CYTOKINES AND T LYMPHOCYTES IN TRANSPLANTATION

TARGETS FOR IMMUNOTHERAPY

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CYTOKINEN EN T LYMFOCYTEN IN TRANSPLANTATIE
DOELEN VOOR IMMUNOTHERAPIE

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Aan mijn ouders

Cytokines and T lymphocytes in transplantation
Targets for immunotherapy

Contents

Chapter 1

General introduction

Introduction

Organ transplantation has become an established therapy in patients suffering from diseases leading to organ failure. Table I shows the number of organ transplants performed in the Eurotransplant area from 1990 to 1993. The use of potent immunosuppressive drugs and effective protocols for prevention and treatment of infectious diseases have contributed to improved outcome of clinical organ transplantation. Nowadays, one-year patient and graft survival after kidney transplantation is 85 to 95% (1). However, the potent immune response to transplanted tissues leading to acute graft rejection remains a major problem.

Table I Number of organ tranplants in the Eurotransplant area* from 1990 to 1993**

	1990	1991	1992	1993
Kidney***	3171	3395	3101	3294
Heart	682	803	751	766
Lung	50	74	109	118
Liver***	576	715	765	878
Pancreas/Kidney	62	70	62	91

* Austria, Belgium, Germany, Luxemburg, the Netherlands
** Data from the Annual Report 1993, Eurotransplant Foundation
*** Including combined organ transplantation

Graft rejection

Incompatibility of donor and recipient Major Histocompatibility Complex (MHC) proteins results in the extremely potent immune response known as alloreaction. The MHC is a complex of highly polymorphic genes encoding the MHC class I and class II molecules. These molecules present intracellularly processed peptides to T lymphocytes and thereby initiate T cell mediated immune responses against pathogens. MHC class I molecules, that are expressed on virtually all nucleated cells, present peptides to $CD8^+$ T cells, whereas MHC class II molecules, expressed on B lymphocytes, macrophages and other antigen-presenting cells (APC), present peptides to $CD4^+$ T cells. Physiologically, T lymphocytes recognize their antigen, via their T cell receptor (TCR), only in the context of self MHC (MHC restriction).

T cell responses to allogeneic MHC (alloMHC) molecules are extremely potent due to the high frequency of alloreactive T cells (2). Alloreactivity results from crossreactive recognition of alloMHC-peptide complexes by antigen specific TCR. TCR of alloreactive T cells interact with a great variety of peptides presented in the context of allogeneic MHC molecules and may

also bind directly to the allogeneic MHC molecule (2).

The most common form of TCR is a heterodimer composed of two glycoprotein chains, α and ß, linked by a disulfide bond. The affinity of TCR for MHC/peptide complexes seems to be extremely low (Kd of 10^{-4} to 10^{-5} M) and therefore adhesion molecules may play a significant role in initiating T cell-APC interaction (3). Examples of adhesion molecules are CD2 and LFA-1 on the T cell surface and their ligands LFA-3 and ICAM-1 on the APC. If the TCR encounters its particular MHC-peptide complex, the T cell receives a signal via the TCR-associated CD3 complex. This complex consists of several invariant polypeptide chains (δ, ε, γ, ζ, η) and initiates a cascade of intracellular events leading to T cell activation. Other molecules on the T cell and APC contribute to T cell activation. CD4 and CD8 molecules on T cells act as co-receptors, not only by binding MHC class II and MHC class I molecules, respectively, but also by potentiating intracellular signaling. Besides the TCR/CD3 complex derived signal (signal 1), a costimulatory signal (signal 2) is required for T cell activation. T cells, which only receive signal 1, have *in vitro* been shown to become anergic. Co-stimulatory signals may be provided by interaction of CD80 on APC and CD28/CTLA-4 on T cells (4).

Recognition of donor MHC molecules by recipient T lymphocytes results in intracellular signaling events, involving activation of protein tyrosine kinases and the phosphatidylinositol pathway, followed by increased intracellular Ca^{2+} and activation of protein kinase C. This pathway eventually leads to expression of DNA binding proteins that initiate cytokine gene transcription. Via an unknown distinct pathway, CD28 signal transduction leads to cytokine mRNA stabilization (5). The activated alloreactive T cells produce IL-2 and express IL-2R leading to clonal expansion of these cells. Production of IL-2 and other cytokines leads to recruitment and activation of $CD8^+$ cytotoxic T cells, macrophages, NK cells and B lymphocytes. These effector cells cause, via cell-cell interactions and their soluble products, tissue destruction, leading to graft loss (6, 7).

Immunosuppression

Graft loss is inevitable if alloreactivity is not suppressed by immunosuppressive agents. Currently, immunosuppressive protocols are based upon the combined use of multiple immunosuppressive agents which may potentially interfere with distinct steps of the rejection process, including antigen recognition, T cell cytokine production, cytokine activity and T cell proliferation (8). Table II shows examples of immunosuppressive drugs and antibodies frequently used in clinical transplantation. In experimental settings many other drugs and monoclonal antibodies (mAb) have been and are being evaluated for their immunosuppressive capacity. Among these are mizorbine, RS-61443, 15-deoxyspergualin, brequinar sodium and mAb against LFA-1, ICAM-1, CD4 and IL-2R.

Table II Immunosuppressive agents and their site of action in alloresponses

Alloresponse stage	Immunosuppressive agent
Antigen recognition	OKT3, ATG, ALG
T cell cytokine production	CsA, FK506, corticosteroids
Cytokine activity	Rapamycin
T cell proliferation	Azathioprine, corticosteroids

Why continue searching for new immunosuppressive agents?

As one-year patient and graft survival have increased enormously the last years, long-term problems have gained importance (1). Chronic graft rejection appears more difficult to control than acute rejection. Furthermore, the required life-long immunosuppressive treatment leads to serious problems, such as opportunistic infections, increased incidence of malignancies and drug-related complications (e.g. cyclosporin A (CsA) nephrotoxicity or steroid-related metabolic changes). So, improved immunosuppressive agents should be non-toxic and effective in preventing both acute and chronic rejection. To overcome problems associated with long-term non-specific immunosuppression, strategies should be designed to induce allospecific tolerance. Therefore, the search for new immunosuppressive strategies continues. In our study, we have focussed on anti-CD3 mAb and on strategies interfering with cytokines.

Why monoclonal antibodies?

Monoclonal antibodies are agents that specifically and with high affinity interact with e.g. cell surface molecules. These target molecules determine which cell population the mAb will affect and in which functions they will interfere. The isotype of mAb determines what effector mechanisms of the recipient may be used and therefore the effect of mAb treatment, such as target cell coating or target cell depletion.

Why anti-CD3 mAb?

The CD3-complex, which is noncovalently associated with the TCR, is involved in intracellular signal transduction after antigen binding by the TCR. Since the description of OKT3 (9), a mouse IgG2a mAb against human CD3, a vast amount of literature has appeared on the *in vitro* and *in vivo* properties of anti-CD3 mAb. *In vitro*, OKT3 can inhibit T cell proliferative responses to soluble antigens, the development of CTL and allogeneic cytotoxicity. Two mechanisms may be responsible for these immunosuppressive effects. First, mAb may block the TCR binding site and thus recognition of the alloantigen. Second, anti-CD3 mAb induce co-modulation of CD3 and TCR. These modulated cells are immunologically incompetent. *In vivo*, anti-CD3 mAb result in coating and modulation of the TCR/CD3 complex, T cell depletion and possibly T cell non-responsiveness (10, 11). These mechanisms are responsible for the successful use of OKT3 for immunosuppression of clinical organ allograft rejection.

Side effects of anti-CD3 mAb

The first injection of OKT3 is followed by severe clinical symptoms, such as fever, chills, nausea, diarrhea and headache. These "first dose reactions" are caused by massive release of cytokines (IL-2, IFN-γ, TNF-α, IL-6), which are probably produced by activated T cells.

Another complication of OKT3 treatment, as treatment with all mouse mAb, is the development of human-anti-mouse-antibodies. These may abrogate the effectiveness of OKT3 treatment.

Why cytokine-directed therapy?

Cytokines produced by many cell types, such as T cells, macrophages and NK cells, may influence the rejection process, as we describe in chapter 2.1. Table III illustrates some cytokine effects that may contribute to this process.

Because of their central role in graft rejection, $CD4^+$ T cells and the cytokines they produce have been studied widely in rejection and acceptance of allografts. $CD4^+$ T lymphocytes can be subdivided into at least two subsets, T helper(Th)1 and Th2 cells, based on their cytokine production pattern (12). Th1 cells, which produce IL-2, IFN-γ and TNF-ß, play a role in delayed type hypersensitivity (DTH) reactions and cellular cytotoxicity, whereas Th2 cells, which produce IL-4, IL-5, IL-6 and IL-10, are effective stimulators of B cell differentiation and antibody production. These two Th subsets can regulate each others proliferation and function (13). While IFN-γ inhibits Th2 cell proliferation and antagonizes IL-4 effects, IL-10 inhibits Th1 cytokine production. Graft rejection is thought to be mediated by Th1 cells, that may stimulate DTH and CTL activity. On the other hand, suppression of alloreactive Th1 cells by Th2 cells may lead to graft acceptance (14).

Cytokine-directed strategies

Immunosuppression may be achieved by neutralizing pro-inflammatory cytokines (Table III) by administration of anti-cytokine mAb or soluble cytokine receptors. Alternatively, "skewing" of the alloreactive T cell response towards a Th2 dominated response may inhibit graft rejection and even induce graft tolerance. *In vitro*, "skewing" of T cell differentiation towards one of the Th subsets can be achieved by varying the cytokine environment. For example, IFN-γ (Th1, NK cells) and IL-12 (macrophages, B cells) promote Th1 cell differentiation, whereas IL-4 (Th2) enhances Th2 cell development (15). Changing the *in vivo* cytokine environment by anti-cytokine mAb or cytokines, may have a similar effect.

Table III Cytokine effects that may contribute to graft rejection

Cytokine	Source	Effects
IL-1	macrophages	T cell activation
IL-2	T cells	T cell proliferation, CTL activation
IL-6	T cells, macrophages	T cell activation
IL-12	B cells, macrophages	NK cell activation, Th1 cell differentiation
IFN-γ	T cells, NK cells	CTL, NK cell and Mϕ activation, increased MHC expression
TNF-α	macrophages, NK cells	Mϕ activation, increased MHC class I expression

Aim of this study

In this study we explored different ways to induce suppression of graft rejection in order to find regimens yielding effective immunosuppression and possibly graft acceptance, with minimal toxicity. We studied the effect of T lymphocyte and cytokine-directed therapies on mouse skin graft rejection. Skin transplantation in mice is a technically easy procedure and the rejection process can easily be monitored. As in vascularized organ transplants, rejection is mediated by T lymphocytes. Because of our interest in the role of Th cells in rejection, we have chosen an MHC class II disparate strain combination, as rejection in this strain combination is mediated by $CD4^+$ T cells (16).

A review on the role of cytokines in clinical and experimental transplantation (chapter 2.1) illustrates that cytokines may be targets for immunotherapy. In chapter 2.2 we compare cytokine-directed strategies for immunosuppression with T lymphocyte-directed strategies. We conclude that in our model the use of anti-T cell mAb is significantly more effective than treatment with Th2 cytokines in combination with neutralization of IFN-γ, a Th1 cytokine. Treatment with anti-CD3 mAb resulted in better skin graft survival than anti-CD4 mAb treatment.

The effective immunosuppression by anti-CD3 mAb was illustrated by the long-term skin graft survival after treatment with 17A2, a rat IgG2b anti-CD3 mAb (chapter 3.1). In contrast to OKT3, the clinically used anti-CD3 mAb, this anti-CD3 mAb had no side effects. In chapter 3.2 we studied the immunosuppressive effects of three different anti-CD3 mAb, 145-2C11, 17A2 and KT3. These mAb were equally effective in prolonging skin graft survival and all induced modulation of the TCR/CD3 complex and T cell depletion. One of these mAb (145-2C11) induced strong cytokine release and morbidity, whereas the other two did not. In chapter 3.3 these three anti-CD3 mAb served as a model to elucidate the mechanism of anti-CD3 mAb-associated side effects.

In chapter 4, we briefly discuss our results concerning the effectiveness of cytokine and T cell-directed immunosuppressive strategies. Finally, we speculate on future perspectives of anti-CD3 mAb treatment.

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Chapter 2

Cytokines in transplantation

Cytokines in transplantation

CHAPTER 2.1

Invited review

Cytokines in clinical and experimental transplantation

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Allograft rejection is a complex process, which requires interactions between different cell types and a variety of soluble factors, such as cytokines. In this review we discuss the role of cytokines in the induction and effector phases of the rejection process and in the induction and maintenance of allospecific graft tolerance. Furthermore, we discuss the feasibility of clinical graft function monitoring by measuring cytokines and the possibilities for intervention in the cytokine network in order to inhibit graft rejection and eventually obtain graft acceptance.

Key words: Cytokines, Graft rejection, Monoclonal antibody treatment, Th1–Th2 subsets, Tolerance

Introduction

Cytokines are proteins, which act as soluble mediators and regulators of immune responses. They perform their actions by binding to specific membrane-bound receptors and act in a paracrine or autocrine fashion. Different cell types, not only leukocytes, can produce the same cytokine or react to the same cytokine. So, one cytokine can have a range of differential effects on various different target cells (pleiotropy). Furthermore, two different cytokines can have the same effect (redundancy). Cytokines are able to positively or negatively influence the production and function of one another. The complexity of the cytokine network should be kept in mind, while interpreting experimental or clinical findings as discussed in this review.

One of the immune responses in which cytokines are considered to play an important role is allograft rejection. The process of rejection has been studied carefully and starts to become more clear.[1–3] During the induction phase of rejection, T-cell receptor (TcR) recognition of the foreign MHC molecule results in T-cell activation, expression of the interleukin (IL)-2 receptor (R) and production IL-2, which in an autocrine fashion induces clonal expansion of the activated T cells. During T cell activation, soluble (s)IL-2R are released.[4] It is thought that $CD4^+$ T lymphocytes are essential for initiation of graft rejection and $CD8^+$ T cells are more important in the effector phase of rejection.[5] In this phase, production of IL-2 and other cytokines induces influx and proliferation of $CD8^+$ cytotoxic T cells, natural killer (NK) cells and macrophages. This inflammatory process will lead to tissue destruction and dysfunction of the graft. Cytokines can influence this process at several levels (Table 1). IL-1, produced by macrophages, acts as an accessory signal from the antigen presenting cell (APC) for T cell proliferation.[6,7] IL-2 is a growth factor for T lymphocytes, induces T lymphocyte cytotoxicity and stimulates NK cell activity.[8] Interferon (IFN)-γ activates macrophages, cytotoxic T cells and NK cells and induces increased expression of MHC class I and II molecules in this way increasing the allogenicity of the graft.[9] Tumour necrosis factor (TNF)-α increases the MHC class I expression and activates neutrophils.[10–12] Apart from these activities, cytokines may have direct cytotoxic effects on the grafted tissue.[13]

Since $CD4^+$ T cells are of such importance for graft rejection, T-helper (Th)1–Th2 subsets have been studied for their potential role in regulating the rejection process. Both in mice and in human, different Th cell clones have been described, which can be distinguished by the different sets of cytokines they produce.[14,15] Th1 cells produce IL-2, IFN-γ and

Table 1. Cell sources of cytokines relevant in transplantation

	CTL[a]	Th1	Th2	NK	B cell	Monocytes/ macrophages	Other[b]
IL-1					+	+	+
IL-2	+	+					
IL-4			+				
IL-5			+				
IL-6			+			+	+
IL-10			+			+	
IFN-γ	+	+		+			
TNF-α	+	+	+	+		+	+
TNF-β	+	+		+			+

[a]CTL, cytotoxic T lymphocytes.
[b]Other cells producing these cytokines are fibroblasts, epithelial cells etc.

lymphotoxin (LT, also known as TNF-β), while Th2 cells produce IL-4, IL-5, IL-6 and IL-10. Other cytokines, such as IL-3 and TNF-α, are produced by both Th subsets.

Different functions have been assigned to the different Th subsets. Th1 cells mediate delayed-type hypersensitivity (DTH), anti-viral and anti-tumoral cytotoxic effects, while Th2 cells provide help for B cell differentiation and antibody formation. These two Th subsets are able to influence each others cytokine production and proliferation. IFN-γ, produced by Th1 cells, inhibits Th2 cell proliferation and cytokine production.[16] IFNγ also antagonizes the activity of IL-4 in B cell activation and isotype production.[14] The Th2 cytokine IL-10, inhibits the production of Th1 cytokines while IL-4 inhibits Th1 cell proliferation.[17,18] Since cellular cytotoxicity plays an important role in allograft rejection, Th1 cells are thought to dominate in this process. On the other hand, the suppression of these allospecific Th1 cells by Th2 cells could be a mechanism of graft acceptance. Altered cytokine gene expression and production during graft rejection provides a logical target to redirect the observed shifts in the Th1–Th2 balance interfering with the cytokine network.

To further elucidate the role of cytokines in clinical and experimental allograft rejection and survival, we first focus on the detection of cytokines during the rejection process and during graft acceptance. Then we describe the approaches that have been used to interfere in the cytokine network and future prospects for therapeutic intervention.

Detection of cytokines during graft rejection

There are two reasons for detecting cytokines during allograft rejection. The first is to find a reliable and preferably non-invasive method to monitor graft function. If certain cytokines are found to be early predictors of acute rejection this enables us to start rejection therapy as soon as possible and to monitor the success of this therapy. The second reason is to learn more about which cytokines play a role in the rejection process and therefore might be good targets for immunomodulation. We think that therapeutic intervention in the earliest (induction) phase of rejection is preferable to intervention in the effector phase in order to redirect the immune response and achieve long-term allograft survival. Therefore, we consider cytokines appearing in the induction phase of more importance to serve as targets for immunomodulation than cytokines appearing late in rejection.

The advantage of animal models for graft rejection is that proper controls, including syngeneic grafts and non-grafted tissue can be examined for cytokine expression. Furthermore, in general, no immunosuppressive drugs are being used in these models.

In the clinical setting, immunosuppressive drugs are routinely used for the prevention of organ transplant rejection. Immunosuppressive drugs like cyclosporin A (CsA) and glucocorticoids can inhibit TNF and IL-6 production,[19–21] while the anti-CD3 mAb OKT3 and anti-thymocyte globulin (ATG) can increase serum IL-6 and TNF-α levels.[22–24]

Table 2 summarizes the cytokines described to be involved in allograft rejection.

