

**INTERACTION OF VASCULAR
ENDOTHELIAL CELLS WITH
CD8⁺ T-CELLS IN VIVO**

Inauguraldissertation

zur

Erlangung der Würde eines Doktors der Philosophie

vorgelegt der

Philosophisch-Naturwissenschaftlichen Fakultät

der Universität Basel

von

Beatrice Bolinger

aus Kaiseraugst, Aargau

St.Gallen, 2008

Genehmigt von der Philosophisch-Naturwissenschaftlichen Fakultät
auf Antrag von

Prof. Dr. med. et phil. E. Palmer
Fakultätsverantwortlicher

PD Dr. Burkhard Ludewig
Dissertationsleiter

Prof. Dr. phil. A. Rolink
Korreferent

Basel, den 22.04.2008

Prof. Dr. Hans-Peter Hauri
Dekan

Index

Index	3
1 Summary	6
2 Zusammenfassung	8
3 Introduction	10
3.1 General principles of immune activation.....	10
3.1.1 Antigen-presenting cells	11
3.1.2 T-cell activation	12
3.1.3 T-cell tolerance	13
3.1.3.1 Central tolerance.....	13
3.1.3.2 Peripheral tolerance	14
3.2 Transplantation	17
3.2.1 Alloreactivity	17
3.2.1.1 Molecular basis of alloreactivity	19
3.2.2 Graft versus host disease (GVHD)	20
3.2.3 Allograft rejection.....	20
3.2.3.1 T-cell activation in acute and chronic rejection	20
3.2.3.2 Chronic rejection	21
3.2.3.3 Biology of chronic vascular rejection.....	22
3.2.4 Cytomegalovirus infection.....	23
3.2.5 Immunologic mechanisms of chronic vascular rejection.....	24
3.2.5.1 Animal models	24
3.2.5.2 The role of CD4 ⁺ T-cells	25
3.2.5.3 The role of CD8 ⁺ T-cells	25
3.2.5.4 The role of CD4 ⁺ CD25 ⁺ Foxp3 ⁺ regulatory T-cells.....	26
3.2.5.5 The role of endothelial cells in acute and chronic rejection.....	26
3.2.6 Therapy of allograft rejection	30
3.2.7 Tolerance induction in transplantation.....	30
3.3 A transgenic model to investigate the cognate interaction of CD8 ⁺ T-cells with vascular endothelial cells expressing a minor histocompatibility antigen.....	34
4 Aims of the study	35

5	Results	37
5.1	Immunologic ignorance of vascular endothelial cells expressing minor histocompatibility antigen	37
5.1.1	Abstract	38
5.1.2	Introduction	39
5.1.3	Materials and Methods	41
5.1.4	Results	45
5.1.4.1	CD8 T-cell tolerance in Tie2-LacZ mice	45
5.1.4.2	EC-independent peripheral CD8 ⁺ T-cell tolerance	49
5.1.4.3	EC fail to directly activate naive CD8 ⁺ T-cells in vivo	51
5.1.4.4	Immunologic ignorance of antigen expressing EC in vascularized organ transplants	53
5.1.5	Discussion	55
5.1.6	Supplementary data	56
5.1.7	Acknowledgments	61
5.2	Dendritic cell-activated, endothelial cell-specific CTL recognizing a minor histocompatibility antigen rapidly induce transplant vasculopathy	62
5.2.1	Abstract	63
5.2.2	Introduction	64
5.2.3	Materials and Methods	65
5.2.4	Results	68
5.2.4.1	β-gal-specific immune responsiveness in heart transplant recipients	68
5.2.4.2	CTL-induced vascular rejection after DC immunization	71
5.2.4.3	Virus-induced vascular pathology in transplanted Tie2-LacZ hearts	73
5.2.5	Discussion	76
5.2.6	Acknowledgments	77
6	Discussion	78
6.1	Can EC activate naive CD8 ⁺ T-cells?	78
6.2	Can EC become target cells of activated CTL?	82
6.3	CD4 ⁺ T-cell help	86
6.4	CD4 ⁺ CD25 ⁺ Foxp3 ⁺ regulatory T-cells	88
7	Appendix	91

7.1	References.....	91
7.2	Figure legend	108
7.3	Abbreviations.....	109
7.4	Acknowledgements.....	113
7.5	Bibliography	115
7.6	Curriculum Vitae	116

1 Summary

Transplantation of organs and cells saves and prolongs thousands of lives every year. Surgical techniques were significantly improved but major problems remain, in particular the host's immune system. Despite advances in immunosuppressive therapies, chronic allograft rejection still occurs which is characterized by intimal thickening in the arteries and the replacement of graft parenchyma, a phenomenon called chronic transplant vasculopathy (CTV). Within three years after transplantation 45% of transplant patients are affected by CTV which leads to the failure of allografts of about 5% each year post transplantation. The reasons for its development and the mechanistic basis inducing CTV are still not clearly understood.

In graft versus host disease (GVHD) and vascular rejection of solid organ transplants, vascular endothelial cells (EC) have been recognized as important targets for alloreactive cytotoxic T-lymphocytes (CTL) and the presence of CTL has been associated with CTV. Therefore, T-cell-mediated immunity and subsequent inflammation appear to be important features of the initiation and progression of CTV. The contribution of EC to CD8⁺ T-cell activation and therefore their role in the development of chronic vascular rejection is still controversially discussed.

For that reason and the fact that after transplantation of vascularised organs EC are the first graft cells encountered by host lymphocytes, the detailed interaction of vascular EC with CD8⁺ T-cells has been assessed *in vivo* in the first part of our study, in order to find out whether EC are able to activate or tolerize naive CD8⁺ T-cells. Using a transgenic mouse model with beta-galactosidase (β -gal) expression confined to the vascular endothelium (Tie2-LacZ mice) and the help of β -gal TCR transgenic CD8⁺ T-cells (Bg1 mice), the capacity of EC presenting a minor histocompatibility antigen (mhAg) to induce a CD8⁺ T-cell response was studied. We could show that mhAg presentation on EC was ignored by CD8⁺ T-cells and was neither sufficient to activate nor to tolerize CD8⁺ T-cells. Moreover, the mhAg was cross-presented by BM-derived CD11c⁺ DC and led to spontaneous activation of β -gal-specific CD8⁺ T-cells in Tie2-LacZ mice. This identifies the priming of mhAg-specific CD8⁺ T-cells via DC as the critical step in the generation of alloimmune responses. Furthermore, no β -gal-specific CD8⁺ T-cell activation was induced after transplantation of fully vascularised heart or liver grafts from Tie2-LacZ mice into non-transgenic recipients confirming that CD8⁺ T-cell responses against mhAg cannot be initiated by EC.

In the second part of the study the major aim was to develop an experimental system that facilitates *in vivo* studies on the interaction of EC with activated CTL in a heart transplantation model. To this end, Tie2-LacZ hearts were heterotopically transplanted into C57BL/6 recipients. Tie2-LacZ hearts were accepted and showed no vascular inflammatory changes or neointima formation until day 100 post transplantation. Repetitive priming with β -gal peptide loaded DC induced a long-term β -gal-specific CTL response resulting in the induction of vascular inflammatory disease with neointima formation and vascular occlusion. Infection with β -gal recombinant mouse cytomegalovirus (MCMV-LacZ) however, led to a shorter activation of β -gal-specific CTL and thus to a less significant vascular inflammation in Tie2-LacZ hearts. Taken together, we suggest that it is the prolonged presentation of mhAg within secondary lymphoid organs that is responsible for the activation of EC-specific CTL and that activated CTL recognize thereafter mhAg specifically expressed on EC, leading to the development of chronic vascular rejection.

