 mg/ml streptomycin and 10% human A^+ serum, in a total volume of 200 µl per well. Controls used were monocytes, VC or DC alone, and lymphocytes in the presence of 10-50 µg/ml phytoheamagglutinin (PHA) (Wellcome Diagnostics, UK). Cultures were performed in

triplicate. On day 5, thymidine incorporation was measured by adding 0.5 µCi 3H-thymidine (specific activity = 185 GBq/mmol) to each well, then harvesting 16 hours later. Scintillation was counted on an LKB 1205 Betaplate liquid scintillation counter (Wallac, Turku, Finland).

IL-12/IL-10 production

Monocytes and VC were placed in 24-well plates (Nalgene Nunc) at a concentration of $5x10^5$ cells/ml and cultured for 24 hours in RPMI$^+$ containing ultraglutamine (2 mM, g/ml, Biowhitaker) and Biowhitaker), penicillin/streptomycin (100 U/ml, 100 serum free medium supplement (SF-1; Corning Costar Europe, Badhoevedorp, the Netherlands). To stimulate IL-10 production, the culture fluid contained staphylococcus aureus cowan 1 strain (SAC) (1:5000, Calbiochem, La Jolla, CA, USA). The IL-12 production was stimulated by SAC (1:5000) and γ-IFN (1000 U/ml, Biomedical Primate Research Centre, Rijswijk, the Netherlands). The production was measured by ELISA as indicated by the manufacturer (IL-10: ELISA Pelikine, CLB, Amsterdam, the Netherlands and the IL-12 (p 70): Eli-pair, Diaclone, Besançon, France).

RESULTS

Exposing monocytes to dexamethasone prior to an overnight non-adherent culture resulted – similar to T3 exposure – in a population of CD14 dim/negative and a population of CD14 positive VC. The CD14 positive cells were – as in the case of T3 exposure – poor stimulators in (allogeneic) MLR and we therefore concentrated on the CD14 dim/negative VC.

Figure 1 shows the FACS analysis of the cells. The DEX- exposed cells had a significant lower HLA-DR, CD86 and CD83 expression as compared to T3-exposed cells and mean fluorescence indexes (MFI) of 618, 107 and 39 versus 910, 290 and 100 (averages, $p<0.05$, $n=7$) were respectively found. CD14 was higher expressed, but not statistically significant.

Figure 2 shows the allogeneic T cell stimulatory capacity of the VC generated from DEX-, T3- and DHEA-exposed monocytes as compared to the original non-exposed monocytes. It can clearly be seen that the T cell stimulatory capacity of all the CD14dim/negative populations is higher than the non-exposed monocytes, but the DEX-VC were clearly much lower than the T3-VC, while the DHEA-VC reached the highest T cell stimulatory capacity (yet this experiment was only performed 3 times and significances could therefore not be calculated).

Fig. 3 shows the IL-10 and IL-12 production of the DEX-VC and T3-VC as compared to this cytokine production of the original monocytes.

The IL-10 production capability of the CD14 dim/negative VC populations was higher than that of the original monocytes and was practically equal in the case of DEX- and T3-VC. Interesingly the IL-12 production capability of the DEX-VC was almost completely shut down, while that of the T3-VC was comparable to that of the original monocytes (yet again this experiment was only performed 3 times and significances could again not be calculated).

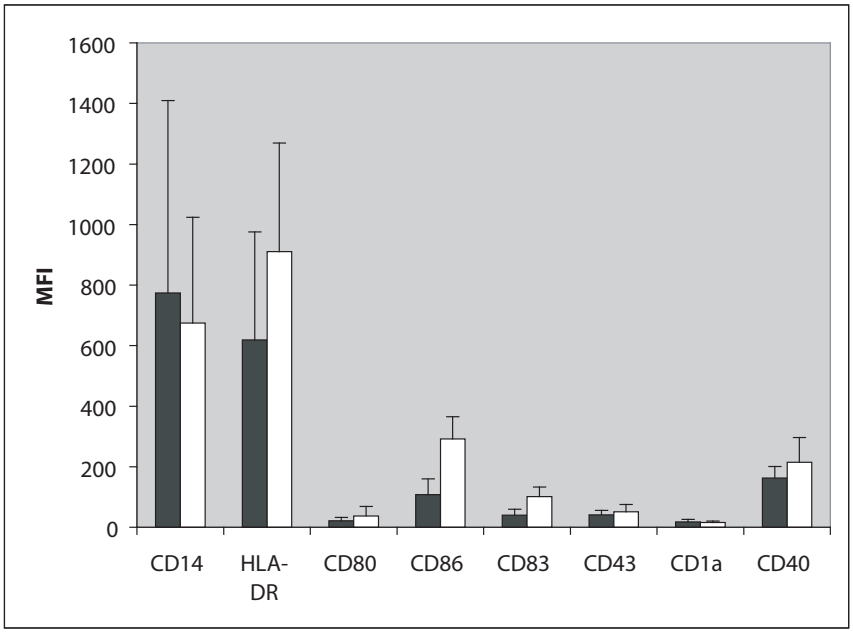

Figure 1. The FACS analysis of the CD14 negative/dim veiled cells. Means of the mean fluorescence intensities (MFI) and standard deviations are shown. Black bars represent dexamethasone stimulated cultures, white bars represent cultures in the absence of dexamethasone.

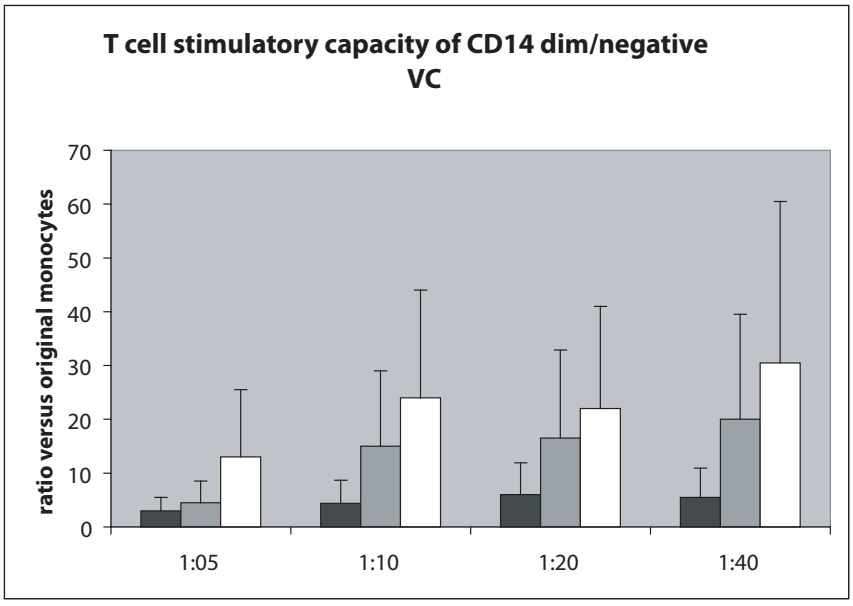

Figure 2. The T cell stimulatory capacity of CD14 negative/dim veiled cells. The increase in T cell stimulatory capacity is shown relative to the original monocytes. Black bars represent dexamethasone exposed cells, gray bars represent T3 exposed cells and white bars represent DHEA-exposed cells.

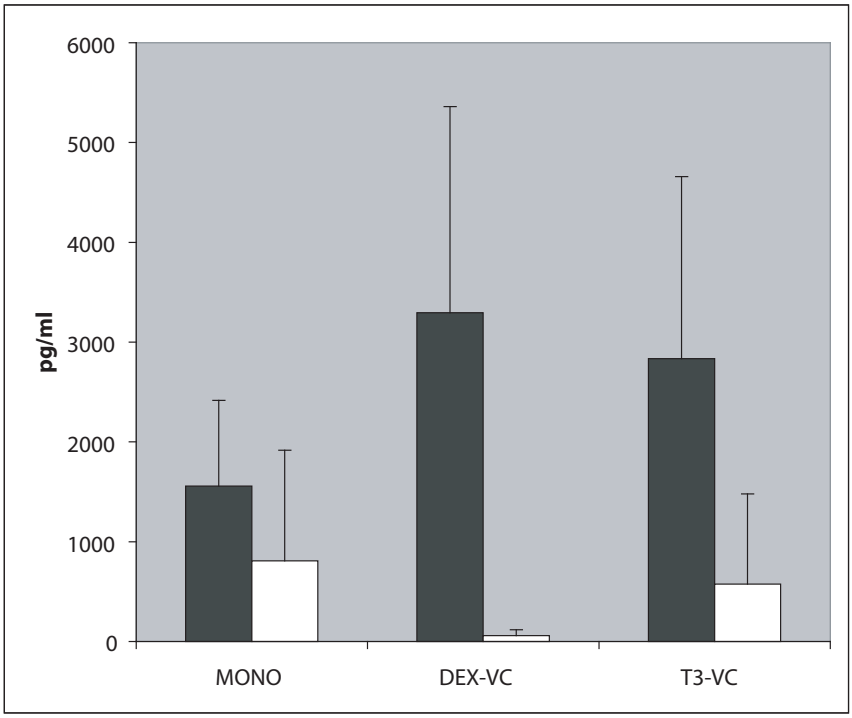

Figure 3. The cytokine production of veiled cells (VC) exposed to dexamethasone (DEX) or T3 in comparison to that of the original monocytes. The black bars represent the IL-10 production, while the black bars represent the IL-12 production. Means and standard deviations are shown.

CONCLUSIONS

Dexamethasone exposure of monocytes prior to a non-adherent overnight culture resulted in veiled cells that

a. had a lower expression of the DC specific marker CD83 and a low expression of the stimulatory MHC class II and CD86 molecules (as compared to T3-exposed cells)
b. had a lower accessory capability in allogeneic MLR (as compared to T3- and DHEA-exposed cells), and
c. had a very low IL12 production capability (as compared to T3-exposed cells).

These veiled cells generated after exposure of monocytes to dexamethasone lend support to the view that this hormone hampers the generation of immunostimulatory APC from monocytes (see chapter 4).

Chapter 5

1-α, 25-Dihydroxyvitamin D_3 ($1,25(OH)_2 D_3$) hampers the maturation of fully active immature dendritic cells from monocytes

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ABSTRACT

Objective: To study the effects of the active metabolite of vitamin D3, $1,25(OH)_2D_3$, an immunomodulatory hormone, on the generation of so-called immature dendritic cells generated from monocytes (Mo-iDC).

Design and methods: Human peripheral blood monocytes were cultured to iDC in the presence of GM-CSF and IL-4 for one week, with or without the extra addition of 10^{-8} M $1,25(OH)_2D_3$ to the culture. We examined their phenotype (CD14, CD1a, CD83, HLA-DR, CD80, CD86 and CD40 expression) by FACS analysis and their T cell stimulatory potential in allogeneic MLR (allo-MLR). Additionally we investigated their in vitro production of IL-10, IL-12 and TGF-ß by ELISA.

Results: $1,25(OH)_2D_3$, when added to monocytes in culture with GM-CSF and IL-4, hampered the maturation of Mo-iDC. Firstly the phenotype of the $1,25(OH)_2D_3$-differentiated DC was affected with a impaired down-regulation of the monocytic marker CD14 and a impaired upregulation of CD1a, CD83, HLA-DR, CD80 and CD40. CD86 was expressed on more $1,25(OH)_2D_3$-differentiated DC. Secondly, the T cell stimulatory capability of $1,25(OH)_2D_3$-differentiated DC was upregulated from that of the original monocytes to a lesser degree than DC differentiated without $1,25(OH)_2D_3$ when tested in allogenic mixed lymphocyte reaction (MLR). With regard to the production of cytokines, SAC-induced IL-10 production, although not enhanced, remained considerably high in $1,25(OH)_2D_3$-differentiated DC, whereas it was strongly downregulated in DC generated in the absence of $1,25(OH)_2D_3$. SAC/IFNγ-induced IL-12 production was clearly upregulated in both types of DC from that of the original monocytes and TGF-ß production downregulated.

Conclusion: Our data confirm earlier reports that $1,25(OH)_2D_3$ hampers the maturation of fully active immunostimulatory MHC-classII+,CD1a+,CD80+ DC from monocytes. Our data supplement the other reports in showing that the expression of CD86 was upregulated in $1,25(OH)_2D_3$-differentiated DC, while the capability for IL-10 production remained high. Collectively, this is in line with earlier descriptions of suppressive activities of this steroid-like hormone regarding the stimulation of cell-mediated immunity.

INTRODUCTION

1α, 25-Dihydroxyvitamin D_3 ($1,25(OH)_2D_3$) is a steroid hormone known for its ability to regulate calcium metabolism. The presence of the vitamin D_3 receptor in almost all types of immune cells and the ability of $1,25(OH)_2D_3$ to affect immune cell function in vitro is indicative of other actions of this hormone. The ability of $1,25(OH)_2D_3$ to stimulate cell differentiation has been well characterized. For instance, this hormone can inhibit proliferation and induce differentiation of benign cells such as keratinocytes, malignant cells such as prostate, breast and colon adenocarcinoma cells and various leukemic cells.

$1,25(OH)_2D_3$ also plays a role in the differentiation of benign cells of the myeloid lineage. The differentiation of immature monocytes toward mature macrophages has been demonstrated to be fostered by this hormone in many reports (4,5,6). The hormone enhances macrophage-type activities such as phagocytosis and killing of bacteria, adherence and chemotaxis (7,8). $1,25(OH)_2D_3$ is also known for its capability to induce TGF-beta production in monocytes and other cell types (9). Although TGF-beta is commonly considered as a tolerance-inducing or immunosuppressive cytokine, it has great plasticity and its action on immune cells can be inhibiting or promoting, depending upon cell type, differentiation/activation status and environment. (10). Thus, its manner of regulating immune function is heavily context dependent.

Data on the effects of $1,25(OH)_2D_3$ on the accessory cell function of monocytes/macrophages demonstrate a decreased antigen presenting capability together with a reduced MHC class II antigen expression (11,12). Dendritic cells (DC), highly specialized antigen presenting accessory cells (APC) capable of stimulating naïve T cells, can be generated from monocytes by culturing for one week under plastic-adherent conditions in the presence of GM-CSF and IL-4. The resulting socalled immature DC (iDC) express dendritic-type markers such as CD1a, CD83, MHC Class II, CD40, CD80 and CD86, while downregulating the expression of CD14. Their accessory cell function is also greatly increased. Recent reports indicate that the differentiation of DC is inhibited by $1,25(OH)_2D_3$. Markers typical of iDC (CD1a, MHC Class II) are expressed to a lesser degree, and T cell stimulatory capability is reduced. However, conflicting data exists regarding the expression of some costimulatory molecules. Piemonti (2) found that the expression of CD86 was inhibited in the presence of $1,25(OH)_2D_3$, while that of CD80 was not significantly affected. This is in contrast to data from Berer (3), who found that CD86 expression was unaffected by $1,25(OH)_2D_3$ while upregulation of CD80 was prevented. Immature DCs can be further differentiated into mature DCs in vitro by culturing with LPS, TNF-α, IL-1 or CD40-ligand (13,14). In this mature stage, the capacity for antigen uptake is lost, and the cell becomes an expert at antigen presentation (15,16). Maturing DCs are also reportedly affected by $1,25(OH)_2D_3$: decreases in IL-12 production and an increase in IL-10 production have been reported (1,2), however there are –to date- no accounts regarding the

effect of $1,25(OH)_2D_3$ on the production of IL-10 and IL-12 in iDCs. The effect of $1,25(OH)_2D_3$ on the production of the immunoregulatory growth factor TGF-ß by DCs is also unknown.

In order to further investigate these effects of the immunoregulatory hormone $1,25(OH)_2D_3$ on myeloid DC differentiation, we cultured human peripheral blood monocytes to iDC in the presence of GM-CSF and IL-4 for one week, with the addition of $1,25(OH)_2D_3$ to the culture. We examined the phenotype (CD14, CD1a, CD83, HLA-DR, CD80, CD86 and CD40 expression) and T cell stimulatory potential in allogeneic MLR (allo-MLR) of these immature DC, and investigated their production of IL-10, IL-12 and TGF-ß while still in their immature state.

MATERIALS AND METHODS

Isolation of monocytes from peripheral blood

Monocytes were isolated from the peripheral blood of healthy blood donors via well-accepted methods: Heparinized blood diluted with an equal volume of phosphate buffered saline (PBS) was distributed over Ficoll Isopaque (density 1.077g/ml, Pharmacia, Uppsala, Sweden) and centrifuged for 15 minutes at 1000 g. Cells collected from the interface and washed were then suspended in RPMI 1640 with 25mM HEPES and L-glutamine (GIBCO, USA), supplemented with 100U/ml penicillin G, 0.1 mg/ml streptomycin (both Seromed, Biochrom, Berlin, Germany) and 10% FCS (Bio Whitaker, USA) (hereafter known as RPMI 1640^+) added. This cell suspension was distributed over Percoll (density 1.063 g/ml, Pharmacia, Uppsala, Sweden), then centrifuged for 40 minutes at 400 g. Cells collected from the interface were washed and suspended in RPMI 1640^+. Monocyte purity was determined by non-specific esterase staining (NSE) (27). Cell suspensions containing 80% or more monocytes were frozen following standard procedures and stored in liquid nitrogen, providing a bank for experiments. Monocytes purified by elutriator centrifugation were also used (courtesy of CLB, Amsterdam), in order to confirm results obtained via Ficoll/Percoll gradient separation.

Culture of DCs from peripheral blood monocytes

DC were obtained via the well-established method first described by Sallusto and Lanzavecchia (13). Briefly, monocytes were cultured for one week at 37°C, 5% CO_2, 100% humidity at a concentration of $3x10^5$ cells/ml in RPMI 1640^+ with 800 U/ml GM-CSF and 1000 U/ml IL-4. Feeding of the cultures took place every two days, by removing 500 µl culture fluid and replacing this with 1 ml of fresh medium with cytokines. In order to test the effects of exposure to $1,25(OH)_2D_3$ on the monocyte-to-DC transition, this hormone as added at an optimal concentration of 10^{-8} M to monocytes in RPMI 1640 culture medium (without FCS) and incubated for thirty minutes at 37°C, 5% CO_2, 100% humidity after which FCS (10%), GM-CSF (800U/ml) and IL-4 (1000 U/ml) were added to the culture. $1,25(OH)_2D_3$ was also fed to the cultures every two days along with fresh medium and cytokines.

Flow cytometry and (immuno)cytochemistry

For analysis of marker expression by flow cytometry, all cell populations were stained by incubating for 10 minutes with mouse anti-human FITC- or PE-conjugated monoclonal antibodies, then washing three times. The monoclonal antibodies used were My4 (CD14, Beckman Coulter, Hialeah, Florida, USA), CD1a (Beckman Coulter, Hialeah, Florida, USA), HLA-DR (Becton Dickinson, USA) B7.1 (CD80, Becton Dickinson, San Jose, CA, USA), B7.2 (CD86, PharMingen, Los Angeles, CA, USA), CD83 (Immunotech, Marseilles, France) and CD40 (Serotec, Oxford, England). Immediately following the staining, cells were analyzed on a FACScan (Becton Dickinson).

Mixed leukocyte reaction (MLR)

Allogeneic MLRs were performed in order to measure the accessory capability of the various DC populations generated. Responder T lymphocytes were obtained from healthy donors and isolated following standard procedures with Ficoll-isopaque, Percoll density gradient centrifugation, and nylon wool adherence (Leuko-Pak, Fenwall Laboratories, Il, USA). Non-adhering cells recovered were greater than 90% CD3 positive. $1.5x10^5$ responder cells were cultured in 96-well, flat-bottom microtitre plates (NUNC A/S International, Denmark) with different numbers of irradiated (2000 rad) stimulator cells (monocytes or DC) to achieve stimulator-to-T cell ratios of 1:5, 1:10, 1:20 and 1:40. The culture medium used was RPMI 1640 with 25mM HEPES and L-glutamine, supplemented with 100U/ml penicillin G, 0.1 mg/ml streptomycin and 10% human A^+ serum, in a total volume of 200 µl per well. Controls used were monocytes or DC alone, and lymphocytes in the presence of 10-50 µg/ml phytoheamagglutinin (PHA) (Wellcome Diagnostics, UK). Cultures were performed in triplicate. On day 5, thymidine incorporation was measured by adding 0.5 µCi 3H-thymidine to each well, then harvesting 16 hours later. Scintillation was counted on an LKB 1205 Betaplate liquid scintillation counter (Wallac, Turku, Finland).

IL-12, IL-10 and TGFβ production

DC were placed in 24-well plates (Nunc) at a concentration of 5.10^5 cells/ml and cultured for 24 hours in $RPMI^+$ containing ultraglutamine (2mM, BioWhitakker), penicillin/streptomycin (100 U/ml, 100 mcg/ml, BioWhitakker) and serum free medium supplement (Pepro Tech). To stimulate IL-10 production, the culture fluid contained staphylococcus aureus cowan 1 strain (SAC) (1:5000, calbiochem, La Jolla, CA, USA). The IL-12 production was stimulated by SAC (1:5000) and γ-IFN (1000U/ml, Biomedical Primate Research Centre, Rijswijk, the Netherlands). The production of cytokines was measured by ELISA as indicated by the manufacturer (IL-10 ELISA Pelikine, CLB, Amsterdam, the Netherlands; the IL-12 Eli-pair, Diaclone, Besançon, France; and the TGFβ1 ELISA, Biosource, Europe SA).