Detection of systemic cytokines: In the clinical setting a lot of effort has been made to find a single or few cytokines in the serum, urine or bile, that permit monitoring graft rejection. In kidney, liver or lung transplantation, serum levels of IL-6,[25,25,26] TNF-α,[27–29] IL-2, and sIL-2R[30–34] have been shown to increase in relation to graft rejection. Serial monitoring was necessary, since not the absolute levels of IL-6 or sIL-2R, but rather a rise of these levels indicated the development of rejection.[25,26,31–35] However, the measured cytokine levels have low sensitivity and specificity regarding rejection diagnosis. TNF-α, IL-2 and sIL-2R levels were found to be elevated in some cases of stable graft function.[29–31,36] IL-6, TNF-α and sIL-2R levels were increased in the early postoperative period and elevations of IL-6, TNF-α or sIL-2R levels were found during episodes of infection.[25,26,27,29–34] Yoshimura *et al.*[25] suggested that serum IL-6 levels or even better the ratio of IL-6 to CsA trough value may discriminate rejection from CsA nephrotoxicity. Possibly, measurements of TNF-α[49] or IL-2[30] in the urine of renal allograft recipients or IL-6[37] in the bile, as shown in a rat hepatic allograft model, will have more value in discriminating allograft rejection from infection or non-immunological graft dysfunction. These studies demonstrate that systemic cytokine measurements are not reliable enough to differentiate rejection from infection. Therefore, they cannot be used for monitoring and histological tissue analysis remains the standard for diagnosis of rejection. Whether these cytokines are involved in the mechanism of allograft rejection, as has been suggested in a few of the above-mentioned studies, is in our opinion doubtful. Based on their paracrine and autocrine activity, the detection of cytokines in the periphery is not likely to reflect the local activities within the graft. It is much more likely that cytokines

Table 2. Detection of cytokines implicated in allograft rejection

	Cytokines	References
Systemic	IL-2, sIL-2R, IL-6, TNF-α	23, 25–35
In situ animal models	IL-2, IL-4, (IFN-γ), (TNF-β)	44, 45
In situ clinical transplantation	IL-2, IL-5, IL-6, IFN-γ, TNF-α	46–50

that are systemically present, such as TNF-α and IL-6 are involved in the inflammatory process of an ongoing rejection process and are rather nonspecific markers in the serum. Interference with these cytokines could diminish the development of inflammation, but a more preferable target for immunomodulation would be those particular cytokines, that are involved in the induction phase of the local rejection process in order to create a new immunological balance and graft acceptance.

In situ *detection of cytokines during graft rejection:* In order to get more insight into which cytokines are involved in the rejection process, cytokines have been detected in the graft in experimental and clinical settings. For *in situ* detection of cytokines several techniques have been used. Northern blot analysis, reverse transcription (RT)-PCR and *in situ* hybridization were used to detect cytokine mRNA within the graft. In sponge matrix allografts, i.e. sponges containing allogeneic cells, the exudate was tested for cytokine proteins. Furthermore, the local production of cytokines was investigated using immunohistochemistry or cultures of graft-infiltrating cells (GIC). All of these techniques have their own advantages and disadvantages. For example, if cytokine mRNA is to be detected, one cannot conclude that the protein is also present, since the production of cytokines can be regulated at post-transcriptional level. *In situ* hybridization indicates the localization of cytokine production and is also rather sensitive, but is a difficult and time-consuming technique. RT-PCR is an extremely sensitive method, but this method gives no information about the cell-type that produces the detected cytokines. Immunohistochemistry is able to demonstrate the cytokine-producing cells. However, most cytokines are not retained in the cytosol, but are directly secreted after translation. This may cause false negative results. On the other hand, it is difficult to discriminate between the cytokine producing cells and the target cells for these cytokines. Especially interpretations concerning Th1–Th2 imbalances should be made very carefully. This also counts if cytokines are detected in supernatants of cultured graft infiltrating cells. It has been shown that culture conditions, such as added cytokines, can dictate the outcome of the cytokine pattern produced.[38–42] The analysis of mRNA of cultured cells is also unreliable, since manipulating the cells can cause not only induction of a particular cytokine mRNA, but also the level of instable cytokine mRNA to decline.[43]

Two studies in a murine cardiac allograft model[44,45] used the extremely sensitive reverse transcription PCR method to detect cytokine mRNA in normal tissue, isografts and allografts at several days after grafting. Their results were quite similar. The cytokines that were detectable could be divided into three groups. The first group of cytokines can be detected in normal tissue, isografts and allografts and consists of IL-1β, IL-5, IL-6, TNF-α and TGF-β. The second group, including IL-1α and IL-3, is expressed in both isografts and allografts. These two groups seem to represent cytokines which are induced in response to the grafting procedure and are not correlated with the rejection process itself. The last and probably the most important group consists of IL-2 and IL-4. These cytokines are only present in allografts, which suggests they play a role in allograft rejection. However, the two studies show inconsistent data on two cytokines, TNF-β and IFN-γ. Dallman *et al.*[44] showed that TNF-β can only be detected in allografts, while Morgan *et al.*[45] also detected this cytokine in isografts. IFN-γ, however, was only found in allografts by Morgan *et al.*, while Dallman *et al.* detected IFN-γ mRNA in normal tissue and in isografts as well, though the levels were lower than in the allografts. This difference in cytokine pattern remains unexplained. Interestingly, Dallman *et al.* found that IL-2, IL-4 and TNF-β, the cytokines that are expressed only in the allografts, were all transcribed transiently, their expression being maximal at 4–5 days after transplantation. Thereafter the expression declined before clinical signs of rejection appeared, suggesting these cytokines play a role in the initiation of rejection.

In the clinical setting *in situ* detection of cytokines in rejecting allografts has been compared with grafts without signs of rejection. *In situ* hybridization of human renal biopsies showed that normal kidney tissue or biopsies from patients with stable graft function do not significantly express IL-6, TNF-α or IFN-γ transcripts.[46] During acute rejection, however, the grafts showed significant levels of IL-6 mRNA, but not TNF-α or IFN-γ mRNA. The IL-6 mRNA was expressed in many different cell types, such as glomerular cells, tubular epithelium and vascular endothelium. The significance of the IL-6 production by these nonimmune cells remains unclear. Using the same technique on irreversibly rejected kidney grafts, TNF-α mRNA could be detected in macrophage-like infiltrating cells.[47] Immunohistochemistry showed that these cells actually produced TNF-α protein. Glomerular cells and tubular epithelium cells probably were target cells for TNF-α, since TNF-α protein could be detected, but no TNF-α mRNA was found in these cells. The results of mRNA detection during allograft rejection may be influenced by the organ investigated. In human liver allografts IL-1β, IL-4, IL-6 and TNF-α mRNA was expressed in rejecting allografts and in grafts without signs of rejection.[48] Only IL-5 mRNA was associated with liver graft rejection. All the above-mentioned data are derived from grafts, in which the rejection process had developed quite far, as graft dysfunction was already apparent, and the rejection sometimes even irreversible. So, the

cytokines detected may play a role in the graft rejection, but may also be rather nonspecific mediators of tissue inflammation and destruction. Two studies, using RT-PCR on fine-needle aspirates, were able to detect IL-2 mRNA[49] and IFN-γ mRNA[50] in samples, taken before clinical rejection. Since the expression was transient, sequential analysis is required to use this method to predict graft rejection.

Detection of cytokines during allograft acceptance: In animal models, the induction of allograft tolerance has succeeded by using several different protocols, such as a preoperative donor-specific transfusion (DST) or anti-CD4 monoclonal antibody (mAb) treatment.[51–53] Determination of the changes in the cytokine profile in these accepted grafts compared with rejecting allografts, may clarify the mechanism by which this state of tolerance is maintained and may indicate ways to manipulate the immune system in order to obtain tolerance. Strangely, intragraft events, which have been ascribed to cytokines, such as mononuclear cell infiltration, allospecific cytotoxic T cells and elevated expression of MHC class I and II antigens, are still apparent in tolerized grafts[54] and renal allografts with stable graft function.[55]

In a rat kidney allograft model, in which tolerance was induced by DST, graft infiltrating cells from tolerant rats were unable to produce IL-2 *in vitro*, expressed lower levels of the IL-2R and showed lower proliferation in response to IL-2 than cells from untreated rats.[56] In a comparable model, tolerized rat heart allografts showed a lower expression of IL-2 and IFN-γ mRNA and a different kinetics of these messages than rejecting allografts.[57] Strong evidence that the downregulation of IL-2 or IFN-γ mRNA plays a role in the development of graft tolerance is provided by the fact that simultaneous administration of IL-2 or IFN-γ and the DST abrogates the tolerizing effect. It is still unclear whether this downregulation of Th1 cytokines is the mechanism of tolerance induction or whether Th2 cells play a role in suppressing these Th1 cytokines.

After induction of tolerance, using several different strategies, it has been shown that IL-2 and IFN-γ mRNA were downregulated, whereas IL-4 and IL-10 (Th2 cytokines) mRNA expression remained at the same level or even increased.[58] However, there is no proof that the source of these cytokines is indeed T lymphocytes. Recently, it has been shown, that IL-10 mRNA is detectable in many organs of a normal mouse and is expressed at the same level in nude mice or SCID mice, suggesting independence of T and B cells.[59]

Anti-CD4 mAb treatment has resulted in graft acceptance in several animal models. Partial depletion of $CD4^+$ T cells in mice resulted in a higher expression of IL-4 mRNA and a lower expression of IFN-γ mRNA, as detected by *in situ* hybridization in purified $CD4^+$ T cells.[60] In other studies, using anti-CD4 mAb, long term renal allograft survival and abrogation of accelerated cardiac rejection were accompanied by diminished IL-2 expression and preserved IL-4 expression, suggesting that the Th1–Th2 dichotomy does play a role in immunosuppression.[61,62] Additional evidence is provided by the finding that adoptive transfer of a bm12-specific Th2 cell line to B6 mice resulted in prolongation of bm12 skin grafts, whereas BALB/c third party grafts were rejected.[63] It is interesting to note that IL-4 can be detected both early in the rejection process[14] and during graft acceptance. This finding awaits further clarification.

Strategies interfering with cytokine effects

CsA and FK506 are important immunosuppressive drugs, that are widely used for preventing organ allograft rejection. It is known for several years now that their immunosuppressive mechanism is based on inhibition of T cell signal transduction pathways leading to activation of cytokine gene expression.[64] Besides these established regimens, alternative ways to interfere with cytokine effects have been studied (Table 3).

Monoclonal or polyclonal antibodies: Since cytokines have been shown to be associated with allograft rejection, several strategies have been studied to interfere with cytokine function in order to prolong graft survival.

Monoclonal and polyclonal antibodies against cytokines have been shown to prolong allograft survival in several animal models. Anti-IFN-γ mAb blocked MHC class II disparate skin graft rejection in mice, but had no effect on MHC class I disparate skin graft rejection.[65] Anti-IL-2,[66] anti-TNF-α and anti-LT antibodies[67–69] were able to prolong rat cardiac allograft survival, when given as the only therapy. In the same model, anti-IFN-γ antibodies alone failed to prolong graft survival compared with untreated controls.[70,71] However, in combination with CsA, anti-IFN-γ antibodies prolonged allograft survival in a synergistic way.[70,72] This synergistic action with CsA, that permits lowering of the CsA dose, thereby decreasing its potential nephrotoxicity, is also seen using anti-TNF-α antibodies.[68,69] Furthermore, it has been shown that combining anti-LT with anti-TNF-α

Table 3. Strategies interfering with cytokine effects

Modulator	Targets	References
CsA, FK506	IL-2	64
Antibodies	IL-2, IFN-γ, TNF-α, TNF-β, $IL\text{-}2R^+$ cells	65–69, 76–85
Cytokine conjugated toxins	$IL\text{-}2R^+$ cells	86, 87
Soluble receptors	IL-1, IL-4	88, 89
Cytokines (IL-4, IL-10)	Th2 subset	98, Dallman *et al.*

mAb ameliorates graft survival, compared with anti-TNF-α mAb treatment alone.[68]

Although these antibody treatments directed against single cytokines have some effect, graft survival times are not impressive. One of the reasons for this finding could be the property of cytokines to mediate local, i.e. in the graft, short-distance effects. Therefore the local antibody concentration might not be sufficient to neutralize its target cytokine. However, there is evidence that the neutralizing antibodies indeed reach the graft, since ^{125}I-labelled anti-IL-2 mAb was detectable in the graft.[69] Furthermore, local effects of the mAb, such as diminished mononuclear cell infiltration, no expression of MHC class I antigens and modification of immunohistologic staining pattern of TNF-α, were readily visible.[67,69,72] Another explanation for the disappointing results with cytokine-directed antibodies is the redundancy of the cytokine system. Neutralizing the activity of one cytokine probably makes another take over. The striking redundancy of the cytokine system was again demonstrated by the finding, that IL-2 and IL-4 knock-out mice were less affected in the development and function of their T cell system than expected.[73,74]

Another approach to interfere in the cytokine network, by using mAb directed against the IL-2R (CD25), has been shown to be more successful. Not only the number of publications on the use of this mAb, but also the fact that this treatment is already used in clinical trials, are indicative for its success. Anti-IL-2R mAb therapy differs from anti-cytokine mAb therapy in that it acts on IL-2R bearing cells and not just on soluble proteins. Since high-affinity IL-2R is only expressed on activated T cells and not on resting T cells, this approach seems more specific than other established immunosuppressive therapies using ATG, OKT3 or CsA.[75] The mechanism of the anti-IL-2R induced immunosuppression is still not clear. Depletion of IL-2R$^+$ cells, modulation of the IL-2R or blocking of the IL-2-IL-2R interaction have been proposed to play a role.[76–79] In animal models anti-IL-2R mAb have been used to inhibit GVHD and organ allograft rejection.[76,79–81] Prospective clinical trials have shown that anti-IL-2R treatment is equally effective as ATG for the prevention of renal allograft rejection.[82–84]

The use of mouse or rat mAb in clinical transplantation is hampered by the development of human anti-antibodies, that subsequently lead to high clearance rate of the mAb and abrogation of their effect. Therefore, 'humanized' mAb have been produced, combining the rodent complementarity-determining regions with constant regions and framework of human antibodies. A 'humanized' anti-IL-2R mAb, that was less immunogenic and had a longer half-life than its murine form, significantly prolonged cardiac allograft survival in cynomolgus monkeys.[85] As 'humanized' mAb were being developed, molecular engineering offered an alternative approach for selectively attacking IL-2R$^+$ cells. Cytotoxic substances, such as *Pseudomonas* exotoxin (PE) or *Diphtheria* toxin were coupled to IL-2, thereby targeting and killing IL-2R$^+$ cells. IL-2-PE40 and DAB486-IL-2 were able to inhibit allograft survival in animal models.[86,87]

Soluble cytokine receptors: In biological fluids of both animals and humans, cytokine binding proteins have been found, that later appeared to be soluble forms of cytokine receptors, like sIL-2R, sIL-4R and sTNFR. They generally have the same binding affinity for their ligand as the membrane receptors and therefore are able to competitively inhibit cytokine binding to membrane receptors and subsequently their effects on target cells. Soluble cytokine receptors are considered to be naturally occurring cytokine inhibitors and have the advantage of higher affinity and being non-immunogenic over neutralizing mAb. Their potential as therapeutic agents in inflammatory disease and sepsis has been shown. In a cardiac allograft model in mice, sIL-1R and sIL-4R have been shown to somewhat prolong the allograft survival.[88,89]

The efficacy of treatment with soluble cytokine receptors is based on scavenging the relevant cytokine. Therefore, it is crucial to have the soluble cytokine receptor present in the serum with a long half-life. The linking of two sTNFR molecules to the Fc portion of a single human IgG1 molecule resulted in a dimeric form of sTNFR with significantly higher affinity for TNF than the monomeric sTNFR. Moreover, this complex is detectable in serum during 4 – 5 days, being significantly increased over unbound sTNFR. This dimeric sTNFR was very effective in *in vivo* neutralizing endogenous TNF and protecting mice from lethal endotoxaemia.[90,91] Since anti-TNF mAb have been shown to be effective in prolonging allograft survival,[67,69] this agent might have a similar or even more potent effect on allograft rejection. Using soluble cytokine receptors, one should keep in mind that they are also capable of acting as cytokine carriers, protecting them from proteolytic cleavage, prolonging their half-live in the circulation and therefore having an agonistic instead of an antagonistic effect.[92] The ratio in the presence of the cytokine and its soluble receptor probably determines the biological outcome.

Strategies for Th1–Th2 'skewing': The assumption that Th1 cells are responsible for rejection, whereas Th2 cells may act as suppressor cells and induce graft acceptance has led to the idea that 'skewing' of the Th1–Th2 ratio towards Th2 dominance might inhibit graft rejection. *In vitro* studies have shown that cytokines can direct the differentiation of Th cells to one of the subsets. In the presence of IFN-γ Th1 cell development is enhanced,[58] whereas IL-4 enhances

the development of Th2 cells.[89,91,93] IL-10 does not seem to direct bulk cultures towards Th2 cytokine producing cells, though anti-IL-10 mAb did induce Th1-like cells.[93] Treatment of allograft recipients with IL-4 or IL-10 could have the same 'skewing' effect. Furthermore, IL-10 not only inhibits the production of Th1 cytokines, but also has anti-inflammatory properties by inhibiting IL-1, IL-6 and TNF-α synthesis by macrophages.[94] *In vivo* interference in the dominance of one of the Th subsets has already succeeded in a number of inflammatory models. Giving anti-IL-4 mAb before or within the first week of *Leishmania major* infection rendered a susceptible mouse strain resistant to the parasite,[95] but anti-IL-10 mAb had no effect. The other way around, IL-4 promoted the Th2 response to the parasite, but did not render the infected recipient susceptible.[96] Anti-IFN-γ mAb did render *Trypanosoma cruzi* resistant mice susceptible to the infection.[97] In an allogeneic graft model, the use of anti-IFN-γ or anti-IL-2 mAb has not been very effective in prolonging allograft survival, as we described earlier. There have been two groups reporting on the effect of administration of Th2 cytokines, IL-4 and IL-10, on allogeneic responses. Though cardiac allograft rejection (M. Dallman, personal communication) and enlargement of the draining lymphnode[98] were inhibited, no tolerance was induced. Furthermore, systemic administration of these cytokines could have some drawbacks, such as stimulation of B lymphocytes, antibody-production and increased incidence of infections. Also, there is evidence that systemic administration of cytokines, in this case IFN-γ, may lead to a downregulation of endogenously produced cytokine.[99]

Prospects for therapeutic strategies: Strategies directed against a single cytokine in the form of mAb, soluble cytokine receptors or other agents are not likely to be successful in inhibiting graft rejection. Molecular engineered proteins, on the other hand, such as the dimeric form of sTNFR, may be useful to inhibit the intragraft inflammation and systemic effects of graft rejection. Other than in parasitic models, systemic administration of cytokines will probably not be able to interfere with the local processes leading to graft rejection. The increasing knowledge on requirements for T cell activation and the different signalling pathways leading to cytokine production and T cell subset activation, should enable us to interfere with this process in order to establish allospecific tolerance. T cell activation requires besides TcR-MHC interaction costimulatory signals coming from interaction of cell surface molecules on the APC and the T cells.[100,101] Indeed, *in vitro* studies have shown that TcR signalling in the absence of costimulatory signals results in T cell anergy.[102,103] Furthermore, this anergy can be induced in Th1 clones but not in Th2 clones.[104–106] The CD28-B7 interaction seems to play a critical role in the costimulation of T cells.[101,107] *In vivo* blocking of this interaction inhibits cardiac allograft rejection[108] and induces long term survival of pancreatic xenografts.[107] Further studies in *in vitro* and animal models for tolerance should lead to strategies resulting in anergy of allospecific Th1 cells and induction of allospecific 'suppressor' Th2 cells and thereby to allospecific tolerance.

Concluding remarks

Cytokines are involved in the allograft rejection process. However, the relevance of systemic cytokine measurements in order to predict graft rejection is limited, since elevated cytokine levels are found both during rejection and infection. In the induction phase of rejection, a major role is probably played by IL-2 and IFN-γ together with or in balance with IL-4. During the effector phase, many different cytokines may mediate the inflammation. The local rejection process is not easily inhibited by systemic administration of anti-cytokines or cytokines. Intervention in intercellular signalling may lead to a new immunological balance and a state of graft tolerance, based on Th1 anergy and/or Th2 suppression.