2 Zusammenfassung

Durch die Transplantation von Organen oder Zellen werden jedes Jahr Menschenleben gerettet. Trotz medikamentöser Fortschritten zur Unterdrückung des Immunsystems entwickeln im Falle einer Knochenmarkstransplantation 60-70% der behandelten Patienten eine chronische Immunreaktion gegen das eigene Gewebe. Auch bei Organtransplantationen ist der Langzeiterfolg nicht garantiert und es kommt häufig zu spätem Organversagen. Chronische Abstossungsreaktionen, welche sich über Monate oder Jahre entwickeln, stellen den Hauptgrund für die Abstossung transplantierten Organe dar. Während der chronischen Abstossungsreaktion kommt es zur Verdickung der Intima der Arterien und zum Austausch von Transplantatparenchym, ein Prozess, der auch chronische Transplantatvaskulopathie genannt wird. Innerhalb der ersten drei Jahre nach der Transplantation entwickeln 45% der Transplantationspatienten solche vaskuläre Veränderungen, welche meist zum Organversagen führen. Diese Abstossungsreaktionen kommen in einer Häufigkeit von ungefähr 5% pro Jahr nach der Transplantation vor. Die Ursachen und die mechanistischen Grundlagen dafür sind nicht vollständig verstanden.

Es wurde jedoch gezeigt, dass bei der Abstossungsreaktion vaskuläre Endothelzellen (EC) wichtige Zielzellen zytotoxischer T Zellen sind. Weiter konnte eine Korrelation zwischen dem Vorhandensein zytotoxischer T Zellen und dem Erscheinen chronischer Transplantat Vaskulopathie gezeigt werden. Die T Zellen-induzierte Immunität und die sich daraus entwickelnde Entzündung scheinen wichtige Faktoren für die Entstehung und Entwicklung vaskulärer Veränderungen zu sein. Welche Rolle EC im Bezug auf die CD8⁺ T Zellen-Reaktivität und somit in der Entwicklung chronischer Abstossungsreaktionen haben, wird kontrovers diskutiert.

Aus diesen Gründen und weil EC die ersten Zellen des transplantierten Organs sind, welche von Empfänger T Lymphozyten getroffen und erkannt werden, wurde im ersten Teil dieser Arbeit die Interaktion vaskulärer EC mit naiven CD8⁺ T Zellen am Tiermodell untersucht. In dieser Studie wurden transgene Mäuse verwendet, bei welchen die Expressierung des Modelantigens Beta-galactosidase (β -gal) auf die vaskulären EC beschränkt ist (Tie2-LacZ Maus). Zusätzlich standen T Zell Rezeptor transgene β -gal-spezifische CD8⁺ Zellen zur Verfügung. Es wurde untersucht, ob EC, die ein nominales Antigen präsentieren, eine CD8⁺ T Zell-Antwort generieren können. Es konnte gezeigt werden, dass nominales Antigen von CD8⁺ T Zellen ignoriert wurde und weder zu einer Aktivierung, noch zu einer Tolerisierung führte. Weiter wurde gezeigt, dass die Kreuz-Präsentation des Antigens durch

CD11c⁺ dendritische Zellen (DC) zur spezifischen Aktivierung und Proliferierung der CD8⁺ T Zellen führte. Diese Tatsache lässt das Priming der Antigen-spezifischen CD8⁺ T Zellen durch DC als einen kritischen Schritt bei der Generierung einer Immunantwort erkennen. Außerdem wurde auch im Transplantationsmodell, bei welchem Tie2-LacZ Herzen oder Lebern in Wildtyp-Mäuse transplantiert wurden, keine CD8⁺ T Zell-Aktivierung beobachtet. Dies bestätigt, dass EC nicht fähig sind, eine CD8⁺ T Zell-Antwort gegen ein nominales Antigen zu induzieren.

Im zweiten Teil unserer Studie war das Hauptziel, ein experimentelles System, welches die Untersuchungen der Interaktion von EC mit aktivierten zytotoxischen T Zellen im Transplantationsmodell des Herzens erleichtert, zu entwickeln. Dazu wurden Tie2-LacZ Herzen heterotop in C57BL/6 Mäuse transplantiert. Diese Herzen wurden von den Wildtyp-Mäusen akzeptiert und bis 100 Tage nach der Transplantation konnten keine antigenspezifischen Gefäßveränderungen beobachtet werden. Durch die Aktivierung von β -gal-spezifischen CD8⁺ zytotoxischen T Zellen mittels Injektion β -gal-Peptid beladener DC kam es zu vaskulären Entzündungen und der Bildung von Neointima mit kompletter Gefäßokklusion. Diese entzündlichen Veränderungen waren nach der Infektion der Mäuse mit dem β -gal-rekombinanten Maus-Cytomegalievirus weitaus weniger ausgeprägt. Zusammengefasst kann gesagt werden, dass die verlängerte Präsentation von Antigen in sekundären lymphatischen Organen für die Aktivierung von EC-spezifischen zytotoxischen T Zellen verantwortlich ist. Aktivierte zytotoxische T Zellen erkennen daraufhin das Antigen, welches durch EC exprimiert und präsentiert wird, und tragen so zur Entstehung einer chronischen Abstoßungsreaktion bei.

3 Introduction

In a first part of this work, the general principles of immune activation, in particular, T-cell reactivity, were summarized. Furthermore, problems of organ and cell transplantation, as well as the role of vascular EC and CD8⁺ T-cells in allotransplantation, were described in order to be acquainted with the present knowledge concerning the interaction of vascular EC with CD8⁺ T-cells.

3.1 General principles of immune activation

The main role of the immune system is to protect the individual from infections. To fulfil this goal, two major components of the immune system, the innate and the adaptive immunity, work together. Innate immunity is considered as the less specific part and provides the first line of defence against infectious agents. It comprises molecular and cellular mechanisms that are not pathogen-specific but are present before onset of the infection. On the other hand the adaptive immune system, regarded as the specific part, only comes into play when there is antigenic challenge to the organism. A specific response can be observed within five or six days after the initial contact with the antigen and is characterized by its high specificity for the antigen and the ability to provide immunologic memory. Furthermore, the recognition of billions of single structures is possible through the capability of the adaptive immune system to generate a remarkable diversity in recognition molecules. Moreover, it has the capacity to discriminate self from non-self (Billingham et al., 1953; Burnet and Fenner, 1949; Burnet, 1957).