STATISTICAL ANALYSIS

Data were collected in an Excell File. Statistical analysis was carried out between sets of measurements (n=>6, see for exact details the result section and the legends of the table and figures) in the presence or absence of 10^{-8} M $1,25(OH)_2D_3$ using the parametric T test (two-tailed, unpaired) provided by this computer programme.

RESULTS

$1,25(OH)_2 D_3$ influences the morphology and phenotype of DC

Dose response curves revealed that an optimal dose of 10^{-8}M $1,25(OH)_2D_3$ reduced the percentage of cells with cellular protrusions(veils and dendrites) from 84±8% (non-vit D-exposed cultures) to 63±9% in the culture to which $1,25(OH)_2D_3$ was added (mean ± sd, p=0.002, n=10).

The phenotype of the DC in culture was also affected by the addition of $1,25(OH)_2D_3$ (Table 1), resulting in a still high expression of the monocyte marker CD14 (typically downregulated during culture in GM-CSF and IL-4 alone). The expression of the DC markers CD1a and CD83 (typically upregulated during culture in GM-CSF and IL-4 alone) was still low in DC differentiated in the presence of $1,25(OH)_2 D_3$. The intensity of the expression of the antigen-presenting molecule HLA-DR (typically upregulated during culture in GM-CSF and IL-4 alone) remained low in $1,25(OH)_2D_3$-differentiated cells (Table 1). The addition of $1,25(OH)_2D_3$ to the culture also resulted in significant changes in the expression of the costimulatory molecules CD80 (lower expression), CD86 (higher expression) and CD40 (lower expression). In sum, the phenotype of the vit D3-generated DC more or less still resembled that of the original monocytes with a persistent high CD14, low CD1a, low CD83, low CD80 and low CD40 expression. The high CD86 expression of vit D_3-generated DC is remarkable: the percentage of CD86+ DC remained significantly higher and comparable to that of monocytes, while their mean fluorescence intensity was much higher than that of monocytes, which only have a dull expression (Table 1).

Functional differences induced by $1,25(OH)_2D_3$ in DC culture

Dendritic cells were co-cultured with allogeneic T cells in mixed leukocyte reactions (MLR) in order to assess their capability to stimulate T cell proliferation. Dendritic cells generated under the influence of $1,25(OH)_2D_3$ displayed a greatly reduced capability to stimulate T cells to proliferate (Fig. 1). At a stimulator-to-T cell ratio of 1:5, the capability of $1,25(OH)_2 D_3$-generated DC to stimulate T cell proliferation was significantly lower than that of the control. This impairment of stimulatory ability was evident at all ratios tested (p values ranging from p=0.01 to p=0.02, n=7-8). It is also worthy to note that the T cell stimulating capacity of the original monocytes was clearly and significantly lower than that of both DC populations.

Table 1. Effect of $1,25(OH)_2D_3$ on the phenotype of monocyte-derived dendritic cells

	Percentage of positive cells			Mean Fluorescence Intensity		
		Monocytes	Dendritic cells		Monocytes	Dendritic cells
		–Vit D3	+Vit D_3		–Vit D_3	+Vit D_3
CD14	76 ± 5	14 ± 4[b]	49 ± 6	2980 ± 712	280 ± 230[b]	640 ± 414[b]
CD83	3 ± 2	8 ± 4	4 ± 2	75 ± 40	150 ± 63[b]	116 ± 38
CD1a	2 ± 2	24 ± 8[b]	3 ± 1	19 ± 13	270 ± 356[b]	60 ± 25[a,b]
HLA-DR	73 ± 6	80 ± 9	68 ± 9	285 ± 97	870 ± 570[b]	490 ± 350
CD80	5 ± 1	28 ± 6[b]	15 ± 4	50 ± 34	290 ± 130[b]	134 ± 102[a,b]
CD86	75 ± 5	54 ± 5[b]	64 ± 6[a]	84 ± 24	400 ± 270[b]	595 ± 600[b]
CD40	82 ± 7	94 ± 2	71 ± 12[a]	84 ± 32	710 ± 297[b]	320 ± 230[a,b]

FACS analysis of immature DC populations (n=10 populations for each condition of 10 different donors) generated in the absence or presence of $1,25(OH)_2D_3$; FACS analysis was also performed on the original monocyte populations (n=7). Not only the mean percentage of positive cells (i.e. cells with a signal over that of the IgG control) is given, but also the mean fluorescence intensity ± standard deviation. [a] $p<0.05$ vs dendritic cells in the absence of vit D_3 [b] $p<0.05$ vs original monocytes, T test Excel computer programme.

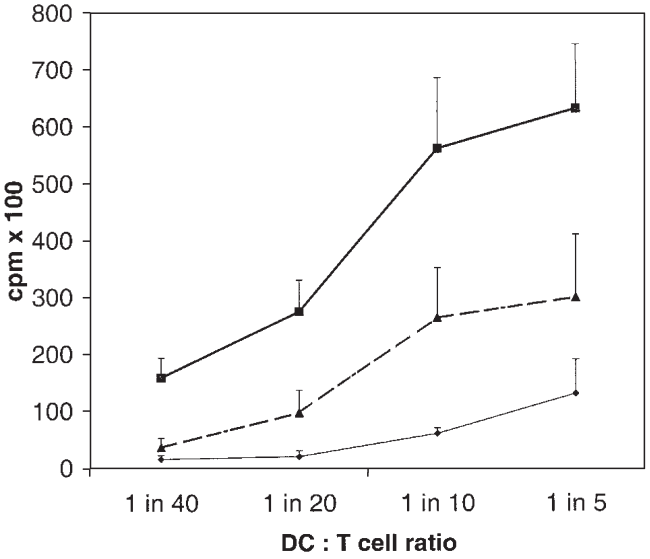

Figure 1. The T cell stimulatory capacity in allo-MLR of the original monocytes (◆) and monocyte-derived DCs generated in the absence (■) or presence (▲) of $1,25(OH)_2D_3$. Mean counts per minute (c.p.m.) of [^{3}H]thymidine incorporation are given (± s.d.) for various DC:T cell ratios in allo-MLR, i.e. 1:40, 1:20, 1:10 and 1:5. Differnces between the three conditions are statistically significant at all ratios (*P* values varying from 0.01 to 0.02, n = 7-8 experiments using a different donor in each experiment; *t*-test, EXCEL computer program).

IL-10, IL-12 and TFGβ production

When stimulated for 24 hrs with staphylococcus aureus cowan strain 1 (SAC), a well known inducer of IL-10 production, dendritic cells generated in the presence of $1,25(OH)_2D_3$ produced more of the immunosuppressive cytokine IL-10 than the control, non-Vit D-exposed DC (1227±221 pg/ml vs 337±83 pg/ml, respectively; mean±sem; $p<0.01$, n=13 and 9 experi-

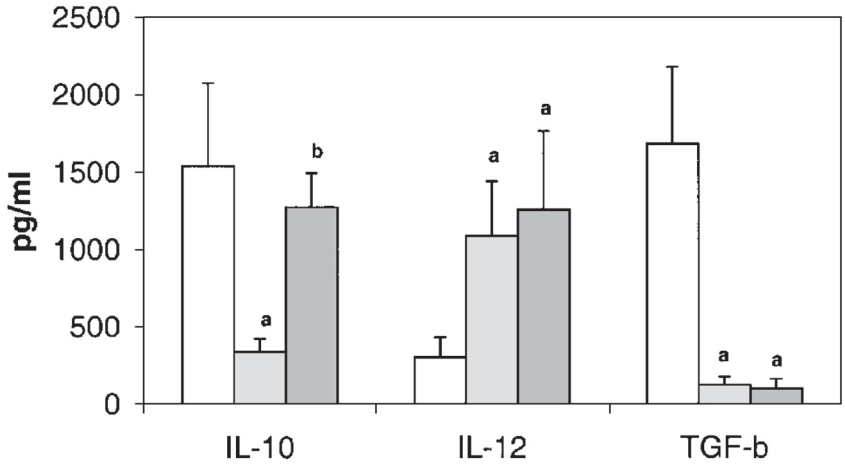

Figure 2. The SAC-stimulated Il-10, the Sac/IFN-γ-stimulated IL-12, and the TGF-β production of original monocytes (white bars) and of monocyte-derived DCs generated in the absence (light grey bars) or presence (dark-grey bars) of $1,25(OH)_2D_3$. Production rates are given as mean pg/mL/5x10^5 cells (± s.e.m.). a, $P < 0.05$ versus original monocytes; b, $P < 0.05$ versus DCs generated in the absence of $1,25(OH)_2D_3$. For the exact number of experiments see the text (n= 9-13); *t*-test, EXCEL computer program.

ments and donors respectively) (Fig. 2). The IL-10 production of the original monocytes was under such circumstances 1540 ± 534 pg/ml (mean ± sem, n=12) (Fig. 2). When SAC was not used IL-10 production was in all instances considerably lower and variable, and therefore tested in fewer cases. Differences stayed more or less the same, allthough not reaching statistical significance anymore due to the rather high variability between experiments. Values for VitD3 exposed DC were 94±27 pg/ml (mean±sem, n=4), non-exposed DC 26±26 pg/ml (n=6), while monocytes produced 174 ± 56 pg/ml (n=8) Il-10.

The presence of $1,25(OH)_2D_3$ in the culture during differentiation of DC from monocytes did not significantly affect their capability to produce IL-12. Under conditions of a 24 hr stimulation by SAC and interferon gamma (IFN-γ) (wellknown inducers of IL-12 production) 1088±351 pg/ml IL-12 were produced by control DC vs 1056±652 pg/ml by DC generated under the influence of $1,25(OH)_2D_3$ (mean±sem, p=0.31, n=10 and 7 respectively) (Fig. 2).

The original monocytes produced less IL-12 under these conditions, i.e. 301 ± 129 pg/ml (mean ± sem, n=17) (Fig. 2). Omitting SAC and IFN-γ from the cultures led to an almost nil production of IL-12 in all instances (< 10 pg/ml).

The TGFβ production of $1,25(OH)_2D_3$-generated and control DC did also not differ, and was in both instances very low when compared to the original monocytes: $1,25(OH)_2D_3$-generated DC produced 98 ± 65 pg/ml TGFβ (mean ± sem) (n=4) over a 24 hr period, control DC 121 ± 57 pg/ml (n=4), and original monocytes 1680 ± 500 pg/ml (n=7). SAC stimulation during the 24 hr production period had no effects.

DISCUSSION

Recent reports indicate a role of $1,25(OH)_2D_3$ on DC development. When monocytes are exposed to $1,25(OH)_2D_3$ during their differentiation to immature DC, the resulting cells are less capable of stimulating T cell proliferation (1,2,3). High expression of the monocyte marker CD14 is maintained, while CD1a expression is reduced (2,3). The upregulation of MHC Class II and CD40 is prevented, while conflicting results have been reported regarding the effects of $1,25(OH)_2 D_3$ on the costimulatory molecules CD80 and CD86. While Piemonti (3) found that CD86 expression was inhibited and CD80 expression was unaffected by the presence of $1,25(OH)_2D_3$, Berer et al (2000) found that CD86 expression was not affected by $1,25(OH)_2D_3$ while upregulation of CD80 was prevented. Differences in the endocytic activity of DCs diferentiated in the presence of $1,25(OH)_2D_3$ were also reported by these two groups. There are no reports on the effects of $1,25(OH)_2D_3$ on IL-10 and IL-12 production by immature DCs, however maturing DCs were affected by the presence of $1,25(OH)_2D_3$, as reflected in decreases in IL-12p75 and IL-12p70 production upon exposure to CD40 ligand (1,2), and an upregulation in IL-10 production (1). This suppressive action of $1,25(OH)_2D_3$ on DC development is in accordance with the numerous reports of a downregulation of MHC Class II expression in mononuclear cells exposed to this hormone (11,12,17). There are no previous reports indicating whether or not this suppressive action of $1,25(OH)_2D_3$ is reflected in the TGF-ß production by DCs.

Our data are largely in accordance with this picture of $1,25(OH)_2 D_3$ as an immunosuppressive agent for DC development. We confirmed that $1,25(OH)_2D_3$ hampers the differentiation of monocytes into DC, generating a population of immature DC with a reduced capability to induce T cell proliferation. These immature DC generated whilst being exposed to $1,25(OH)_2D_3$ differed in phenotype from classical immature DC. A significant reduction in expression of the CD1a and CD40 antigens was seen in the $1,25(OH)_2D_3$-exposed cells. Expression of the costimulatory molecule CD80 was inhibited, as has been reported by Piemonti (3), however expression of the costimulatory molecule CD86 was increased, in contrast to Piemonti's and Berer's reports of an unchanged and decreased level of expression of this marker in the presence of $1,25(OH)_2D_3$ respectively. A significantly higher CD14 expression was seen in the $1,25(OH)_2D_3$-exposed cells, which concurs with a high level of CD14 antigen expression in mononuclear phagocytes exposed to $1,25(OH)_2D_3$, noted by numerous investigators. A reduction in CD1a antigen expression was also reported by Dam (18) in experiments applying calcipotriol to normal human skin.

Although $1,25(OH)_2D_3$ has been shown to inhibit IL-12 production by activated macrophages and mature DC (19), contributing to the immunosuppressive capability of this hormone, we did not find such an effect in immature DC. IL-10 secretion by maturing DC was recently reported to be enhanced when $1,25(OH)_2D_3$ was added to the culture during LPS-induced maturation (1). In our experiments we used SAC as a stimulus and also found a

higher production of IL-10 in $1,25(OH)_2D_3$-differentiated iDC comparable to the production of their original monocyte precursors. In contrast SAC-induced IL-10 production by DC generated without $1,25(OH)_2D_3$ was clearly downregulated as compared to that of the original monocytes. Although it has been demonstrated that calcitriol inhibits epithelial cell growth possibly by inducing the synthesis of TGF-ß (20), and $1,25(OH)_2D_3$ displays identical actions on cell growth and differentiation as TGF-ß, we saw no increase but rather a decrease in the production of TGF-ß by both DC populations in comparison to monocytes. Mercier et al (21) have reported an inhibition in rat liver epithelial cell growth by calcitriol, which was accompanied by a reduction in TGF-ß synthesis. In our hands, the presence of $1,25(OH)_2D_3$ appeared to have no effect on TGF-ß production by immature DC, giving no evidence for the existence of a paracrine/autocrine loop in the inhibitory effect of $1,25(OH)_2D_3$.

Much in vitro investigation has shed light on the presence and role of $1,25(OH)_2D_3$ in immune regulation. Monocytes, macrophages and activated lymphocytes all express the vitamin D receptor (VDR). $1,25(OH)_2D_3$ is also produced by activated monocytes and macrophages, and has been demonstrated to enhance the antimicrobial function of macrophages (7,8). Natural killer cell activity is enhanced as well by this hormone (8). $1,25(OH)_2D_3$ has also been demonstrated as stimulating T suppressor cell function, in vivo as well as in vitro (22,23), and is known to inhibit both T- and B lymphocyte proliferation (24,25,26) as well as Ig production (25). $1,25(OH)_2D_3$ also inhibits the production of the growth-promoting lymphokine IL-2 (24,27,28), which was discovered to be the mechanism mediating the inhibition of lymphocyte proliferation (26,29). Activated T lymphocytes can serve as direct targets for $1,25(OH)_2D_3$ (30,31), however the effects of $1,25(OH)_2D_3$ on these cells are also the results of its actions on monocytes and macrophages in their role as APCs (32). $1,25(OH)_2D_3$ has been demonstrated as both inhibiting (27,33) and promoting (28) the IL-1 production of monocytes and macrophages. The production of IL-12, important in the development of Th1-biased immune responses, has been found to be inhibited by $1,25(OH)_2D_3$ in both monocytes (34) and activated macrophages and mature DC (19). With regard to in vivo effects, immune-modulating properties of $1,25(OH)_2D_3$ and its analogues have been demonstrated in various animal models for autoimmunity. For instance, Mathieu et al (23,35,36) showed that $1,25(OH)_2D_3$ and several of its analogues are able to reduce the incidence of type 1 diabetes in the NOD mouse. Similarly, using the murine model for multiple sclerosis, experimental allergic encephalitis (EAE), $1,25(OH)_2D_3$ was shown to prevent the initiation of disease by myelin basic protein (37). Although the use of $1,25(OH)_2D_3$ in organ transplantation has not been highly successful in preventing graft rejection (partly due to complications of hypercalcaemia at the required dosage), various analogues of $1,25(OH)_2D_3$ have been demonstrated to be highly effective in prolonging graft survival without the hypercalcaemic effect of $1,25(OH)_2D_3$ itself (38,39,40).

In conclusion, exposure to the immunoregulatory hormone $1,25(OH)_2D_3$ during monocyte-to-DC maturation results in our hands in immature DC with a reduced expression of CD1a,

CD80 and CD40, a reduced capability in the MLR and a remaining high capability for IL-10 production, all factors likely accounting for the immunosuppressive nature of this hormone in T cell stimulation. Apparently (also considering the data previously reported in the literature on the effects of this hormone) $1,25(OH)_2D_3$ skews monocyte differentiation away from APC development and toward the direction of mature phagocytosing macrophages. This hormone thus likely favours the development of a strong aspecific, innate immune reaction, over that of an antigen-specific immune response.

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Chapter 5A

The effects of 1,25 (OH)2 Vitamin D3 exposure on the generation of veiled accessory macrophages from monocytes

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INTRODUCTION

In chapter 3 of this thesis we described that apart from classical DC, a population of veiled accessory cells could be generated from monocytes.

In chapter 4A, we examined the effects of a short exposure of dexamethasone on the generation of these veiled accessory cells from monocytes. After seeing clear effects compatible with the immunosuppressive nature of this hormone, and since 1,25(OH)2Vitamin D3 had clear effects on the generation of classical DC from monocytes, we studied here whether exposure of monocytes to 1,25(OH)2Vitamin D3 would influence their capability to turn into veiled accessory macrophages.

MATERIALS AND METHODS

Isolation of monocytes from peripheral blood

Monocytes were isolated from the peripheral blood of healthy blood donors via well-accepted methods: Heparinized blood diluted with an equal volume of phosphate buffered saline (PBS) was distributed over Ficoll Isopaque (density 1.077 g/ml, Pharmacia, Uppsala, Sweden) and centrifuged for 15 minutes at 1000 g. Cells collected from the interface and washed were then suspended in RPMI 1640 with 25 mM HEPES and L-glutamine (GIBCO, USA), supplemented with 100 U/ml penicillin G, 0.1 mg/ml streptomycin (both Seromed, Biochrom, Berlin, Germany) and 10% FCS (Biowhitaker, Walkersville, MD, USA) (hereafter known as RPMI 1640^{+}) added. This cell suspension was distributed over Percoll (density 1.063 g/ml, Pharmacia), then centrifuged for 40 minutes at 400 g. Cells collected from the interface were washed and suspended in RPMI 1640^{+}. Monocyte purity was determined by non-specific esterase staining (NSE) (26). Cell suspensions containing 80% or more monocytes were frozen following standard procedures and stored in liquid nitrogen, providing a bank for experiments. Monocytes purified by elutriator centrifugation (4) were also used in a limited series of experiments (courtesy of CLB, Amsterdam, the Netherlands) in order to confirm results obtained via Ficoll/Percoll gradient separation.

Generation of VC

Monocytes were removed from liquid nitrogen and quickly thawed in a 37°C water bath. These cells were then washed twice in RPMI 1640 (without FCS) (5 minutes, 500 g) and suspended under plastic-nonadherent conditions (polypropylene tubes, Falcon, Becton Dickinson, San Jose, CA, USA) at a concentration of $2x10^{6}$/ml in RPMI 1640 (without FCS). The monocyte suspension was thereafter exposed to 1,25(OH)2D3 at a concentration of 10-8M at 37°C, 5% CO_2, 100% humidity for 30 minutes. This was followed by washing in RPMI 1640^{+} (5 minutes,

500 g) and further culturing in RPMI 1640^{+} at 37°C, 5% CO_2, 100% humidity under plastic non-adherent conditions for 16 hours (overnight).

Following the 16 hour culture period, cells were centrifuged (5 minutes, 500 g) and resuspended in RPMI 1640^{+}. Cells were then examined under light microscopy at a magnification of 400x. VC were defined as large cells with actively moving cytoplasmic processes or veils. Viability was checked using trypan blue exclusion. Time-lapse cinematographic studies were also carried out. For this purpose cells were kept in a microincubator (37°C, 5% CO_2) on an inverted microscope (Axiovert, Zeiss, Germany) attached to a video camera (Sony, Japan) and a time lapse video recorder (Panasonic, Matsushita Electric Industrial CO., Osaka, Japan). The video recording interval was set at 0.18 sec and the tape running speed at 2.599 mm/sec. After recording, the images were analyzed using a computer (Acorn Computers Ltd., Cambridge, UK).