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CHAPTER 2.2

T lymphocyte and cytokine-directed strategies for inhibiting skin allograft rejection in mice

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A major goal of transplantation research is the development of strategies to inhibit allograft rejection and even better, to induce allospecific tolerance. For this purpose, animal models have been widely used and it has become clear that skin allograft rejection may be the most difficult one to influence. However, using a combination of depleting and non-depleting anti-CD4 and anti-CD8 monoclonal antibodies (mAb), Waldmann *et al.* were able to induce allospecific tolerance (1). In other transplant models, the use of anti-CD4 mAb or donor-specific transfusion (DST) has also been successful in inducing tranplantation tolerance. It has been shown that during this state of tolerance the cytokine mRNA pattern in the graft is different from that in rejecting allografts. A diminished IL-2 and IFN-γ mRNA expression was found in tolerized rat cardiac allografts (2) and Dallman *et al.* (3) showed that graft infiltrating cells were unable to produce IL-2. Others found that IL-2 and IFN-γ mRNA expression was downregulated, while IL-4 and IL-10 mRNA expression remained similar or even increased in a tolerized graft (4). This cytokine pattern correlates with a suppressed production of Th1 cytokines and an increased production of Th2 cytokines. Whether this upregulation of Th2 cytokines is an epiphenomenon of Th1 cell anergy or whether it is the reason for the suppressed Th1 cytokine expression is still not clear. IL-4 and IL-10 can direct the *in vitro* differentiation of T cells towards Th2 cells, while IFN-γ enhances Th1 cell development (5). These *in vivo* and *in vitro* findings suggest that it may be possible to inhibit allograft rejection by *in vivo* 'skewing' to an allospecific Th2 response.

In an MHC class II disparate skin graft model in mice, the effect of elevated levels of the Th2 cytokines IL-4 and IL-10 on skin allograft survival was examined, using alginate encapsulated cytokine-gene transfected cell lines. It has been shown that in such a strain combination $CD4^+$ T cells mediate the rejection (6). Furthermore, we studied the effect of the combination of this treatment with a concomitant neutralization of IFN-γ. These cytokine-directed therapies were compared to more established treatments, such as anti-CD4 mAb and anti-CD3 mAb.

Materials and methods

Mice

C57BL/Ka BL-1 ($H\text{-}2^b$) and B6.C-$H\text{-}2^{bm12}$ ($H\text{-}2^{bm12}$) mice were bred at

the Department of Immunology of the Erasmus University, Rotterdam.

Cell lines

We used the following hybridomas: anti-CD3 (17A2; rat IgG2b) (7), anti-CD4 (YTS191.1; rat IgG2b) (8) and anti-IFN-γ (XMG1.2; rat IgG1) (9). The mAb were purified from hybridoma culture supernatant by protein G (Pierce Europe, Oud-Beijerland, The Netherlands) affinity chromatography. The cytokine-gene transfected cell lines, LT1-IL-4 (10) and J558-IL-10 were kindly provided by drs. R.L. Coffman and K.W. Moore (DNAX Research Institute, Palo Alto, CA).

Experimental protocol

In order to obtain elevated levels of cytokines *in vivo*, the different cytokine-gene transfected cells were encapsulated in alginate, as we have previously described (11). These encapsulated cells, $2x10^6$ LT1-IL-4 and $2x10^6$ J558-IL-10 cells, were injected subcutaneously the day before skin grafting. This cell dose has been shown to affect *in vivo* immune responses in other models (11). The purified anti-IFN-γ mAb, XMG1.2, was administered intraperitoneally (ip) at 1 and 8 days after skin grafting, at a dose of 1 mg. This dose is capable of neutralizing IFN-γ effects *in vivo* (12). A combination of these protocols was also used. In the experiments using the T cell-directed strategies, C57BL/Ka BL-1 mice were treated ip with 350 μg of purified anti-CD3 mAb, 17A2 or anti-CD4 mAb, YTS191.1 the day before grafting. FACS analysis demonstrated that this dose of anti-CD3 or anti-CD4 mAb induced depletion of their target cells for at least two weeks (data not shown). Furthermore, the 17A2-producing hybridoma ($2x10^6$ cells/mouse) was administered in alginate-encapsulated form. Control mice received no treatment, as we have shown that mice did not react to empty capsules or encapsulated mock-transfected cells (11).

Skin grafting

Tail skin of bm12 donors was grafted to the dorsal thorax of C57BL/Ka BL-1 recipients using a modification of the method of Billingham and Medawar (13). Grafts were considered rejected when no viable donor skin was detectable.

Statistical analysis

Graft survival was analyzed using the Mann-Whitney test. Probability less than 0.05 was considered significant.

Results

Treatment of the C57BL/Ka BL-1 recipients with XMG1.2, the anti-IFN-γ mAb significantly prolonged bm12 skin graft survival as compared to the untreated control group (Fig.1A). Administration of alginate-encapsulated IL-

4- and IL-10-gene transfected cells alone had no effect on graft survival. Furthermore, when combining these treatments no additive effect of IL-4 and IL-10 was observed on the prolonged graft survival induced by XMG1.2.

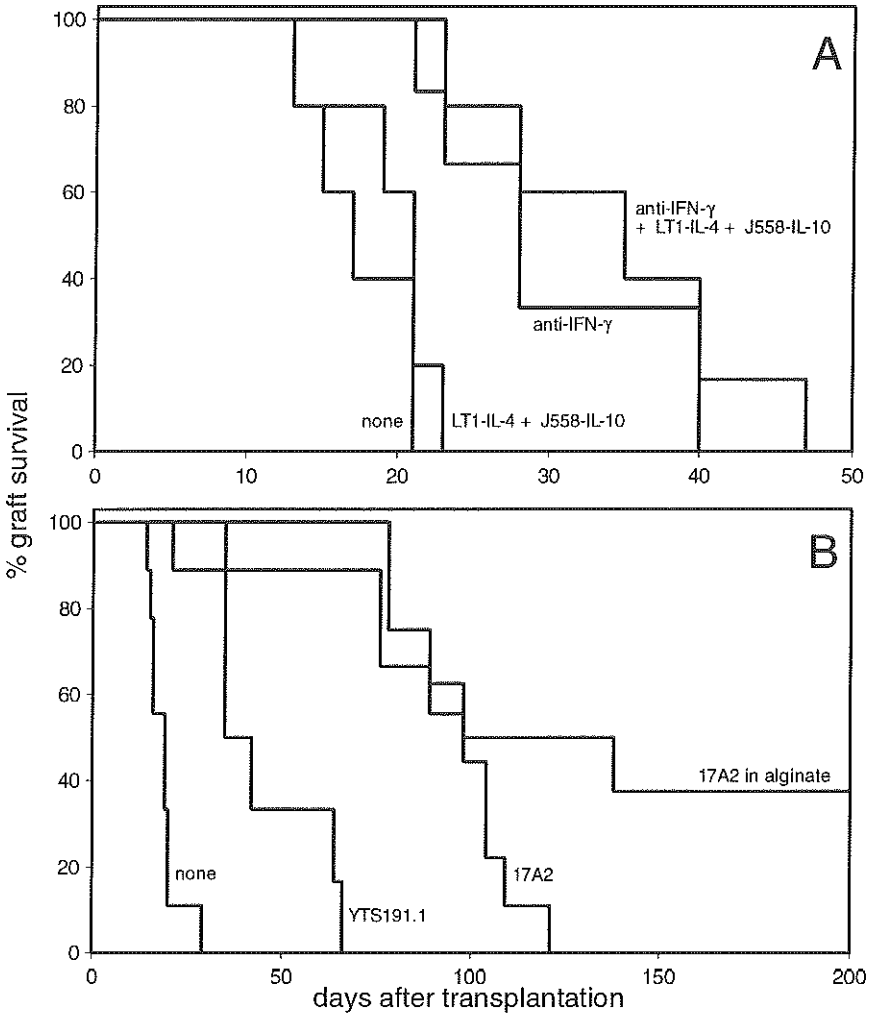

Figure 1 Effect of T cell- or cytokine-directed strategies on skin allograft survival. C57BL/Ka BL-1 mice were grafted with bm 12 tail skin. **A.** Mice were treated with LT1-IL-4 and J558-IL-10 cells in alginate on day-1 (n=5), or 1 mg anti-IFN-γ (XMG1.2) on day 1 and day 8 after grafting (n=6) or a combination of these treatments (n=5). Control mice received no treatment (n=5). **B.** The day before grafting C57BL/Ka BL-1 mice were treated with 350 µg of anti-CD4 (YTS191.1) (n=6), 350 µg anti-CD3 (17A2) (n=9) or $2x10^6$ 17A2 hyridoma cells in alginate (n=10). Control mice received no treatment (n=9).

Figure 1B shows that administration of a single dose of 350 µg of anti-CD4 mAb or anti-CD3 mAb also resulted in a significant prolongation of the MHC class II disparate skin graft survival as compared to the untreated control group. The anti-CD3 mAb was more effective in prolonging skin graft

survival than the anti-CD4 mAb and the anti-IFN-γ mAb, even when the anti-CD3 mAb producing hybridoma cells were encapsulated in alginate and administered ip the day before skin grafting.

Discussion

This study demonstrates that treatment with an anti-IFN-γ mAb prolonged MHC class II disparate skin graft survival, while administration of IL-4 or IL-10 had no effect. Anti-CD4 mAb and anti-CD3 mAb were also effective in prolonging skin allograft survival. Furthermore, anti-CD3 mAb treatment, as a single dose of 350 μg or as alginate encapsulated hybridoma cells, proved to be the most effective in this model.

Using the same strain combination, treatment with anti-IFN-γ mAb has already been shown to block skin graft rejection (14). However, graft rejection did occur when treatment was stopped, suggesting anti-IFN-γ mAb may not have a long term effect on graft survival. The present study shows that systemic administration of Th2 cytokines has no effect on skin graft survival. Though it has not been possible to detect cytokines in the serum of the treated mice, alginate-encapsulated cytokine-gene transfected cells have earlier been shown to affect immune responses (11, 15). Furthermore, alginate-encapsulated 17A2 hybridoma cells induced long-term skin allograft survival in the same model. Since we believe that this technique leads to elevated systemic cytokine levels, we have two explanations for the lack of immunosuppressive effect. First, the cytokines did induce Th2 'skewing' of the allospecific T cells, but these cells are not capable of inducing graft tolerance. Secondly, systemic administration of cytokines can not influence intragraft T lymphocyte activation and differentiation, a process known to be dependent on intimate cellular interactions. In this model single doses of anti-CD4 mAb or anti-CD3 mAb were able to prolong skin allograft survival. Graft survival was significantly better after anti-CD3 mAb treatment than after anti-CD4 mAb. The reason for this might be that anti-CD3 mAb do not only deplete T cells, but also induce modulation of the TcR/CD3 complex. In the context of the 'two-signal' theory for T cell activation, the interaction of these 'disabled' T cells with the alloantigen-bearing antigen presenting cells might lead to incomplete T cell activation or even the induction of anergy.

Together, these data suggest that interference in intercellular signaling probably is a better way to induce long term allograft survival than systemic administration of cytokines or anti-cytokine mAb.

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Chapter 3

T lymphocytes in transplantation

T lymphocytes in transplantation

CHAPTER 3.1

A Rat Anti-Mouse CD3 Monoclonal Antibody Induces Long-Term Skin Allograft Survival Without Inducing Side Effects

A.C.T.M. Vossen, G.J.M. Tibbe, A. van Oudenaren, A.E.C.M. Vredendaal, R. Benner, and H.F.J. Savelkoul

OKT3, a mouse IgG2a monoclonal antibody (MAb) directed against human CD3 is well known for its strong immunosuppressive properties, but also for its severe cytokine-related side effects.[1,2] Newly developed anti-CD3 MAb are currently being tested for their side effects and clinical usefulness. However, it is not yet clear whether these MAb will be as equally effective as OKT3 in preventing and treating organ allograft rejection. A mouse model will be useful to study the properties of anti-CD3 MAb which are important for immunosuppression versus the properties responsible for the severe side effects related to early systemic cytokine release. We studied the immunosuppressive properties of a rat anti-mouse CD3 MAb, 17A2,[3] and the cytokine release induced by this MAb in comparison to the hamster anti-mouse CD3 MAb, 145-2C11. This hamster MAb is known for its immunosuppressive effect on skin allograft rejection and for the induction of severe morbidity, based on the release of TNF-α, IFN-γ, and IL-2.[4]

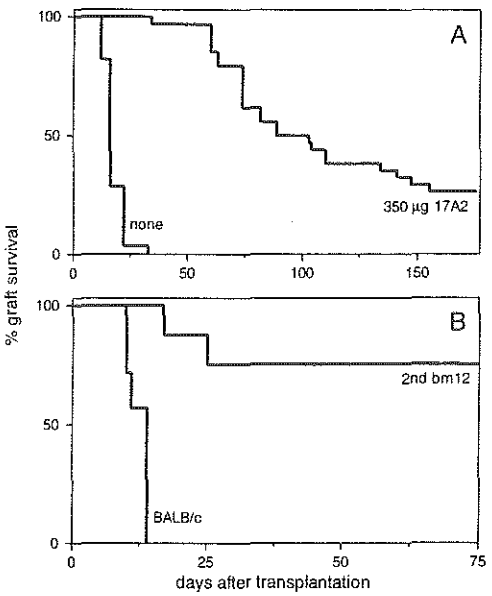

Fig 1. Effect of 17A2 on skin allograft survival. **(A)** C57Bl/Ka mice (n = 34) received 350 µg 17A2 the day before grafting of a bm12 skin. Control mice (n = 28) received no treatment. **(B)** Those C57Bl/Ka mice with long-term (>175 days) bm12 graft survival received a second bm12 skin (n = 8) together with a third party BALB/c graft (n = 7). The two mice rejecting their second bm12 graft also rejected their first bm12 graft.

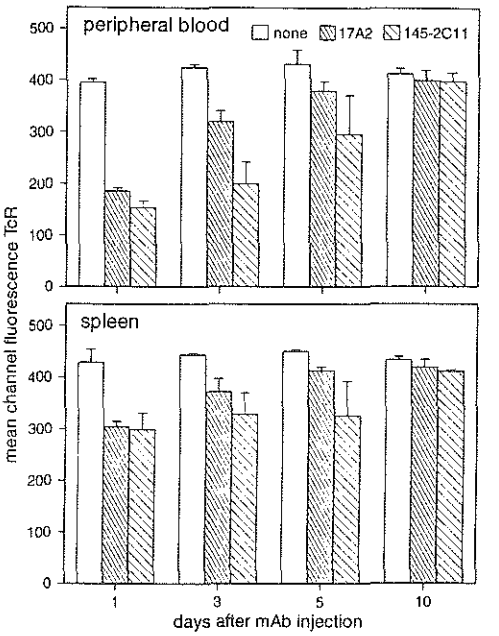

Fig 2. C57BL/Ka mice received 100 µg of 17A2 or 145-2C11. On days 1, 3, 5, and 10, peripheral blood cells (upper panel) and spleen cells (lower panel) were double stained for *Thy-1* and TcRαβ. The results represent the mean ± SD of the mean channel fluorescence of TcRαβ within the *Thy-1*$^+$ population of three mice. Control mice (n = 3) received no treatment.

MATERIALS AND METHODS

The anti-CD3 MAb were purified from culture supernatants by protein G chromatography (Pierce Europe, Oud-Beijerland, The

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Table 1. Cytokine Release in Serum After Anti-CD3 MAb Injection

Anti-CD3 MAb	17A2			145-2C11		
Time (h)	TNF-α (U/mL)	IFN-γ (ng/mL)	IL-6 (U/mL)	TNF-α (U/mL)	IFN-γ (ng/mL)	IL-6 (U/mL)
1	41.9 ± 16.1	1.7 ± 1.5	745.3 ± 40.4	349.8 ± 37.1*	1.7 ± 1.7	776.5 ± 120.0
2	2.1 ± 0.6	1.7 ± 1.6	918.0 ± 65.5	81.1 ± 10.3*	2.5 ± 1.8	1043.5 ± 165.7
4	<1.5	1.0 ± 1.6	519.5 ± 452.5	2.1 ± 1.9	8.4 ± 1.4*	441.0 ± 375.7
8	<1.5	0.2 ± 0.5	72.5 ± 56.6	<1.5	2.9 ± 2.6	577.3 ± 347.4*
24	<1.5	ND	7.1 ± 2.7	<1.5	ND	113.3 ± 97.2*

C57Bl/Ka mice were injected intravenously with 10 μg of 17A2 or 145-2C11. At 1, 2, 4, 8, and 24 hours after injection, five mice were killed for serum detection of TNF-α, IFN-γ, and IL-6. The results represent the mean serum levels ± SD. ND = not determined.
*$P < .05$.

Netherlands). C57BL/Ka mice received different doses of purified anti-CD3 MAb intraperitoneally (IP) of a MHC class II incompatible bm12 skin graft a day before transplantation. After long-term survival (>175 days) of the first graft, the mice received a second bm12 graft together with a third-party skin graft (BALB/c). T-cell depletion and modulation of the TcR/CD3 complex in spleen and peripheral blood after MAb treatment were studied by flow cytometric analysis. To study the cytokine release induced by the two anti-CD3 MAb we injected 10 μg intravenously (IV) in the tail vein and took serum samples at 1, 2, 4, 8, and 24 hours after MAb injection (n = 5). TNF-α was determined by a cytotoxicity assay using WEHI-164 (clone 13) cells. IFN-γ was measured with a specific sandwich ELISA, and for IL-6 measurement we used the B9 proliferation assay.

RESULTS

To compare the immunosuppressive effects of the two anti-CD3 MAb we had to give small doses, since a single dose of 50 μg or more of 145-2C11 resulted in a 100% mortality of the skin-grafted mice. Injection of 17A2 did not induce any morbidity. At doses of 5 to 25 μg, the two MAb were equally effective in prolonging skin allograft survival (data not shown). A single dose of 350 μg of 17A2 induced long-term skin graft survival in 20% of recipients (Fig 1A). These mice accepted a second bm12 graft, while rejecting a third-party graft (Fig 1B). The two mice rejecting their second bm12 graft rejected their first bm12 graft at the same time. Both MAb induced strong T-cell depletion (data not shown) and modulation of the TcR/CD3 complex (Fig 2). Injection of 17A2 induced significantly less TNF-α release than 145-2C11 (Table 1) and induced no IFN-γ release in contrast to 145-2C11. Both MAb induced a strong IL-6 release. These high IL-6 levels were more persistent in the 145-2C11 treated group.

DISCUSSION

In this study we show that effective immunosuppression is possible without inducing side effects. Using a single dose of 350 μg of the rat IgG2b anti-CD3 MAb 17A2 we could induce long-term skin allograft survival. The basis of this tolerance still needs to be elucidated. Treatment with 17A2 induced strong T-cell depletion (data not shown) and modulation of the TcR/CD3 complex as also occurs after 145-2C11 injection. Rapid T-cell lysis, therefore, does not seem to be responsible for the side effects. 145-2C11 induced a stronger TNF-α release than 17A2, which confirms its role in the side effects.[5] IFN-γ was also released after 145-2C11 injection. It is unclear, however, whether IFN-γ is associated with the side effects of 145-2C11 as Ferran et al could not influence the morbidity by giving an anti-IFN-γ MAb.[5] FcγR-binding by the anti-CD3 MAb has been suggested to play a role in their T-cell activating properties.[6] 17A2 is significantly less mitogenic than 145-2C11 (data not shown), which suggests this MAb binds another FcγR or the same FcγR with a lower affinity. Further data on FcR binding, T-cell activation, and cytokine-release by these anti-mouse CD3 MAb should indicate the anti-CD3 MAb properties responsible for side effects and for immunosuppression and provide a basis for development of anti-CD3 MAb for clinical use.