The major mediators of the adaptive immune system are B- and T-lymphocytes. B-lymphocytes produce specific antibodies upon activation, which primarily bind extracellular agents and neutralize the pathogen directly. CD4⁺ T-lymphocytes provide either direct help to B cells, involving CD40L:CD40 interaction and the production of cytokines, or they facilitate CD8⁺ T-cell expansion and activate macrophages by the production of cytokines, such as IL-2, IFN γ and TNF. CD8⁺ T-cells are the cytotoxic T-cells which have the ability to directly kill the infected cells via cell-cell interaction. By the secretion of IFN γ and other cytokines they exert further effector function. The main targets of T-cells are peptides derived from infectious agents or intracellular antigens bound to major histocompatibility complexes (MHC). Peptide antigens have to be processed, which implies the digestion of the protein into peptides and presentation on MHC molecules by antigen presenting cells (APC), since the T-cell receptor (TCR) recognizes foreign only in the combination with self. There

are two types of MHC molecules: MHC class I molecules, expressed on almost all nucleated cells and recognized by CD8⁺ T-cells and the MHC class II molecules, expressed on professional APC such as dendritic cells (DC), macrophages and B-lymphocytes. MHC II molecules are recognized by CD4⁺ T-cells.

3.1.1 Antigen-presenting cells

Strictly speaking, all nucleated cells could be assigned as APC since they all express MHC I or MHC II molecules. But according to convention, cells presenting peptide on MHC class I molecules to CD8⁺ T-cells are referred to as target cells. Only cells that display peptide via class II molecules are called professional APC. Professional APC are hematopoietic cells whereby three types of cells belonging to APC exist: DC, macrophages and B-lymphocytes. DC, a minor cell population in lymphoid tissues, are considered the prototypic professional APC (Banchereau et al., 2000; Guermonprez et al., 2002; Ludewig et al., 1998a; Steinman and Cohn, 1973; Steinman, 1991). They are the most significant and potent APC, able of inducing T-cell responses following infection or organ transplantation. DC constitutively express high levels of both class I and II MHC molecules and members of the co-stimulatory B7 family. Therefore, they are more potent in antigen presentation than B-lymphocytes and macrophages, which both have to be activated prior to acquiring the ability of antigen presentation. Furthermore, DC have the unique capacity to migrate to the periphery, the site of inflammation where they act as sentinels for pathogen-derived structures (Pulendran et al., 2001). As soon as they sense such structures through their pattern recognition receptors, such as Toll-like receptors, they undergo a maturation process including up-regulation of costimulatory molecules (Banchereau and Steinman, 1998). The exposure to proinflammatory cytokines and the ligation of the costimulatory molecule CD40 also lead to their activation (Bennett et al., 1998). During maturation, DC capture antigen, internalize it, either by phagocytosis or by endocytosis and thereafter modify their homing receptors (Roake et al., 1995). After having fully matured, they transport antigen from the periphery to local lymphoid tissue and present the processed antigen on their membrane bound to MHC molecules to CD4⁺ and CD8⁺ T-cells. Specific T-cells recognize this complex and interact with the DC. Thereafter DC produce costimulatory signals, inducing complete activation of the T-cells. There is accumulating evidence that DC play a key role in T-cell immunity. For example, in a mouse model, CD11c⁺ DC depletion was accompanied by a complete absence of primed CTL after infection with *Listeria monocytogenes* or with

Plasmodium yoelli (Jung et al., 2002), confirming that DC have the ability to prime naive T-lymphocytes during inflammation or infection.

However, non-hematopoietic cells, i.e., vascular EC, have also been shown to act as APC. EC express MHC class I molecules and can induce the expression of MHC class II molecules upon inflammation via proinflammatory cytokine such as IFN γ (Marelli-Berg et al., 2000; Pober et al., 1997) and thereby display antigen to CD8⁺ and CD4⁺ T-lymphocytes. The antigen presenting function of EC works only for short periods of time during a sustained inflammatory response, therefore EC are called “semiprofessional” APC. EC present antigen to T-cells via the direct pathway, in the context of allograft transplantation where they display allo-MHC-peptide complexes, or via the indirect pathway which involves cross-presentation of antigens (Pober et al., 1997; Epperson and Pober, 1994; Rose, 1998; Bagai et al., 2005a; Limmer et al., 2000; Limmer et al., 2005; Valujskikh et al., 2002a). Although EC have the capacity to stimulate allogeneic CD8⁺ T-cells and memory allogeneic CD4⁺ T-cells, can they neither activate naive alloreactive CD4⁺ T-cells *in vitro* and *in vivo* nor are they able to activate resting T-cells specific for a minor histocompatibility antigen (mhAg) *in vitro* (Bagai et al., 2005a; Kreisel et al., 2002b; Kreisel et al., 2004; Marelli-Berg et al., 2000; Marelli-Berg et al., 2001). There is even evidence for EC to induce T-cell tolerance (Berg et al., 2006; Limmer et al., 2000; Limmer et al., 2005; Marelli-Berg et al., 2000). However, their capability of being a semiprofessional APC, regardless whether they induce T-cell activation or tolerization, is of main importance in the context of solid organ and bone marrow (BM) transplantation.

Nonetheless, not only EC have been shown to induce T-cell tolerance, but DC too. While they activate T-cells during inflammation and infection, they are immature under steady state conditions and induce peripheral T-cell tolerance, see Figure 1 (Abbas and Sharpe, 2005) (Probst et al., 2005; Probst et al., 2003; Steinman et al., 2003b; Steinman et al., 2003a; Steinman, 2003).

3.1.2 T-cell activation

Naive T-lymphocytes circulate via blood through secondary lymphoid organs and home with the help of CD62L and CCR7 expression (Sallusto et al., 1999). Without previous activating stimulus they are not able to enter peripheral non lymphoid tissue. They need antigen presentation by professional APC within the secondary lymphoid organs, referred to as signal 1, and thereafter suitable costimulation, referred to as signal 2, to be activated and to differentiate into full effector T-cells (Lakkis et al., 2000; Salomon and Bluestone, 2001). These interactions are based on cell-cell contact which demands the special microenvironment

of organized lymphoid tissue that provides the necessary inflammatory milieu with antigen location, cytokines and interleukins, and enhances the chance of specific antigen encounters (Zinkernagel et al., 1997). After interaction of the TCR with the APC, CD28 and CD40L expression on T-cells is up-regulated and costimulation by engagement of one or more T-cell surface receptors with their specific ligands (CD40, CD80, CD86) on APC is provided. Differentiated T-cells are preferentially recruited to sites of inflammation. Via the expression of adhesion molecule ligands (L-selectin, LFA-1, VLA-4, Mac-1) they make contact to adhesion molecules, such as P-selectin, E-selectin, VCAM-1 and ICAM-1 on the activated endothelium and enter the periphery, to fulfil their task to protect the individual from infections (Campbell et al., 1998; Rao et al., 2007).

3.1.3 T-cell tolerance

T-cell development is a highly coordinated process with the main goal of reaching a maximal degree of diversity and of removing all T-lymphocytes that recognize self-antigen to prevent autoimmune diseases. A major mechanism in accomplishing self-tolerance is the elimination of potentially self-reactive T-lymphocytes during their development in the thymus. This negative selection of T-cells, referred to as central tolerance, has been shown to play a key role (Kappler et al., 1987).