The cell suspension was then further purified and separated using large cell columns and a mini-MACS (Magnetic Cell Separation System, Miltenyi Biotec GmbH, Bergisch Gladbach, Germany) with mouse anti-human CD3 (Becton Dickinson, USA), CD19 (Coulter, Hialeah, Florida, USA), and CD56 (Becton Dickinson) monoclonal antibodies and goat anti-mouse magnetic beads (Miltenyi Biotec GmbH) to deplete contaminating T cells, B cells and NK cells. Separation into $CD14^{bright}$ and $CD14^{dim/-}$ cells was performed via mini-MACS using CD14 (Coulter) monoclonal antibody and goat anti-mouse magnetic beads (Miltenyi Biotec GmbH).

Flow cytometry and immunocytochemistry

For analysis of marker expression by flow cytometry, all cell populations were stained by incubating for 10 minutes with mouse anti-human FITC- or PE-conjugated monoclonal antibodies, then washing three times. The monoclonal antibodies used were My4 (CD14, Coulter), CD1a (Coulter), B7.1 (CD80), B7.2 (CD86), (both from PharMingen, San Diego, CA, USA). Immediately following the staining, cells were analyzed on a FACScan (Becton Dickinson).

The marker pattern of monocytes, VC and DC was also investigated by immunocyto-chemistry. Cytospins were prepared using a Cytospin apparatus (Nordic Immunological Laboratories, the Netherlands). These were air-dried for 1 hour, then fixed in 100% acetone (Merck, Darmstadt, Germany) for 10 minutes. Indirect immunoperoxidase staining was performed with the monoclonal antibodies My4 (CD14, Coulter) and HLA-DR (Becton Dickinson). Application of rabbit-anti-mouse IgG peroxidase-labelled conjugate (1:50, Dako A/S, Glostrup, Denmark) in 1% normal human pool serum followed incubation with the monoclonal antibodies (RT, 1h). Cytospins were developed with a 0.05% 3,3'- diaminobenzidine (DAB, Sigma) solution containing 0.01% hydrogen peroxide for 3-5 minutes.

All cytospin preparations were mounted in DePeX mounting medium (Gurr, BDH Limited, Poole, England).

Mixed leukocyte reaction (MLR)

Allogeneic MLRs were performed in order to measure the accessory capability of the various APC populations generated. Responder T lymphocytes were obtained from healthy donors and isolated following standard procedures with Ficoll-isopaque, Percoll density gradient centrifugation, and nylon wool adherence (Leuko-Pak, Fenwall Laboratories, IL, USA). Non-adhering cells recovered were greater than 90% CD3 positive. $1.5x10^5$ responder cells were cultured in 96-well, flat-bottom microtitre plates (Nalgene Nunc International, Rochester, NY, USA) with different numbers of irradiated (2000 rad) stimulator cells (monocytes, VC or DC) to achieve stimulator-to-T cell ratios of 1:5, 1:10, 1:20 and 1:40. The culture medium used was RPMI 1640 with 25 mM HEPES and L-glutamine, supplemented with 100 U/ml penicillin G, 0.1 mg/ml streptomycin and 10% human A^+ serum, in a total volume of 200 µl per well. Controls used were monocytes, VC or DC alone, and lymphocytes in the presence of 10-50 µg/ml phytoheamagglutinin (PHA) (Wellcome Diagnostics, UK). Cultures were performed in triplicate. On day 5, thymidine incorporation was measured by adding 0.5 µCi 3H-thymidine (specific activity = 185 GBq/mmol) to each well, then harvesting 16 hours later. Scintillation was counted on an LKB 1205 Betaplate liquid scintillation counter (Wallac, Turku, Finland).

IL-12/IL-10 production

Monocytes and VC were placed in 24-well plates (Nalgene Nunc) at a concentration of $5x10^5$ cells/ml and cultured for 24 hours in $RPMI^+$ containing ultraglutamine (2 mM, g/ml, Biowhitaker) and Biowhitaker), penicillin/streptomycin (100 U/ml, 100 serum free medium supplement (SF-1; Corning Costar Europe, Badhoevedorp, the Netherlands). To stimulate IL-10 production, the culture fluid contained staphylococcus aureus cowan 1 strain (SAC) (1:5000, Calbiochem, La Jolla, CA, USA). The IL-12 production was stimulated by SAC (1:5000) and γ-IFN (1000 U/ml, Biomedical Primate Research Centre, Rijswijk, the Netherlands). The production was measured by ELISA as indicated by the manufacturer (IL-10: ELISA Pelikine, CLB, Amsterdam, the Netherlands and the IL-12 (p 70): Eli-pair, Diaclone, Besançon, France).

RESULTS

Exposing monocytes shortly to 1,25(OH)2D3 prior to an overnight non-adherent culture resulted (similarly to T3 and dexamethasone) in a stimulation of cells forming veils and interestingly the number of cells forming veils after the overnight culture was higher in the case of the pulse with 1,25(OH)2D3 as compared to the pulse with T3 (Fig 1). Also similarly the 1,25(OH)2D3 pulse resulted in a population of CD14 dim/negative and a population of CD14 positive VC. As was the case with T3 and dexamethasone exposure, the CD14 positive cells were poor stimulators in allogeneic MLR and we therefore concentrated on the CD14 dim/negative VC.

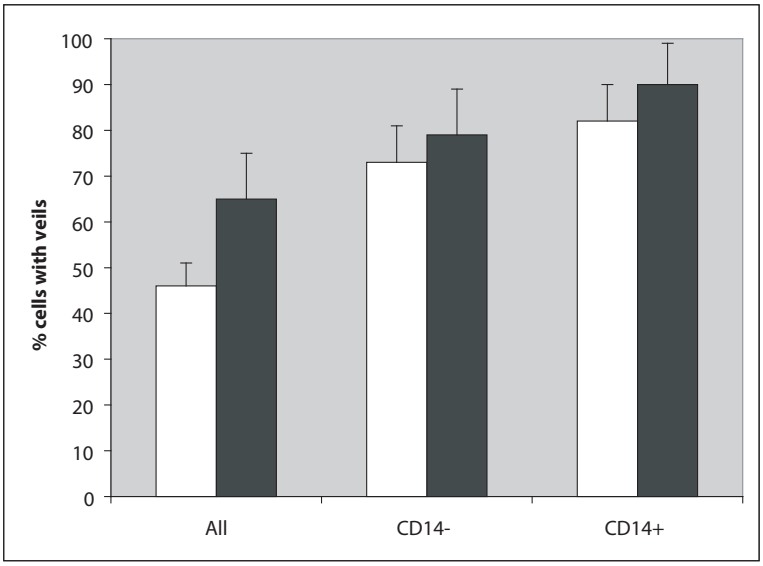

Figure 1. This figure shows the percentage of cells (all cells, or MACS separated CD14- and CD14 + cells) with long, cytoplasmic, moving extensions after an overnight culture of either T3 (white bars) or D3 (black bars) pulsed monocytes (n=11, mean and standard deviations are shown, differences between T3 and D3 are in all cases signficant, i.e. $p<0.05$, Wilcoxon)

Figure 2 shows the FACS analysis of the cells. The 1,25(OH)2D3-exposed cells expressed similar levels of HLA-DR, CD1a, CD80 and CD 86 than the T3-exposed cells, however the expression level of CD14 tended to be higher (in Figure 2 statistical significance was not reached, yet in another small series of experiments, n=4, significance was reached).

Figure 3 illustrates the allogeneic T cell stimulatory capacity of the VC generated from 1,25(OH)2D3- and T3-exposed monocytes. At a stimulatory-to-T cell ratio of 1:5 it can be seen that T3-VC were better stimulators of T cell proliferation than 1,23(OH)2D3-VC (mean 53013,s. d 35862 cpm vs mean 34899,s.d 22414 cpm, p=0.01).

Figure 4 shows the IL-10 and IL-12 production of the 1,25(OH)2D3-VC and the T3-VC as compared to the cytokine production of the original monocytes. The IL-10 production capability of both VC populations was higher than that of the original monocytes, but the IL-12 production capability of both the 1,25(OH)2D3-VC and the T3-VC was lower and that of the 1,25(OH)2D3-VC the lowest (although not reaching statistical significance. This pattern of IL-10 and Il-12 production is in keeping with the pattern as described in Chapter 3.

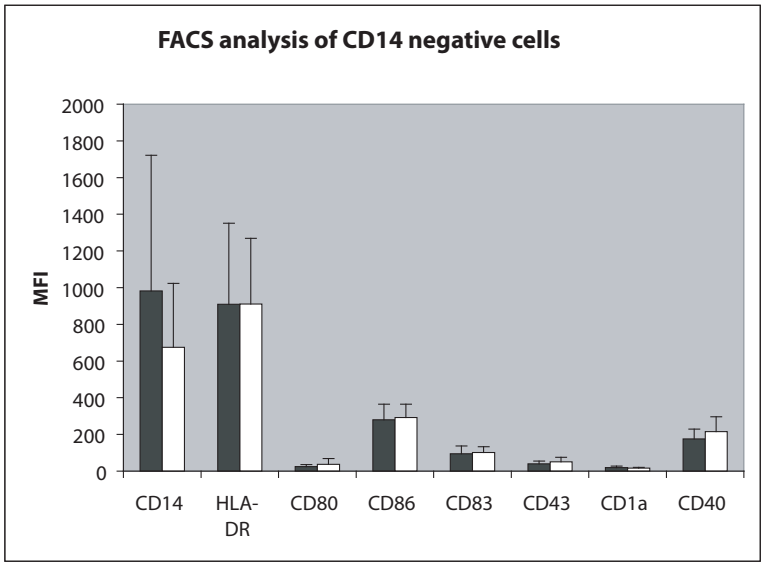

Figure 2. This figure shows the mean fluorescence intensity (MFI) of the CD14 negative monocyte-derived cells (MACS separated) after overnight culture and a T3 (white) or D3 (black) pulse (n=7, mean and standard deviations are shown)

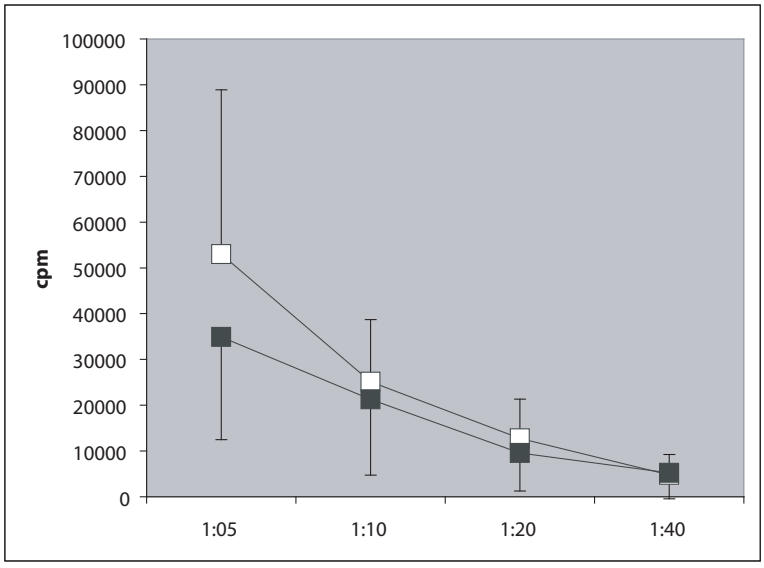

Figure 3. The accessory capability of CD14 negative monocyte-derived cells (MACS separated) after overnight culture and a T3 (white) or D3 (black) pulse as measured in allogeneic MLR (n=10, mean and standard deviations are shown). $P<0.01$ at a ratio of 1:5 (VC:T cell)

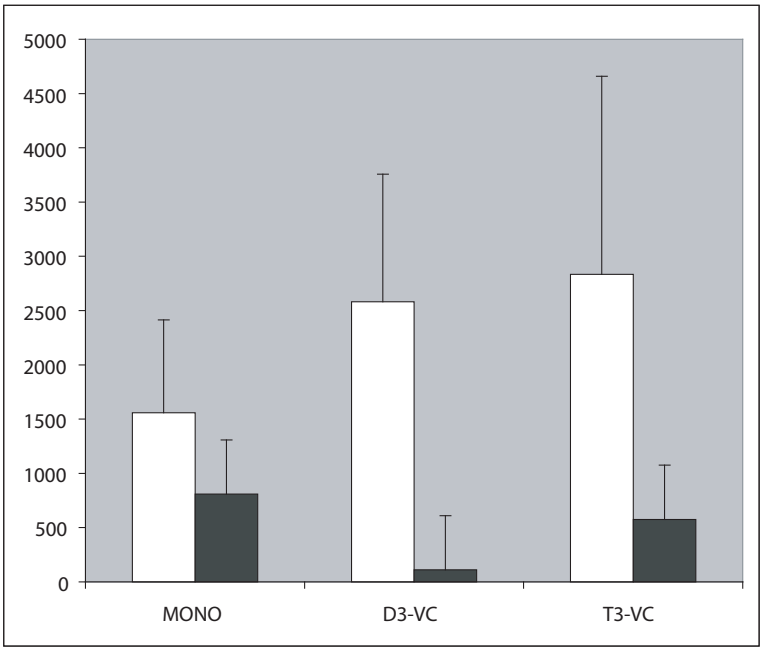

Figure 4. The IL-10 (white) and IL-12 (black) production of original monocytes, CD14 negative D3-VC and T3-VC (n=4, means and standard deviations are shown)

CONCLUSIONS

Exposure of monocytes to 1,25(OH)2D3 prior to a non-adherent overnight culture resulted in veiled cells that

a. tended to express higher levels of CD14
b. had a lower accessory capability in allogeneic MLR and
c. a very low IL-12 production capability

(as compared to T3-exposed cells)

These results are compatible with other reports that 1,25(OH)2D3 hampers the generation of fully immunostimulatory APC from monocytes and is engaged in suppressive activities in cell-mediated immunity (see chapter 5).

Chapter 6

A defective adherence of monocytes to fibronectin in thyroid autoimmunity has consequences for cell polarization and the development of veiled cells

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ABSTRACT

Blood monocytes of patients with thyroid autoimmune disease (TAID) show a defective capability to rearrange their cortical actomyosin cytoskeleton ('polarize') in response to chemoattractants. Since such cytoskeletal rearrangements also take place after adherence of monocytes to fibers of the extracellular matrix (ECM), it is not surprising that monocytes are primed for chemoattractant stimulation after fibronectin (FN) adherence, showing an enhanced polarization toward chemoattractants.

We investigated the adherence capability of blood monocytes of TAID patients to a FN matrix, and their integrin expression and chemoattractant-induced polarization both before and after FN adherence. Since cytoskeletal rearrangements are also needed for the development of veils during the transition of monocytes into veiled antigen-presenting (VCs), we additionally investigated such transition of FN-adherence monocytes of TAID patients.

Adherent and non-adherent monocyte populations from TAID patients and healthy controls were subjected to a polarization test with the chemoattractant fMLP (or MCP-1), FACS analyses (FITC-labelled FN, CD29, CD49e, d, b, and a) and tested for their capability to develop into veiled APC.

Monocytes of healthy individuals showed an improved chemoattractant-induced cell polarization after FN adherence, not reflected by TAID monocytes, in which chemoattractant-induced polarization worsened. Monocytes of healthy individuals upregulated CD49e and d integrins and their capability to bind FITC-labelled FN after adherence to a FN-coated plate, as well as enhancing their capability to generate T cell-stimulatory VCs. Monocytes of TAID patients did not.

These data indicate that integrin- (and chemokine)-mediated functions are hampered in monocytes of TAID patients. Because, integrin action is pivotal to processes such as monocyte adherence to endothelial cells, uropod formation, migration into tissues and differentiation into APC and macrophages, these defects might underly immune dysbalances important in thyroid autoimmune development.

INTRODUCTION

We previously reported in patients with thyroid autoimmune disease (1,2) a hampered response of monocytes to change shape from a round to a triangular motile form when stimulated with the chemoattractant N-formyl-methionyl-leucyl-phenalanine (fMLP). The process of shape change, also called cell polarization, is based on a rearrangement of the interior cytoskeleton of the cell (3). Chemoattractants and chemokines such as fMLP induce such rearrangement by binding to serpentine G-protein coupled receptors in the cytomembrane of monocytes. This stimulates a complex cascade of 2nd messenger systems including the phosphatidyl inositol (PI) cycle and the Rho-family of GTP-ases, which results - amongst other things - in a coupling of f-actin and other cytoskeletal building blocks to the membrane of the cell (4). In this way a submembranous myoactin cell cortex is formed, enabling the cell to change shape (5).

A similar formation of submembranous cortical myoactin filaments does occur after contact of monocytes with the extracellular matrix (ECM). Monocytes adhere to the ECM via integrins (6). The integrins are a family of noncovalent heterodimeric complexes situated in the cell membrane and consisting of an α and a β chain. Integrins do not only act as intermediary molecules linking the ECM fibers to the interior cytoskeleton, they also act as receptors triggering 2nd messenger systems like the PI cycle and the Rho-family of GTP-ases (7). It is thus not surprising that adherence of monocytes to an ECM matrix synergizes with chemoattractant signaling. Fibronectin (FN)-adhered monocytes have been reported to respond to chemoattractants with an enhanced cell polarization (8,9). A concerted action between integrin signaling and chemoattractant stimulation is thus of prime importance for the diapedesis of monocytes from the bloodstream into the tissues and their differentiation in the various monocyte-derived cells.

Here we describe an investigation on the chemoattractant-induced cell polarization of monocytes from 20 patients with recently diagnosed thyroid autoimmune disease (10 patients with hyperthyroid Graves' disease; 10 patients with hypothyroid autoimmune thyroiditis). Monocytes had either been or not been adhered to a fibronectin (FN)-coated surface prior to fMLP or MCP-1 chemoattractant stimulation.

Several integrins can bind to FN, however of the α chains CD49d and CD49e are primarily involved (6,7). Of the β chains CD29 is involved. Because of the molecular heterogeneity of integrin receptors for FN binding, we labeled FN with FITC and studied in addition to CD49 and CD29 expression, the expression of "fibronectin-receptors", i.e. the binding of FITC-labeled FN to patient and control monocytes before and after FN adherence.

Since rearrangements of the cortical actomyosin cytoskeleton are also needed for the development of the cell protrusions characteristic of veiled cells during their differentiation from monocytes (10), we also studied the capability of patient monocytes to develop into accessory veiled cells (VCs) before and after FN adherence. We previously reported on

a method to generate considerable numbers of VCs from monocytes (11,12). Crucial in this method of VC generation is the prevention of monocyte oxidase stimulation, via strictly plastic non-adherent culture conditions and the presence of reducing substances like BHA or iodinated compounds (e.g. metrizamide, reverse T3 and T3) to improve the yield of VCs (11,12). The procedure results in a veiled cell population of which 30-45% of the cells have veiled protrusions, starting from a suspension of round or oval-shaped monocytes. These generated VCs show a strong MHC class II positivity, a decreased expression of the monocyte marker CD14, S100 positivity (a marker of Langerhans dendritic cells), a decreased phagocytic capability, and a clearly enhanced stimulator capability in T cell proliferation assays indicating their improved accessory cell function (11,12). These in vitro-generated monocyte-derived VC thus resemble in many morphological and functional aspects the cells in vivo known to belong to the heterogenous group of dendritic cells (DC)(13). We previously showed that the generation of VCs from monocytes and their function is disturbed in patients with an organ-specific autoimmune disease, like autoimmune thyroid disease(1,2,14).

PATIENTS AND METHODS

Patients and controls

Heparinized blood (70 mls) was obtained via venapuncture from the following groups of individuals:

a. Ten recently diagnosed hyperthyroid Graves' patients (9 females, one male, ages 50 ± 13 (mean ± standard deviation) age-ranges 23-69 years) visiting the Department of Internal Medicine of the Zuiderziekenhuis, Rotterdam. None of the patients received medication at the time of blood collection. All patients had raised serum free thyroxin (fT4) levels and decreased serum thyroid stimulating hormone (TSH) levels. Patients had a diffuse non-nodular appearance of the thyroid on palpation, scan or ultrasonography. TSH-receptor antibodies (Brahms Diagnostics, Berlin, Germany) were positive in 8, thyroid anticytoplasmic antibodies (inhouse indirect immunofluorescence assay) were positive in 7.
b. Ten recently diagnosed hypothyroid patients (9 females, one male; ages 53 ± 14 years, age ranges 40-75 years) visiting the Department of Internal Medicine of the Zuiderziekenhuis, Rotterdam. None of the patients received medication at the time of blood collection. All patients had lowered serum fT4 levels and raised serum TSH levels. All had positive thyroid anticytoplasmic antibodies. TSH-receptor antibodies were positive in none.
c. Twenty healthy controls (15 females, 5 males) consisting of laboratory and hospital staff, ages 34±8 years; age range 21-52 years.

All patients and controls gave informed consent to donate blood for this investigation. The protocol had been approved by the Medical Ethical Committee of the Erasmus University Hospital Dijkzigt.

Monocyte isolation

Peripheral blood mononuclear cell separation was performed using Ficoll Paque density gradient centrifugation (density 1.077 g/ml; Pharmacia, Uppsala, Sweden). The cells were washed twice in RPMI 1640 culture fluid enriched with 10% fetal calf serum (FCS; Gibco, Breda, the Netherlands). Aliquots of 40-80 x 10^6 cells were stored at −150°C in a solution of 10% DMSO (Dimethylsulphoxide) in RPMI 1640 supplemented with 10% FCS.