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CHAPTER 3.2

SUPPRESSION OF SKIN ALLOGRAFT REJECTION IN MICE BY ANTI-CD3 MONOCLONAL ANTIBODIES WITHOUT CYTOKINE-RELATED SIDE-EFFECTS[1]

OKT3, a murine IgG2a monoclonal antibody (mAb)* against the human CD3 antigen, is becoming increasingly important for prevention and treatment of organ allograft rejection (*1, 2*). Since the first clinical use of OKT3 in 1980 (*3*), there have been several studies on the mechanism of immunosuppression of anti-CD3 mAb treatment (*4–6*). Besides coating of the CD3 molecule and T cell depletion, OKT3 treatment results in modulation, i.e. disappearance of the TcR/CD3 complex from the cell surface. Modulated T cells were demonstrated to be functionally inactive (*4–6*). The first administration of this mAb, however, is accompanied by severe morbidity. The clinical symptoms, including fever, chills, nausea, vomiting, and headache have been associated with a systemic release of several cytokines, probably caused by T cell activation (*7–9*). There is a need for anti-CD3 mAb that are equally effective in rejection treatment as OKT3 but do not induce side-effects.

In order to study the basis of the side-effects of anti-CD3 mAb treatment we used three different anti-CD3 mAb: two rat mAb, an IgG2b (17A2) and IgG2a (KT3) and a hamster mAb (145-2C11) (*11–13*). We first established the immunosuppressive efficacy of these mAb in a skin allograft model in mice. The hamster mAb is in its effect very similar to OKT3. Treatment with this mAb can suppress skin allograft rejection in mice, but also induces early systemic release of cytokines, such as TNF, IFN-γ, and IL-2, resulting in severe morbidity and even mortality (*9, 10*). Remarkably, the two rat mAb did not induce any morbidity or mortality. We compared the in vivo T cell depletion and TcR/CD3 modulation and the cytokine release induced by these equally immunosuppressive mAb.

To compare the capacity of the three anti-CD3 mAb to suppress skin allograft rejection, C57BL/Ka BL-1 (H-2^b) mice were injected i.p. with a single dose of 0.5 μg, 10 μg, or 50 μg of 17A2 (rat IgG2b), KT3 (rat IgG2a) or 145–2C11 (hamster) mAb. Monoclonal antibodies were purified from hybridoma culture supernatant by protein G (Pierce Europe, Oud-Beijerland, The Netherlands) affinity chromatography (Savelkoul et al., manuscript submitted). The amount of endotoxin was measured using the Limulus amebocyte lysate (LAL) micromethod, as previously described (*14*). The day after injection, all mice were skin grafted with a MHC class II disparate skin graft (H-2^{bm12}) using a modification of the method of Billingham and Medawar (*15*). A dose of 0.5 μg of the different mAb did not prolong mean survival time as compared with the untreated control group (data not shown). A dose of 10 μg, however, did significantly prolong skin graft survival (Fig. 1). All three anti-CD3 mAb were equally effective in prolonging skin allograft survival. At the higher dose of 50 μg, graft survival of the rat mAb–treated groups was not significantly better (data not shown). At this dose, the hamster anti-CD3 mAb induced severe morbidity (piloerection, hypomotility, and diarrhea) in all skin-grafted mice, resulting in death within 3 days of injection. Doses up to 1 mg of the two rat anti-CD3 mAb did not induce any morbidity or mortality.

To investigate the effect of the three anti-CD3 mAb on $CD4^+$ and $CD8^+$ T cells in vivo, we treated nongrafted C57BL/Ka BL-1 mice with 100 μg of mAb. Doses of 70 to 400 μg of 145–2C11 have previously been shown to be equally effective in T cell depletion (*10*). In nongrafted mice, this dose induced mortality in only 30% of the mice, in contrast to a 100% mortality in skin-grafted mice. We measured the degree of T cell subset depletion in spleen, lymph nodes, and peripheral blood on days 1, 3, 5, 10, and 20 after mAb injection by flow cytometry using a FACScan (Becton Dickinson). To detect coating due to previously injected anti-CD3 mAb, we directly stained the samples with FITC-coupled Ab against rat Ig or hamster Ig. To study the degree of modulation of the TcR/CD3 complex, we compared the mean channel fluorescence of Thy-1.1^+ cells stained with anti-CD3 FITC of the treated groups and the untreated control group. To exclude interference of CD3 coating in the expression of CD3, we also stained with anti-TcRαβ FITC. Flow cytometric analysis of the $CD4^+$ T cell subset showed that all three mAb induced an early and equally strong depletion of the $CD4^+$ T cells in the peripheral blood (Fig. 2A). In lymph nodes (data not shown) and spleen (Fig. 2C) the depletion of $CD4^+$ T cells was delayed as compared with the peripheral blood. At days 10 and 20, the hamster mAb–treated group showed a significantly stronger $CD4^+$ T cell depletion than the rat mAb–treated groups. As compared with the $CD4^+$ T cell depletion, the depletion of the $CD8^-$ cell subset was less pronounced. At day 3 a sudden increase of the percentage of $CD8^+$ T cells was found. The hamster mAb induced $CD8^-$ T cell depletion in the peripheral blood on days 1, 5, 10, and 20, in contrast to the two rat mAb, which showed significant depletion on days 1 and 20 only (Fig. 2B). In the spleen (Fig. 2D) the hamster mAb induced a significantly stronger $CD8^+$ T cell depletion than the rat mAb. Only on days 1 and 3 could we detect anti-CD3 coated cells in peripheral blood, lymph nodes, and spleen of the rat mAb–treated mice. The percentage of coated cells was higher in the KT3 (IgG2a)–treated group (80–100%) than in the 17A2 (IgG2b)–treated group (30–55%). In the group treated with hamster mAb, we could not detect any cell coating on all days tested (data not shown). In all anti-CD3 mAb treated groups, there was a strong modulation of CD3 and TcRαβ (data not shown). The modulation was stronger in

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* Abbreviations: FcR, FC receptor; IFN, interferon; Ig, immunoglobulin; IL, interleukin; mAb, monoclonal antibody; TcR, T cell receptor; TNF, tumor necrosis factor.

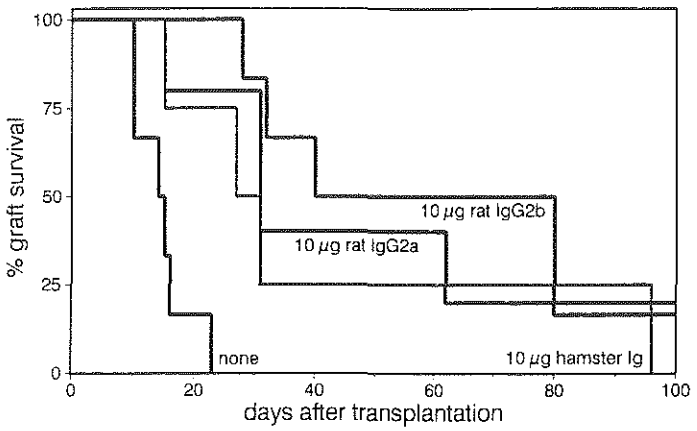

FIGURE 1. Effect of anti-CD3 mAb on skin allograft survival. C57BL/Ka BL-1 mice were skin-grafted with bm12 tail skin. The day before skin grafting, groups of mice were treated with 10 μg of 17A2 (rat IgG2b) mAb (n=6), KT3 (rat IgG2a) mAb (n=5), or 145-2C11 (hamster) anti-CD3 mAb i.p. (n=4). Control mice received no treatment (n=6).

the peripheral blood than in the spleen. By day 5, modulation of the TcR/CD3 complex was still detectable in the hamster mAb–treated mice, in contrast to the rat mAb–treated mice. On day 10 TcR/CD3 modulation could no longer be detected.

The cytokine release in the serum induced by the three mAb was studied at 1, 2, 4, 8, and 24 hr after mAb injection. For the detection of IFN-γ we used a specific ELISA with XMG 1.2 (*16*) as a coating mAb and R46 A2 (*17*) as a second

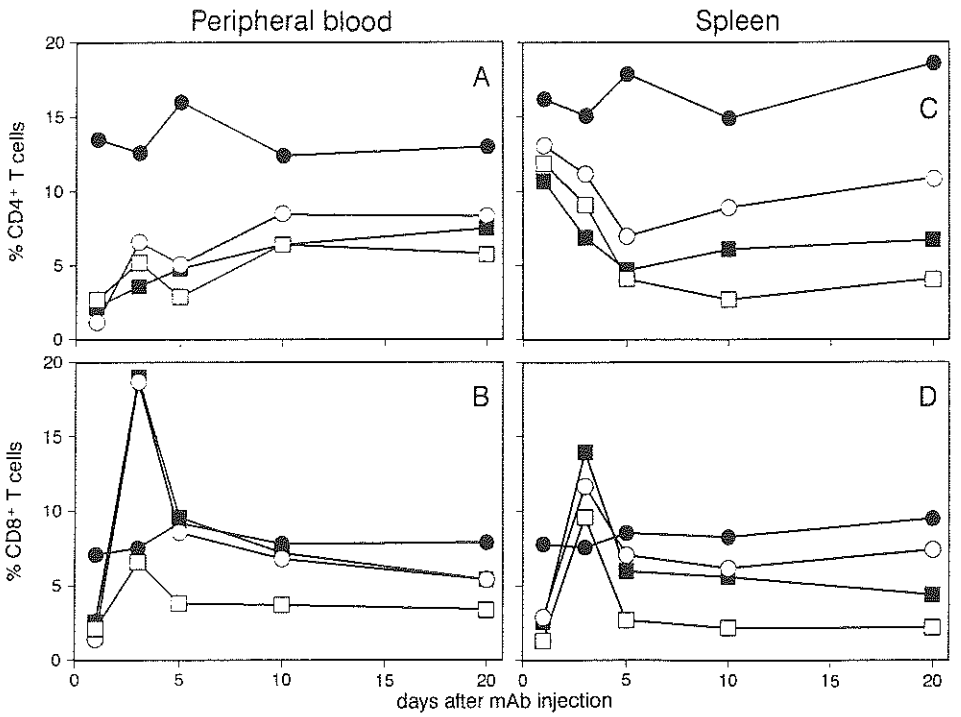

FIGURE 2. Effect of anti-CD3 mAb on T cell subsets in peripheral blood and spleen. C57BL/Ka BL-1 mice received 100 μg of rat IgG2b (open circles), rat IgG2a (closed squares), or hamster anti-CD3 mAb (open squares) i.p. On days 1, 3, 5, 10, and 20 peripheral blood (A, B) and spleen cells (C, D) were double-stained for Thy-1.1 and CD4 (A, C) or CD8 (B, D). The results represent the mean percentage of $CD4^+$ or $CD8^+$ cells of 3 mice. Control mice (n=3; closed circles) received no treatment.

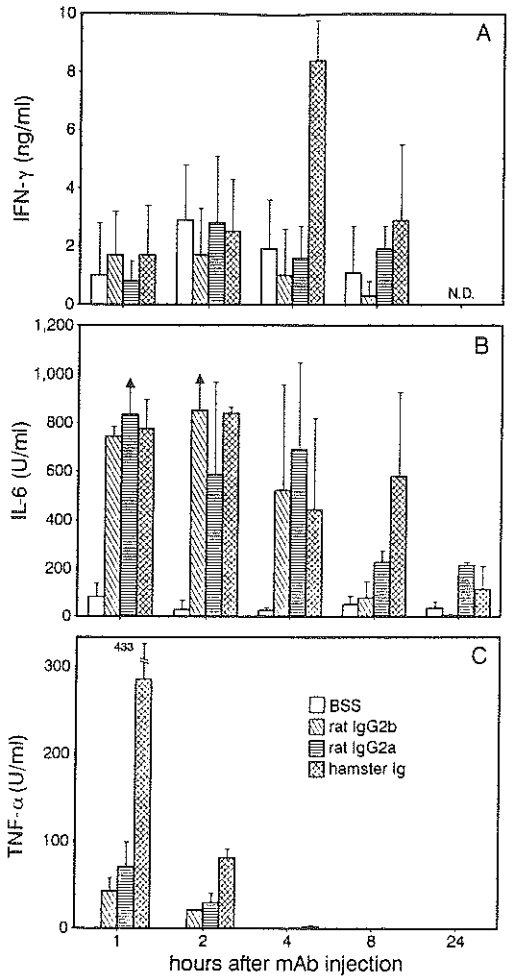

FIGURE 3. Effect of anti-CD3 mAb on the serum levels of IFN-γ, IL-6, and TNF-α. C57BL/Ka BL-1 mice received 10 μg of rat IgG2b, rat IgG2a or hamster anti-CD3 mAb i.v. Control mice received 0.5 ml of BSS. 1, 2, 4, 8, and 24 hr after mAb injection; 5 mice per group were killed for serum detection of IFN-γ (A), IL-6 (B), and TNF-α (C). The results represent the mean serum levels ± SD. (ND) not determined.

step mAb. To measure IL-6 levels in the serum (*18*), the IL-6-dependent cell line B9 was used, while TNF-α levels were determined by a cytotoxicity assay on WEHI164 clone 13 cells (*19*). The proliferative or cytotoxic activity was measured with the MTT assay (*20*). In the samples of all groups low levels of IFN-γ were detected (Fig. 3A). However, at 4 hr after injection the IFN-γ levels of the hamster mAb–treated group were significantly higher than in the other 3 groups. Figure 3B shows that all anti-CD3 mAb induced an equally strong IL-6 release detectable from 1 hr until 8 hr after injection. TNF-α (Fig. 3C) was induced by all three mAb, with a peak at 1 hr after injection. The hamster mAb induced a significantly stronger TNF release than the two rat mAb. Since the amount of endotoxin in the injected mAb preparations was the same (<0.1 ng), the differences in cytokine release can not be attributed to a difference in endotoxin contamination.

This study shows that two rat anti-CD3 mAb, 17A2 (*11*) and KT3 (*12*)—which in vitro cross-compete with the hamster anti-CD3 mAb 145–2C11 (*13*)—are extremely effective in suppressing skin allograft rejection, but do not induce any morbidity or mortality. All three mAb induced T cell depletion and modulation of the TcR/CD3 complex. This depletion and modulation were stronger and more persistent in the hamster mAb–treated mice. Injection of a single dose of 10 μg of the different mAb induced in all groups an early systemic IL-6 release. The hamster mAb, in contrast to the two rat mAb, induced the release of TNF-α and IFN-γ, which can therefore be related to the severe side-effects seen in mice treated with this mAb.

Administration of OKT3 in humans and 145–2C11 in mice has been shown to induce strong T cell depletion and modulation of the TcR/CD3 complex (*4, 6, 10*). In the first days after treatment with the two rat mAb our flow cytometric analysis showed the same T cell depleting effects. This suggests that rapid cell lysis is not necessarily involved in anti-CD3 mAb–induced side-effects (*7*). T cell depletion is probably due to opsonization and complement fixation, followed by cell lysis by the mononuclear phagocytic system. Modulation of the CD3 molecule has been shown to be dependent on FcR binding on monocytes (*22*). Our finding that 145–2C11 induced a stronger and more persistent T cell depletion and modulation of the TcR/CD3 complex than the two rat mAb could therefore be caused by a stronger complement fixation or FcR binding. We found in all groups that $CD4^+$ T cell depletion was more profound than $CD8^+$ T cell depletion. This may be due to a stronger CD3 expression on the $CD4^+$ T cell subset (*23*).

The "first-dose reactions" to OKT3 treatment and 145–2C11 treatment have been associated with an early systemic release of cytokines, including IL-2, IL-3, TNF-α, IFN-γ, and IL-6 (*8, 9, 24*). Ferran et al. tried to elucidate the causal relationship between the release of the different cytokines, induced by 145–2C11 and the severe side-effects induced by this mAb (*21*). Administration of anti-TNF-α mAb significantly decreased the physical reaction to 145–2C11. Our finding that 145–2C11 induced a strong TNF-α release in contrast to the rat mAb again confirms the role of TNF-α in anti-CD3 mAb–induced side-effects. The hamster mAb also induced a release of IFN-γ, in contrast to the two rat mAb. It is unclear to what extent this cytokine is associated with the side-effects. Ferran showed that treatment of 145–2C11 injected mice with anti-IFN-γ mAb did not improve their physical condition (*21*). Furthermore, these authors found that treatment with anti-TNF-α mAb even induced an increase in IFN-γ levels. Though the release of IL-6 after anti-CD3 treatment has been related to the "first-dose reactions" (*21, 24*), our results show that IL-6 release also occurred after rat mAb injection. As these mAb did not induce any morbidity, it is unlikely that IL-6 mediates the side effects.

It is not yet clear which cells are responsible for the production of these cytokines. Evidence is accumulating that not only T cell activation but also activation of FcR-bearing

monocytes, induced by crosslinking of these cells via the Fc portion of anti-CD3 mAb, plays a role in the morbidity caused by anti-CD3 mAb. In vitro, OKT3 has been shown to induce IFN-γ production by lymphocytes (*25*). Furthermore, in vitro cross-linking of Fc receptors on monocytes induces release of IL-1, IL-6 and TNF-α (*26–28*). Parlevliet et al. (*29*) showed that the in vivo administration of an IgA switch variant of OKT3, which does not interact with the human FcR, induced fewer side-effects in chimpanzees than the IgG2a OKT3. Alegre et al. (*30*) created an anti-CD3 mAb that had a lower affinity for the FcR on monocytes due to a single amino acid mutation in the Fc portion of a "humanized" OKT3 mAb. In vitro, this mAb seemed to retain the immunosuppressive properties of OKT3, but induced much less T cell proliferation, activation, and cytokine release. These results suggest that the use of anti-CD3 mAb with a lower affinity for the FcR might provide good immunosuppression without the severe first-dose reactions. However, in clinical practice it is difficult and time-consuming to establish the immunosuppressive efficacy of these newly developed mAb. Since the present study demonstrates that effective anti-CD3 mAb treatment is possible without the induction of any morbidity, the two rat-anti-CD3 mAb together with the hamster mAb provide a good system in which to study the properties that are responsible for the side effects of anti-CD3 mAb in a mouse model. Additional in vitro and in vivo studies should elucidate the role of FcR-binding as well as activation of T lymphocytes and monocytes in these side effects. Such studies will help to optimize anti-CD3 mAb treatment in clinical organ allograft transplantation.

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CHAPTER 3.3

Soluble TNF receptor release after anti-CD3 monoclonal antibody treatment in mice is not related to TNF-α release.

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Summary

The involvement of TNF-α in the release of soluble TNF receptors was assessed in mice, treated with anti-CD3 monoclonal antibodies. After treatment with three different anti-CD3 monoclonal antibodies, we simultaneously studied serum levels of TNF-α, soluble TNF receptor P55 and P75. All three anti-CD3 monoclonal antibodies triggered release of both soluble TNF receptors, whereas only one anti-CD3 monoclonal antibody triggered TNF-α release. These data demonstrate that in our model soluble TNF receptor release is independent of TNF-α release.