3.1.3.1 Central tolerance

T-cell progenitors migrate from the BM to the thymus, where they undergo maturation. Maturation includes rearrangements of the germ-line TCR genes and the expression of various membrane markers. Developing T-cells in the thymus, the so called thymocytes, proliferate and differentiate along determined pathways to become functionally distinct subpopulations of mature T-cells. During this process they are submitted to positive and negative selection. Whereas positive selection permits the survival of T-cells with a TCR able of recognizing self-MHC molecules, negative selection eliminates T-cells with too high affinity towards self-MHC plus self-antigens. These self-antigens consist of those that are ubiquitous expressed, and those who are restricted to a few tissues, referred to as tissue-specific antigens (TSAs). TSAs are expressed on medullary thymic epithelial cells (mTECs) (Kyewski and Derbinski, 2004) and BM-derived DC which capture the TSAs from mTECs (Gallegos and Bevan, 2004; Speiser et al., 1989) and present it to the thymocytes.

However, central tolerance seems to be incomplete and potentially self-reactive T-cells escape negative selection and are released into the periphery where they could become

activated by professional APC, presenting self-antigens, and thereafter induce autoimmunity. In particular, T-cells with low avidity for self-antigen can escape central tolerance (Zehn and Bevan, 2006) and it is possible that other self-proteins, for which tolerance is required, may not access the thymus. Therefore, it is not surprising that further mechanisms in the periphery control self reactivity.

3.1.3.2 Peripheral tolerance

The induction of peripheral tolerance is an imperative physiological process necessary to supplement central tolerance (Kurts et al., 1997; Probst et al., 2003). Particularly at sites of infection, where maturing DC process and present self- and non-self-antigens at the same time, efficient tolerance mechanisms are of main importance.

One major mechanism of peripheral tolerance is the induction of T-cell tolerance via steady-state DC. DC that have not been activated through inflammation or infection have an immature phenotype. Although they efficiently capture antigen, they lack high expression of co-stimulatory molecules and do not express CCR7. Despite the fact that they deliver signal 1 under steady state conditions, they lack the ability to deliver signal 2, resulting in T-cell tolerance. (Banchereau and Steinman, 1998). This has been shown to be mediated through PD-1 and CTLA-4 (Probst et al., 2005; Sakaguchi et al., 2006b). Upon recognition of self-antigens presented by DC, T-cells undergo proliferation but are ultimately deleted from the peripheral T-cell repertoire.

Moreover, DC have also been shown to induce tolerance by the activation of CD4⁺CD25⁺Foxp3⁺ regulatory T-cells (Sakaguchi, 2004). CD4⁺CD25⁺Foxp3⁺ regulatory T-cells (Tregs) are known to be essential in many processes of the immune system. They play a crucial role in the maintenance of maternal tolerance to the foetus (Aluvihare et al., 2004), in the prevention of autoimmunity (Sakaguchi et al., 2006a) and in the inhibition of antitumor immunity (Beyer and Schultze, 2006). Furthermore, they are important in the regulation of immunity to viral and parasite infections (Belkaid et al., 2006; Rouse et al., 2006). Tregs develop in the thymus where they get positively selected (Bensinger et al., 2001). It has been shown that the Treg repertoire is enriched in auto specific cells (Fisson et al., 2003; Hsieh et al., 2004). This is consistent with what can be found looking at their central function in controlling autoreactive T-lymphocytes. However, Tregs become specifically activated by the presentation of autoantigens through DC in secondary lymphoid organs (Fisson et al., 2003; Samy et al., 2005) and need IL-2 for their proliferation. In part, the effector mechanisms used by Tregs are mediated by CTLA4, expressed by Tregs, and its ligation with CD80 and CD86

on T-lymphocytes. Additionally, the suppression requires responsiveness of effector T-cells to TGF- β and the secretion of IL-10 by Tregs (Asseman et al., 1999; Fahlen et al., 2005).

Furthermore, there is accumulating evidence for the tolerogenic capacity of EC. Liver sinusoidal EC (LSEC) have been shown to induce CD8⁺ T-cell tolerance to soluble, oral and tumour-derived antigens (Berg et al., 2006; Limmer et al., 2000; Limmer et al., 2005) and isolated murine lung EC negatively regulated CD8⁺ T-cell function (Marelli-Berg et al., 2000).

However, there is a third way of peripheral tolerance, which is immunologic ignorance where peripheral antigen is not visible to T-lymphocytes. Whether CD8⁺ T cell ignore a peripheral antigen or become tolerized has been shown to be a matter of antigen dose. When the tissue-specific antigen was expressed at low concentration, no cross-presentation by DC could be observed and indeed, there was no CD8⁺ T-cell response indicating that the CD8⁺ T-cell compartment remained ignorant. In mice expressing antigen at higher doses the antigen was cross-presented and led to the peripheral deletion of specific CD8⁺ T-cells (Kurts et al., 1999). It has further been demonstrated that CD8⁺ T-cells need the presence of the antigen in order to become tolerized, indicating that they are rather anergized than fully deleted (Lees et al., 2006).

Nevertheless, it has been shown that peripheral tolerance mechanisms cannot entirely recompense for inefficiencies in central tolerance since deficiency of AIRE, the autoimmune regulator that controls the gene expression of peripheral antigens in TEC leading to central tolerance, resulted in autoimmune syndrome (Anderson, 2002; Villasenor et al., 2005). However, it is obvious that negative selection is not sufficient either to eliminate all potentially autoreactive T cells since the absence of the development and action of regulatory T-cells leads to the development of autoimmunity (Piccirillo and Shevach, 2004).

Taken together, both central and peripheral tolerance mechanisms play a major role in inducing specific self-tolerance. Nevertheless, only peripheral mechanisms are involved in the induction of tolerance towards foreign antigens, such as allo-antigens in the case of transplantation where the induction of specific allogeneic tolerance is a major goal to prevent transplant rejection because it is not accompanied by a general immunosuppression of the patient.