Monocytes were isolated from the separated and deep-frozen lymphoid cells: an enrichment for the monocytes in the Ficoll-Isopaque isolated fraction was obtained by Percoll gradient centrifugation: after washing, the Ficoll-isolated pellet containing both monocytes and lymphocytes was resuspended in RPMI 1640 10% FCS and carefully layered on top of an equal volume of Percoll 1.063 (Pharmacia, Diagnostics AC, Uppsala, Sweden). After centrifugation (40 min, 450 g) the cells were collected from the interface, washed twice in the culture fluid (10 min, 500 g) and counted. The suspension almost always contains 80-95% monocyte-specific esterase positive cells, and only suspensions showing this yield of monocytes were used. The monocyte suspension was either directly used for the monocyte polarization assay and for FACS analysis, or for further adherence to fibronectin-coated surfaces.

Preparation of fibronectin-adhered monocytes

Monocytes were suspended at a density of 2x10^6 cells/ml in supplemented RPMI medium; aliquots (2.5 ml) were then dispensed into 50-mm tissue culture dishes (Nunc, Denmark) that had been precoated with varying concentrations of human fibronectin (CLB, Amsterdam, the Netherlands) in a total volume of 2.0 ml of phosphate buffered saline (PBS) for 2 h at 37°C. A coating concentration of 20 µg/ml of fibronectin was found to be optimal, and this concentration was used in subsequent assays. The monocytes were incubated in these fibronectin-coated wells at 37°C for 1 h in a 5% CO_2-95% air atmosphere. Nonadherent monocytes were vigorously washed off with ice-cold PBS (2 x 2 ml).

Firmly fibronectin-adherent monocytes were removed by incubating the cells in 2.0 ml PBS enriched with 3mM EDTA for 45 min. at 4°C, followed by further removal using a rubber policeman. This procedure was repeated twice with RPMI 1640, cells were washed, concentrated by centrifugation and counted using a hemocytometer.

We checked the lymphocytic contamination of the fibronectin-adhered cells by studying the expression of monocyte-esterase and markers such as CD3 (for contaminating T cells), CD19 (for contaminating B cells) and CD56-CD16 (for contaminating NK cells): the lymphocytic contamination of the monocytes after fibronectin adherence was very low: the population contained >90% of cells staining for monocyte-esterase, while contamination with $CD3^+$

T cells was as low as 2%, and the B- and NK-cell contamination as low as 5%. The fibronectin nonadherent monocytes were considerably contaminated with other populations of cells (around 15% of T cells and 15% of NK cells) and were not further used in our assays.

The polarization assay

For the polarization assay, aliquots (0.2 ml) of the various suspensions containing $2x10^5$ monocytes were added to 12x75 mm polypropylene tubes (Falcon Labware Division of Becton Dickinson, Oxford, CA, USA) containing 0.05 ml of either medium, N-formyl-methionyl-leucyl-phenylalanine (fMLP) in medium to reach a final concentration of 10 nm, or MCP-1 (gift dr. A. Montovani). The tubes were incubated at 37°C in a waterbath for 15 min. The incubation was stopped by addition of 0.25 ml ice-cold 10% formaldehyde in 0.05% PBS (pH 7.2). The cell suspensions were kept at 4°C until counting of polarized cells in a hemocytometer using an ordinary light microscope (magnification 250x). The test was read blindly by two experienced persons; 200 cells were counted, manually, from each tube. A monocyte was "polarized" if any of the following occurred: a change to an elongated or triangular shape, broadened lamallopodia, and/or membrane ruffling. The responsiveness of a monocyte population was expressed as the percentage of polarized cells in the presence of the chemoattractant minus the percentage of polarized cells in the absence of the chemoattractant. On previous occasions we verified that contaminating lymphocytes (in occasional preparations present in up to 20%) do not polarize under these conditions. The percentage of polarized monocytes was calculated as follows: *(% cells polarized due to chemoattractant / % monocyte-specific esterase positive cells) x 100%*.

The mean value of the two values recorded blindly by the two experienced observers was taken as the final value. Although this method seems to be a subjective microscopical quantification, it has been proven to be the most sensitive and reliable polarization assay as compared to morphometric and flow cytometric methods (15). This has also been our experience, with intra- and interassay variation hardly ever exceeding 5% polarization (n>100). If the data were discrepant in this way (less than 2% of assays), outcomes were not used and the assay was carried out again.

FACS analysis

The various monocyte fractions were analyzed using a FACScan flow cytometer (Becton Dickinson) for the following markers using the following monoclonal antibodies: CD49a (VLA1, Serotec, Oxford, England); CD49b (VLA2, Serotec); CD49d (VLA4, Serotec); CD49e (VLA5, Serotec); CD29 (Beckman Coulter, Hialeah, FL, USA) and FITC-labeled fibronectin. Fibronectin had been labeled with FITC (Sigma, St. Louis, USA) according to Bergquist et al. (16). Mononuclear cells were incubated with the FITC-labeled fibronectin and monoclonal antibodies for the detection of the membrane-bound integrins according to earlier described standard

FACS analysis techniques (17). The monoclonal antibodies had been directly conjugated with fluorescein isothiocynate (FITC) or phycoerythrin (PE) by the manufacturer.

Generation of veiled cells (VC)

Monocytes were suspended under plastic-nonadherent conditions (polypropylene tubes, Falcon, Becton Dickinson, USA) at a concentration of $2x10^6$/ml in RPMI 1640 (without FCS). T3 (Sigma) was added to the cell suspension at a concentration of $2x10^{-10}$ M to improve the yield of VCs. This suspension was then incubated at 37°C, 5% CO_2, 100% humidity for 30 min. This was followed by washing in FCS supplemented RPMI 1640 culture fluid (5 min, 500 g), and further culturing in the same culture fluid at 37°C, 5% CO_2, 100% humidity under non-adherent conditions for 16 hrs (overnight).

Following the 16 hr culture period, cells were centrifuged (5 min, 500 g) and resuspended in FCS supplemented RPMI 1640. Cells were then examined under light microscopy at a magnification of 400x. Veiled cells were defined as large cells with actively moving cytoplasmic processes or veils, which can easily be identified (see Fig.1). Measurements were performed blindly, and intra-assay variation remained below 2% (n>50). Inter-assay variation ranged from 1-6%.

Allogeneic MLRs were performed in order to measure the accessory capability of the generated VCs. Responder T lymphocytes were obtained from healthy donors and isolated following standard procedures with Ficoll-isopaque, Percoll density gradient centrifugation, and nylon wool adherence (Leuko-Pak, Fenwall Laboratories, Il, USA). Non-adhering cells recovered were more than 90% CD3 positive. $1.5x10^5$ responder cells were cultured in 96-well, flat-bottom microtiter plates (NUNC A/S International, Denmark) with different numbers of irradiated (2000 rad) stimulator cells (VC) to achieve stimulator-to-T cell ratios of 1:5 and 1:10. The culture medium used was RPMI 1640 with 25mM HEPES and L-glutamine, supplemented with 100U/ml penicillin G, 0.1 mg/ml streptomycin and 10% human A^+ serum, in a total volume of 200 µl per well. Controls used were VC alone, and lymphocytes in the presence of 10-50 µg/ml phytoheamagglutinin (PHA) (Wellcome Diagnostics, UK). Cultures were performed in triplicate. On day 5, thymidine incorporation was measured by adding 0.5 µCi 3H-thymidine to each well, then harvesting 16 hrs later. Scintillation was counted on an LKB 1205 Betaplate liquid scintillation counter (Wallac, Turku, Finland).

Statistical analysis

For statistical testing, the Mann-Whitney test was performed (two-tailed, Instat program). $P<0.05$ was considered significant.

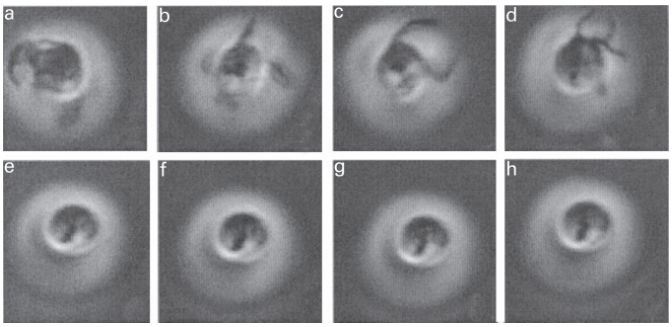

Figure 1. Time-lapse cinematographic pictures of a living monocyte-derived veiled cell (a-d) and an unchanged monocyte (e-h) in the same suspension (see for generation of these cells materials and methods). Pictures are taken 20-30 seconds apart. Magn. x1000. Note the constant changes in shape of the veiled cell extanding and withdrawing long, thin cytosplasmic extensions (a-d), this in contrast to the rigid round shape of the unchanged monocyte (e-h).

RESULTS

The chemoattractant-induced cell polarization of monocytes of thyroid autoimmune patients is defective and does not improve, but worsens after fibronectin adherence

Monocytes of thyroid autoimmune patients were as capable of adhering to fibronectin (FN)-coated surfaces as circulating monocytes of healthy control individuals. A mean value of 47% ± 10% (mean ± standard deviation, n=20) adhering monocytes was found in healthy controls, whereas thyroid autoimmune patients showed a value of 48% ± 10% (n=20). There was also no difference between the purities of the monocyte suspensions of thyroid autoimmune patients and of healthy controls neither before nor after FN adherence, values ranged from 80-95% monocyte-esterase positive cells before and over 95% monocyte-esterase positive cells after FN adherence.

We went on to test the chemoattractant-induced polarization of monocytes of thyroid autoimmune patients and healthy controls before and after FN adherence. As can be seen from Fig. 2, percoll-purified monocytes of thyroid autoimmune patients were hampered in their polarization response towards fMLP before FN adherence (verifying our earlier data, ref. 1): Values of 27% (mean, s.d. 11, n=20) vs. 35% (s.d. 6, n=17) of polarized monocytes were found in thyroid autoimmune patients and healthy controls respectively (p=0.005). Both hypothyroid and hyperthyroid patients showed this phenomenon.

After adherence to FN, the monocytes of healthy controls had a higher polarization capability as compared to the original monocytes: 45% (s.d. 5%, n=17) of the cells now responded to the chemoattractant fMLP (Fig. 2). The monocytes of thyroid autoimmune patients, on the contrary, became (after FN adherence) extremely poor in their polarization capability, and only 18% of polarized monocytes (s.d. 11, n=20) were found when stimulated with fMLP (Fig. 2).

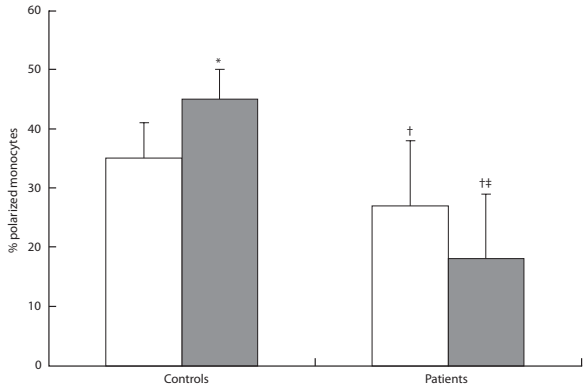

Figure 2. The percentage of monocytes that show a cytoskeletal rearrangement (cell polarization) under the influence of the chemoattractant fMLP. Means ± s.d. are shown of monocytes before (open columns) and after (gray dotted columns) fibronectin (FN) adherence of healthy controls (n=17) and thyroid autoimmune patients (n=20). *p<0.01 vs monocytes prior to FN adherence, † p<0.01 vs monocytes of healthy controls, ‡ p<0.001 vs FN-adhered monocytes of healthy controls

In a few experiments we used the chemokine MCP-1 as a polarization-inducing signal. Monocytes of healthy controls again showed a good polarization response to MCP-1 (40-42%, at optimal MCP-1 dosage 20 ng/ml) after FN adherence. In 3 patients tested (2 hypo, 1 hyper) this response was again very low (11%, 29% and 9% respectively), reinforcing the observation of a poor capability of the patient monocytes to rearrange their cytoskeleton upon a chemoattractant stimulus, particularly after FN adherence. It must also be noted that we found MIP1α and RANTES to be poor inducers of monocyte polarization both in healthy controls and patients.

The upregulation of fibronectin receptors on monocytes of thyroid autoimmune patients is hampered after fibronectin adherence

Firstly it must be noted that the expression of "fibronectin receptors", of the various CD49 markers and of CD29 was "dull" on monocytes and at a moderate Mean Fluorescence Intensity (MFI) level of 60-150 as compared to the MFI expression levels we have seen previously on e.g. dendritic cells (MFIs of 500-2000).

Although we had expected that FN-adherence would have acted as a method to separate 'fibronectin receptor' positive monocytes from 'fibronectin receptor' negative monocytes, we observed (Table I) that the percentage of original monocytes that were capable to adhere to a FN-coated surface did not match with the percentage of original monocytes capable of binding FITC-labelled FN or expressing the relevant FN-binding CD49d, e and CD29 integrins. Also the expression of such binding sites and integrins was still considerable in the fraction of FN-nonadhered monocytes (both numerically and regarding the intensity of expression). Moreover the number of FITC-FN, CD49d, e and CD29 positive cells in the FN-adhered fraction was certainly not enriched to 90- 100%, though it was significantly increased for FITC-FN,

Table I. Fibronectin-related integrin expression (percentage of monocytes positive in FACS analysis) of fibronectin-adhered and non-adherent monocytes of healthy donors (n=10)

	Original monocytes	Fibronectin-adhered cells	Fibronectin-nonadherent cells
Fibronectin-adhering cells	47 ± 10%		
Fibronectin-FITC$^+$	36 ± 16%	55 ± 20%[a]	16 ± 10%[b]
CD49d$^+$	84 ± 12%	93 ±4%[a]	68 ± 12%[b]
CD49e$^+$	67 ± 18%	86 ± 10%[a]	42 ± 14%[b]
CD29	70 ± 23%	51 ± 19%	n.t.
CD49b$^+$	55 ± 17%	73 ± 7%	32 ± 12%[b]
CD49a$^+$	14 ± 9%	17 ± 10%	8 ± 5%

[a] $p<0.05$ vs original monocytes.
[b] $p<0.05$ vs fibronectin-adhered monocytes
n.t. = not tested

CD49d and CD49e positive cells (see Table 1). The number of CD29 positive cells was even lower in the FN-adhered fraction, but values did not reach statistical significance. There were also no difference with regard to the MFI intensity of integrin expression after FN adherence. Furthermore, repetitive steps of FN-adherence did not alter these integrin expression data in a noteworthy manner. These observations are in line with the previously described plasticity of integrin expression with regard to the number and avidity / affinity of these receptors after binding to ECM proteins (6, 7). This process of regulation of integrin expression is called "inside-out" signalling.

Table 1 also shows that CD49a and CD49b (α integrin chains important in laminin binding) were not significantly upregulated after adherence to FN, though there were significant differences with the FN-nonadherent cells for CD49b.

Fig. 3 gives the data for the percentages of patient monocytes with "fibronectin receptors" (able to bind FITC-labeled fibronectin) before and after FN adherence in relation to values of healthy control monocytes. Also the percentages of monocytes expressing the CD49e, CD49d, CD29, CD49b and CD49a integrins are shown. Before FN adherence there were no differences between healthy controls and thyroid autoimmune patients with regard to the percentage and expression levels of monocytes expressing FITC-FN binding sites or CD49e, d, and b integrins. Remarkably, more monocytes of thyroid autoimmune patients expressed CD49a. The number of CD29-expressing monocytes was lower, but this did not reach statistical significance. Fig 3 also shows that - although monocytes of thyroid autoimmune patients adhered equally well to FN-coated surfaces (see before) - after FN adherence there were fewer monocytes from these patients with "fibronectin receptors" as compared to healthy control monocytes. The same phenomenon was seen for CD49e positive monocytes. CD49d expression was raised to the same extent in patient and control monocytes after FN adherence. CD29 and CD49b expression were neither significantly altered before nor after FN adherence in patients and controls. CD49a expression stayed high after FN adherence in monocytes of thyroid autoimmune patients as compared to those of healthy controls.

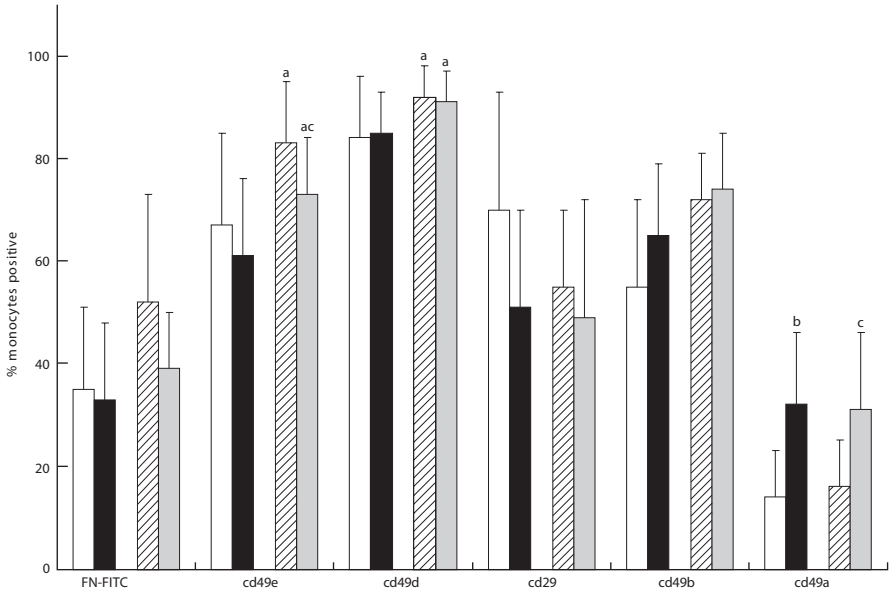

Figure 3. The percentages of monocytes positive in FACS analysis for "fibronectin receptors" (FN-FITC, see text), CD49e, CD49d, CD29, CD49b and CD49a integrins. Mean ± s.d. are given of original monocytes (first two columns of a set) and of FN-adhered monocyts (last two dotted columns of a set) of healthy controls (open columns) and patients (gray columns). a=p<0.05 vs monocytes before FN adherence, b=p<0.01 vs normal monocytes of healthy controls, c=p<0.05 vs FN-adhered monocytes of healthy controls.

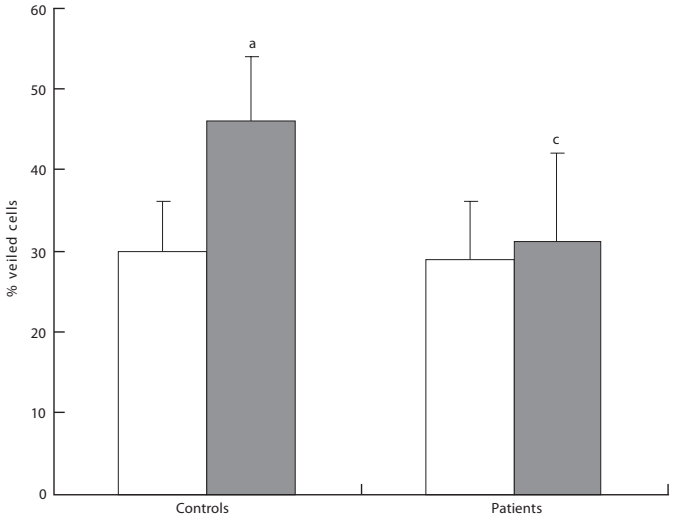

Figure 4. The percentages of veiled cells (VCs) generated from blood monocytes prior to (open columns) and after (gray,dotted columns) FN adherence. Means ± s.d. are shown of healthy controls before FN adherence (n=4) and after FN adherence (n=8), and of thyroid autoimmune patients under both conditions (n=20). a=p<0.01 vs monocytes before FN adherence, c=p<0.001 vs FN-adhered monocytes of healthy controls.

The development of functionally active veiled cells from fibronectin-adherent monocytes is lower in thyroid autoimmune patients

On an earlier occasion we reported that the recovery of veiled cells (VCs) from normal, non-fibronectin-adhered monocytes is in the same range in Graves' patients as in healthy controls (1). We experienced the same observation in a few patients tested (n=4) in the present series of experiments, and values in the range of 22-37% of cells with long, actively moving veils could be generated from monocytes both in patients and healthy controls (Fig. 4). Fig. 4 also shows that the percentages of VCs that could be generated from monocytes was higher in healthy controls after FN adherence of the cells, and values of 46% ± 8% (n=8) were obtained. These generated VC were good stimulators of the T cell proliferation in an allogeneic MLR (Fig. 5).