Introduction

Tumor necrosis factor (TNF-α) is a pleiotropic cytokine that is involved in various infectious and noninfectious inflammatory diseases (1). At low concentrations, TNF-α is an important mediator of the host defence against pathogens, whereas at high concentrations it causes shock and disseminated intravascular coagulation, leading to organ failure and death (2). TNF-α mediates its effects by binding to specific cell surface receptors, which are expressed on virtually all cell types. Two distinct transmembrane TNF receptors (TNFR) have been identified, one of 55 kDa (TNFR-P55) and one of 75 kDa (TNFR-P75). Soluble forms of both receptors (sTNFR) can be produced by proteolytic cleavage (3). These sTNFR bind TNF-α with the same affinity as their transmembrane equivalents and competitively inhibit *in vitro* biological effects of TNF-α (4). As sTNFR have been detected in serum of healthy controls (5) and at higher concentrations in patients with septic shock (6), cancer (7) and rheumatoid arthritis (8), it has been suggested that they provide a natural protection mechanism against the disastrous systemic effects of TNF-α (9). If sTNFR are protective by neutralizing TNF-α, TNF-α itself will be a likely trigger for their release. It has been shown that TNF-α is capable of triggering sTNFR release in mice (10). However, the mechanism of release of these sTNFR is still unclear.

Anti-CD3 monoclonal antibody (mAb) treatment in both man (OKT3)

and mice (145-2C11) induces elevated levels of TNF-α (11, 12) and both forms of sTNFR (13). TNF-α plays a key role in the side effects of anti-CD3 mAb (14). To get more insight into the role of TNF-α in the release of sTNFR, we studied sTNFR release after treatment with three anti-CD3 mAb in mice. One of these anti-CD3 mAb, 145-2C11, induces strong *in vivo* release of TNF-α, whereas the other two anti-CD3 mAb, 17A2 and KT3, induce only minimal increase of TNF-α levels (12). We studied serum kinetics of TNF-α and both sTNFR-P55 and P75 after treatment with the three anti-CD3 mAb.

Materials and methods

Mice

C57BL/Ka BL-1 (H-2^b) mice were bred at the Department of Immunology of the Erasmus University Rotterdam. Mice were kept in light-cycled rooms and had access to acidified water and pelleted food ad libitum. The microbiological status of the mice fulfilled the "specific pathogen free V" criteria of the Dutch Veterinary Inspection, as described in the Dutch law on animal experiments.

Monoclonal antibodies

We used the anti-CD3 mAb 17A2 (rat IgG2b) (15), KT3 (rat IgG2a) (16) and 145-2C11 (hamster Ig) (17). Monoclonal antibodies were purified from hybridoma culture supernatant by protein G affinity chromatography (Pierce Europe, Oud-Beijerland, The Netherlands), as previously described (18). The amounts of endotoxin were measured using the Limulus amebocyte lysate (LAL) micromethod (19). Endotoxin contamination was similar for the different mAb preparations and never exceeded 25 EU/mg mAb.

Experimental protocol

C57BL/Ka BL-1 mice were injected i.v. with 10 μg of 17A2, KT3 or 145-2C11 mAb, dissolved in 0.5 ml BSS (n = 10). Control mice received 0.5 ml BSS alone (n = 10). At 1, 4 and 24 hours after mAb injection, blood was obtained from 5 mice per group via a small incision in the lateral tail vein. At 2 and 8 hours after mAb injection, blood samples were obtained from the other 5 mice of the groups. Blood samples were left to clot overnight at 4°C, centrifuged and serum was aliquotted and stored at -20°C.

Detection of serum TNF-α

Serum TNF-α levels were determined by a cytotoxicity assay on WEHI-164 clone 13 cells (20). Briefly, trypsinized WEHI-164 cells were seeded in flat-bottomed tissue microtiter plates (Falcon, Becton Dickinson, Lincoln Park, NJ) at 1 x 10^4 cells/100 μl RPMI 1640 medium (Gibco, Paisley, UK) supplemented with 10% heat-inactivated FCS, L-glutamine (4 mM), ß-mercaptoethanol ($5x10^{-5}$ M), penicillin (100 IU/ml) and streptomycin (50

μg/ml). After overnight cell adherence at 37°C, serum samples and actinomycin D (1 μg/ml) were added and incubated for 24 hours at 37°C. Samples were serially diluted and assayed in triplicate. As a standard we used recombinant human TNF-α. Cytotoxic activity was measured with the MTT assay (21).

Detection of sTNFR

For measurement of mouse sTNFR-P55 and sTNFR-P75, a sandwich ELISA was used as described previously (10). Briefly, immunoassay plates (Nunc-Immuno Plate Maxisorp, Roskilde, Denmark) were coated with polyclonal rabbit antibodies against mouse sTNFR-P55 and sTNFR-P75, respectively. Plates were incubated with serially diluted samples of recombinant mouse sTNFR-P55 and sTNFR-P75, respectively, and with test samples. After a washing step, biotin-labeled rabbit anti-sTNFR-antiserum was added. After incubation and washing, plates were incubated with peroxidase-labeled streptavidin (DAKO, Glostrup, Denmark). As peroxidase-substrate 3,3',5,5'-tetramethylbenzidine (Kirkegaard & Perry Laboratory, Gaithersburg, MD) was used. Photospectrometry (450 nm) was performed using a micro ELISA autoreader. The ELISA had a lower detection limit of 5 pg/ml for sTNFR-P55 and 50 pg/ml for sTNFR-P75.

Statistical analysis

Analysis of variance was used for comparison of the serum levels of TNF-α and both sTNFR of the different groups. Probability (p) values below 0.05 were considered statistically significant.

Results

Serum TNF-α levels after anti-CD3 mAb treatment

As shown in Figure 1, the hamster anti-CD3 mAb, 145-2C11, induced a strong TNF-α release at 1 hour after mAb injection. Two hours after 145-2C11 mAb injection, TNF-α levels were still significantly higher than in all other groups. At later time points no TNF-α could be detected in the serum samples. In contrast, after 17A2 (rat IgG2b) and KT3 (rat IgG2a) mAb treatment no increase in serum TNF-α levels could be detected as compared with TNF-α levels after control treatment (BSS). Thus, 145-2C11 was the only anti-CD3 mAb that induced serum TNF-α release.

Serum sTNFR levels after anti-CD3 mAb treatment

To study whether sTNFR release is related to TNF-α release, the same serum samples were used to measure TNF-α and sTNFR levels. At 1 hour after mAb injection, all three anti-CD3 mAb induced a significant increase in serum levels of both sTNFR-P55 (Fig. 2) and sTNFR-P75 (Fig. 3) as compared to BSS. There was no difference in the sTNFR levels between the groups treated with the different anti-CD3 mAb. At two hours after mAb injection, only 145-2C11 treated mice showed significantly increased levels of both sTNFR. At 4 hours, serum levels of both sTNFR were significantly elevated in all anti-CD3 mAb treated mice, whereas at 8 hours sTNFR levels were significantly higher in the 145-2C11 treated group than in the groups treated

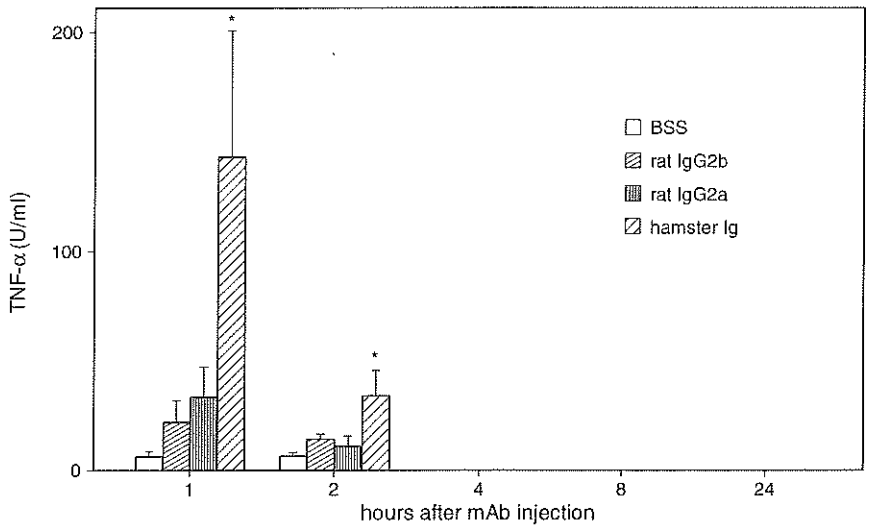

Figure 1 Effect of anti-CD3 mAb on the serum levels of TNF-α. C57BL/Ka BL-1 mice received 10 μg of rat IgG2b 17A2, rat IgG2a KT3 or hamster anti-CD3 145-2C11 mAb i.v. Control mice received 0.5 ml of BSS. At 1, 2, 4, 8 and 24 hours after mAb injection, serum samples were obtained from 5 mice per group for detection of TNF-α by WEHI bioassay. The results represent the mean serum levels ± SD. * $p < 0.05$ as compared to BSS treated group.

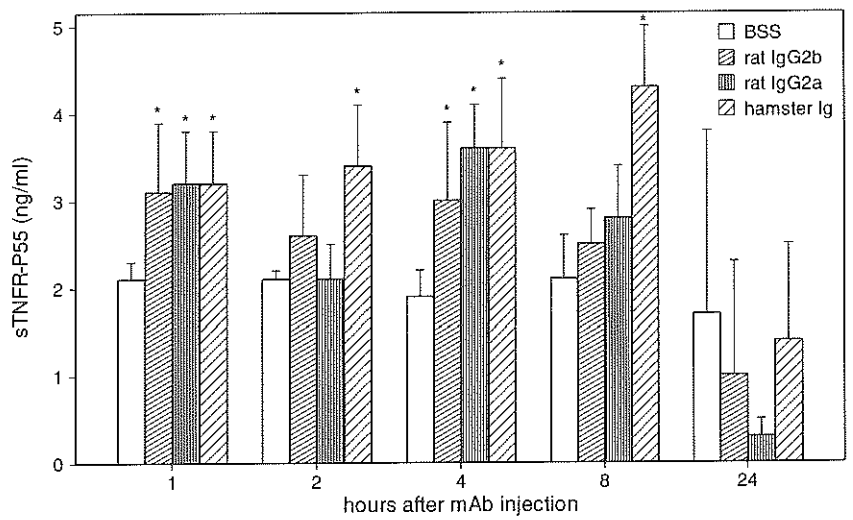

Figure 2 Effect of anti-CD3 mAb on the serum levels of sTNFR-P55. The same serum samples as used for TNF-α detection (Fig. 1) were used for detection of sTNFR-P55 by ELISA. The results represent the mean serum levels ± SD. * $p < 0.05$ as compared to BSS treated group.

with 17A2 or KT3 mAb. From these data we conclude that all three anti-

CD3 mAb induce elevated serum levels of both sTNFR-P55 and sTNFR-P75. Peak levels of both sTNFR were similar in all anti-CD3 mAb treated groups, but were more sustained after 145-2C11 treatment than after treatment with 17A2 or KT3 mAb.

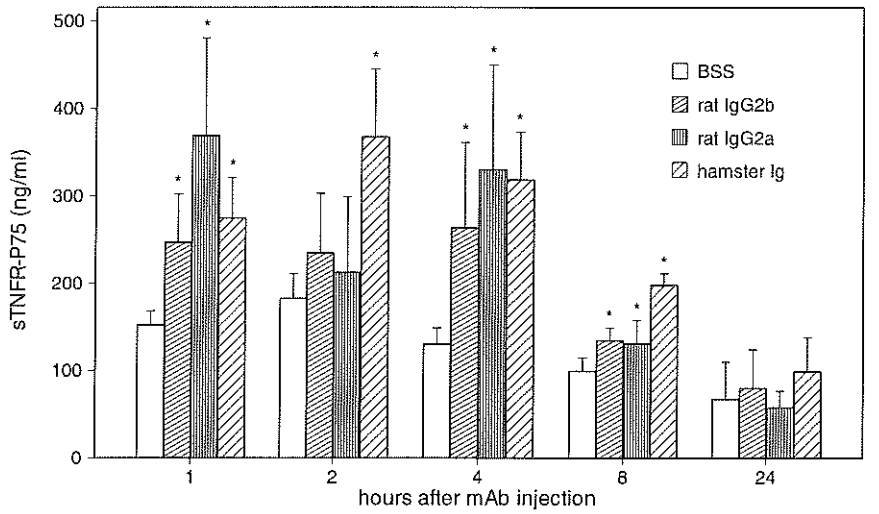

Figure 3 Effect of anti-CD3 mAb on the serum levels of sTNFR-P75. The same serum samples as used for TNF-α detection (Fig. 1) were used for detection of sTNFR-P75 by ELISA. The results represent the mean serum levels ± SD. * $p < 0.05$ as compared to BSS treated group.

Discussion

In this study we show that 145-2C11, a hamster anti-mouse CD3 mAb, induces strong release of both TNF-α and sTNFR. However, two anti-CD3 mAb 17A2 and KT3, that did not induce TNF-α release, did trigger the release of both sTNFR-P55 and sTNFR-P75, suggesting that in our model sTNFR release is independent of TNF-α.

The difference in TNF-α release by the three anti-CD3 mAb is related to their difference in interaction with mouse Fcγ receptors (FcγR) (Vossen *et al.*, submitted). By binding FcγR via its Fc portion, 145-2C11 is capable of crosslinking CD3 molecules, leading to T cell activation and TNF-α production.

Treatment with 145-2C11 mAb has previously been shown to trigger sTNFR release (13). Surprisingly, in the present study sTNFR levels were approximately ten times higher in both anti-CD3 mAb treated and control mice, whereas TNF-α levels were lower. This may be due to the different mouse strains used, i.e. C57BL/Ka BL-1 mice and DBA/2 mice, respectively or to a different microbiological status. In accordance with our results, sTNFR release in DBA/2 mice was relatively independent of TNF-α levels. Inhibition of TNF-α release by several agents, including anti-TNF mAb, only

partly inhibited sTNFR release (13).

Our study shows that TNF-α itself is not essential for the release of soluble forms of its receptors. Release of sTNFR by anti-CD3 mAb may be based on their capacity to induce protein kinase C (PKC) activation by binding to CD3 molecules on T lymphocytes. It has been shown that PKC activation by phorbol esters results in a rapid decrease of transmembrane TNFR (22). PKC activation probably triggers proteolytic cleavage of TNFR and thereby release of sTNFR. This would imply that in our model T lymphocytes are the major source of sTNFR. It is possible that TNF-α is responsible for our finding that 145-2C11 mAb induced more prolonged release of both sTNFR than the other two anti-CD3 mAb.

Release of sTNFR may be protective against the effects of TNF-α. *In vivo* administration of sTNFR could protect mice from the lethal effects of TNF-α (23). Physiologically, downregulation of membrane TNFR may even be more important for protection than neutralization of TNF-α. This is demonstrated by the fact that circulating sTNFR-P55 is unable to neutralize the high levels of TNF-α found after e.g. endotoxemia. Whenever extremely high levels of TNF-α are found, such as after treatment with 145-2C11 mAb, sTNFR release does not seem to offer enough protection. Treated mice show several signs of disease, such as hypothermia, hypomotility and diarrhea (24). It is possible that at this high TNF-α to sTNFR ratio, TNF-α is not neutralized but stabilized by the sTNFR. The potential of sTNFR to stabilize TNF-α and thereby prolong TNF-α activity has been shown *in vitro* and *in vivo* (25, 26).

In conclusion, in our model the release of sTNFR-P55 and sTNFR-P75 is independent of TNF-α, as both TNF-inducing and non-TNF-inducing anti-CD3 mAb trigger sTNFR release. Release of sTNFR may be protective against effects of TNF-α, though it could not prevent pathophysiology after 145-2C11 treatment.

Acknowledgment

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CHAPTER 3.4

Fc receptor-binding of anti-CD3 monoclonal antibodies is not essential for immunosuppression, but triggers cytokine-related side effects

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Abstract

A major drawback to the use of OKT3, a mouse anti-CD3 mAb, as immunosuppressive agent is the associated cytokine release syndrome. We used a mouse model to elucidate the properties of anti-CD3 mAb responsible for these cytokine-related side effects. We have previously demonstrated that the hamster anti-CD3 mAb 145-2C11 induced strong *in vivo* cytokine release and morbidity, whereas two rat anti-CD3 mAb 17A2 and KT3 did not. In the current study, we show that *in vitro* mitogenic capacity of soluble anti-CD3 mAb correlates with their *in vivo* induction of side effects. *In vitro* mitogenesis and *in vivo* TNF-α release and weight loss, induced by anti-CD3 mAb, could be inhibited by the anti-FcγR mAb 2.4G2, indicating that FcγR binding of anti-CD3 mAb is responsible for their mitogenic properties and for their induction of side effects. Importantly, the two non-mitogenic rat anti-CD3 mAb were equally capable of suppressing skin allograft rejection as the mitogenic hamster anti-CD3 mAb, suggesting FcγR binding of anti-CD3 mAb is not essential for their immunosuppressive properties. This is supported by our *in vivo* demonstration that administration of 2.4G2 did not interfere with immunosuppression of skin allograft rejection by 145-2C11. These findings suggest that clinical use of non-mitogenic anti-CD3 mAb will result in effective immunosuppression without cytokine-related side effects.

Introduction

The murine anti-CD3 mAb OKT3 is widely used for prevention and treatment of clinical organ allograft rejection because of its known immunosuppressive properties (1, 2). However, treatment with this mAb is accompanied by severe clinical symptoms, such as fever, chills, nausea, vomiting, headache, and diarrhea. Occasionally, more serious reactions, such as pulmonary oedema, aseptic meningitis or seizures are seen (3). These side effects have been related to an early systemic release of several cytokines, such as IL-2, TNF-α, IFN-γ and IL-6 (4, 5), probably produced by activated T lymphocytes (6). This so-called 'cytokine release syndrome' precludes the use of anti-CD3 mAb in autoimmune diseases.

Besides the patients' discomfort, there are other reasons to avoid cytokine release induced by anti-CD3 mAb. It has been shown that rejection treatment with OKT3 results in transiently increased serum creatinine levels. Cytokines are thought to play a role in this OKT3 induced nephrotoxicity (7).

Furthermore, it cannot be excluded that the T cell activation and cytokine release induced by OKT3 enhance production of human anti-mouse antibodies and graft rejection.

Two procedures are currently employed to counteract the cytokine release syndrome of OKT3. The first one involves the use of additional immunosuppressive agents. Both high-dose corticosteroids (8, 9) and pentoxyfylline (10) inhibit cytokine release and the associated clinical syndrome. However, none of the known strategies can completely prevent anti-CD3 mAb induced side effects. An alternative strategy involves the development of new non-activating anti-T cell mAb. Some of these mAb (11-13) induced less cytokine-related side effects, but their immunosuppressive efficacy has not been convincingly demonstrated.

We previously presented a mouse model to elucidate the properties of anti-CD3 mAb responsible for their side effects. The strength of this model is that it is based on three anti-CD3 mAb with similar immunosuppressive capacity in a mouse skin transplantation model (14). Furthermore, all three mAb induced T cell depletion and TCR/CD3 complex modulation (14). One of these mAb, the hamster mAb 145-2C11 (15) triggers strong cytokine release, accompanied by severe physical reactions consisting of piloerection, hypothermia, hypomotility and diarrhea (16, 17). Interestingly, this was not observed with the anti-CD3 mAb 17A2 (rat IgG2b) (18) and KT3 (rat IgG2a) (14, 19).

In the present study, we examined whether differences in epitope recognition and affinity of these three anti-CD3 mAb underly their heterogeneity in capacity to trigger side effects. The role of FcγR was assessed by using an FcγR-blocking mAb, 2.4G2 (20) both *in vitro* and *in vivo*. Our results demonstrate that *in vivo* FcγR binding is responsible for cytokine release and side effects following anti-CD3 mAb treatment. Most importantly, this study provides direct *in vivo* evidence that FcγR-binding of anti-CD3 mAb is not necessary for immunosuppression. Therefore, these data may have important clinical implications.