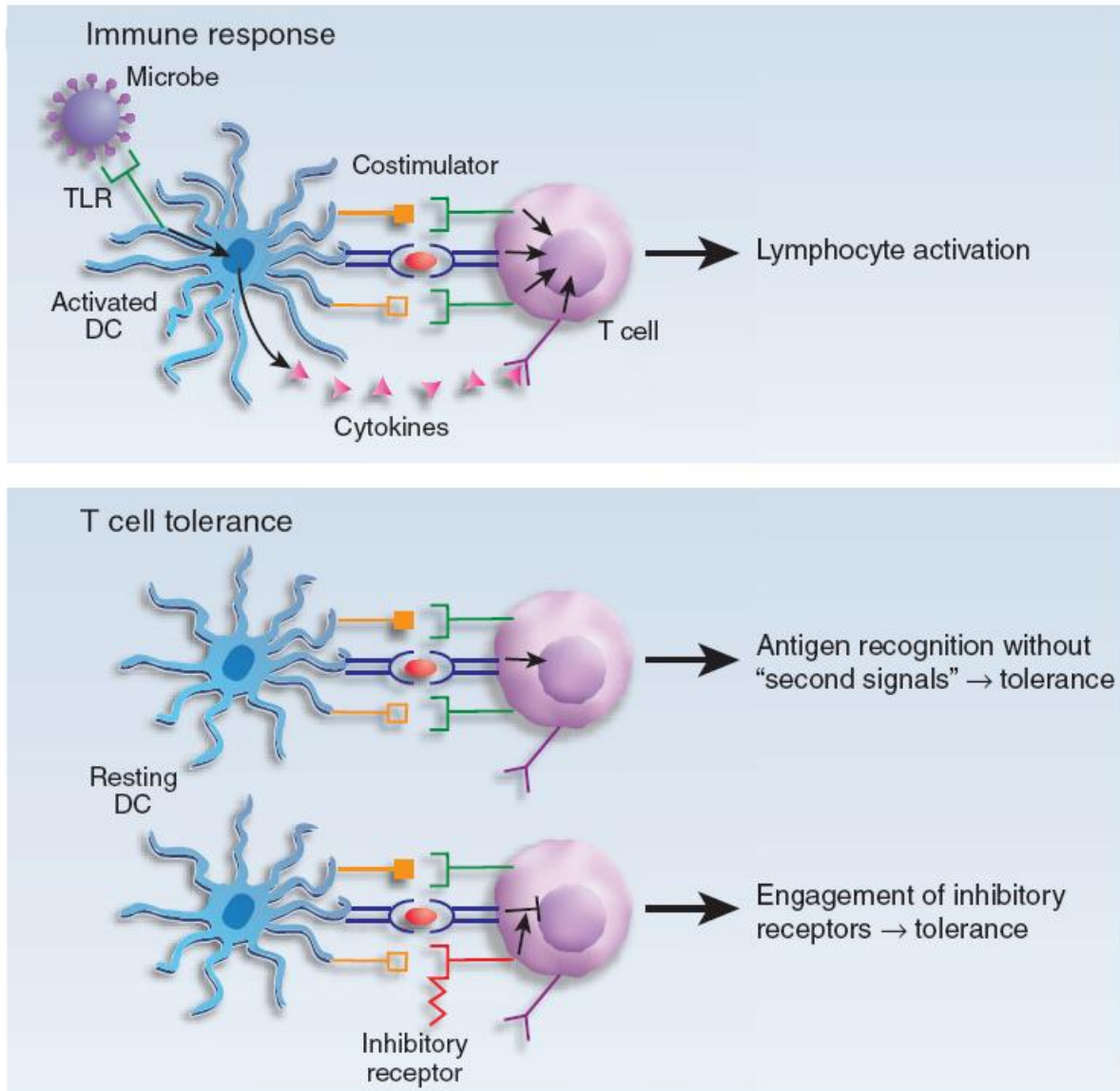


Figure 1: Immune activation and tolerance induction by DC. Top, DC are activated by TLRs and other signals. They display antigens and express costimulatory molecules, which stimulate T-lymphocytes. Bottom, resting, immature DC induce T-lymphocytes tolerance since DC do not express sufficient amounts of costimulatory molecules or they engage inhibitory receptors on the T cells (from (Abbas and Sharpe, 2005)).

3.2 Transplantation

Each year, transplantation of organs and cells between individuals saves or prolongs thousands of lives. Allogeneic BM transplantation for example, displays an extremely effective treatment of malignant blood cell disorders. However, despite improved immunosuppressive therapies, up to 60-70% of patients develop chronic GVHD. And despite advances in surgical techniques for organ transplantations and the better immunosuppressive drugs for acute rejection, successful long-term outcome of transplanted organs is still hampered by late graft failure since the major problem, the immune system, remains. However, transplantations are performed between members of the same species and thus referred to as allotransplants. The immune response to allotransplants is called alloreactivity. It is directed against allelic differences in MHC molecules between host and donor and leads to rejection of the transplanted organ. The immune system uses the same mechanisms evolved to protect the organism from foreign agents to cause rejection from genetically not identical donor grafts. Thus, alloreactivity includes T- and B-cell-mediated responses as well as innate immune responses. Clinically, it manifests as transplant rejection (host-versus-donor) or as GVHD. MHC graft rejection generally depends on both class I and class II antigens together and a rejected vascularised solid organ graft is characterized by its dense infiltrates of host lymphocytes. As a consequence, graft cell necrosis and graft vessel thrombosis are induced and lead to acute rejection, taking place one to two weeks after transplantation. For that reason, the success of all kind of transplants strongly depends at the beginning on the ability to prevent acute rejection by the use of immunosuppressive agents. However, despite advances in immunosuppressive therapies, chronic allograft rejection or chronic GVHD, which develop over months and years, still occur and display in the end the main limitations of long-term survival (Cecka, 1999; Keck et al., 1999).

3.2.1 Alloreactivity

T-lymphocytes mature in the thymus where they undergo positive and negative selection to subsequently recognize self-MHC molecules in combination with non-self peptides. There is no process that selects for, or against, the ability of the TCR to bind to MHC alleles that are not expressed by an individual. However, in the first scenario the TCR makes contact with both the peptide and the self MHC molecule as a complex, and foreign is only recognized in combination with self-MHC (MHC-restriction). On the contrary, the direct interaction of alloreactive T-lymphocytes with the non-self MHC molecule or the foreign peptide or even

with both is not MHC-restricted. Furthermore, direct recognition of the foreign MHC molecules by allogeneic T-cells is defined as the recognition of a major histocompatibility antigen, whereas minor histocompatibility antigens, in the context of allotransplantation, are defined as immunogenic peptides, which derive from cellular proteins from the graft (allorejection) or the host (GVHD), presented on MHC-I molecules and recognized by alloreactive T-cells. Naturally, alloresponses arise in the context of major and minor histocompatibility mismatches between donor and recipient.

However, alloreactive T-lymphocytes have the ability to recognize peptide-MHC complexes that were not encountered during thymic development. This is characterized by lower specificity and binding affinities which goes along with a high precursor frequency that is 100 to 1000 times higher than the precursor frequency of T-cells specific for any single foreign-peptide-self-MHC complex (Lindahl and Wilson, 1977; Suchin et al., 2001). This explains the strong primary immune response where the high frequency of alloreactive T-cells (1 in 10^3 - 10^4) enables their detection. Nonetheless, a comprehensive model of how the peptide-allogeneic-MHC complex contributes to TCR alloreactivity is elusive. Some models suggest that T-cell activation derives entirely from interaction between the TCR and the allogeneic MHC molecules (Bevan, 1984), whereas other models explain the activation by a central role of the peptide (Matzinger and Bevan, 1977). According to these models, allrecognition and conventional recognition are two different mechanisms (Figure 2B). Despite the tendency to distinguish them, many recent findings reveal their similarities. Structural analyses have displayed that, although individual interactions differ, the type of TCR interactions with self and allogeneic MHC molecules are comparable (Figure 2A) and numerous studies have clearly shown the existence of alloreactive T-lymphocytes highly peptide specific (Alexander-Miller et al., 1993; Felix et al., 2007; Heath and Sherman, 1991; Heath et al., 1991; Mazza et al., 2007; Mendiratta et al., 1999; Tallquist et al., 1996; Weber et al., 1995; Whitelegg et al., 2005). Moreover, the ability of alloreactive TCR to identify multiple distinct peptide-allogeneic-MHC complexes, but each with a high degree of specificity, has been shown in several studies (Guimezanes et al., 2001; Mazza et al., 2007; Reiser et al., 2003; Tallquist et al., 1996).