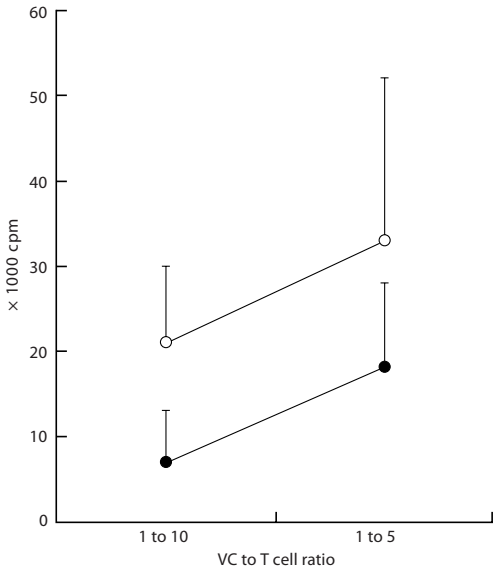

Figure 5. The T cell stimulatory potential of the VC populations generated from FN-adhered monocytes. Data are expressed as the capacity of the VCs to induce T cell proliferation in an allogeneic MLR (3H-thyminidine incorporation, cpm, means ± standard errors). Data are given of 7 healthy controls (○) and of 18 thyroid autoimmune patients (●). The horizontal axis gives the VC toT cell ratios. At both ratios the differences between the controls and the patients are significant (p=0.003 for the 1 to 10 and p=0.02 for the 1 to 5 ratio).

When FN-adhered monocytes of thyroid autoimmune patients were used, the recovery of VCs was much poorer (Fig. 4, 31% ± 10%, $p<0.01$ n=20), and the cell population was less capable to stimulate T cells in allo-MLR (Fig. 5).

It must also be noted that there existed a good correlation between the polarization capability of a given population of FN-adhered monocytes on the one side and its capability to generate VCs and the capability of that VC population to stimulate T cells in allo-MLR on the other side; correlation coefficients of 0.48 and 0.47 were found respectively (n=30,

$p<0.01$). This underlines the notion that the capability of fibronectin-adhered monocytes to rearrange their cortical cytoskeleton is closely linked to the capability of that population to generate veiled T cell-stimulating APCs.

DISCUSSION

Integrins and chemoattractant stimuli play a pivotal role when monocytes enter the tissues from the bloodstream. Via integrin ligation and integrin signaling monocytes are able to firmly adhere to endothelial cells (18), to form uropods (19), to respond more vigorously to chemoattractants (8,9), to migrate through the vessel wall and through the connective tissue via ECM fibers (20) and to differentiate more efficiently into antigen presenting cells (APCs) and macrophages (21). Integrin-induced stimulation of 2nd messengers and rearrangements of the cytoskeleton are essential in many of these processes ("outside-in" signaling, 6,7).

After binding to ECM-fibers, integrin expression itself and the avidity and affinity of integrins to ECM proteins is upregulated (6,7). The significance and biochemical pathways of this so-called "inside-out" signaling are poorly understood (6,7). It has been suggested that "inside-out" signaling plays a role in the ability of migrating cells to align ECM-fibers (22).

Our experiments indeed show that greater numbers of monocytes from healthy controls expressed FN binding sites, CD49e and CD49d integrins after FN adherence. The FN-adhered cells also showed an enhanced chemotactic polarization and an enhanced capability to differentiate into veiled accessory cells.

With regard to monocyte dysfunction in thyroid autoimmune disease, we show here that monocytes of patients with recently diagnosed thyroid autoimmune disease are hampered in these integrin - and chemokine - mediated functions: the cells were less able to rearrange their cytoskeleton after FN adherence, to upregulate the above-named integrins and to generate veiled, T cell-stimulating APCs. Monocytes of both hypo- and hyperthyroid patients showed these defects. It is known that the thyroid hormone status is able to influence the function of monocytes and monocyte-derived cells (23,24). However since both hyper- and hypothyroid patients showed these abnormalities and since we reported on a previous occasion on monocyte cytoskeletal rearrangement disturbances in euthyroid TPO-Ab- positive women (2), we presume that our data indicate that the autoimmune status per se and not the thyroid hormonal status determines the above described monocyte abnormalities.

Together with our earlier report on a poor homotypic clustering capability of veiled/dendritic cells in thyroid autoimmune disease (1), also a function dependent of integrins (25,26), the present data strongly point to the existence of defects in the integrin and chemokine signaling pathways in monocytes and monocyte-derived cells of thyroid autoimmune patients. There are previous reports on a defective and altered expression of integrins on leukocytes

in thyroid autoimmune disease (27,28). However, these reports mainly focus on a defective integrin expression on lymphocytes and the consequences of such defective expression for a hampered lymphocytic adherence to endothelial cells, which has indeed been established for high endothelial venules in the thyroid-draining lymph nodes of thyroid autoimmune patients (29).

It is perhaps difficult to conceive why a general disturbance in monocyte function – as described here- should occur in a disease in which the abnormality of the immune system is an immune response mainly focused toward thyroid gland antigens. In explaining this, the NOD mouse model can be helpful. In the majority of NOD strains, no thyroiditis but only autoimmune insulitis occurs. The NOD mouse is – despite a mainly focussed immune reaction towards islet antigens- characterized by a general immune disturbance including an abnormal development of DC and other APC from precursors, leading to suppressor T cell defects (30-32). Only when the thyroid is manipulated such that monocytes, macrophages and DC are attracted to the thyroid (via iodine intoxication, 33) is thyroid autoimmune disease precipitated. Thus, local factors together with a general immune disturbance lead to full-blown thyroiditis in this animal model. This likely also is the case in humans, as iodine also precipitates autoimmune thyroid disease here.

Integrin and chemoattractant signaling activates a plethora of molecules involved in intracellular signal transmission and cytoskeletal rearrangements. In Wiskott-Aldrich syndrome (WAS), a rare disease characterized by thrombocytopenia, immune dysregulation and also autoimmunity, there is an inherited deficiency of the so-called WAS protein (WASp, reviewed in 34). WASp is a molecule important in the signaling cascade from the cell membrane to the myoactin cytoskeleton, probably at the level of the Rho GTP-ase CDC42 and the cytoskeletal organizing complex Arp2/3. This deficiency leads - amongst other things - to an inability of monocytes to respond to a chemoattractant signal with cell polarization (3). Also the appropriate maturation of dendritic cells (DC) from monocytes is hampered, and WAS-DC are defective in their ability to migrate on ECM matrixes (35). The here-presented data on monocytic defects in thyroid autoimmune patients are reminiscent of those reported in WAS, and therefore urge for a more detailed study on putative molecular abnormalities in the signaling cascade “integrins and chemokine receptors $\rightarrow$ cytoskeletal rearrangements" in monocytes of patients with thyroid autoimmune disease. Interestingly in the NOD mouse an impaired cell membrane targeting of the Grb2-sos complex has been found (36) (The Grb2-sos complex mediates the signal from integrins to the MAP-kinase pathway). In BB-DP rats, another animal model of autoimmune thyroiditis, an abnormal expression of vav was found (37) (Vav mediates the signal from integrin-activated tyrosine kinases to the Rho-family of GTP-ases).

The present report also expands our previous observations on abnormalities of monocyte-derived APCs in thyroid autoimmune patients. Here we show that the generation of VCs from fibronectin-adhered monocytes is lower in patients with a recently diagnosed thyroid autoimmune disease as compared to healthy controls. The here-used method of generating VC from monocytes yields a type of APC which is different from the so-called "immature dendritic cells (DC)" generated according to a method first published by the group of Lanzevecchia (38). In the latter method, monocytes are cultured under plastic-adherent conditions for one week in the presence of GM-CSF and IL-4. The here-generated VC show many characteristics known of dendritic/veiled cells in ex vivo/in vivo situations (13): they are MHC class II positive, have various markers known of Langerhans cells (e.g. S100) and have a clearly increased capability in stimulating T cells as compared to monocytes. They are almost as good as "Lanzavecchia" DC in this respect (11,12). The VCs, however, hardly produce the immunostimulating cytokine IL-12. They do produce large quantities of the immunosuppressive and immunomodulating cytokine IL-10 (to be published). It is thus tempting to speculate that the here-described defects in VC development from integrin-activated monocytes represent defects in the generation of APC with immunosuppressive and tolerating capabilities. Interestingly, similar abnormalities in APC generation exist in BB-DP rats (39) and NOD mice (30). In these animals, these defects indeed lead to a faulty activation of T cells with an immunosuppressive/regulatory capability (30,31,39). Further research is however needed to explore the latter hypothesis.

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Chapter 6A

A normal development of dendritic cells from blood monocytes of patients with autoimmune thyroid disease

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INTRODUCTION

Dendritic Cells (DC) form a heterogenous group of antigen presenting cells (APC) with different lineage backgrounds (lymphoid versus myeloid), with different (local) precursors and with various stages of differentiation and maturation. In the last decade the idea has gained acceptance that DC are not only cells capable of initiating immune responses, but also prime inducers of tolerance [4]. There are a number of non-mutually exclusive ideas about the characteristics and capabilities of the populations of DC that are capable of performing such function [5]: thymus DC [6], so-called "steady state" DC [7] and certain forms of (semi-) mature DC [8] have all been described as capable of inducing tolerance.

In chapter 3 of this thesis we described that apart from classical DC also a population of veiled accessory macrophages exist which are quite similar to classical DC, yet also show differences. Culturing monocytes for 24 hrs while avoiding plastic adherence (polypropylene tubes) and avoiding the activation of NADPH oxidase (blocking agents) results in the generation of such veiled accessory cells (VC). The generated VC were actively moving cells like lymph-borne VC in vivo. The monocyte (mo)-derived VC population existed of $CD14^{dim/-}$ and $CD14^{bright}$ cells. Of these the $CD14^{dim/-}$ VC were as good in stimulating allogeneic T cell proliferation as immature DC (iDC) obtained after one week of adherent culture of monocytes in granulocyte-macrophage-colony stimulating factor (GM-CSF)/interleukin (IL)-4. Although the $CD14^{dim/-}$ VC had a modest expression of the DC specific marker CD83 and were positive for S100, expression of the DC specific markers CD1a, Langerin, DC-SIGN, and DC-LAMP were absent. This indicates that the generated $CD14^{dim/-}$ VC cannot be considered as classical LC/DC.

It was impossible to turn the $CD14^{dim/-}$ mo-derived VC population into typical DC by culture for one week in GM-CSF/IL-4 or LPS. In fact the cells died under such circumstances, gaining some macrophage characteristics before dying. We also found that culturing the the $CD14^{dim/-}$ mo-derived VC in IL-3 let the cells survive while gaining clear macrophage characteristics such as an adhesive capacity and acid phosphatase activity, underscoring the actual macrophage character of the cells.

Abnormalities in the differentiation and maturation of VC from fibronectin-adhered monocytes were documented in Chapter 6 in patients with autoimmune thyroid disease.

Interestingly the non-obese diabetic (NOD) mouse and the BB-DP rat, both models for endocrine autoimmune disease, show aberrancies in the generation of classical DC and macrophages from their precursors, and it is thought that such defects might be causal to the state of defective tolerance in such animals.

With regard to the NOD mouse, the group of Haskins showed that a mixture of DC and macrophages isolated from the spleen were poor in accessory function [11]. In accord with the abnormalities of the DC isolated from the lymph node and spleen of this animal are findings on an abnormal differentiation and maturation of DC from NOD bone–marrow

precursors, particularly when DC are generated in the presence of GM-CSF alone [12-18]. Under such circumstances NOD BM-precursors yield lower numbers of differentiated DC, yet show various signs of pro-inflammation, viz. an enhanced expression of NF-κB [19] and a higher production of interleukin-12 [20].

There exist however also reports for the NOD mouse that doubt these defects in the differentiation of DC from their precursors [21-23].

With regard to the BB-DP rat our group previously showed that lymph node and spleen DC are less differentiated and have an abnormal ("immature") phenotype [24], i.e. a relatively low expression of MHC class II and of co-stimulatory molecules. These lymph node and spleen DC also showed a diminished capability to stimulate the proliferation of T cells, particularly of the suppressor T cell population of the rat: the $RT6^+CD8^+$ T cells.

Here we tested monocytes of patients with a recent onset autoimmune thyroiditis (AIT) for their capability to generate classical DC in culture with GM-CSF/IL-4.

SUBJECTS AND METHODS

Subjects

Heparinised blood (60 mL) was obtained from

1. Recently diagnosed AIT patients (n=28), who visited the outpatient clinic of the Department of Internal Medicine, Medical Center Rijnmond Zuid, Rotterdam, the Netherlands. The mean age was 42.7 ± 12.6 years, ranging from 30.9 to 71.2 years. All patients had high levels of thyroid peroxidase antibodies (TPO-Abs > 400IU/ml).
2. Healthy controls with an absent family history of autoimmune diseases. These individuals consisted of laboratory personnel and students. The controls were divided for some comparisons into two groups to obtain age-matched controls for the two diabetic groups (young group, n=41, mean age 28 ± 6.6 years, ranging from 19.4 to 42.5 years; older group, n=15, mean age 50.1 ± 9.5 years, ranging from 30 to 60.5 years).

Informed consent was obtained from all participants. The research protocol has been approved by the Medical Ethical Committee of the Erasmus MC, Rotterdam, the Netherlands.

MONOCYTE ISOLATION AND GENERATION OF DCS

Ficoll (Pharmacia, Uppsala, Sweden; density 1.077 g/ml) and Percoll (Pharmacia; density 1.063 g/ml) density gradient centrifugation were used to isolate monocytes from heparinized blood and buffycoats (Sanquin Blood bank, Rotterdam, the Netherlands) as described in detail before by Kabel et al [26]. The monocytes were cultured (according to the methods of

Sallusto [27]) at a concentration of $5x10^5$ cells/ml on 24-well culture plates for six days under plastic-adherent conditions in RPMI 1640 with 25mM HEPES and L-glutamine (BioWhittaker Europe, Verviers, Belgium) (hereafter referred to as $RPMI^+$) containing ultraglutamine (UG) (2mM, BioWhittaker), penicillin/streptomycin (P/S) (100U/ml penicillin, 100µg/ml streptomycin, BioWhittaker) and 10% inactivated FCS (FCSi) (BioWhittaker) in the presence of GM-CSF 400U/10^6 cells and IL-4 500U/10^6cells (both Pepro Tech EC, London, England). The cells were incubated at 37^0C, 5% CO_2 and 95% humidity. On day three, half of culture fluid was refreshed with GM-CSF 200U/10^6cells, IL-4 250U/10^6cells. After six days DC were collected by resuspending and washing the wells thoroughly with cold phosphate buffered saline (PBS) pH 7,4 (Biowittaker), with 0,1% bovine serum albumin (BSA) (Bayer, Kankakee, IL, USA) and 3mM ethylene diamine tetraacetic acid (EDTA), pH 8 (Sigma-Aldrich, Steinheim, Switzerland). The cells were counted with 0.1% trypan blue (Sigma Chemical co, St Louis, USA) to assess cell viability.

T cell isolation was performed by washing the pellet in the Percoll gradient twice with PBS/0,1%BSA and incubating the cells with 20µl/10^7 cells anti-CD3 microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany) for 20 minutes on ice. A magnetic cell sorting system (auto MACS sorter, Miltenyi Biotec) was used for the selection of CD3 positive T cells.

Phenotype of DCs

The following monoclonal antibodies (mAbs) were used for flowcytometry: anti-IgG1 FITC, anti-IgG1 PE (both (1:10) Becton Dickinson, San Jose, CA, USA), anti-CD14 FITC (1:250, Beckman Coulter, Hialeah, FL, USA), DC-SIGN PE (1:10, R&D systems, Minneapolis, MN, USA), anti-HLA-DR PE (1:200, Becton Dickinson), anti-CD80 PE (10µl/10^5 cells, Becton Dickinson), anti-CD86 FITC (10µl/10^5 cells, Pharmingen, San Diego, USA), anti-CD40 FITC (10µl/10^5 cells, Serotec, Oxford, England), anti-CD1a PE (1:100, Beckman Coulter), anti-CD18 FITC, anti-CD11b PE (1:5 and 1:10, Becton Dickinson), anti-CD29 FITC (1:160, Beckman Coulter), anti-CD54 PE (ICAM-1) (1:4, Becton Dickinson), anti-CD49a FITC (VLA-1), anti-CD49b FITC (VLA-2), anti-CD49d (VLA-4) (10µl/10^5 cells, Serotec), anti-CD49e FITC (VLA-5) (10µl/10^5 cells, Immunotech), anti-CD3 FITC (1:20, Becton Dickinson), anti-CD19 PE (1:25, Becton Dickinson) and 7AAD (1:250, Molecular probes, Eugene, Oregon, USA).

DC were incubated in polypropylene tubes with mAbs for 15 minutes, then washed twice. The cells were measured immediately following cell staining using a FACScan flowcytometer and analyzed using CellQuestPro (Becton Dickinson, Mountain View, CA, USA). Routinely 10,000 events were collected. Debris and dead cells were gated out on basis of their light scatter properties. In addition, we stained the cells with trypan blue and 7AAD. The gated DC population was devoid of $CD3^+$ and $CD14^+$ cells. Data were expressed as mean ± SD of percentage of positive cells and mean ± SD of mean fluorescence intensity (MFI). The background staining was determined by staining of cells with IgG1-FITC and IgG1-PE alone and subtracted from the values.

Mixed Leucocyte Reaction (MLR)

For the MLR DC were irradiated with 20Gy and placed in flat-bottom 96-wells-plates (Nunc, Roskilde, Denmark) at concentration of $3x10^5$, $1.5x10^5$, $0.75x10^5$ cells/ml RPMI$^+$ containing UG, P/S and A$^+$-serum. We added 100µl of DC and an equal volume of autologous T-cells at concentration of $1.5x10^6$ cells/ml to the wells resulting in a total volume of 200µl per well. As positive control these T-cells were stimulated with phytohemagglutamin (PHA) (Sigma). Culture of DC or T-cells in medium alone served as negative controls. Proliferation was determined after 18 hours of 0.5µCi/well ^{3}H-thymidine addition on day 5. Cells were harvested on filterpapers and radioactivity was counted in a liquid scintillation analyzer (LKB Betaplate, Wallac, Turun, Finland). The values are the mean of triplicates.

STATISTICAL ANALYSIS

Statistical analysis was performed using SpSS 11.0. Paired Student's T-test was used to detect differences between data of untreated and CD54 treated DC experiments. For the other experiments the Mann Whitney U-test was used. P–values lower than 0.05 were considered significant.

RESULTS

The yields of DC, measured as the percentage of generated viable cells of the original number of monocytes put into GM-CSF/IL-4 culture, were comparable for AIT patients and healthy controls. After six days of culture the yield of DC was 39% (mean) ± 21% (standard deviation, SD) for the healthy controls (n=18) and for AI thyroiditis patients 32% ± 24% (n=12). Differences between groups and series of experiments were not statistically significant.

The percentages and expression levels of the markers CD1a, DC-SIGN, HLA-DR, CD80, CD86 and CD40 on the DC of AIT patients were also comparable to those of the healthy controls (Figure 1).

With regard to DC function, the T cell stimulatory capacity in autologous MLR of the phenotypically abnormal DM1 DC was significantly lower. For the DC:T cell ratio 1:10 and 1:20 DM1 patients showed a significantly lower incorporation of ^{3}H-thymidine (Figure 2).

When the T cells of the DM1 patients were stimulated with the mitogen PHA in the absence of DC, they also showed a lower proliferation as compared to healthy control T cells (8568 ± 7508 vs 22099 ± 13550, p=0.03).

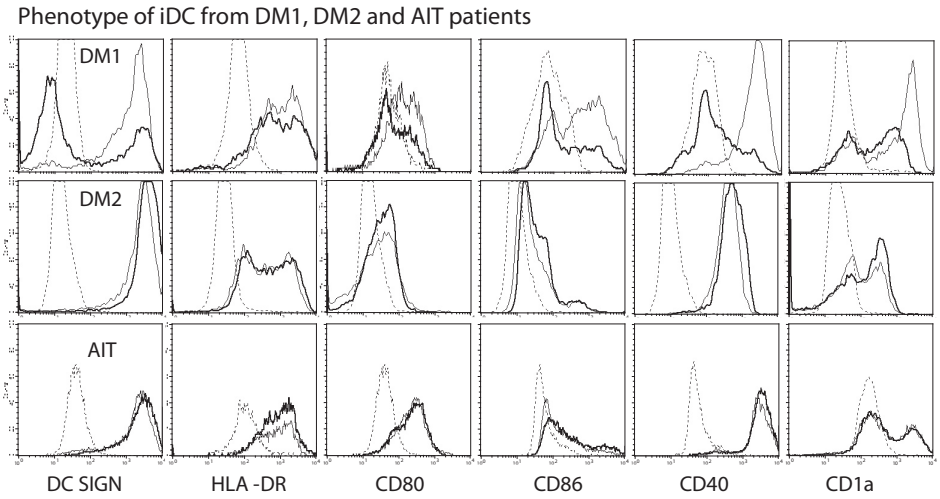

Figure 1. Phenotype of immature DC derived from DM1, DM2, AIT patients and control subjects. The phenotypes (DC markers and costimulatory molecules) of DC from DM1, DM2 and AIT patients (thick lines) are shown in histograms. The thin lines are DC from control subjects and the dashed lines are DC stained with isotype control Abs. DC of DM1 have lower expression of DC-SIGN, CD80, CD86, CD40 and CD1a. DC of DM2 and thyroiditis patients are comparable to controls.