Table I Monoclonal antibodies used in this study

mAb	Isotype	Specificity	Reference
17A2	rat IgG2b	mouse CD3	18
KT3	rat IgG2a	mouse CD3	19
145-2C11	hamster Ig	mouse CD3	15
2.4G2	rat IgG2b	mouse FcγII/III	20
PH2-4a	rat IgG2b	E.coli β-galactosidase	
PH2-104	rat IgG2a	E.coli β-galactosidase	
GL3	hamster Ig	mouse TCRγδ	23
P1.17	mouse IgG2a	unknown	24

Materials and methods

Monoclonal antibodies

The mAb we used in this study are listed in Table I. We used the anti-mouse CD3 mAb 17A2, rat IgG2b (18), KT3, rat IgG2a (19) and 145-2C11,

a hamster mAb (15). 2.4G2 (20), a rat IgG2b mAb directed against mouse FcγRII/III (21), that also binds FcγRI via its Fc-portion (20), and $F(ab')_2$ fragments of 2.4G2 (kindly provided by dr. M. Daëron, Institut Curie, Paris, France (22)) were used to block FcγR-binding of anti-CD3 mAb. As isotype control mAb we used PH2-4a (rat IgG2b), PH2-104 (rat IgG2a), both directed against E.coli ß-galactosidase (kindly provided by dr. J. van Denderen, Department of Immunology, Rotterdam, The Netherlands), and anti-TCRγδ mAb GL3 (hamster Ig) (23). We used P1.17, a mouse IgG2a Ab of unknown specificity (24), to compete for FcγRI-binding.

MAb were purified from hybridoma culture supernatants by protein G (Pierce Europe, Oud-Beijerland, The Netherlands) affinity chromatography, as previously described (25).

Mice

For all *in vitro* experiments and as skin graft recipients we used C57BL/Ka mice ($H\text{-}2^b$). B6.C-$H\text{-}2^{bm12}$ ($H\text{-}2^{bm12}$) mice were used as skin graft donors. All mice were bred at the Department of Immunology of the Erasmus University Rotterdam. Mice were kept in light-cycled rooms and had access to acidified water and pelleted food ad libitum.

Preparation of cell suspensions

C57BL/Ka mice were killed using carbon dioxide. Spleen and lymph node cell suspensions were prepared and washed in BSS. Lymph node cell suspensions were used for flow cytometry and binding studies. Spleen cell suspensions were used for T cell proliferation assays after Ficoll-Hypaque density gradient centrifugation.

Competition flow cytometry

Twenty-five µl of lymph node cell suspensions ($2x10^7$ cells/ml) were incubated with 100, 10 or 1 µg/ml of either one of the anti-CD3 mAb in 96 well round-bottomed microtiter plates (Nunc, Roskilde, Denmark) for 1 hour on ice. Cells were washed three times with PBS, containing 5% FCS and 20 mM sodium azide, and incubated with optimally titrated FITC-labeled 17A2 mAb. After 1 hour incubation on ice, cells were washed thrice and resuspended in isotonic fluid. All samples were analyzed using a FACScan (Becton Dickinson, San Jose, CA). The mean fluorescence intensities (MFI) of CD3-positive cells were compared to those of cells, incubated with buffer and 17A2-FITC. We calculated % inhibition using the following formula:

$$\% \text{ inhibition} = 100 - \left(\frac{\text{MFI competition samples - MFI unlabeled cells}}{\text{MFI 17A2-FITC labeled cells - MFI unlabeled cells}} \times 100 \right)$$

Binding studies

Binding studies of different anti-CD3 mAb were performed on lymph node suspensions, containing ≥ 65 % CD3-positive cells (analyzed by FACScan). Radio-iodination of mAb was performed with $Na^{125}I$ (Amersham, UK) using Iodogen (Pierce) as catalyst. Lymph node cells (5 x 10^6 in 200 µl) were incubated in duplicate with serial two-fold dilutions (starting of 1 x 10^{-7} M) of ^{125}I-anti-CD3 mAb for 4 hours on ice. After incubation, cells were washed three times with cold PBS. Radioactivity of pellets (specific and non-specific binding) and supernatants (free mAb) was counted using a Packard Autogram 500-C spectrometer. Non-specific binding of labeled mAb was determined by adding a 100-fold excess of unlabeled anti-CD3 mAb to a third series of binding reactions. Radioactivity of these samples was subtracted from the total amount of bound radioactivity to determine specific binding. Binding data were analyzed according to Scatchard (26). To determine the number of binding sites per cell, we corrected for the exact number of $CD3^+$ T cells in lymph node suspensions (measured by FACScan). Two experiments with eight concentrations were performed for each mAb.

Proliferation assays

Spleen cells (2 x 10^5/well) were cultured in 200 µl RPMI 1640 medium (Gibco, Paisley, UK) supplemented with 10% heat-inactivated FCS, L-glutamine (4 mM), ß-mercaptoethanol (5x10^{-5} M), penicillin (100 IU/ml) and streptomycin (50 µg/ml). Cells were incubated with different concentrations of anti-CD3 mAb in round-bottomed tissue culture plates (Falcon). Alternatively, cells were cultured on anti-CD3 mAb coated flat-bottomed tissue culture plates (Falcon). After 48 hours of culture at 37°C in a 5% CO_2 incubator, 0.5 µCi [^{3}H]thymidine was added to each well. 18 hours later cells were harvested and [^{3}H]thymidine incorporation was measured in a liquid scintillation counter. All data are expressed as mean cpm of triplicate cultures.

The role of FcγR in anti-CD3 induced mitogenesis was examined by adding 10 µg/ml 2.4G2 mAb or an equivalent molar amount (6.7 µg/ml) 2.4G2 F(ab')$_2$ fragments to culture wells. In other wells, 10 µg/ml P1.17 (mouse IgG2a) (24) was added to compete for high-affinity FcγRI-binding. In control wells, cells were cultured with isotype control mAb or Con A (5 µg/ml).

In vivo blocking of FcγR

The role of FcγR-binding in TNF-α release, weight loss and immunosuppression induced by 145-2C11 was examined by *in vivo* blockade of FcγR. C57BL/Ka mice were injected i.p. with 250 µg of 2.4G2 mAb 12 to 18 hours before injection of 10 µg 145-2C11. Our primary goal was to achieve short term blocking of FcγR, since the effects of 10 µg of 145-2C11 can be detected early after injection (14, 27). This dose of 2.4G2 has previously been shown to inhibit FcγR-mediated sequestration of immune complexes for at least 24 hours (28).

Detection of serum TNF-α

For induction of TNF-α release, 10 μg 145-2C11 mAb were injected i.v. The role of FcγR in TNF-α release was studied by 2.4G2 administration, 12 to 18 hours before 145-2C11 mAb injection. Control mice were injected with 2.4G2 alone or 0.5 ml BSS. At 1, 2, 4, 8, and 24 hours after 145-2C11 mAb injection, 3 or 5 mice per group were killed using carbon dioxide. Blood was obtained via heart puncture in sterile tubes and left to clot overnight at 4°C. After centrifugation, serum samples were aliquotted and stored at -70°C.

Serum TNF-α levels were determined by a cytotoxicity assay on WEHI-164 clone 13 cells (29). Briefly, trypsinized WEHI-164 cells were seeded in flat-bottomed tissue culture plates (Falcon) at 1 x 10^4 cells/100 μl complete medium. After overnight cell adherence at 37°C, serum samples and actinomycin D (1 μg/ml) were added and incubated for 24 hours at 37°C. Cytotoxic activity was measured with the MTT assay (30).

Measurement of body weight

A group of six C57BL/Ka mice was injected i.p. with 10 μg 145-2C11. Other groups received 2.4G2 mAb before 145-2C11 injection or 2.4G2 mAb alone. Body weight was measured daily late in the afternoon. In order to study changes in body weight longitudinally, all mice were tagged. Changes in weight are expressed as percentage of body weight prior to treatment.

Skin grafting

The immunosuppressive effect of 145-2C11 mAb on MHC class II disparate skin allograft rejection was studied by administration of 10 μg mAb the day before grafting. FcγR-blockade was achieved by 2.4G2 mAb injection 12 to 18 hours before 145-2C11 treatment. Control mice received 2.4G2 mAb alone or 0.5 ml BSS.

Tail skin of B6.C-H-2^{bm12} donors was grafted to the dorsal thorax of C57BL/Ka recipients using a modification of the method of Billingham and Medawar (31). Briefly, donor skin was placed on the graft bed, attached at the four corners with Histoacryl (B. Braun, Melsungen, Germany), and covered with a thin film of Nobecutan (Astra Pharmaceutica, Rijswijk, The Netherlands). Mice were bandaged with a paraffin gauze and a plaster cast. After 8 days, the bandage was removed and grafts were scored daily for signs of rejection, such as scab formation, necrosis or loss of hair. Grafts were considered rejected when no viable donor skin was detectable.

Statistical analyses

Induction of proliferation, TNF-α release and weight loss by the three anti-CD3 mAb, was compared via analysis of variance (ANOVA). If ANOVA revealed significant differences, the groups were compared using the Student's *t*-test. Graft survival of groups was compared by Mann Whitney tests. Values of $p < 0.05$ were considered significant.

Results

Binding characteristics of anti-CD3 mAb.

Since differences in epitope recognition or binding affinity by the three anti-CD3 mAb (145-2C11, 17A2, KT3) may underly their differential capacity to induce cytokine-related side effects (14), we examined whether the three anti-CD3 mAb could cross-compete in a FACScan competition assay. Table II shows that incubation of lymph node cells with 100 μg/ml of all three anti-CD3 mAb resulted in complete inhibition of subsequent binding of FITC-labeled 17A2. Though the degree of inhibition slightly differred between the anti-CD3 mAb, all three unlabeled mAb competed effectively for binding of FITC-labeled 17A2 in a dose-dependent manner.

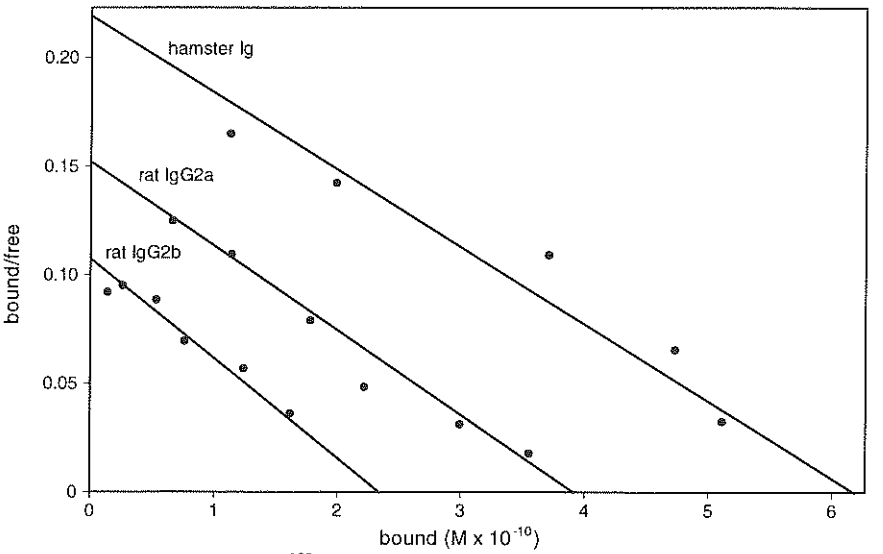

Figure 1 Scatchard analysis of binding of ^{125}I-labeled anti-CD3 mAb to C57BL/Ka lymph node cells. Cells were incubated with different concentrations of ^{125}I-labeled rat IgG2b, rat IgG2a or hamster anti-CD3 mAb. At each mAb concentration the amount of cell bound and free mAb was determined. Data represent the mean of duplicate determinations from one representative experiment.

Analysis according to Scatchard of binding experiments, using ^{125}I-labeled anti-CD3 mAb, resulted in linear Scatchard plots (Fig. 1), suggesting that all three mAb bind a single class of binding sites. The mAb displayed a comparably high affinity with a Kd ranging from 2.5 to 3 x 10^{-9} M. The number of binding sites per cell, however, was different amongst the three mAb. The rat IgG2b mAb 17A2 had ≈10,000 binding sites, KT3 (rat IgG2a) ≈17,000, and 145-2C11 (hamster Ig) ≈20,000 binding sites. These data show that the three anti-CD3 mAb bind the same or overlapping epitopes with comparable affinity.

Table II Competition binding of anti-CD3 monoclonal antibodies

	% Inhibition of 17A2-FITC binding		
Unlabeled mAb (μg/ml)	17A2	KT3	145-2C11
100	100	100	100
10	100	16.1±3.4	34.3±1.2
1	10	0	0

C57BL/Ka lymph node cells were incubated with different concentrations of unlabeled 17A2, KT3 and 145-2C11 mAb. After washing, cells were incubated with optimally titrated 17A2-FITC. The mean fluorescence intensities of cells incubated with unlabeled mAb and subsequently with 17A2-FITC were compared to mean fluorescence intensities of cells incubated with buffer and 17A2-FITC. The results represent mean % inhibition ± SD of three independent experiments.

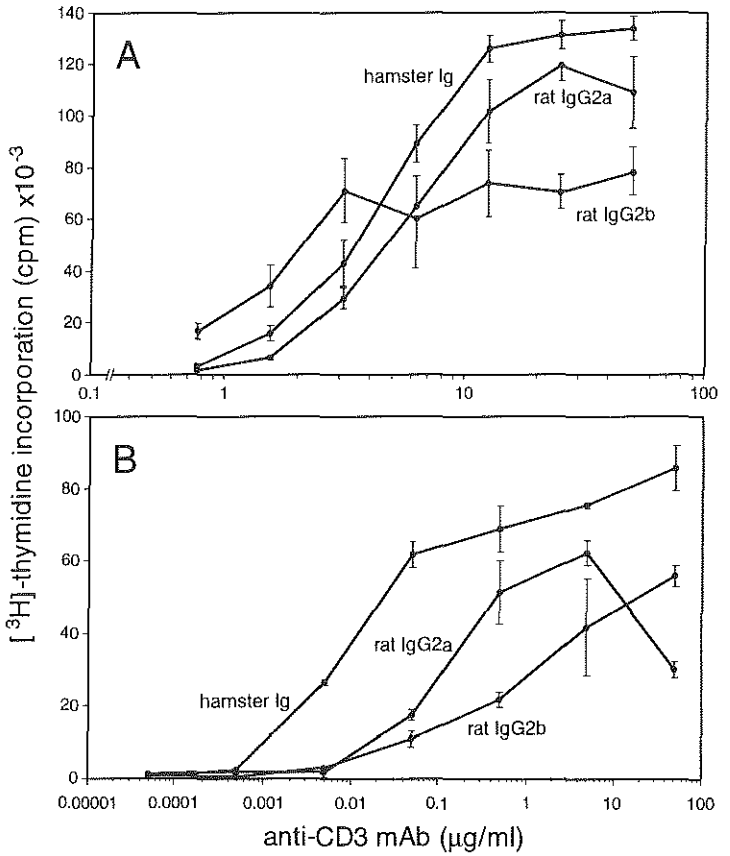

Figure 2 Proliferation induced by anti-CD3 mAb. C57BL/Ka spleen cells were stimulated with different concentrations of immobilized (A) or soluble (B) rat IgG2b, rat IgG2a or hamster anti-CD3 mAb. After 48 hours, 0.5 μCi/well of [^{3}H] thymidine was added for an additional 18 hours of culture. The data represent mean cpm of triplicate cultures ± SD. Experiment was repeated three times, yielding essentially identical results.

T lymphocyte proliferation induced by immobilized and soluble anti-CD3 mAb

In order to induce T cell proliferation, anti-CD3 mAb need to be crosslinked either via FcγR$^+$ cells or through a solid phase. The use of immobilized anti-CD3 mAb enabled the study of their mitogenic properties independent of their FcγR binding capacity (32). Figure 2A shows that, using immobilized anti-CD3 mAb, rat IgG2a and hamster mAb induced comparable T cell proliferation. The proliferation curve of rat IgG2b anti-CD3 mAb was significantly different from that of the two other anti-CD3 mAb. At higher concentrations, 17A2 (rat IgG2b) was significantly less mitogenic than the other mAb, whereas at lower concentrations this mAb was significantly more mitogenic.

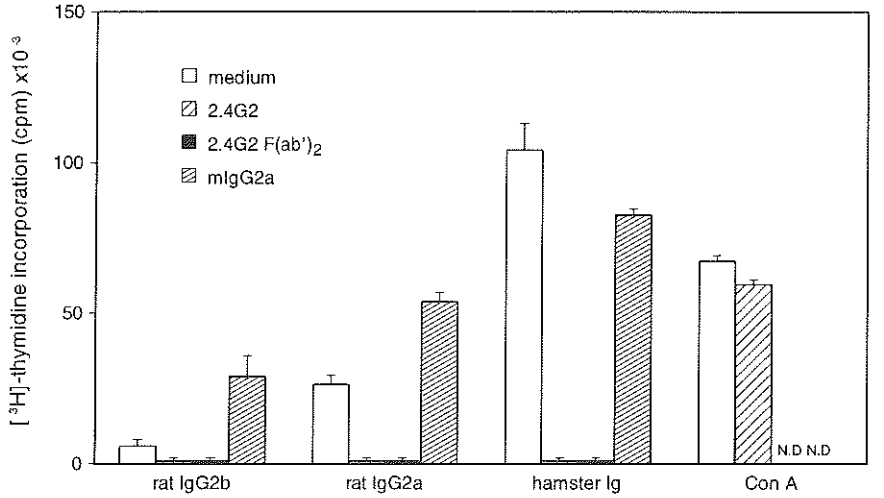

Figure 3 Effect of FcγR-blocking on proliferation induced by soluble anti-CD3 mAb or Con A. C57BL/Ka spleen cells were cultured in medium alone or medium supplemented with 2.4G2 mAb (10 μg/ml), F(ab')$_2$ fragments of 2.4G2 (6.7 μg/ml) or mIgG2a (10μg/ml). T cell mitogenesis was induced by addition of 10 μg/ml rat IgG2b, rat IgG2a or hamster anti-CD3 mAb. After 48 hours, 0.5 μCi/well of [^{3}H]thymidine was added for an additional 18 hours of culture. Data represent mean cpm of triplicate cultures ± SD. N.D = not determined. Similar results were obtained in a second experiment (not shown).

The capacity of soluble anti-CD3 mAb to induce T cell proliferation has been shown to correlate with their extent of interaction with FcγR (33). Soluble rat IgG2b and rat IgG2a anti-CD3 mAb induced comparable T cell proliferations (Fig. 2B). To induce T cell proliferation, high concentrations (consistently 50-100 times higher than 145-2C11 mAb) of rat anti-CD3 mAb were needed. Neither of the isotype control mAb (PH2-4a, PH2-104 and GL3) induced proliferation (data not shown).

Addition of FcγR-blocking mAb (10 μg/ml 2.4G2) completely inhibited proliferation, induced by all three anti-CD3 mAb in soluble form (Fig. 3). This inhibition was specific, since addition of 10 μg/ml of an isotype control mAb (PH2-4a) had no effect on anti-CD3 mAb induced proliferation (data not

shown) and Con A induced proliferation was not affected by 2.4G2 mAb (Fig. 3). Highly purified $F(ab')_2$ fragments of 2.4G2, that only block FcγRII/III binding, had a similar inhibitory effect as intact 2.4G2 (Fig.3). In addition, competition for FcγRI binding by adding 10 μg/ml P1.17 (mouse IgG2a), did not inhibit anti-CD3 mAb induced T cell proliferation (Fig. 3). Thus, mitogenesis of all three anti-CD3 mAb involved FcγRII/III molecules. The ability of low doses of hamster mAb to induce significant T cell proliferation suggests that this mAb has a high affinity for either FcγRII or FcγRIII.