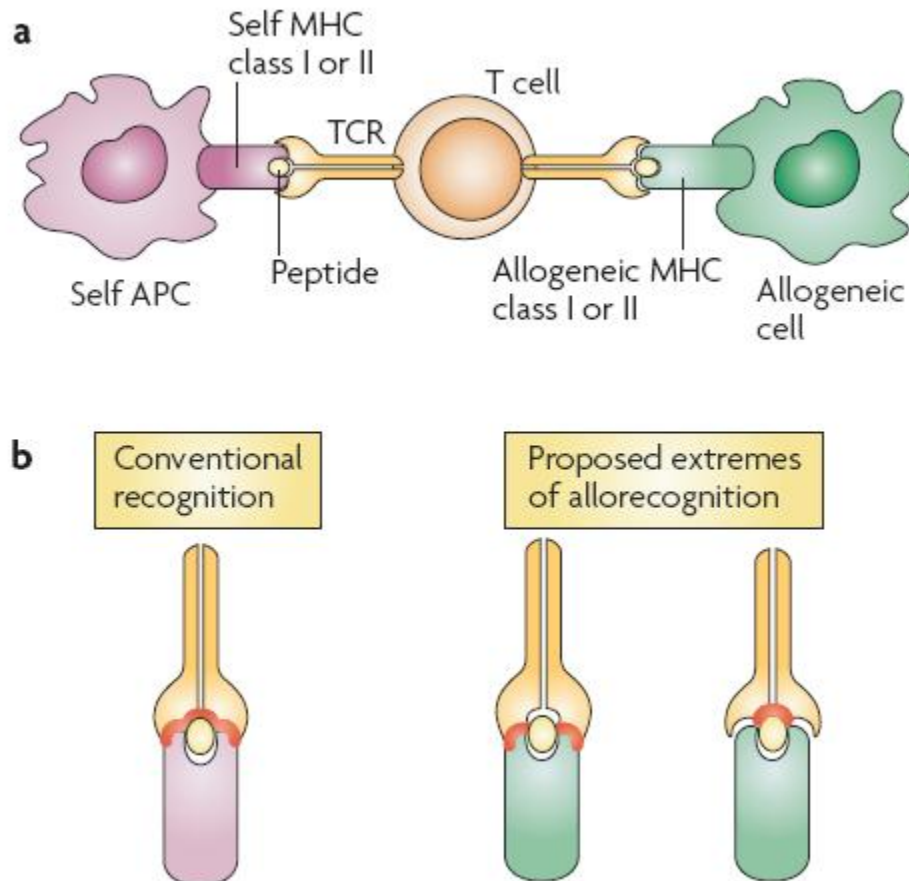


Figure 2: T-cell recognition of conventional and allogeneic complexes. (a) TCRs recognize both conventional peptide-self-MHC complexes and peptide-allogeneic-MHC complexes although these TCRs have never encountered the allogeneic-MHC molecules during thymic development. (b) In conventional recognition, the TCR makes contact with both the peptide and the self MHC molecule. In allorecognition some models suggest that the TCR primarily contacts the MHC molecule or the peptide. However, many alloreactive T-cells seem to interact with the peptide-allogeneic-MHC complex in a manner identical to conventional recognition (from (Felix and Allen, 2007)).

3.2.1.1 Molecular basis of alloreactivity

Within a species and even within individuals, MHC molecules show a huge degree of diversity. They are highly polymorphic. Hundreds of different MHC alleles are present in the human population. These alleles differ in their DNA sequences from one individual to another by 5 to 10%. The location of so many polymorphic amino acids within the binding site for processed antigen strongly suggest that allelic differences contribute to the observed differences in the ability of MHC molecules to interact with a given antigenic peptide. Polymorphism in the MHC α -helical residues can directly influence allorecognition, whereas polymorphism in the peptide-binding groove affects which peptides binds, as well as the overall conformation of the peptide-allogeneic-MHC complex (Bluestone et al., 1992; Chattopadhyay et al., 1994). Each peptide-MHC complex may take up a unique conformation, even when the same peptide is presented by both self and allogeneic MHC molecules.

Consequently, the surface that is recognized by the TCR is intimately linked to both the bound peptide and the MHC molecule.

3.2.2 Graft versus host disease (GVHD)

GVHD is the most severe and common immunological complication of BM transplantation (Horowitz et al., 1990). It occurs when donor T-cells recognize alloantigens on the host cells and mediate an immune response directed against the recipient. Usually, the recipient of the BM transplant is immunologically suppressed before BM grafting is performed. The activation and proliferation of alloreactive T-cells and the following cytokine generation induces inflammatory reactions in the skin, gastrointestinal tract, and liver. In patients with acute and chronic GVHD vascular injury in the skin has been observed (Biedermann et al., 2002), indicating that EC are target cells of allospecific CTL. In severe cases these processes can lead to generalized erythroderma of the skin, gastrointestinal haemorrhage, and liver failure. Immunosuppressive drugs are used in order to inhibit the immune response of the donor. GVHD affects 50-70% of BM-transplant patients and is even induced in HLA-identical BM transplantation where disparities in mAg between the donor and the host lead to the induction of GVHD (Goulmy et al., 1983).

3.2.3 Allograft rejection

Most allograft rejections involve T-cell mediated responses; whereby acute and chronic allograft rejection can be distinguished.

3.2.3.1 T-cell activation in acute and chronic rejection

Due to improved immunosuppression regimen, acute rejection occurs with decreasing incidence after the first three months. Indeed, a high percentage of transplant recipients never experience episodes of acute rejection. However, in allotransplantation APC, responsible for T-cell activation, may potentially originate from either the donor graft (direct) or from the recipient (indirect, semi-direct). Donor APC are tissue-specific and only the determinants expressed on donor APC will also be expressed by parenchymal cells of the graft. After transplantation of vascularised solid organs, donor APC travel to the secondary lymphoid organs of the host where they directly activate naive CD4⁺ and CD8⁺ T-lymphocytes leading to the induction of acute allograft rejection (Hernandez-Fuentes et al., 1999). In the case of vascularised grafts, memory alloreactive T-cells could be directly activated by donor APC in the periphery, e.g., EC, without need of co-stimulation and the environment of secondary lymphoid structures (Perez et al., 1998; Pober et al., 1996; Epperson and Pober, 1994).

Nonetheless, over the time, donor APC are replaced by recipient APC and the indirect pathway becomes more important. This pathway is characterized by the presentation of donor antigens on recipient APC. Host APC enter the graft, pick up donor antigen, particularly allogeneic MHC antigens, and present it on self MHC-I (cross-presentation) and MHC-II molecules to naive T-lymphocytes in secondary lymphoid organs. The indirect pathway plays a major role during chronic rejection (Hornick et al., 2000; Shirwin, 1995). Furthermore, cross-presentation of alloantigen by DC is a phenomenon not restricted exclusively to DC. In the context of allotransplantation EC have also been shown to cross-present antigen (Bagai et al., 2005a). Recently, a third approach for presentation of foreign MHC molecules has been described, the so called semi-direct pathway. In the semi-direct pathway recipient DC are able to acquire intact functional MHC molecules from graft cells and thereafter induce antigen specific T-cell responses (Herrera et al., 2004). However, no matter which way of activation is used, naive cells can only be primed within the microenvironment of the secondary lymphoid organs and are not able to reject an allograft in the absence of those (Lakkis et al., 2000).