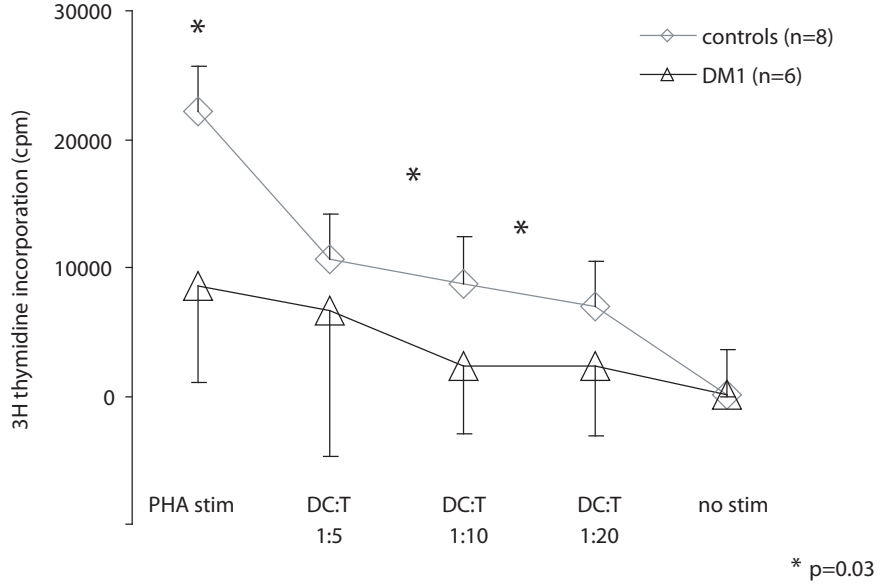

Figure 2. Proliferation of autologous T cells measured by ^{3}H-thymidine incorporation and expressed as counts per minute (cpm) are shown. DC of DM1 patients (n=6, triangles) have lower autologous T cell stimulatory capacity compared to DC of controls (n=8, diamants). T cells of DM1 patients have significantly lower proliferation to PHA stimulus compared to T cells of controls. Data are shown as means and standard deviations of cpm for triplicate samples per individual (* p=0.03).

CONCLUSION

We could neither find abnormalities in the generation of mo-DC of recent onset AIT patients, nor in the expression of the here studied markers and important immune stimulatory molecules of their DC. In a previous study (Chapter 6) we did find that monocytes of AIT patients had a lower capability to arrange their cyto-skeleton when stimulated with a chemo-attractant and were less capable in forming veiled accessory macrophages, particularly when FN-adhered monocytes were used [53].

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Chapter 7

Discussion

GENERAL CONCLUSIONS

Regarding veiled accessory macrophages

The studies performed in Chapter 3 on monocytes using oxygen radical blocking agents and non-adherent conditions illustrate the plasticity of monocyte-derived APC populations by demonstrating that within a period of twenty-four hours, veiled accessory cells (VC) can be generated from human monocytes. These VC are actively moving cells and demonstrate the morphology and some markers typical of classical monocyte-derived DC (i.e.in S-100 expression). They also differ from the DC described in peripheral tissues and in the T cell areas of lymph nodes (the interdigitating cells) by showing an absence of DC-SIGN and CD1a.

Despite this difference, our monocyte-derived veiled macrophage-like cells most likely form part of the heterogeneous population of veiled/dendritic macrophage-like cells in afferent lymph described in the 1970-1980s which drain the peripheral tissues to the draining lymph nodes, since cells with a morphology and movement pattern similar to our in vitro obtained VC formed part of this heterogeneous lymph-borne population. However, detailed studies on such lymph-borne cells using markers developed in the last few decades are required to strengthen or refute such a view.

The VC we generated could be divided based upon their expression of CD14. While the CD14 bright cells were extremely poor accessory cells, the CD14dim/negative population was a very good accessory cell population, able to stimulate T cell proliferation as good as classical immature (i) DC obtained after one week of adherent culture in GM-CSF/IL-4. Interestingly, CD14dim/negative VC expressed S100 strongly, like Langerhans cells in the skin and DC in interstitial tissues (in fact monocyte-derived DC do not). Like monocyte-derived DC, the VC were not reactive to acid phosphatase and expressed MHC Class II, however expression of the DC-specific marker CD83 was modest, and there was hardly or no expression of other DC-specific markers such as CD1a, Langerin, DC-SIGN and DC-LAMP. The production of IL-10 and IL-12 by these veiled accessory cells also differed from iDC, with more IL-10 and less IL-12 produced, with the result that the T cells that were stimulated by these VC produced less IFN-gamma than T cells stimulated by iDC.

It was impossible to keep the VC in culture, or to differentiate them further into typical DC by culturing them with GM-CSF/IL-4 or LPS. Under these conditions, the cells gained some characteristics of macrophages before dying. Culturing the CD14dim/negative VC in IL-3 let the cells survive; the cells now gained clear macrophage characteristics such as an adhesive capacity and acid phosphatase activity, underscoring the actual macrophage character of the cells (unpublished results).

Conclusive hypothesis: Avoiding "danger signals" (plastic adherence and radical formation) monocytes are capable of rapidly transforming into veiled macrophages with an excellent T cell stimulatory capacity. The cytokine profile of these veiled macrophages (high IL-10 and low IL-12 production capability) and their capability to skew the T cell response in a Th2 direc-

tion suggests that the cells are "steady state" APC involved in tolerance induction rather than in eliciting a cell-mediated immune response.

Regarding the effects of hormones on classical DC and veiled accessory macrophages

The studies described in Chapter 4 and 5 demonstrate clear effects of hormones on the differentiation and maturation of classical DC and VC from monocytes, the two APC populations studied in this thesis (Table I and II).

1,25 (OH)2 Vit D3. Specifically, the presence of Vitamin D3 in the cultures caused a down regulation of typical DC markers such as CD1a, CD83, CD80, CD40 and HLA-DR, while up regulating monocyte markers such as CD14 and CD86. Functionally, the resulting DC were less able to stimulate T cell proliferation in the MLR than normal monocyte-derived iDC, and produced large amounts of IL-10 (Table I).

The use of Vitamin D3 in the VC culture resulted in a lower yield of CD14 negative/dim VC with a considerable residual CD14 expression. Such VC had a lowered capability to stimulate T cells and a poorer IL-12 production (Table I).

Table I. The effects of 1,25 $(OH)_2$ Vit D_3 on monocyte-derived APC

	Classical DC	CD 14^- VC
Yield	↓ ($CD1a^+$, $CD83^+$)	↓
Immune stimulatory molecules (DR, CD80/86)	↓	~
T cell stimulatory capacity	↓	↓
Cytokines IL-12	~	↓
IL-10	↑	~

Conclusion: This combination of a down regulation of particularly CD1a, CD80 and CD40 in DC culture, a high residual CD14 expression for VC and DC, a reduced capability in MLR for both APC populations and a high production of IL-10 for iDCs and a low production of IL-12 for VC clearly illustrate the suppressive influence of this hormone on the stimulation of cell-mediated immunity and a skewed differentiation of monocytes away from antigen presenting cells (APC) and toward phagocytosing macrophages, thus favoring a non-specific innate immune response over an antigen-specific response.

Dexamethasone. The influence of dexamethasone in the DC cultures was clearly seen by a down regulation of CD1a, CD40 and CD80 combined with an increase in CD14 expression in the resulting plastic-adherent macrophage-like cells (Table II). Although the ability of these cells to stimulate T cell proliferation in the MLR was not affected, their production of IL-12 was virtually shut down, while their production of IL-10 increased. Therefore, the presence of dexamethasone in the culture during the generation of monocyte-derived DC directed cell development away from DC toward a more macrophage-like cell, possibly indicating a Th-2 biased response.

Exposure of monocytes to dexamethasone also affected their differentiation potential towards veiled accessory macrophage-like cells. The resulting CD14 dim/negative VC had a low expression of important immune stimulatory molecules such as HLA-DR and CD86, a low capability to produce IL-12 and a poor capability to stimulate T cells.

Conclusion: The effects of dexamethasone on the generation of APC population from monocytes are therefore clearly inhibitory as was the case for VitD3.

Table II. The effects of dexamethasme on monocyte-derived APC

	Classical DC	CD 14$^-$ vC
Yield	More mø-like	~
Immune stimulatory molecules (DR, CD80/86)	~ (to ↓)	↓
T cell stimulatory capacity	~	↓
Cytokines IL-12	↓↓	↓
IL-10	↑	~

DHEA. Adding DHEA to the cultures during the differentiation process from monocytes to DC had a contrasting effect to that of dexamethasone, possibly skewing the immune reaction toward a Th-1 type response. The continuous presence of DHEA resulted in monocyte-derived iDC possessing similar functional characteristics to typical non-exposed iDC. While the presence of DHEA did not significantly alter the ability of the resulting cells to stimulate T cell proliferation or their IL-12/IL-10 production, an increase in CD80 expression and decrease in the expression of the immaturity marker CD43 may indicate the emergence of a more mature DC after exposure to this 'immunostimulating' hormone. The effects of DHEA on VC development were not tested in depth, but in a few experiments we noted that the T cell stimulatory capacity of CD14dim/negative VC generated from DHEA-exposed monocytes was stronger than that of T3-exposed monocytes (see addendum 4A).

Conclusion: Our observation supports the view that DHEA has some immuno-stimulatory capacity.

Regarding VC and DC function in patients with autoimmune thyroiditis

The studies in Chapter 6 on monocytes obtained from recently diagnosed thyroid autoimmune disease (TAID) patients indicate abnormalities in integrin- and chemokine-mediated functions. We found the chemoattractant-induced polarization of monocytes (rearrangement of interior cytoskeleton) to be defective in TAID patients, and this defect was more pronounced after fibronectin (FN) adherence. The monocytes of TAID patients were also less able to up regulate specific (CD49d and e) integrins and to generate VC (after FN adherence) that were functionally active in T cell-stimulation (normally, adherence to FN increases the expression of integrins on monocytes and stimulates the VC development from monocytes).

Interestingly, monocytes of autoimmune thyroiditis patients had a normal capability to generate classical monocyte-derived DC when cultured with GM-CSF and IL-4 for one week.

Conclusion: These data indicate that there are in particular aberrancies in monocytes of TAID patients. These aberrancies are in the signaling pathways downstream of the integrins and chemokine-receptors and linked to the cytoskeletal functions of the cell. These aberrancies did not affect the in vitro development of classical DC, but did affect the in vitro development of VC from TAID monocytes.

Taken together with the characteristics of monocyte-derived VC described in chapter 3 (particularly their significant production of IL-10 and the literal shut-down of IL-12), the defects in VC development from fibronectin-adherent (=integrin-activated) monocytes of TAID patients could represent defects in the generation of a subset of APC with immunosuppressive and tolerating capabilities.

Collectively and answering the questions posed in Chapter II of this thesis we have shown here that

a. A population of APC, characterized by veils, could rapidly be generated from monocytes. Such VC were equally potent in stimulating T cells as classical mo-derived iDC, yet were different in marker pattern, cytokine production and Th1/Th2 skewing capability in that they were less potent to stimulate Th1 cells. The VC were also more macrophage-like cells.
b. Hormones modulate the function of both DC and VC. The effects of dexamethasone and 1,25 (OH)2 Vit D3 were inhibitory on the generation of both APC populations from monocytes (making them actually more macrophage-like), while DHEA had a slight immune stimulating effect.
c. The motility of monocytes and the generation of VC from monocytes was disturbed in patients with autoimmune thyroid disease, yet the generation of mo-derived DC was intact.

How do our data on monocyte-derieved APC populations relate to a putative pathogenesis of thyroid autoimmune disease in the human?

To discuss this question we want to address the following items before trying to construct a state of the art hypothesis on the pathogenesis of autoimmune thyroiditis:

a. The histological evidence for a primary role of APC in the pathogenesis of human autoimmune thyroiditis
b. The T cell defects that exist in addition to defects in APC function in animal models and human autoimmune thyroid disease

The histological evidence for a primary role of APC in the pathogenesis of human autoimmune thyroiditis

For histological studies predominantly thyroids of Graves' patients have been available. These specimens are taken late in disease. Interventions that likely influence the infiltration and accumulation of APC, T cells, B cells and scavenger macrophages (such as an anti-thyroid

drug treatment) have often been given. Despite that, the histological picture of the leukocyte infiltration into a Graves' goiter is remarkably similar to that of the late phase leukocyte infiltration into the thyroid of the BB-DP rat. In both situations the glands show areas of intra-thyroidal lymphoid tissue and areas in which the thyroid follicles are largely intact. In Graves' disease such areas of intact parenchyma are composed of hyperplastic and hypertrophic cells (1,2), in BB-DP rats of slightly hypertrophic cells only (3). A few Hashimoto glands have also been examined. In such glands, areas of intra-thyroidal lymphatic tissue are also present, but located next to areas of heavily damaged thyroid parenchyma (1,2). Damage is caused by inflammatory infiltrates of CD8+ cytotoxic lymphocytes, CD4+ lymphocytes and scavenger macrophages.

In areas of Graves' goiters where there are intact follicles, significantly elevated numbers of peri-follicularly-located DC/VC are present (1,2). Such cells are often adherent to the basal surface of the thyrocytes, sending their long cytoplasmic protrusions deeply into the intercellular space in between the thyrocytes (4). Such peri-follicular DC/VC have an immature phenotype with a low expression of the co-stimulatory molecules CD80, CD86 and CD40 (2).

Mature DC with a high expression of such molecules are predominantly present in the larger areas of intra-thyroidal lymphatic tissue and co-localized with activated CD4+ T cells (2). In such areas, high endothelial venules (HEV) are also present. Such venules are characterized by an up regulated integrin expression of their high columnar endothelial cells (1). HEV are responsible for the increased infiltration of (naïve) T and B cells in such areas of intra-thyroidal lymphatic tissue by virtue of their heightened integrin expression (5).

With regard to the discussion on the role of thyrocytes as APC, Tandon et al (6) and Matsuoka et al (7) showed that normal human thyrocytes are not capable of expressing co-stimulatory molecules to a noteworthy extent. There is however some evidence that they might express such molecules in Graves disease (8). In addition, while the co-culture of thyrocytes and T cells alone resulted in a relatively weak T cell proliferation, addition of low numbers of monocytes or APC to the culture led to a clear enhancement of the T cell proliferative response (9).

Collectively, these observations point in the direction of DC/VC and not the thyrocytes playing the most important role in the induction of thyroid autoimmunity, not only in the animal models (see Chapter 1) but also in the human.

The T cell defects that exist in addition to defects in APC function in animal models and human autoimmune thyroid disease

The defects in T cells in the animal models of spontaneously developing autoimmune thyroid disease and/or in autoimmune thyroid patients can be divided into defects in apoptosis and defects in the generation of suppressor T cells.

Defects in apoptosis. The BB-DP rat has a profound peripheral (=non-thymic) T cell lymphopenia. The peripheral lymphopenia of the BB-DP rat is due to an excessive apoptosis of

recent thymus emigrants and is a recessive trait (10). The animals are lymphopenic from birth onwards due to a mutation in one of the Immune-associated nucleotide (Ian) genes on rat chromosome 4, i.e. the Ian-5 gene (11). Ian-5 codes for a protein, which binds to the mitochondrial membrane. The protein protects the cell against apoptosis. Homozygosity for the mutation in the Ian-5 molecule leads to a strongly reduced expression of the anti-apoptotic Ian-5 molecule in T cells and their consequent vulnerability for apoptosis. Interestingly, this affects in particular activated, i.e. RT6+ T cells in the blood and in the peripheral tissues of the BB-DP rat. The activated RT6+ T cell population mainly harbors the suppressor T cells of the rat. Hence the apoptosis disturbance leads in the BB-DP rat to a massive death of recent thymus emigrating T cells but particularly of suppressor T cells.

The T cells of the NOD mouse are also characterized by defects in apoptosis, but –in contrast to the BB-DP rat- the T cells are resistant to apoptosis (12,13). Decallone et al (12) found that NOD T lymphocytes, when compared to non-obese diabetes-resistant (NOR) and healthy control strains, were resistant to activated immune T cell death (AITCD) induced by strong activation stimuli (IL-2 and anti-CD3). The AITCD defect of NOD T cells was characterized by a low Caspase-8 activity and an aberrant transcriptional expression of several AITCD-related molecules in resting NOD T cells (14). Interestingly, this immunological dysfunction of the T cells (which is directly related to peripheral tolerance induction) could be counteracted by treating the mice with 1,25 (OH)2 Vitamin D3 (14).

With regard to the T cells of autoimmune thyroid patients, there is a recent report that shows that the T cells of autoimmune thyroid patients also have a defective apoptosis, similar to the defective apoptosis of the T cells in the NOD mouse model (15).

Defects in T suppressor cells in animal models of thyroid autoimmune disease. The mouse thymectomy models are clear models of the lack of the current most popular regulator T cell population, the CD4+CD25+ T reg cells (16,17). Regulatory CD4+CD25+ T cells comprise 5-10% of all CD4+ T cells, and are further characterized by the constitutive expression of CTLA-4, Fox-P3 and GITR. CD4+CD25+ T cells develop in the thymus via a distinct pathway of thymic selection requiring the expression of endogenous TCR α chains on the cells for selection since CD4+CD25+ T cells are absent in TCR transgenic mice on a RAG-deficient background. In this selection process, the cells are selected that are able to recognize self-antigens expressed in the thymus. A feature of CD4+CD25+ T cells is that in the periphery the cells are "anergic" to TCR ligation, yet capable of suppressing the activation of (bystander) CD4+ and CD8+ cells when these cells are approached by their cognate antigens. Ligation of surface molecules such as CD152 (CLTA-4) and GITR, as well as secretion of anti-inflammatory cytokines, such as TGF-β, IL-4 and IL-10, have all been implicated in the regulation or mediation of the suppressive activity of CD4+ CD25+ T cells (18).

Although relatively little is known about the mechanisms that control the development and/or function of CD4+CD25+ T cells, studies in CD7, CD28 and CD40 deficient (knock-out) mice have clearly shown that co-stimulation, most likely given by professional APC, is essen-

tial for their generation in the thymus and expansion in the periphery. In particular, signaling via the CD80-CD28 route seems to be essential. Studies in C57BL/6 mice have shown that when such co-stimulation via the CD28 and CD7 pathways are not given, that there are decreased numbers of CD4+CD25+ T cells in the periphery and that autoimmune thyroiditis will spontaneously develop. Interestingly, the NOD mouse shows a progressive loss of CD4+ CD25+ T regulator cells during the development of diabetes, while the pathogenicity of CD4+ CD25- effector T cells increases (19). Also, treatment of the NOD mice with Vitamin D receptor (VDR) ligands, mimicking the effects of 1,25(OH)2 D3, increases the number of CD4+CD25+ T cells in the spleen of NOD mice and prevents the development of autoimmune diabetes. There are indications that at least part of the effects are mediated by the effects of the VDR on DC (see also this thesis) and that such modified DC are more able to expand the CD4+CD25+ T cell population. There is also a report that shows that thyroglobulin-specific CD4+CD25+ T cells are activated in mice and experimental autoimmune thyroiditis suppressed, when DC generation is stimulated using a treatment with GM-CSF/IL-4, but not with Flt-3 ligand (20). Taken together, there is ample evidence that there are defects in the recently discovered new sub-set of CD4+CD25+ T regulator cells in the animal models of spontaneously developing autoimmune thyroiditis/diabetes and that a faulty function of APC might at least in part be instrumental to these defects.

Defects in T suppressor cells in patients with thyroid autoimmune disease. What about CD4+CD25+ T cells in patients with autoimmune thyroiditis? There is one recent Japanese report (21) that shows that CD4+ CD25+ T cells are not related to the progression of Hashimoto's disease. Some Japanese patients with Hashimoto's disease develop hypothyroidism and are treated with thyroxine (severe Hashimoto's disease), but most do not throughout their lives (mild Hashimoto's disease). There was no significant difference in CD4+ CD25+ cells between these Hashimoto's disease groups, but the serum titers of the thyroid autoantibodies and the proportion of CD25+ cells within CD8+ cells (activated T cytotoxic cells) were higher in patients with severe Hashimoto's disease than in those with mild Hashimoto's disease. However proper control groups of healthy individuals were not included in this study.

Recently collected data by Strieder et al support a role for CD4+CD25+ T cells in the development of human autoimmune thyroiditis (to be published). We showed that s-IL2R levels and the percentages of CD4+CD25+ T cells/CD4+CD152+ (CTLA-4) T cells are lower in patients with autoimmune thyroiditis and in first degree relatives (FDR) of these autoimmune thyroiditis patients as compared to healthy controls. FDR who smoked and FDR who used oral contraceptives surprisingly had the lowest risk of developing autoimmune thyroiditis, and interestingly these FDR had the highest levels of CD4+CD25+ T cells.

Taken together evidence is emerging that also in the human CD4+CD25+ T cells might play a prominent role in the protection against autoimmune thyroiditis. It is tempting to speculate that the APC defects, as found in this thesis, play a role in the defective generation and sustenance of the CD4+ CD25+ T cells, as they do in the animal models. It must also be

noted that apart from these CD4+CD25+ T regulator cells, there are other T cell populations with regulator function, such as the Tr1 cells (producing IL-10) and the TH3 cells of the gut (producing TGF-β), and little is known about these populations in patients with endocrine autoimmune disease and their regulation by the defective APC.