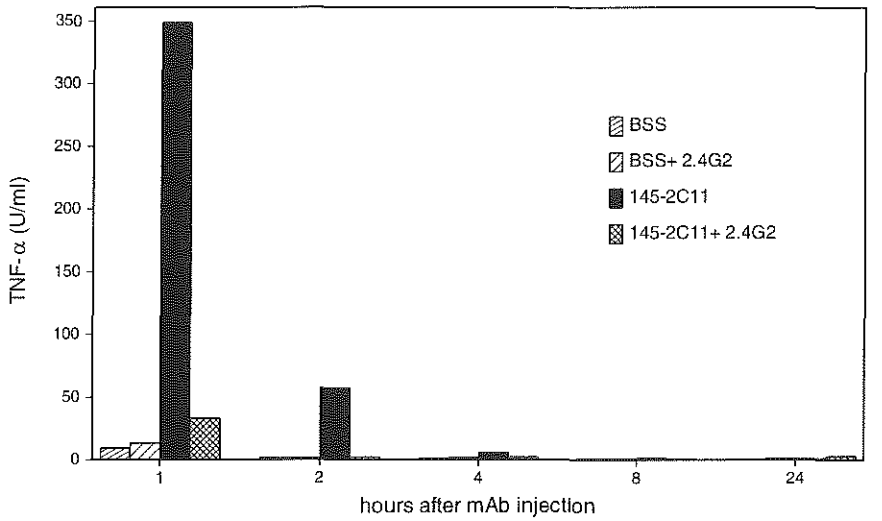

Figure 4 Effect of in vivo anti-FcγR mAb on TNF-α release triggered by 145-2C11 mAb. C57BL/Ka mice received either 10 μg 145-2C11 mAb i.v. (t=0 hr), 250 μg 2.4G2 mAb (t=-12 to -18 hrs) or a combination of 145-2C11 and 2.4G2 mAb. Control mice received 0.5 ml BSS. 1, 2, 4, 8 and 24 hours after treatment, 3 (2.4G2 and BSS groups) or 5 (other groups) mice per group were killed for serum detection of TNF-α. This experiment was performed twice. Results represent the mean serum TNF-α levels (SEM ≤ 15%) from one representative experiment.

In vivo effect of FcγR-blocking mAb on TNF-α release triggered by 145-2C11

Of the three anti-CD3 mAb, only 145-2C11 mAb induced cytokine related side effects (14). TNF-α has been shown to play a crucial role in these side effects (14, 17). *In vivo* administration of 10 μg hamster anti-CD3 mAb, 145-2C11, induced a strong increase in serum TNF-α levels (Fig. 4).

The highest TNF-α serum levels were measured 1 hour after 145-2C11 injection. To determine the role of FcγR in this TNF-α release, we temporarily blocked FcγR by giving 250 μg 2.4G2 mAb 12 to 18 hours before 145-2C11 injection. Treatment with 2.4G2 mAb completely abrogated the rise of serum TNF-α levels. Anti-FcγR mAb itself did not trigger TNF-α release. These results show that binding of 145-2C11 mAb to FcγR bearing cells is necessary for *in vivo* TNF-α release.

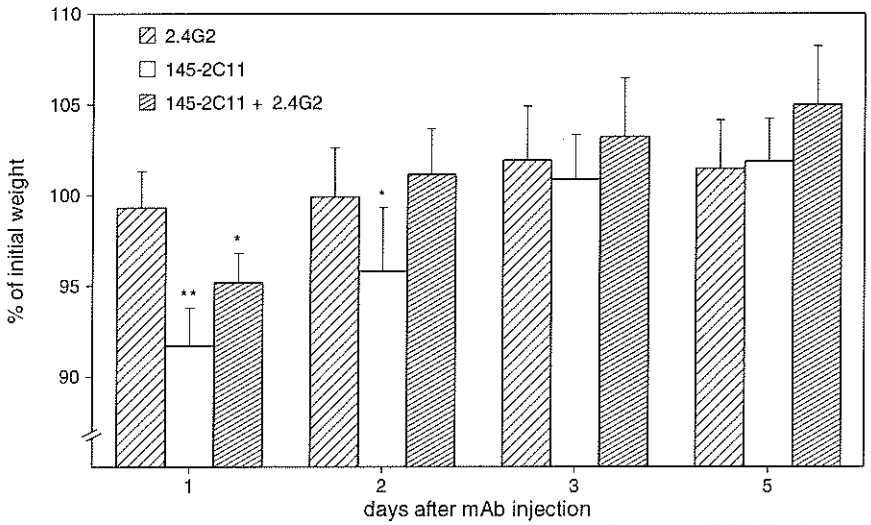

Figure 5 Effect of in vivo FcγR-blockade on weight loss due to treatment with 145-2C11 mAb. C57BL/Ka mice received either 10 μg 145-2C11 mAb i.p. (t = 0 hr), 250 μg 2.4G2 mAb (t = -12 to -18 hrs) or a combination of 145-2C11 and 2.4G2 mAb. Body weight was measured daily. Data are expressed as mean percentage of initial weight ± SD of six mice. * $p < 0.05$, ** $p < 0.001$ as compared to the group treated with 2.4G2 mAb alone.

Effect of in vivo FcγR blockade on weight loss due to 145-2C11

Treatment with 10 μg 145-2C11 mAb resulted in a significant weight loss (Fig. 5). The mice showed signs of morbidity, such as hypomotility and piloerection. 2.4G2 mAb-pretreated mice showed significantly less weight loss ($p < 0.05$) and regained their initial weight two days after 145-2C11 mAb injection, compared to three days in the group treated with 145-2C11 alone. In addition, signs of illness were absent in these mice. No changes in weight were seen in the group that only received 250 μg 2.4G2. These data show that *in vivo* blocking of FcγR-binding of 145-2C11 mAb did not only prevent TNF-α release, but also abrogated cytokine-related illness reflected in inhibition of weight loss.

Effect of in vivo FcγR blocking on 145-2C11-induced immunosuppression of skin allograft rejection

Since FcγR-blockade significantly inhibited the 'cytokine release syndrome' of 145-2C11 mAb, it was essential to determine whether this treatment interferes with the immunosuppressive effect of this anti-CD3 mAb. Therefore, we studied the effect of the different treatment schedules on skin allograft rejection. The day after 145-2C11 injection, C57BL/Ka mice received an MHC class II disparate B6.C-H-2^{bm12} skin graft. As shown in Figure 6, a single dose of 10 μg of 145-2C11 significantly prolonged skin allograft survival, as compared to the untreated control group. The administration of 250 μg 2.4G2 mAb had no effect on the immunosuppression,

induced by 145-2C11. Furthermore, the group that received 2.4G2 mAb alone showed the same graft survival as the untreated control group. These results show that FcγR-mediated binding of anti-CD3 mAb is not essential for immunosuppression.

Discussion

Treatment and prevention of organ allograft rejection using OKT3 has been shown to be very successful (1, 2). However, this treatment is complicated by the OKT3-induced cytokine release syndrome. In this study, we used a mouse model with three anti-CD3 mAb, to characterize the properties of anti-CD3 mAb that are responsible for induction of cytokine-related side effects. Since all three mAb have been shown to be equally immunosuppresive (14), this model allows the study of T cell activating capacities of anti-CD3 mAb independent of their immunosuppressive properties.

Differences in T cell activation by anti-CD3 mAb might be caused by differences in epitope recognition or affinity of the mAb (33, 34). Our results show that the three anti-CD3 mAb recognize similar or closely related epitopes (Table II). This is in agreement with earlier studies (18, 19) showing cross-competition between 17A2, KT3 and 145-2C11 mAb. The hamster mAb 145-2C11 has been shown to be specific for the CD3 epsilon chain (15). The anti-CD3 mAb bound, furthermore, with similar affinity (Kd of 2.5 - 3 x 10^{-9} M) to lymph node cells (Fig. 1). We found a difference in the number of binding sites of the three anti-CD3 mAb, with 17A2 (rat IgG2b) binding ≈10,000 sites per cell, while KT3 (rat IgG2a) bound ≈17,000 and 145-2C11 (hamster Ig) ≈20,000 sites. This difference in binding site number is not reflected in *in vivo* effects of anti-CD3 mAb, as 17A2 and KT3 induced the same degree of T cell depletion, TCR/CD3 modulation, immunosuppression of skin allograft rejection and cytokine release *in vivo* (14).

In vitro mitogenesis and T cell activation by anti-CD3 mAb is dependent on interaction of these mAb with FcγR (35). The finding that 145-2C11 $F(ab')_2$ fragments induce less cytokine release and morbidity in mice than intact 145-2C11 mAb suggests that FcγR binding is also involved in *in vivo* T cell activation (17, 36), though interpretations may be influenced by the short half-life of $F(ab')_2$ fragments. Our results demonstrate in a mouse model that intact non-mitogenic anti-CD3 mAb induced less cytokine-related side effects than mitogenic mAb (14). This difference in mitogenesis is due to differences in FcR-binding capacities of the anti-CD3 mAb, since in immobilized form all three mAb induced T cell proliferation. The mitogenic anti-CD3 mAb 145-2C11 displayed a higher affinity for FcγRII or FcγRIII than the non-mitogenic mAb 17A2 and KT3. The first direct evidence for the role of FcγR binding in the cytokine related side effects of anti-CD3 mAb is provided by our finding that *in vivo* blocking of FcγR binding resulted in complete inhibition of TNF-α release by 145-2C11 mAb and significant inhibition of the weight loss induced by 145-2C11. Some side effects of

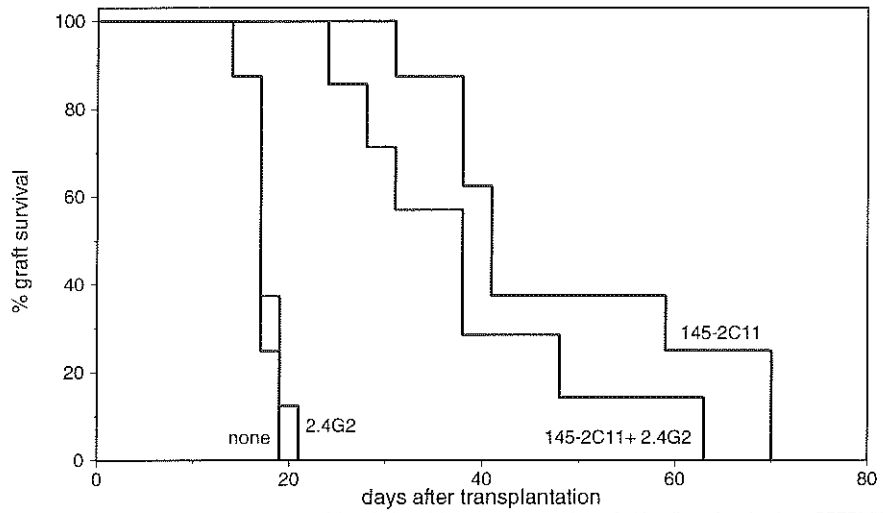

Figure 6 Effect of in vivo FcγR-blockade on 145-2C11 induced immunosuppression of skin allograft rejection. C57BL/Ka mice received an MHC class II disparate B6.C-H-2^{bm12} skin graft. The day before grafting, the mice were treated with 10 µg 145-2C11 (n=8). One group received 250 µg 2.4G2 12 to 18 hours before 145-2C11 administration (n=7) and another received 2.4G2 mAb alone (n=8). Control mice (n=8) received no treatment.

145-2C11 are probably FcγR independent, as even administration of 1 mg 2.4G2 mAb could not prevent transient weight loss (data not shown). As has been shown for OKT3, complement activation might contribute to the 145-2C11-associated side effects (37).

Our previous study showed that the non-mitogenic rat anti-CD3 mAb 17A2 and KT3 are equally effective as the mitogenic 145-2C11 mAb in suppressing skin allograft rejection (14). That FcγR binding of anti-CD3 mAb is not essential for their immunosuppressive properties, is further shown by our finding that *in vivo* blocking of FcγR binding has no effect on the suppression of skin allograft rejection by 145-2C11 mAb. FACScan analysis has shown that treatment with 2.4G2 did not result in depletion of monocytes or macrophages (data not shown), suggesting the effect of 2.4G2 is due to FcγR blockade. Most of the previously published *in vivo* data on the role of FcR binding of anti-CD3 mAb in immunosuppression are based on the use of $F(ab')_2$ fragments of 145-2C11, showing that these $F(ab')_2$ fragments are equally immunosuppressive as intact 145-2C11 mAb. This has been shown in a skin transplantation model in mice and in insulin-dependent diabetes mellitus in mice (38-40). However, the use of $F(ab')_2$ fragments is always compromised by the possible presence of small amounts of contaminating intact mAb. In addition, establishment of efficacy of non-mitogenic anti-CD3 mAb in the clinical setting is time consuming and has ethical limitations. Therefore, our findings concerning immunosuppressive capacity of non-mitogenic anti-CD3 mAb are of great importance.

The immunosuppressive mechanisms of our non-mitogenic anti-CD3 mAb and $F(ab')_2$ fragments of 145-2C11 are probably the same as those of mitogenic anti-CD3 mAb, including T cell depletion, blocking and modulation of the TCR/CD3 complex and the induction of T cell anergy (41-43). It has been suggested that some degree of T cell activation is needed for anergy induction (44). It would be interesting to investigate the signaling capacity of our rat anti-CD3 mAb.

Together, our data suggest that the use of non-mitogenic anti-CD3 mAb would imply the induction of fewer cytokine related side effects, while retaining effective immunosuppression. Though $F(ab')_2$ fragments have been proposed to be useful in the clinical situation, a major drawback of these fragments is their extremely short half-life and thus the necessity of frequent administration. In addition, the production of these fragments requires great care, since even minimal contamination with intact mAb may induce significant T cell activation (35). The use of whole non-mitogenic anti-CD3 mAb would therefore be much more preferable. An IgA switch variant of a murine anti-CD3 mAb, unable to interact with human FcγR, induces significantly less cytokine release and side effects in chimpanzees (12). To date, the number of patients treated with these mAb are insufficient to warrant conclusions on their immunosuppressive efficacy (45). Alegre *et al.* showed that mutations in the Fc portion of a "humanized" OKT3 resulted in a reduced FcγR binding of this mAb. *In vitro*, this mutated mAb also induced significantly less T cell activation than the parental mAb, but displayed the same immunosuppressive properties (46). Recently, this mutated mAb has been shown to be equally effective in suppressing human skin graft rejection as the parental anti-CD3 mAb and OKT3, in SCID mice reconstituted with human splenocytes (47).

Summarizing, interaction of the hamster anti-CD3 mAb 145-2C11 with FcγRII/III is responsible for its cytokine related side effects. From comparison of this mAb with two other anti-CD3 mAb that do not induce any morbidity, we conclude that *in vitro* mitogenesis correlates with *in vivo* cytokine release and morbidity. This finding is relevant for the development of new anti-CD3 mAb. Furthermore, FcγR binding is not essential for immunosuppression by anti-CD3 mAb. Together these data suggest that non-mitogenic anti-CD3 mAb are promising immunosuppressive agents in clinical tissue and organ transplantation and that they may also be useful for treatment of autoimmune diseases.

Acknowledgment

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Chapter 4

General discussion

General discussion

In search of optimal immunosuppressive regimens for prevention and treatment of transplant rejection our study has focussed on cytokine-directed and T lymphocyte-directed strategies. We studied the effect of these strategies on skin allograft rejection in mice.

Cytokine-directed strategies

In chapter 2.1 many cytokines are described that may be involved in transplant rejection. In order to inhibit graft rejection, cytokine neutralizing agents have been tested, such as mAb and soluble cytokine receptors. In chapter 2.2 we demonstrate that mAb against mouse IFN-γ can prolong skin graft survival in an MHC class II disparate strain combination. However, our study (chapter 2.2) and many other studies (reviewed in chapter 2.1) using cytokine neutralizing agents show that these cytokine-directed strategies are not very effective in prolonging graft survival. The complexity of the cytokine network offers some explanations for the ineffectiveness of these strategies. Cytokines act on short range and systemically administered agents may not reach the site where these cytokines mediate their effect. Furthermore, the cytokine system is extremely redundant, i.e. one function can be performed by several cytokines. Thus, neutralizing one cytokine makes another take over. Besides the lack of efficiency, the lack of specificity is one of the disadvantages of these cytokine-directed treatments. Others are their short-lived effectiveness, the need of enormous quantities of mAb or soluble receptors and last but not least the fact that these strategies may have the opposite effect to the one intended. This last-mentioned phenomenon is due to the capacity of some mAb and soluble cytokine receptors to act as cytokine carriers and prolong the half-life and consequently the effect of their target cytokines (1). We conclude that treatment directed against one cytokine may at best result in short-term inhibition of its effects.

Another cytokine-directed strategy involves the creation of a cytokine micro-environment in favor of development of Th2 cells, which could induce non-responsiveness of alloreactive Th1 cells. In chapter 2.2 we tried to do so by treating mice with the Th2 cytokines IL-4 and IL-10. Even in combination with mAb against IFN-γ (Th1), this treatment did not induce long-term graft survival. In other alloresponse models, treatment with IL-4 or IL-10 could only moderately inhibit heart graft rejection (M. Dallman, personal communication) or enlargement of alloreactive lymph nodes (2) and no tolerance could be induced. Ineffectiveness of Th2 promoting cytokine treatment may have several reasons. First, systemic administration of cytokines may fail to modulate the intragraft environment. Cytokines may not reach the graft, may be consumed or neutralized by e.g. soluble cytokine receptors. Secondly, assuming Th2 cell development is enhanced by administration of Th2 cytokines, these cells may not be capable of inhibiting graft rejection. Possibly,

allospecific tolerance is only based on Th1 cell anergy and detection of Th2 cytokines is just an epiphenomenon. Alternatively, Th1 cell anergy is required in order for Th2 cells to maintain this state of allospecific tolerance. Thus, systemic Th2 cytokine treatment does not seem capable of inducing long-term non-responsiveness and in addition may have side effects, such as stimulation of antibody-formation and inhibiton of inflammatory responses against pathogens. These data, however, do not exclude that the Th1-Th2 paradigm plays a role in transplant rejection and acceptance and that other ways may be found to effectively influence this balance.

Use of anti-CD3 mAb for treatment of transplant rejection

In clinical and experimental transplantation anti-CD3 mAb induce effective immunosuppression. However, their use is complicated by cytokine-related side effects and the production of antibodies against the anti-CD3 mAb (xenosensitization).

Side effects of anti-CD3 mAb treatment

In chapter 3.4 we show that the capacity of anti-CD3 mAb to induce *in vitro* T cell proliferation (mitogenesis) and *in vivo* cytokine-related side effects correlates with their affinity for FcγR. Pretreatment of the mice with anti-FcγR mAb 2.4G2 could prevent TNF-α release and weight loss induced by 145-2C11 mAb. These results provide direct evidence for the role of FcγR binding in cytokine release and the associated morbidity. FcR-binding of Fc portions of anti-CD3 mAb can lead to cross-linking of CD3 molecules, causing T cell activation and cytokine release. We have shown that non-mitogenic anti-CD3 mAb are capable of inducing effective immunosuppression without cytokine-related side effects (chapters 3.1 and 3.2). In addition, blocking of FcγR by 2.4G2 mAb treatment had no effect on anti-CD3 mAb induced immunosuppression of skin graft rejection (chapter 3.4).