Nevertheless, although acute rejection can be prevented by immunosuppressive therapy, chronic rejection developing over months and years and characterized by the replacement of graft parenchyma still develops. It leads to the failure of allografts of about 5% each year posttransplantation and frequently necessitates retransplantation (Cecka, 1999; Keck et al., 1999). It is important to point out that this rate has not been altered by the introduction of new immunosuppressive drugs compared to the tremendous reduction in the rate of acute allograft rejection.

3.2.3.2 Chronic rejection

There are two different forms of chronic rejections. The first is defined as chronic parenchymal cell rejection where graft failure is due to progressive immune-mediated fibrotic replacement of graft parenchyma. The second is called chronic vascular rejection and is caused by progressive immune-mediated host response to the blood vessels of the solid organ graft followed by stenosis of the arteries, arterioles, and capillaries. This process eventually leads to replacement fibrosis of the graft parenchyma (Libby and Pober, 2001). This progressive luminal narrowing of the graft arteries is frequently seen in cardiac transplantation (Julius et al., 2000). It is a multifactorial incident and develops over months to years and is only observed in allogeneic but not syngeneic cardiac grafts, demonstrating a central role of alloreactivity in the development of these lesions. Since the progressive loss of

lumen mainly affects conduit arteries, it can also be called arteriosclerosis, graft arteriosclerosis, or transplant vasculopathy (Mitchell and Libby, 2007; Libby and Pober, 2001). CTV is the main cause of long-term allograft dysfunction and late graft loss in heart and kidney transplantation (Nankivell et al., 2003; Weis and von Scheidt, 1997) and it may lead to sudden death, myocardial infarction, or dump in cardiac function (Valantine, 2003).

3.2.3.3 Biology of chronic vascular rejection

The arterial wall contains three distinct layers (Figure 3: Arterial remodelling in chronic vascular rejection (from(Libby and Pober, 2001)).A). The innermost layer, the intima, consists of a monolayer of endothelial cells which are positioned on extracellular matrix and occasional smooth muscle cells. The intima is situated ablumenally on the internal elastic lamina. The next layer is the tunica media and consists of arterial smooth muscle cells within an elastin- and collagen-rich extracellular matrix. The next layer is the external elastic lamina. The adventitia, the outermost layer of the artery, consists of myofibroblasts, autonomic nerve endings, few lymphocytes, and extracellular matrix.

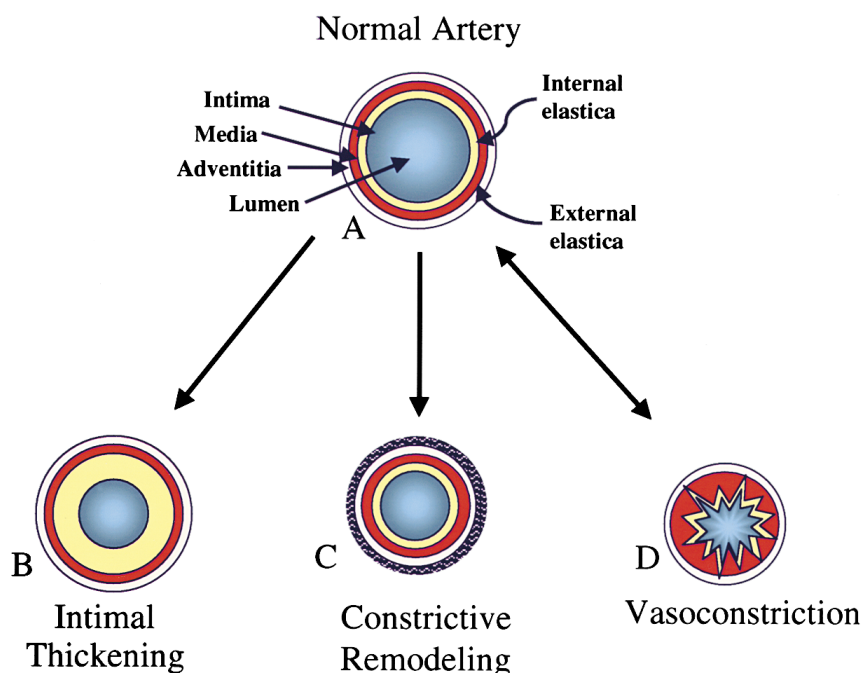


Figure 3: Arterial remodelling in chronic vascular rejection (from(Libby and Pober, 2001)).

Vascular lumen loss in transplant vasculopathy is mostly due to the thickening of the intima (Figure 3B), characterized by intimal accumulation of mononuclear cells, particularly in the early stage (Salomon et al., 1991), and vascular smooth muscle cells which are both mainly host derived (Hillebrands et al., 2001; Shimizu et al., 2001). Furthermore, myofibroblasts accumulate and the expansion of extracellular matrix has been observed. As a consequence of inflammation at the sites of injury, adhesion molecules such as ICAM-1 and VCAM-1 are upregulated on the endothelium (Ardehali et al., 1995), thus leukocytes are recruited and colonize the thickened intima of the graft (Hruban et al., 1990; Salomon et al., 1991; Russell et al., 1994b). The central role of T-cells and EC in the development of CTV has been further proven by the significance of the ICAM-1:LFA-1 pathway (Russell et al., 1995; Russell et al., 1994b). However, it is important that EC are preserved in long-term allografts (Rifle et al., 2006) and thus are able to act as on-going targets of an alloresponse. Mediators, such as IFN γ secreted by lymphocytes, mediate further injury and enhance extracellular matrix synthesis and MHC class II expression on EC. IFN γ -blocking with monoclonal antibodies clearly demonstrated that IFN γ is required for the development of CTV because lesions did not build up in its absence (Nagano et al., 1997; Nagano et al., 1998; Russell et al., 1994d). IFN γ is mainly produced by activated CD4⁺ T-cells but also by activated CD8⁺ T-cells and macrophages (Munder et al., 1998) that, once they are activated, can be significant to maintain an environment where CTV advances (Nagano et al., 1998). Further risk factors for CTV development are frequency and severity of acute rejection. In addition, differences in MHC and ineffective immunosuppression augment the risk (Isobe et al., 2006). Furthermore, nonimmunologic factors, such as the origin of the donor graft from a cadaveric donor or diseases like hyperlipidemia, diabetes, hypertension, or high donor age, are also known to increase the risk of CTV (Caforio et al., 2004; Kemna et al., 1994; Vassalli et al., 2003). In addition, cytomegalovirus infection has been demonstrated to promote CTV (Potena and Valantine, 2007; Valantine, 2004). Taken together, these findings confirm the overall impact of various vascular wall insults that finally lead to intimal hyperplasia.

3.2.4 Cytomegalovirus infection

It is well known that transplant patients undergo immunosuppressive treatment to prevent the potent rejection response. As a consequence, an array of side effects, such as defects in the control of pathogens (e.g., viral reactivation), can arise. However, accumulating evidence supports the hypothesis that viral infections play an imperative role in the pathogenesis of solid organ allograft rejection. In clinical and experimental models an association of viral

infection has been shown. Most of the studies centred their attention on Cytomegalovirus (CMV) as the initiating infectious agent.