A state of the art hypothesis on the etio-pathogenic mechanisms of human autoimmune thyroiditis

Taking the data from the literature and the here-presented data together, it can be concluded that there exists in animals and patients with autoimmune thyroiditis a plethora of (sometimes subtle) aberrancies in the function of thyrocytes, monocytes, macrophages, DC/VC and T cells. From these aberrancies a general blueprint for the etio-pathogenesis of destructive autoimmune thyroiditis can be constructed (see Figure 1). In this blueprint the first step is that APC are attracted to the thyroid in larger numbers. Instead of staying in a "steady state tolerogenic" mode the APC become "activated" as in danger situations and start to trigger T- and B-cells to mount an immune response towards auto-antigens after arrival in the draining lymph node. The basis for this aberrant switch from "steady state tolerogenic mode" towards "danger-associated immune response eliciting mode" lies in the various aberrancies of the thyrocytes, monocytes, macrophages, DC/VC and T cells. However it is important to note that the various specific thyrocyte, APC and T cell aberrancies clearly differ between the three animal models for destructive autoimmune thyroiditis (e.g. T suppressor cell defects in the BB-DP rat due to an enhanced T cell apoptosis, AITCD defects in the NOD mouse due to a reduced T cell apoptosis). This tells us that there clearly exist different etio-pathogenic routes for the development of destructive autoimmune thyroiditis. It is likely that - as in the animal models – also in patients different combinations of various and different (and perhaps even human-specific) inborn polymorphisms for thyrocyte, APC and T cell function in combination with environmental factors contribute to the loss of tolerance to thyroid autoantigens and to a susceptibility for thyroid autoimmunity. Interesting in this respect is that we were unable to find in patients the defects in DC development so characteristic of the BB-DP rat and the NOD mouse. In humans, however, we detected a similar defect in the generation of veiled macrophages and perhaps this lead to a similar inability to stimulate T suppressor cells sufficiently.

Whether a heterogeneity in etio-pathogenesis is also the case for Graves' disease remains unknown. Spontaneously developing animal models of the disease would be helpful in unraveling this question.

If there are indeed various and different routes that lead to destructive autoimmune thyroiditis, and if one wants to attempt to correct the underlying immune abnormalities to prevent the development or progression of destructive autoimmune thyroiditis, then different therapeutic approaches need to be carried out in patients who differ in such routes. Obviously a more precise definition of cellular immune aberrancies is required in individual cases of destructive autoimmune thyroiditis, before therapies that aim at correcting the immune disturbances could even be tested successfully. With regard to preventive therapies, several

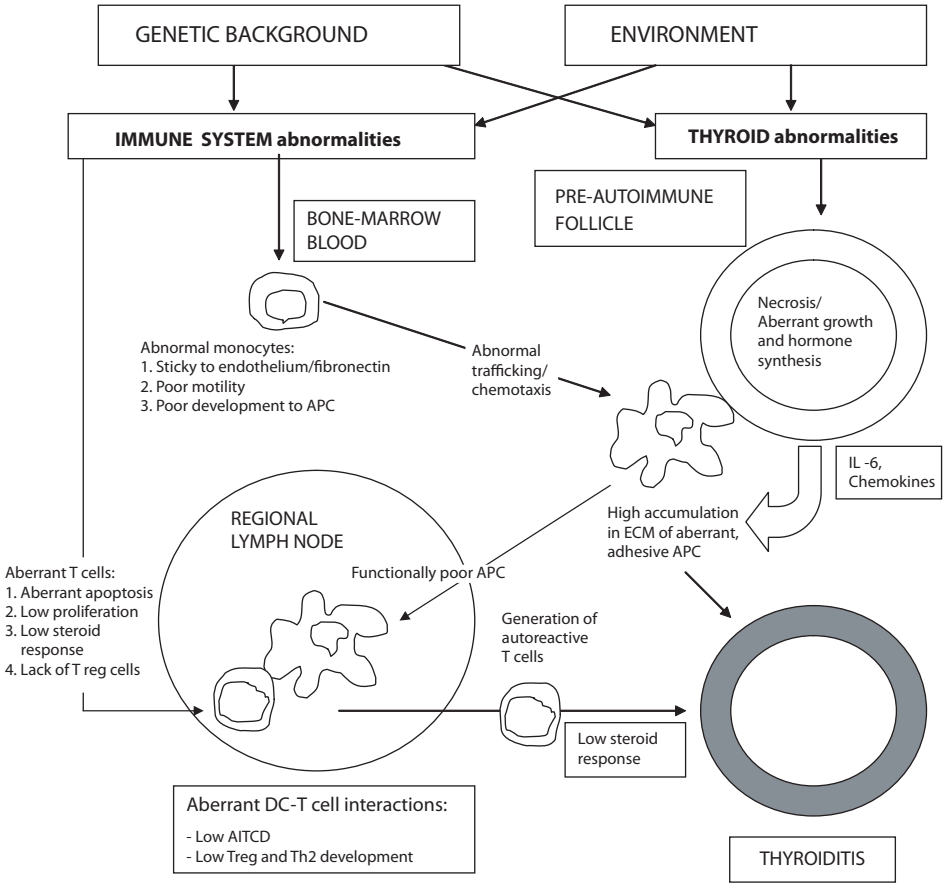

Figure 1. The general blueprint for the pathogenesis of the various phases of autoimmune thyroiditis

are emerging. This thesis shows that interventions with hormones, such as with DHEA and its metabolites, with glucocorticoids and with Vitamin D3 analogs (lacking effects on the calcium metabolism, but retaining their tolerance-inducing effects) hold promise for the future, as do vaccination protocols with "tolerogenic" DC or approaches to stimulate regulator T cells.

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Summary

Two polar forms of autoimmune reactivity to the thyroid gland are known, a catabolic and an anabolic form. The catabolic form is best known as destructive autoimmune thyroiditis subdivided in Hashimoto goiter and atrophic thyroiditis, while the anabolic form is generally referred to as Graves' disease. Immune responses to thyroid specific autoantigens form the basis of these autoimmune thyroid diseases. The most important antigens are thyroperoxidase (TPO), thyroglobulin (Tg) and the TSH receptor. Antibodies to TPO and Tg are found in high titres in the serum of the majority of patients with the catabolic forms of autoimmune thyroid disease, i.e. Hashimoto goiter and atrophic thyroiditis. Although such antibodies to TPO and Tg are good markers of Hashimoto goiter and atrophic thyroiditis, macrophages activated by TPO- and Tg-specific T helper-1 (Th 1) cells, cytotoxic CD8+ T cells and apoptotic interactions between thyrocytes and these inflammatory cells are believed to play a more prominent role in the actual destruction of the thyroid parenchyma. Antibodies to the TSH receptor are a hallmark of the anabolic form of autoimmune thyroid disease, i.e. Graves´disease, and cause the stimulation of thyrocytes resulting in the hyperfunction and growth of thyrocytes characteristic of this autoimmune disorder.

Prior to the disease development and autosensitization an accumulation of antigen presenting cells (APC), i.e. of dendritic cells (DC) and macrophages (mφ) is observed in the thyroid gland around the follicles (as observed in the animal models of the disease). It is generally thought that these APC originate from blood monocytes that have entered the thyroid gland and have differentiated into DC or mφ. On the basis of their normal physiological role, these cells presumably take up self-antigens (TPO, Tg and the TSH receptor) and process these into peptides, which they present, after a so-called "steady-state" or "homeostatic" trafficking to the draining lymph nodes, to T lymphocytes in the para-cortical area of these nodes. Normally this leads to tolerance induction (an immunological non-reactivity) to these self antigens via the induction of T regulatory cells. However in the case of thyroid autosensitization and the development of thyroid autoimmune disease, not T regulatory cells, but erroneously T effector lymphocytes and B cells become activated to these self antigens and thyroid autoimmunity is induced.

The focus of this thesis is on the role of APC in autoimmune thyroid disease. Although almost all cells can act as antigen presenting cells to some degree, there are cells in the immune system that excel in this function, specifically B cells, accessory macrophages and above all, the dendritic cell. Dendritic cells are critically involved in the initiation of primary T cell responses and the generation of T cell dependent autoantibody formation.

Langerhans cells in the skin are prototypic DC and it is now well accepted that the Langerhans cells of the skin pick up foreign antigens, process these antigens and travel with these – as so-called veiled cells - via the lymph to draining lymph nodes to populate the T cell areas of these nodes as interdigitating cells. The interdigitating cells act in presenting the transported and processed antigens to the surrounding T cells, and stimulate these lymphocytes to proliferate and to differentiate. In the case of a foreign ("danger") signal the T cells are

programmed for an effector response, in the case of self-antigens ("non-danger") the T cells are programmed for a tolerogenic response.

Langerhans cells, veiled cells, interdigitating cells and similar antigen transporting and presenting cells from other sites are generally referred to as dendritic cells (DC) since these cells share many morphological and functional characteristics with the so-called dendritic cells initially described by Steinman in the spleen. Cells belonging to the dendritic cell group are the most potent antigen transporting and presenting cells, and have considerable potential for use in immunotherapy and tolerance induction. The population of dendritic cells is heterogeneous. Lymphoid dendritic cells orginate from the thymus whereas plasmacytoid dendritic cells have a plasmacytoid precursor in the peripheral blood while myeloid dendritic cells originate form CD34+ precursors and from CD14+ circulating monocytes.

In vitro generation of antigen presenting cells from monocytes with a morphology and function reminiscent of lymph-borne veiled cells was already described by Drexhage et al. in 1989. In chapter 3 this method has been further explored and the impact of diphenylene iodonium (DPI) and ascorbate, both nicotinamide adeninedinucleotide phosphate (NADPH) inhibitors, to enhance transition of monocytes into veiled cells has been studied. Monocytes were cultured for 24 hours while avoiding adherence to polypropylene tubes and avoiding activation of NADPH oxidase. The generated veiled cells were actively moving cells like lymph-borne veiled cells in vivo. The monocyte-derived veiled cell population consisted of $CD14^{dim/-}$ and $CD14^{bright}$ cells. Of these the $CD14^{dim/-}$ veiled cells were as good in stimulating allogeneic T cell proliferation as prototypic immature dendritic cells obtained after one week of adherent culture of monocytes in granulocyte-macrophage-colony stimulating factor (GM-CSF) and interleukin (IL)-4. This underscores the accessory cell function of the monocyte derived $CD14^{dim/-}$ veiled cells. Although the $CD14^{dim/-}$ veiled cells had a modest expression of the dendritic cell specific marker CD83 and were positive for the Langerhans cell marker S-100, expression of the dendritic cell specific markers CD1a, Langerin, DC-SIGN, and DC-LAMP were absent. This indicates that the here generated $CD14^{dim/-}$ veiled cells can not be considered as classical dendritic cells. It was not possible to transform the $CD14^{dim/-}$ monocyte-derived veiled cells population into typical dendritic cells by culturing for one week in GM-CSF/IL-4 or LPS. Under these circumstances, all cells died while presenting some characteristics of macrophages.

The IL-12 production from monocyte-derived $CD14^{dim/-}$ veiled cells was lower, whereas the production of IL-10 was higher as compared to immature dendritic cells. Consequently the T cells which were stimulated by these monocyte-derived veiled cells produced less interferon gamma in comparison to T cells stimulated by immature dendritic cells (hence there was a skewing of the T cell response away from Th1 cells).

In conclusion the method described in chapter 3 generates $CD14^{dim/-}$ veiled accessory cells which are not classical dendritic cells but appear to be more related to the macrophage series. They must thus be considered as a potent subpopulation of the accessory macrophages.

Chapters 4 and 5 are dealing with the effect of hormones on the generation of classical dendritic cells and veiled accessory macrophages. Hormones with a known or suspected effect on autoimmune reactivity were selected.

Dehydroepiandrosterone (DHEA) is one of the adrenal steroid hormones. Although no major endocrine function has been ascribed to DHEA, it has been suggested that DHEA plays a role in age-related processes such as fat depot distribution and neurodegeneration. DHEA is considered as an immunostimulating steroid hormone of which the effects on the development of dendritic cells are unknown. The effects of DHEA tend to oppose those of the other adrenal glucocorticoid hormone cortisol. Glucocorticoids are known to suppress the immune response at different levels and have recently been shown to modulate the development of dendritic cells, thereby influencing the initiation of the immune response. Variations in the duration of exposure to and doses of glucocorticoids however, have resulted in conflicting effects on the development of dendritic cells.

In chapter 4, the effects of a continuous high level of exposure to the adrenal steroid DHEA on the generation of immature dendritic cells from monocytes, as well as the effects of the opposing steroid dexamethasone (DEX) on this development were studied.

The continuous presence of DHEA (10^{-6} M) in GM-CSF/IL-4-induced monocyte-derived dendritic cells cultures resulted in immature dendritic cells with a morphology and functional capabilities similar to those of typical immature dendritic cells, such as T cell stimulation and IL-12/IL-10 production, but with a slightly altered phenotype of increased CD80 and decreased CD43 expression which are markers of maturity.

The coninuous presence of DEX at a concentration of 10^{-6} M in the monocyte/DC cultures resulted in the generation of plastic-adherent macrophage-like cells in place of typical immature dendritic cells, with increased CD14 expression, but decreased expression of the typical dendritic cell markers CD1a, CD40 and CD80. These cells were strongly reactive for acid phosphatase, but equally capable of stimulating T cell proliferation as immature dendritic cells. The production of IL-12 by these macrophage-like cells was virtually shut down, whereas the production of IL-10 was significantly higher than that of control immature dendritic cells.

The continuous presence of a high level of glucocorticoids during the generation of immature dendritic cells from monocytes thus modulates this development away from dendritic cells towards a macrophage-like cell with equally strong accessory capabilities, yet the combination of a low CD80 expression and a shutdown of IL-12 production suggests the possibility of DEX-generated cells preferably initiating a Th2-biased response. These effects by DEX on dendritic cells development contrast with those by DHEA, which resulted in a more typical dendritic cell although possessing a phenotype possibly indicating a more mature state of the cell.

In chapter 4a the effects of DEX on the generation of veiled accessory macrophages from monocytes are described. The findings were compatible with the view expressed above that this hormone hampers the generation of Th1 skewing immunostimulatory APC from

monocytes; the generated VC were less capable to stimulate T cells and produced less of the cytokine IL-12.

1α, 25-Dihydroxyvitamin D_3 ($1,25(OH)_2D_3$) is a steroid hormone known for its ability to regulate calcium metabolism. The presence of the vitamin D_3 receptor in almost all types of immune cells and the ability of $1,25(OH)_2D_3$ to affect immune cell function in vitro is indicative of other actions of this hormone. The ability of $1,25(OH)_2D_3$ to stimulate cell differentiation has been well characterized. For instance, this hormone can inhibit proliferation and induce differentiation of benign cells such as keratinocytes, malignant cells such as prostate, breast and colon adenocarcinoma cells and various leukemic cells.

In chapter 5, the impact of $1,25(OH)_2D_3$ on the generation of immature dendritic cells from monocytes has been studied. Human peripheral blood monocytes were cultured to immature dendritic cells in the presence of GM-CSF and IL-4 for one week, with or without the extra addition of 10^{-8} M $1,25(OH)_2D_3$ to the culture. Their phenotype (CD14, CD1a, CD83, HLA-DR, CD80, CD86 and CD40 expression) was studied by FACS analysis and their T cell stimulatory potential in allogeneic mixed lymphocyte reaction (allo-MLR) was assessed. The in vitro production of IL-10, IL-12 and TGF-ß by ELISA was investigated.

$1,25(OH)_2D_3$, when added to monocytes in culture with GM-CSF and IL-4, hampered the maturation of monocytes into immature dendritic cells. Firstly the phenotype of the $1,25(OH)_2D_3$-differentiated dendritic cells was affected with a impaired down regulation of the monocytic marker CD14 and a impaired up regulation of CD1a, CD83, HLA-DR, CD80 and CD40. CD86 was expressed on more $1,25(OH)_2D_3$-differentiated dendritic cells. Secondly, the T cell stimulatory capability of $1,25(OH)_2D_3$-differentiated dendritic cell was up regulated from that of the original monocytes to a lesser degree than dendritic cells differentiated without $1,25(OH)_2D_3$ when tested in allogenic mixed lymphocyte reaction (MLR). With regard to the production of cytokines, SAC-induced IL-10 production, although not enhanced, remained considerably high in $1,25(OH)_2D_3$-differentiated dendritic cells, whereas it was strongly down regulated in dendritic cells generated in the absence of $1,25(OH)_2D_3$. SAC/IFNγ-induced IL-12 production was clearly up regulated in both types of dendritic cells from that of the original monocytes and TGF-ß production down regulated.

These data confirm earlier reports that $1,25(OH)_2D_3$ hampers the maturation of fully active immunostimulatory MHC-classII+,CD1a+,CD80+ dendritic cells from monocytes. Our data supplement the other reports in showing that the expression of CD86 was up regulated in $1,25(OH)_2D_3$-differentiated dendritic cells, while the capability for IL-10 production remained high. Collectively, this is in line with earlier descriptions of suppressive activities of this steroid-like hormone regarding the stimulation of cell-mediated immunity.

In Chapter 5 a the effect of $1,25(OH)_2D_3$ on the generation of veiled accessory macrophages from monocytes is described. Exposure of monocytes to $1,25(OH)_2D_3$ prior to an overnight non-adherent cell culture resulted in the generation of veiled cells that had a very low IL-12 production capability and a poor T cell stimulatory capacity as compared to veiled cells

generated in the presence of iodinated compounds. These data underscore that $1,25(OH)_2D_3$ is a hormone hampering the maturation of fully active Th1 skewing APC from monocytes.

Blood monocytes of patients with thyroid autoimmune disease show a defective capability to rearrange their cortical actomyosin cytoskeleton ('polarize') in response to chemoattractants. Since such cytoskeletal rearrangements also take place after adherence of monocytes to fibers of the extracellular matrix, it is not surprising that monocytes are primed for chemoattractant stimulation after fibronectin adherence, showing an enhanced polarization toward chemoattractants.

In chapter 6, the adherence capability of blood monocytes from patients with thyroid autoimmune disease to a fibronectin matrix, and their integrin expression and chemoattractant-induced polarization both before and after fibronectin adherence were studied. Since cytoskeletal rearrangements are also needed for the development of veils during the transition of monocytes into veiled accessory macrophages, the transition of fibronectin-adhered monocytes of patients with autoimmune disease of the thyroid was investigated as well.

Adherent and non-adherent monocyte populations from patients with autoimmune disease of the thyroid and healthy controls were subjected to a polarization test with the chemoattractant fMLP (or MCP-1), FACS analyses (FITC-labelled fibronectin, CD29, CD49e, d, b, and a) and tested for their capability to develop into veiled antigen presenting cells.

Monocytes of healthy individuals showed an improved chemoattractant-induced cell polarization after fibronectin adherence, not reflected by monocytes from patients with autoimmune disease of the thyroid, in which chemoattractant-induced polarization worsened. Monocytes of healthy individuals upregulated CD49e and d integrins and their capability to bind FITC-labelled fibronectin after adherence to a fibronectin-coated plate, as well as enhancing their capability to generate T cell-stimulatory veiled cells. Monocytes of patients with autoimmune disease of the thyroid did not.

These data indicate that integrin- (and chemokine)-mediated functions are hampered in monocytes from patients with autoimmune disease of the thyroid. Because, integrin action is pivotal to processes such as monocyte adherence to endothelial cells, uropod formation, migration into tissues and differentiation into antigen presenting cells and macrophages, these defects might underly immune dysbalances important in thyroid autoimmune development.

In chapter 6a it is described that the generation of prototypic DC from monocytes (via culture in GM-CSF and IL-4) was not altered in patients with autoimmune thyroiditis.

In conclusion, a population of antigen presenting cells, characterized by veils, could be generated in 24 hours from monocytes. Such veiled cells were equally potent in stimulating T cells as classical monocyte-derived immature dendritic cells, yet were different in marker pattern, cytokine production and Th1/Th2 skewing capability in that they were less potent to stimulate Th1 cells. The veiled cells were also more macrophage-like cells.

Hormones modulate the function of both dendritic cells and veiled cells. The effects of dexamethasone and $1,25(OH)_2D_3$ were inhibitory on the generation of both antigen presenting cells populations from monocytes (rendering them actually more macrophage-like), while DHEA had a slight immune stimulating effect.

The motility of monocytes and the generation of veiled accessory macrophages from monocytes was disturbed in patients with autoimmune thyroid disease, particularly after adherence to fibronectin. The generation of monocyte-derived dendritic cells was intact.

This thesis thus shows aberrancies in the APC function of monocyte-derived veiled macrophages in patients with autoimmune thyroiditis (particularly after fibronectin adherence) that may play a role in the erroneous tuning of the immune system towards self reactivity and that interventions with hormones, such as with Vitamin D_3 analogues, which lack effects on the calcium metabolism but retain effects on APC development, holds promise for the future of patients with autoimmune thyroid disease.