In man, xenosensitization is a major complication of treatment with murine mAb, since human anti-mouse antibodies may enhance mAb clearance and abrogate their immunosuppressive efficacy. Coadministration of immunosuppressive agents, such as azathioprine or cyclosporin A, decreases the sensitization frequency (3). It has been suggested that anti-CD3 mAb-induced production of cytokines, in particular IL-4, enhances the development of anti-antibodies (4). It is conceivable that treatment with non-mitogenic anti-CD3 mAb will lead to reduced sensitization. The humoral response to mitogenic anti-CD3 mAb in mice has been shown to be stronger than to non-mitogenic $F(ab')_2$ fragments of these mAb (5). However, it cannot be excluded that this difference is caused by their difference in half-life. It would be interesting to study the antibody response to the non-mitogenic anti-CD3 mAb that were used in our study. A promising strategy to minimize xenosensitization is the development of "humanized" antibodies (6). These antibodies consist of murine complementarity determining regions (CDR), i.e.

antigen-binding sites build into human antibodies. For instance, "humanized" mAb with CDR of OKT3 have been generated (7). Moreover, by two amino-acid substitutions in the C_{H2} region of this "humanized" OKT3, a mAb has been created with significantly lower affinity for FcR (8). This non-mitogenic mAb has all properties to induce effective immunosuppression with minimal toxicity.

As FcR binding is not essential for immunosuppression and is responsible for cytokine-related side effects (chapter 3.4), the use of $F(ab')_2$ fragments of anti-CD3 mAb has to be considered. However, $F(ab')_2$ fragments have two major disadvantages over non-mitogenic anti-CD3 mAb. First, due to their extremely short serum half-life, frequent administrations would be required to achieve the same level of cell coating or modulation found after administration of intact anti-CD3 mAb. Secondly, preparation of $F(ab')_2$ fragments requires great care, as contamination with minimal amounts of intact anti-CD3 mAb can result in significant T cell activation. Thus, for clinical immunosuppression non-mitogenic anti-CD3 mAb are preferable to $F(ab')_2$ fragments of mitogenic anti-CD3 mAb.

Immunosuppression by anti-CD3 mAb

Non-mitogenic anti-CD3 mAb are capable of inducing effective immunosuppression with minimal toxicity (chapters 3.1 and 3.2). They may prove to be promising immunosuppressive agents for clinical use. Whether anti-CD3 mAb are only capable of inducing generalized (non-specific) immunosuppression or whether they can also induce allospecific tolerance is unclear. Several effects of anti-CD3 mAb may contribute to both non-specific, i.e. concerning all T cells, and specific, i.e. concerning alloreactive T cells only, immunosuppression.

Short-term non-specific immunosuppression results from T cell depletion and blocking of the interaction of TCR with alloantigens on APC by coating and subsequent modulation of the TCR/CD3 complex. In chapters 3.1 and 3.2 we showed that both non-mitogenic and mitogenic anti-CD3 mAb induced T cell depletion and modulation of the TCR/CD3 complex.

Long-term non-specific immunosuppression has been found after recovery of T cell number and TCR/CD3 expression (9). This is probably based on the capacity of anti-CD3 mAb to induce intracellular signal transduction as TCR signaling without costimulatory signaling results in T cell anergy (10). In addition, chronic anti-CD3 mAb stimulation *in vitro* resulted in T cell anergy whether or not costimulatory signals were provided (11).

For the induction of long-term allospecific tolerance, interaction of allospecific TCR with alloMHC is a prerequisite. In chapter 3.1 we show that in a small percentage of the anti-CD3 mAb treated mice allospecific tolerance was achieved. Anti-CD3 mAb may contribute to allospecific tolerance induction by simply delaying TCR-alloantigen interaction. It has been shown that after approximately two weeks donor APC in the graft have disappeared and are replaced by recipient APC. These recipient APC may present al-

loMHC peptides in the context of self MHC to T lymphocytes. It has been suggested that this indirect presentation of allopeptides may lead to nonresponsiveness of T cells (12). In addition, TCR signaling by anti-CD3 mAb may change TCR structure or function, which may lead to an altered reaction to subsequent interaction with alloantigens, resulting in T cell anergy (13). Furthermore, anti-CD3 mAb might skew alloreactive T cells to Th2 cell development. Aside from the cytokine micro-environment, the antigen density on the APC can direct Th cell development (14). Low antigen density preferably induces Th2 cell development and high antigen density induces Th1 cell development. Anti-CD3 mAb treatment leads to TCR/CD3 complex modulation that lowers the number of TCR-alloantigen interactions, which might favor Th2 cell development. These alloreactive Th2 cells can suppress alloreactive Th1 cells, which might lead to graft acceptance.

Future prospects

Protocols based on anti-CD3 mAb, leading to effective suppression of graft rejection and possibly to graft acceptance, should consist of administration of a dose of non-mitogenic, non-FcR-binding anti-CD3 mAb that results in coating and modulation of TCR/CD3 complexes on all T cells. This treatment should begin on the day of transplantation. It is not clear yet how long this treatment has to be continued. After the period of non-specific immunosuppression, lowering of anti-CD3 mAb dose should enable contact between TCR and alloantigens and redirect the alloresponse to a state of allospecific anergy and/or suppression. Possible interference of other immunosuppressive agents with anti-CD3 mAb induced immunosuppression should be investigated. Both non-mitogenic and mitogenic anti-CD3 mAb are capable of inducing T cell depletion, coating and modulation of the TCR/CD3 complex (chapters 3.1 and 3.2) and signal transduction (15). Whether both of them are capable of inducing allospecific tolerance needs further study. Such studies will contribute to the development of clinical protocols leading to effective immunosuppression and possibly graft acceptance.

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Summary

Summary

Many different diseases may lead to severe dysfunction of organs, such as kidney or heart. Nowadays, organ transplantation is an established therapy in patients suffering from organ failure. However, incompatibility of donor and recipient Major Histocompatibility Complex (MHC) proteins generally leads to rejection of the transplanted organ. Rejection is prevented by treating the recipient with strong immunosuppressive agents. However, these agents also suppress immune responses against pathogens and tumor cells and therefore cause an increased incidence of infections and malignancies among transplant recipients. Furthermore, many of these agents have other side effects, such as nephrotoxicity in the case of cyclosporin A.

In our study, we investigated the effectiveness and side effects of several immunosuppressive strategies. For this purpose we used a skin transplantation model in mice. The immunosuppressive therapies we studied were either directed against cytokines or against T lymphocytes. T lymphocytes play a key role in the induction of transplant rejection, whereas cytokines, i.e. soluble factors produced by T lymphocytes and many other cell types, are important for communication between the cells involved in rejection. Two subsets of T helper (Th) cells are believed to be differentially involved in transplant rejection. Th1 cells, producing a.o. IL-2 and IFN-γ, are believed to induce rejection, whereas Th2 cells, producing a.o. IL-4 and IL-10, can suppress Th1 cell function and may therefore inhibit graft rejection and even induce transplant acceptance.

In chapter 2.1, we review studies on the role of cytokines in clinical and experimental transplantation. In clinical transplantation, serum levels of various cytokines have been used for monitoring graft function. Increased levels of for instance IL-2, IL-6 or TNF-α have been found during transplant rejection. However, as these levels are also increased during infection, measurements of these cytokines cannot be used to reliably predict rejection. Using animal models, intragraft detection of cytokine mRNA has shown that expression of IL-2, IL-4 and IFN-γ mRNA is related to graft rejection. During transplant acceptance, decreased expression of IL-2 and IFN-γ (Th1) mRNA was found, whereas IL-4 and IL-10 (Th2) mRNA expression was stable or increased as compared to mRNA expression in rejecting grafts. In experimental transplantation, several cytokine-directed strategies, such as anti-cytokine monoclonal antibodies (mAb) or soluble cytokine receptors, have been tested for their immunosuppressive effectiveness. However, none of these strategies was very effective in prolonging allograft survival. Other strategies aimed at changing the Th subset balance. However, the few studies attempting Th2 "skewing" have not been successful in inhibiting graft rejection. In chapter 2.2 we show that treatment of mice with anti-IFN-γ mAb prolonged skin graft survival with about 15 days as compared to untreated control mice. Treatment with Th2 cytokines IL-4 and IL-10 had no effect on skin graft survival. T lymphocyte-directed strategies, using anti-CD4 mAb or anti-

CD3 mAb were able to prolong skin graft survival with about 25 days and about 70 days, respectively. From these data we conclude that in our model T lymphocyte directed strategies are more effective than cytokine directed strategies in inhibiting graft rejection and that anti-CD3 mAb cause strong immunosuppression.

In chapter 3 we focus on anti-CD3 mAb treatment of skin allograft rejection. OKT3, a mouse anti-CD3 mAb, is a potent immunosuppressive agent used in humans to prevent and treat organ graft rejection. However, administration of OKT3 is accompanied by severe side effects, which are related to systemic cytokine release. In mice a similar anti-CD3 mAb has been described. The hamster anti-CD3 mAb 145-2C11 can suppress skin allograft rejection in mice, but also induces cytokine release that is accompanied with severe morbidity. In chapters 3.1 and 3.2 we show that two rat anti-CD3 mAb, 17A2 and KT3, are equally capable of suppressing skin allograft rejection as 145-2C11 mAb. Treatment with 17A2 mAb even induced long term graft survival in a small percentage of treated mice and these mice also accepted a second skin graft, while rejecting a third party graft (chapter 3.1). All three anti-CD3 mAb caused T cell depletion and modulation of the TCR/CD3 complex. In contrast to 145-2C11 mAb, 17A2 and KT3 mAb did not cause any morbidity in mice. We studied release of TNF-α, IFN-γ and IL-6 after injection of the three anti-CD3 mAb and found that TNF-α and IFN-γ release were related to anti-CD3 mAb induced side effects (chapters 3.1 and 3.2). In chapter 3.3 we studied serum soluble TNF receptor levels (sTNFR) after anti-CD3 mAb treatment. sTNFR are thought to be protective against TNF-α effects. As only 145-2C11 mAb induced TNF-α release and all three anti-CD3 mAb induced release of sTNFR-P55 and P75, we conclude that in our model sTNFR release is independent of TNF-α. In chapter 3.4 we studied the basis of the anti-CD3 mAb induced side effects. We show that interaction of anti-CD3 mAb with Fcγ receptors (FcγR) is responsible for mitogenesis, TNF-α release and the related morbidity. Furthermore, we demonstrate that FcγR binding is not essential for immunosuppression by anti-CD3 mAb.

From the studies presented in this thesis we conclude that T lymphocyte-directed treatment of transplant rejection is more effective than cytokine-directed treatment. Anti-CD3 mAb are strong immunosuppressive agents and, if non-mitogenic, safe to use. Further research should prove whether non-mitogenic anti-CD3 mAb are also effective in prevention and treatment of clinical transplant rejection.

Samenvatting

Samenvatting

Verschillende ziekten kunnen leiden tot een ernstig gestoorde functie van organen zoals de nieren en het hart. Orgaantransplantatie is een tegenwoordig veel toegepaste behandeling voor patiënten met orgaanfalen. Echter, het verschil in weefselantigenen van de donor en de recipiënt kan afstoting van het getransplanteerde orgaan veroorzaken. Afstoting wordt tegengegaan door de recipiënt te behandelen met middelen die de immunologische afweer sterk onderdrukken, ook wel immunosuppressieve middelen genoemd. Deze middelen onderdrukken echter ook de afweer tegen bacteriën, virussen en tumorcellen. Hierdoor krijgen transplantaatrecipiënten vaker infecties en tumoren. Bovendien hebben vele van deze middelen nog andere bijwerkingen, zoals nieraantasting bij cyclosporine A behandeling.

In onze studie hebben we verschillende immunosuppressieve strategieën onderzocht op hun werkzaamheid en hun bijwerkingen. Hiervoor hebben we gebruik gemaakt van een huidtransplantatie model in de muis. De bestudeerde immunosuppressieve behandelingen waren gericht tegen òf cytokinen, òf T-lymfocyten. T-lymfocyten zijn essentieel voor transplantaatafstoting, terwijl cytokinen (oplosbare factoren, die zowel door T-lymfocyten als door andere cellen kunnen worden geproduceerd) een belangrijke rol spelen in de communicatie tussen de cellen die betrokken zijn bij de afstoting. Twee soorten T-helper (Th) cellen zouden op een verschillende manier betrokken zijn bij transplantaatafstoting. Th1 cellen, die o.a. IL-2 en IFN-γ produceren, zouden verantwoordelijk zijn voor afstoting, terwijl Th2 cellen, die o.a. IL-4 en IL-10 produceren, door onderdrukking van Th1 cellen transplantaatafstoting zouden remmen en zelfs acceptatie van het transplantaat zouden bewerkstelligen.

In hoofdstuk 2.1 geven we een overzicht van eerder gepubliceerd onderzoek naar de rol van cytokinen in klinische en experimentele transplantatie. In klinische transplantatie heeft men de serumspiegels van verschillende cytokinen gebruikt om de transplantaatfunctie te controleren. Tijdens transplantaatafstoting konden verhoogde serumspiegels van o.a. IL-2, IL-6 en TNF-α worden aangetoond. Omdat deze spiegels ook verhoogd waren bij infectie, kunnen deze cytokine bepalingen niet gebruikt worden om transplantaatafstoting te voorspellen. In proefdieren was de IL-2, IL-4 en IFN-γ mRNA expressie in het transplantaat gerelateerd aan afstoting. Tijdens acceptatie van het transplantaat werd verlaagde IL-2 en IFN-γ (Th1) mRNA expressie gevonden, terwijl de IL-4 en IL-10 (Th2) mRNA expressie gelijk bleef of verhoogd was t.o.v. de mRNA expressie tijdens afstoting. In experimentele transplantatie zijn verschillende cytokine-gerichte strategieën, zoals anti-cytokine monoklonale antistoffen (mAs) en oplosbare cytokine receptoren, getest op hun werkzaamheid. Deze therapieën waren echter geen van alle erg effectief in het verlengen van de transplantaatoverleving. Andere strategieën hadden tot doel de balans tussen de verschillende Th subsets te veranderen. De weinige pogingen tot Th2 verschuiving konden transplantaat-

afstoting niet remmen. In hoofdstuk 2.2 laten we zien dat behandeling van muizen met anti-IFN-γ mAs de transplantaatoverleving verlengde met ongeveer 15 dagen t.o.v. de onbehandelde controlegroep. Behandeling met Th2 cytokinen IL-4 en IL-10 had geen effect op de transplantaatafstoting. T-lymfocyt-gerichte behandeling met anti-CD4 of anti-CD3 mAs kon de transplantaatoverleving verlengen met respectievelijk ongeveer 25 en ongeveer 70 dagen. Hieruit concluderen wij dat in ons model T-cel-gerichte behandeling effectiever is in de remming van transplantaatafstoting dan cytokine-gerichte behandeling, en dat anti-CD3 mAs sterke immunosuppressie veroorzaken.

In hoofdstuk 3 gaan we dieper in op de behandeling van huidtransplantaatafstoting m.b.v. anti-CD3 mAs. OKT3 is een muis anti-CD3 mAs met sterk immunosuppressieve eigenschappen, dat wordt gebruikt voor preventie en behandeling van klinische orgaantransplantaatafstoting. Toediening van OKT3 gaat echter gepaard met ernstige bijwerkingen die gerelateerd zijn aan systemisch aantoonbare cytokinen. Voor de muis is een vergelijkbaar anti-CD3 mAs beschreven. De hamster anti-CD3 mAs 145-2C11 kan huidtransplantaatafstoting in de muis remmen, maar veroorzaakt ook cytokineproduktie en de daarmee gepaard gaande ziekteverschijnselen. In de hoofdstukken 3.1 en 3.2 laten we zien dat twee rat anti-CD3 mAs, 17A2 en KT3, even goed in staat zijn de transplantaatafstoting te remmen als 145-2C11 mAs. Behandeling met 17A2 mAs resulteerde zelfs in langdurige transplantaatoverleving in een klein percentage van de behandelde muizen. Deze muizen accepteerden ook een tweede transplantaat, terwijl ze een transplantaat van een andere donor als van het eerste transplantaat gewoon afstootten (hoofdstuk 3.1). De drie gebruikte anti-CD3 mAs veroorzaakten T-cel depletie en modulatie van het TCR/CD3 complex (hoofdstukken 3.1 en 3.2). In tegenstelling tot 145-2C11 mAs veroorzaakten 17A2 en KT3 geen ziekteverschijnselen in de muis. We hebben de serumconcentraties van TNF-α, IFN-γ en IL-6 gemeten na toediening van de drie anti-CD3 mAs en vonden dat de produktie van TNF-α en IFN-γ gerelateerd was aan de bijwerkingen (hoofdstukken 3.1 en 3.2). In hoofdstuk 3.3 onderzochten we de serumspiegels van oplosbare TNF receptoren (sTNFR) na anti-CD3 mAs behandeling. Deze sTNFR zouden bescherming bieden tegen TNF-α effecten. Aangezien TNF-α produktie alleen werd gevonden na 145-2C11 mAs behandeling en sTNFR produktie na behandeling met alle drie de anti-CD3 mAs, concluderen we dat in ons model de produktie van sTNFR onafhankelijk is van de TNF-α produktie. In hoofdstuk 3.4 hebben we onderzocht wat de basis is van de bijwerkingen van anti-CD3 mAs. In dit hoofdstuk tonen we aan dat *in vitro* de interactie van anti-CD3 mAs met Fcγ receptoren (FcγR) verantwoordelijk is voor T-cel proliferatie. *In vivo* is deze interactie verantwoordelijk voor de TNF-α produktie en de hieraan gerelateerde ziekteverschijnselen. Verder blijkt FcγR binding niet noodzakelijk voor immunosuppressie door anti-CD3 mAs.

Uit de resultaten, zoals beschreven in dit proefschrift, concluderen wij dat T-cel-gerichte behandeling effectiever is dan cytokine-gerichte behande-

ling van transplantaatafstoting. Anti-CD3 mAs zijn sterk immunosuppressief en, indien niet-mitogeen, veilig in gebruik. Verder onderzoek moet uitwijzen of niet-mitogene anti-CD3 mAs ook effectief zijn in de preventie en behandeling van transplantaatafstoting in de kliniek.

Abbreviations

ALG	anti-lymphocyte globulin
APC	antigen presenting cell(s)
ATG	anti-thymocyte globulin
BSS	balanced salt solution
CD	cluster of differentiation
cpm	counts per minute
CsA	cyclosporin A
CTL	cytotoxic T lymphocyte
DST	donor-specific transfusion
DTH	delayed-type hypersensitivity
ELISA	enzyme-linked immunosorbent assay
$F(ab')_2$	divalent antigen-binding fragment of immunoglobulin
FACS	fluorescence activated cell sorter
FcR	Fc receptor
FCS	fetal calf serum
FITC	fluorescein isothiocyanate
GVHD	graft-versus-host disease
IFN	interferon
Ig	immunoglobulin
IL	interleukin
i.p.	intraperitoneal
i.v.	intravenous
Kd	dissociation equilibrium constant
LPS	lipopolysaccharide
LT	lymphotoxin
mAb	monoclonal antibody
MFI	mean fluorescence intensity
MHC	major histocompatibility complex
mRNA	messenger ribonucleic acid
MTT	3 - [4,5 - dimethylthiazol-2-yl] - 2,5 diphenyltetrazolium bromide
n	number in study or group
NK	natural killer
p	probability
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
RT	reverse transcription
SCID	severe combined immunodeficiency disease
SD	standard deviation
sIL-2R	soluble IL-2 receptor
sTNFR	soluble TNF receptor
TcR/TCR	T cell receptor
Th	T helper
TNF	tumor necrosis factor

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