Samenvatting

Auto-immuunziektes van de schildklier kunnen zowel een katabole als anabole vorm aannemen. De katabole vorm staat bekend als destructieve auto-immuun thyreoïditis onder te verdelen in Hashimoto struma en atrofische thyreoïditis. De anabole vorm van auto-immuun thyreoïditis refereert in het algemeen aan de ziekte van Graves. Immuunreacties op schildklierspecifieke auto-antigenen vormen de basis van de genoemde schildklierziektes. De belangrijkste antigenen zijn thyreoperoxidase (TPO), thyreoglobuline (Tg) en de TSH receptor. Antilichamen tegen TPO en Tg worden in hoge concentraties gevonden in het serum van patiënten met katabole vormen van auto-immuunziektes van de schildklier, te weten Hashimoto struma en atrofische thyreoïditis. Alhoewel deze antilichamen tegen TPO en Tg goede merkstoffen zijn voor Hashimoto struma en atrofische thyreoïditis, spelen macrofagen geactiveerd door TPO- en Tg-specifieke T helper 1 (Th1) cellen, cytotoxische CD8+ T cellen en apoptotische interacties tussen schildkliercellen en deze inflammatoire cellen een belangrijkere rol in de destructie van schildklierparenchym. Antilichamen tegen de TSH-receptor zijn de hoeksteen van de anabole vorm van auto-imuunziekten van de schildklier, te weten de ziekte van Graves, en stimuleren schildkliercellen leidend tot hyperfunctie en groei van schildkliercellen die karakteristiek zijn voor deze auto-immuunaandoening.

Voorafgaand aan het ontstaan van de ziekte en de autosensitisatie vindt een ophoping van antigeen presenterende cellen (APC), oftewel dendritische cellen (DC) en macrofagen in de schildklier plaats rondom de follikels (althans in diermodellen). In het algemeen wordt aangenomen dat deze APC ontstaan uit bloedmonocyten die de schildklier zijn binnengedrongen en zijn gedifferentieerd tot DC of macrofagen. Conform de normale fysiologie nemen deze cellen eigen antigenen (TPO, Tg en de TSH receptor) op en verwerken deze tot peptiden die worden gepresenteerd, na een zogenaamde “steady state” of “homeostatisch” transport naar de drainerende lymfklieren, aan T lymfocyten in de paracorticale velden van deze lymfklieren. Normaliter leidt dit tot tolerantie-inductie (immunologische non-reactiviteit) voor deze eigen antigenen door middel van inductie van regulerende T-cellen. Echter, in het geval van autosensitisatie van de schildklier en het ontstaan van auto-immuunziekten van de schildklier, worden in plaats van de regulerende T-cellen, effector T-cellen en B-cellen geactiveerd tegen deze eigen antigenen en wordt aldus auto-immuniteit van de schildklier geïnduceerd.

Dit proefschrift richt zich op de rol van antigeen presenterende cellen bij auto-immuunziektes van de schildklier. Alhoewel vrijwel alle cellen in zekere mate kunnen functioneren als antigeen presenterende cellen, excelleren sommige cellen van het immuunsysteem hierin, dit zijn met name B-cellen, ‘accessory’ macrofagen en bovenal, de dendritische cel. Dendritische cellen spelen een sleutelrol bij de initiatie van de primaire T-cel reactie en de vorming van T-cel afhankelijke auto-antilichamen.

Langerhans cellen in de huid zijn protoype DC en inmiddels is het algemeen aanvaard dat Langerhans cellen in de huid vreemde antigenen oppakken, vervolgens verwerken en zich met deze antigenen verplaatsen – als zogenaamde sluiercellen - via de lymfe naar de

drainerende lymfklieren om zich te nestelen als interdigiterende cellen in T-cel velden van deze lymfklieren. De interdigiterende cellen presenteren de getransporteerde en verwerkte antigenen aan de omringende T-cellen aan en stimuleren deze lymfocyten om te prolifereren en te differentieren. In het geval van een vreemd ('danger') signaal worden de T-cellen geprogrammeerd voor een effector respons terwijl in het geval van eigen antigenen ('non-danger') de T-cellen worden geprogrammeerd voor een tolerantierespons.

Langerhans cellen, sluiercellen, interdigiterende cellen en vergelijkbare antigeen transporterende en presenterende cellen worden in het algemeen dendritische cellen genoemd (DC) omdat deze cellen vele morfologische en functionele eigenschappen delen met de zogenaamde dendritische cellen die in eerst instantie werden beschreven door Steinman in de milt. Cellen die behoren tot de groep van dendritische cellen zijn de meest krachtige antigeen transporterende en presenterende cellen en kunnen potentieel worden toegepast in immunotherapie en tolerantie-inductie. De familie van dendritische cellen is heterogeen. Lymfoïde dendritische cellen worden gevormd in de thymus in tegenstelling tot plasmacytoide dendritische cellen die een plasmacytoïde voorloper hebben in het perifere bloed terwijl myeloïde dendritische cellen afstammen van CD34+ voorlopers en van CD14+ circulerende monocyten.

In vitro differentiatie van monocyten tot antigen presenterende cellen met een morfologie en functie die zich verhouden tot die van sluiercellen in lymfe werd door Drexhage cum suis beschreven in 1989. In hoofdstuk 3 wordt deze methode verder onderzocht en wordt de invloed van diphenyleen iodonium (DPI) en ascorbaat, beide nicotinamide adeninedinucleotide fosfaat (NADPH) remmers, op de vorming van sluiercellen uit monocyten bestudeerd. Monocyten werden gedurende 24 uur gekweekt in polypropyleen reageerbuizen en activatie van NADPH oxidase werd voorkomen. De gevormde sluiercellen bewogen actief zoals 'sluiercellen in lymfe in vivo. De monocyt afgeleide sluiercel populatie bestond uit $CD14^{dim/-}$ en $CD14^{bright}$ cellen. De $CD14^{dim/-}$ sluiercellen stimuleerden allogene T-cel proliferatie even krachtig als prototype immature dendritische cellen die waren verkregen na een één week durende adherente kweek van monocyten in aanwezigheid van granulocyte-macrophage-colony stimulating factor (GM-CSF) en interleukine 4 (IL-4). Dit benadrukt de 'accessory' functie van de van monocyten afgeleide $CD14^{dim/-}$ sluiercellen. Alhoewel de $CD14^{dim/-}$ sluiercellen een redelijke expressie vertoonden van de voor dendritische cellen specifieke merkstof CD83 en een positieve expressie vertoonden van de Langerhans cel specifieke merkstof S-100, was expressie van voor dendritische cellen specifieke merkstoffen CD1a, Langerin, DC-SIGN en DC-LAMP afwezig. Dit impliceert dat de gevormde $CD14^{dim/-}$ monocyt afgeleide sluiercellen niet kunnen worden beschouwd als klassieke dendritische cellen. Het was niet mogelijk om de $CD14^{dim/-}$ sluiercellen om te vormen tot typische denritische cellen door middel van een één week durende kweek in GM-CSF/IL-4 of LPS. Onder deze omstandigheden gingen alle cellen dood nadat ze enkele karakteristieke aspecten van macrofagen hadden vertoond.

De IL-12 productie van monocyt afgeleide $CD14^{dim/-}$ sluiercellen was lager terwijl de productie van IL-10 hoger was dan die van immature dendritische cellen. Als gevolg hiervan produceerden de T-cellen die waren gestimuleerd door deze monocytafgeleide sluiercellen minder interferon gamma in vergelijking met T-cellen die gestimuleerd waren door immature dendritische cellen (oftewel de T-cel respons ging voornamelijk in de richting van Th1 cellen)

Concluderend kan worden gesteld dat de methode die wordt beschreven in hoofdstuk 3 $CD14^{dim/-}$ gesluierde 'accessory' cellen oplevert die geen klassieke dendritische cellen zijn maar meer lijken op macrofagen. Deze cellen moeten dus worden beschouwd als een krachtige subpopulatie van 'accessory' macrofagen.

De hoofdstukken 4 en 5 richten zich op de effecten van hormonen op de vorming van klassieke dendritische cellen en gesluierde 'accessory' macrofagen. De onderzochte hormonen hebben een bekend of vermoed effect op auto-immuniteit. Dehydroepiandrosteron (DHEA) is één van de steroïde hormonen van de bijnier. Alhoewel er geen duidelijke endocriene functie wordt toegeschreven aan DHEA, is gesuggereerd dat DHEA een rol speelt in verouderingsprocessen zoals vetverdeling en neurodegeneratie. DHEA wordt beschouwd als een immuunstimulerend steroïd hormoon waarvan de effecten op de ontwikkeling van dendritische cellen onbekend zijn. DHEA zou de werking van het andere glucocorticoïde hormoon van de bijnier, cortisol, tegen gaan. Glucocorticoïden onderdrukken de immuunrespons op verschillende niveau's. Onlangs is aangetoond dat deze hormonen de ontwikkeling van dendritische cellen moduleren waardoor de initiatie van de immuunrespons wordt beinvloed. Variatie van de blootstelling aan en variatie van de doses glucocorticoïden heeft echter geleid tot tegenstrijdige effecten op de ontwikkeling van dendritische cellen.

In hoofdstuk 4 worden de effecten van een continue hoge spiegel van DHEA en dexamethason (DEX) op de ontwikkeling van monocyten tot immature dendritische cellen gerapporteerd.

De continue aanwezigheid van DHEA (10^{-6} M) in GM-CSF/IL-4 geïnduceerde monocyt afgeleide dendritische celculturen leidde tot immature dendritische cellen met morfologische en functionele aspecten die vergelijkbaar waren met die van typische immature dendritische cellen. Hierbij moet worden gedacht aan T-cel stimulatie en IL-12/IL-10 productie. Het fenotype was enigszins afwijkend zich uitend in verhoogde CD80 expressie en verlaagde CD43 expressie, beide merkstoffen voor maturiteit.

De continue aanwezigheid van DEX met een concentratie van 10^{-6} M in de monocyt/DC kweken resulteerde in de vorming van aan plastic adherente macrofaagachtige cellen in plaats van typische immature dendritische cellen. Deze macrofaagachtige cellen vertoonden een verhoogde CD14 expressie maar een verlaagde expressie van typische dendritische cel merkstoffen zoals CD1a, CD40 en CD80. Deze cellen vertoonden verder een sterke reactie met zure fosfatase maar een capaciteit om T-cellen te stimuleren die vergelijkbaar was met die van immature dendritische cellen. De productie van IL-12 door deze macrofaagachtige

cellen was vrijwel nihil terwijl de productie van IL-10 significant hoger was dan die van immature dendritische cellen.

De continue aanwezigheid van een hoge concentratie glucocorticoïden gedurende de vorming van immature dendritische cellen uit monocyten verschuift derhalve de ontwikkeling tot dendritische cellen naar een ontwikkeling tot macrofaagachtige cellen met vergelijkbare sterke accessoire capaciteiten. Echter, de combinatie van een lage CD80 expressie en een afwezige IL-12 productie suggereert de mogelijkheid van DEX-gevormde cellen die bij voorkeur een Th2 respons initieren. Deze effecten van DEX op de ontwikkeling van dendritische cellen contrasteren met die van DHEA die resulteerden in meer typische dendritische cellen alhoewel het fenotype mogelijk wees op een meer mature status van de cel.

In hoofdstuk 4a worden de effecten van DEX op de vorming van gesluierde 'accessory' macrofagen vanuit moncyten bescheven. De bevindingen waren compatibel met het eerder beschreven fenomeen dat DEX de vorming belemmert van immuunstimulerende antigen presenterende cellen (die de immunrespons in de richting van Th1 cellen sturen) uit monocyten. De gevormde sluiercellen waren minder goed in staat om T-cellen te stimuleren en produceerden minder IL-12.

1α, 25-Dihydroxyvitamin D_3 ($1,25(OH)_2D_3$) is een steroid hormoon dat het calcium metabolisme reguleert. De aanwezigheid van de vitamine D_3 receptor in vrijwel alle immuuncellen en het vermogen van $1,25(OH)_2D_3$ om de functie van immuuncellen in vitro te beinvloeden zijn duidelijke aanwijzingen dat dit hormoon ook andere functies heeft. Stimulatie van celdifferentiatie door $1,25(OH)_2D_3$ is duidelijk vastgelegd. Bijvoorbeeld, dit hormoon kan de proliferatie remmen en de differentiatie tot goedaardige cellen induceren van cellen zoals keratinocyten, kwaadaardige cellen zoals prostaat-, borst- en dikke darm kanker cellen en verscheidene leukemie cellen.

In hoofdstuk 5 wordt de invloed van $1,25(OH)_2D_3$ op de vorming van immature denritische cellen uit monocyten bestudeerd. Humane perifere monocyten uit bloed werden gekweekt tot immature dendritische cellen in de aanwezigheid van GM-CSF en IL-4 gedurende één week met of zonder toevoeging van 10^{-8} M $1,25(OH)_2D_3$. Het fenotype van deze cellen, te weten CD14, CD1a, CD83, HLA-DR, CD80, CD86 en CD40 expressie, werd bestudeerd middels FACS analyse en de T-cel stimulerende potentie werd in een 'allogenic mixed lymfocytes reaction' (allo-MLR) bepaald. Verder werd de in vitro productie van IL-10, IL-12 en TGF-β gemeten door middel van een ELISA.

Wanneer $1,25(OH)_2D_3$ werd toegevoegd aan monocyten in een kweek met GM-CSF en IL-4, dan werd de rijping van monocyten tot immature dendritische cellen belemmerd. Ten eerste was het fenotype van $1,25(OH)_2D_3$ gedifferentieerde dendritische cellen aangedaan met een gestoorde 'down-regulation' van de monocyt merkstoffen CD14 en een verstoorde 'upregulation' van CD1a, CD83, HLA-DR, CD80 en CD40. Expressie van CD86 was verhoogd in $1,25(OH)_2D_3$ gedifferentieerde dendritische cellen. Ten tweede, de capaciteit van $1,25(OH)_2D_3$ gedifferentieerde dendritische cellen om T-cellen te stimuleren (getest in een 'allogenic

mixed lymphocyte reaction') was in mindere mate verhoogd ten opzichte van de oorspronkelijke monocyten dan dendritische cellen gedifferentieerd zonder $1,25(OH)_2D_3$. Ten aanzien van de productie van cytokines bleef de SAC geïnduceerde IL-10 productie van $1,25(OH)_2D_3$ gedifferentieerde dendritische cellen hoog terwijl het duidelijk verlaagd was in dendritische cellen gevormd in afwezigheid van $1,25(OH)_2D_3$. De SAC/IFNγ geïnduceerde IL-12 productie was duidelijk verhoogd en de TGF-β productie was verlaagd in beide types dendritische cellen in vergelijking met oorspronkelijke monocyten.

Deze gegevens bevestigen eerder publicaties dat $1,25(OH)_2D_3$ de rijping van monocyten tot actieve immuunstimulerende MHC-Class II+, CD1a+, CD80+ dendritische cellen belemmert. Deze data vullen eerdere publicaties aan door te laten zien dat de expressie van CD86 hoger was in $1,25(OH)_2D_3$ gedifferentieerde dendritische cellen terwijl de capaciteit om IL-10 te produceren hoog bleef. Dit bevestigt voorgaande rapportages betreffende de suppressie van cellulaire immuniteit door dit type steroidachtige hormonen.

In hoofdstuk 5a wordt het effect van $1,25(OH)_2D_3$ op de vorming van gesluierde 'accessory' macrofagen vanuit monocyten beschreven. Blootstelling van monocyten aan $1,25(OH)_2D_3$ voorafgaande aan een niet-adherente celkweek gedurende de nacht resulteerde in de vorming van sluiercellen met een zeer lage productie van IL-12 en een zwakke T-cel stimulerende capaciteit in vergelijking met sluiercellen gevormd in de aanwezigheid van gejodeerde stoffen. Deze bevindingen onderstrepen dat $1,25(OH)_2D_3$ de rijping van actieve antigeen presenterende cellen (die de immuunrespons in de richting van Th1 cellen sturen) gevormd uit monocyten belemmert.

Monocyten uit het bloed van patiënten met auto-immuunziekten van de schildklier vertonen een verminderde capaciteit om hun corticale actomyosine cytoskelet aan te passen (te polariseren) als reactie op chemoattractantia. Aangezien dergelijke aanpassingen ook plaats vinden nadat monocyten zich hebben vastgekleefd aan de extracellulaire matrix, is het niet verbazend dat monocyten verhoogd gevoelig zijn voor chemoattractant stimulatie na adherentie aan fibronectine zich uitend als een versterkte polarisatie op chemoattractantia.

In hoofdstuk 6 worden de adherentie capaciteit aan een fibronectine matrix, de expressie van integrinen en de chemoattractant geïnduceerde polarisatie van monocyten uit het bloed van patiënten met een schildklier auto-immuunziekte bepaald voor en na fibronectine adherentie. Aangezien aanpassingen van het cytoskelet ook noodzakelijk zijn tijdens de ontwikkeling van sluiercellen vanuit monocyten, werd deze differentiatie van fibronectine adherente monocyten van patiënten met autoimmuunziekten van de schildklier eveneens bestudeerd.

Adherente en niet-adherente monocyten populaties van patiënten met auto-immuunziekten van de schildklier en gezonde personen werden onderworpen aan een polarisatietest met de chemoattractant fMLP (of MCP-1) en aan FACS analyses met FITC-gelabellmerkte fibronectin, voor CD29 en voor CD49e, d, b en a). Tevens werd de capaciteit van deze cellen om te ontwikkelen tot een gesluierde antigeen presenterende cel getest.

Monocyten van gezonde personen vertoonden een verbeterde 'chemoattractant' geïnduceerde cel polarisatie na adherentie aan fibronectine terwijl de monocyten van patiënten met auto-immuunziekten van de schildklier een verslechterde polarisatie lieten zien. Monocyten van gezonde personen regelden CD49e en d integrines op, evenals hun vermogen om FITC-gemerkte fibronectine te binden na adherentie aan een plaat bedekt met fibronectine. Tevens was de capaciteit om T-cel stimulerende 'veiled cells' te vormen versterkt. Monocyten van patiënten met auto-immuunziekten van de schildklier vertoonden deze eigenschappen niet.

Deze data impliceren dat integrine- (en chemokine)- gemedieerde functies gestoord zijn in patiënten met auto-immuunziekten van de schildklier. Aangezien integrine activiteit van essentieel belang is voor processen zoals adherentie van monocyten aan endotheliale cellen, uropod vorming, migratie in weefsels en differentiatie tot antigeen presenterende cellen en macrofagen, zouden deze gestoorde functies ten grondslag kunnen liggen aan belangrijke verstoorde immunologische evenwichten in auto-immuunziekten van de schildklier.

In hoofdstuk 6a wordt beschreven dat de vorming van prototype dendritische cellen uit monocyten (door middel van een kweek in GM-CSF en IL-4) niet verschilde tussen patiënten met auto-immuunziekten van de schildklier en gezonde personen.

Concluderend kan worden gesteld dat een populatie van antigeen presenterende cellen, gekarakteriseerd door sluiers, kon worden gevormd uit monocyten in 24 uur. Dergelijke sluiercellen stimuleerden T-cellen even krachtig als klassieke monocyt afgeleide immature dendritische cellen. Echter, het patroon van merkstoffen, de cytokine productie en de Th1/Th2 sturingscapaciteit waren verschillend. De sluiercellen vertoonden minder sterke stimulatie van Th1 cellen en ze leken meer op macrofaagachtige cellen.

Hormonen moduleerden zowel de functie van dendritische cellen als sluiercellen. De effecten van dexamethason en $1,25(OH)_2D_3$ remden de vorming van zowel antigeen presenterende cel populaties vanuit monocyten (veranderden monocyten in macrofaagachtige cellen) terwijl DHEA een beperkt immuunstimulerend effect had.

De motiliteit van monocyten en de vorming van gesluierde 'accessory' macrofagen uit monocyten was verstoord in patiënten met auto-immuunziekten van de schildklier vooral na adherentie aan fibronectine. De vorming van monocyt afgeleide dendritische cellen was normaal. Dergelijke APC afwijkingen spelen mogelijk een rol in de verstoring van het immuunsysteem richting zelfreactiviteit. Interventies met hormonen, zoals vitamine D_3 analogen, die geen effect hebben op het calcium metabolisme maar wel op de ontwikkeling van antigeen presenterende cellen, bieden een perspectief voor patiënten met auto-immuunziekte van de schildklier.

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CURRICULUM VITAE

Martha Olwyn Canning werd geboren op 19 mei 1961 in Boston, Massachusetts. In Wolfville in Nova Scotia studeerde zij biologie aan de Acadia University, werkte vervolgens bij het Department of Pathology en het Maine Cytometry Research Insitute in Portland, Maine waarna ze moleculair biologisch onderzoek verrichtte bij het SSDZ in Delft. In 1993 begon ze als Assistent in Opleiding onder supervisie van Prof. dr. H.A. Drexhage bij de afdeling Immunologie van de Erasmus Universiteit Rotterdam (hoofd: Prof. Dr. R. Benner). Martha is getrouwd en is de moeder van twee dochters.

Martha Olwyn Canning was born on May 19, 1961 in Boston, Massachusetts. She studied biology at Acadia University in Wolfville, Nova Scotia, then worked at the Department of Pathology and Maine Cytometry Research Institue in Portland, Maine followed by molecular biological research at the SSDZ in Delft, the Netherlands. In 1993 she commenced PhD research under Prof. dr. H.A. Drexhage at the Department of Immunology, Erasmus University Rotterdam, the Netherlands (Chair: Prof. Dr. R. Benner). Martha is married and is the mother of two daughters.