CMV is a ubiquitous herpesvirus which persists in the host. Primary CMV infection is frequently asymptomatic. Cells like myeloid lineage cells, smooth muscle cells, and EC are crucial sites of CMV replication and latency (Jarvis and Nelson, 2002). In heart transplant recipients, CMV infection is a common finding (Potena and Valantine, 2007). Moreover, in a mouse model with the closely related murine CMV (MCMV) EC have also been shown to be sites of viral latency, particularly small vessels and capillaries harbour the MCMV genome (Koffron et al., 1998). Recent studies demonstrate the impact of CMV infection on the pathogenesis of allograft rejection, CTV and, long-term graft outcome. CMV-related CTV manifests as intimal thickening and constrictive vascular remodelling (Potena et al., 2003). Even in the absence of CMV replication, CMV interacts with inflammatory pathways and mechanisms of immune-regulation of recipients that subsequently leads to graft damage inducing acute and chronic rejection (Potena and Valantine, 2007). It has further been shown that only parts of the virus and very low viral burden have the capacity to disrupt the subtle equilibrium between graft, host and the immune system (Boehme et al., 2006; Carlquist et al., 1999; Compton et al., 2003; Tu et al., 2006). Moreover, in rat renal transplant recipients, rats with RCMV infection showed significantly reduced renal function measured in serum creatinine levels and had considerable inflammatory cell infiltration compared to rats without RCMV infection. Furthermore, chemokines, such as RANTES, MCP-1, MIP-1 α , and the IP-10, were up-regulated (Soule et al., 2006). However, recent studies evaluating the outcome of CMV prophylaxis in solid organ recipients demonstrate improved survival and lower rejection episodes in patients with anti-CMV treatment (Hodson et al., 2005; Potena et al., 2006).

3.2.5 Immunologic mechanisms of chronic vascular rejection

3.2.5.1 Animal models

Recent studies of transplantation rejection and its mechanisms have predominantly been performed in mice. Although transplantation of hearts or other grafts requires difficult microsurgery, the availability of useful reagents, well characterized inbred strains, and transgenic mice have made them to become the preferred experimental model. The most frequently employed form for heart transplantation at the moment is the heterotopically grafting of whole hearts into the abdomen of recipient mice. In these cardiac grafts, the ventricular chambers do not fulfil pumping function, although the coronary arteries are

perfused. This leads to the development of an intracavitary thrombus. Nevertheless, transplantation of allogeneic hearts in recipients with MHC disparities induces the development of graft arteriosclerosis that resembles human lesions in many characteristics.

3.2.5.2 The role of CD4⁺ T-cells

In chronic rejection, CD4⁺ T-cells are activated by the recognition of alloantigens, mostly by the indirect (via host lymphocytes) pathway. It is known that activation of CD4⁺ T-cells requires the expression of MHC class II and costimulatory molecules by APC. After activation they release proinflammatory cytokines that support the generation of CD8⁺ CTL responses and the activation of DC. Cytokines such as TNF, LT and IFN γ , lead to the induction of inflammatory infiltrates. Indeed, in the absence of TNF-receptors on the graft, CTV has not developed (Suzuki et al., 2003). Furthermore, clearly reduced arterial lesions are seen in IFN γ -deficient mice grafted with an allogeneic heart graft (Nagano et al., 1997). Moreover, the activation state of multiple cell types and the regulation of MHC and costimulatory molecule expression is affected by IFN γ . In addition, IFN γ influences the production of other cytokines, chemokines and adhesion molecules and the extra cellular matrix. However, CD4⁺ T-lymphocytes provide CD4⁺ T-helper functionality by the provision of signals that promote differentiation and activation of alloantibody-producing B-cells. T-helper cells (Th) activate macrophages unspecifically leading to direct tissue damage (Black, 1999; Lowry, 1996). Nonetheless, in the absence of antigen elimination, i.e., after transplantation, CD4⁺ T-cells and macrophages stay activated and continue to release cytokines which promote the growth of stromal cells and fibrosis which, afterwards, leads to chronic rejection (Black, 1999; Salomon et al., 1991).

3.2.5.3 The role of CD8⁺ T-cells

During chronic transplant rejection, donor antigen may be directly presented by donor cells, expressing MHC class I molecules, i.e., graft EC, (Biedermann and Pober, 1999; Biedermann and Pober, 1998) or it may be cross-presented to CD8⁺ T-cells by host APC, such as DC and EC. Cross-presentation of alloantigen by EC has been shown in a mouse model with transplanted skin where anti-H-Y monospecific H-2b-restricted MataHari CD8⁺ T-cells rejected H-2k male skin grafts on female recipients (Valujskikh et al., 2002a). This indicates that donor H-Y antigens are processed and presented by recipient EC and thus, CD8⁺ T-cells were activated by a minor histocompatibility difference only. This rejection was clearly IFN γ -dependent. However, by their ability of producing IFN γ , CD8⁺ T-cells support the

general inflammatory environment and the activation of leukocytes (Fischbein et al., 2002). But their main role in chronic graft rejection is direct cytolysis of parenchymal and vascular cells which express MHC class I molecules-donor-antigen-complexes (Rosenberg and Singer, 1992). CTL are the major effector cells in rejection. This has been confirmed in a mouse aortic allograft model where absence of CD8⁺ T-cells in MHC-I knockout recipients, prevented the development of chronic rejection (Sun et al., 2001). It is important to point out that effector cell triggering occurs in response to any target expressing the particular peptide-MHC complex. Even very low antigen concentrations are sufficient for their induction while activation of CD8⁺ T-cells requires costimulatory signals offered by only a few APC and triggering of at least 20-50% of the TCR. (Lassila et al., 1988; Medzhitov and Janeway, Jr., 1998; Ridge et al., 1998; Valitutti et al., 1996).

3.2.5.4 The role of CD4⁺CD25⁺Foxp3⁺ regulatory T-cells

Regulatory T-cells are one of the most powerful mechanisms to induce antigen-specific self-tolerance (Joffre et al., 2004). This feature may have important implications for transplantation since the induction of alloantigen-specific tolerance would be the treatment of choice to evade graft rejection and GVHD. However, in an experimental model transplanted allogeneic BM has been protected from rejection by host T-cells via the injection of CD4⁺CD25⁺ regulatory T-cells cultured *ex vivo* (Joffre et al., 2004). In this study specific CD4⁺CD25⁺Foxp3⁺ T-cells could be cultivated and expanded *in vitro* by stimulation with host-type APC and in the presence of high IL-2 concentrations and the addition of TGF-β. In a subsequent study, CD4⁺CD25⁺Foxp3⁺ regulatory T-lymphocytes could even prevent acute and chronic rejection of skin and heart allografts (Joffre et al., 2008). Mice treated with clinically acceptable levels of irradiation got long-term tolerance to BM and subsequent skin and cardiac allografts after transfer of regulatory T-cells stimulated *in vitro* with alloantigen. Furthermore, acute rejection could be prevented by regulatory T-cells specific for directly presented antigen, whereas for the prevention of chronic rejection regulatory T-cells specific for both directly and indirectly presented alloantigens had to be used. In the same context, Krupnick et al. showed that allogeneic presentation by EC led to the generation of CD4⁺CD25⁺Foxp3⁺ regulatory T-cells (Krupnick et al., 2005). Tolerance induced by regulatory T-cells is IFNγ-dependent (Thebault et al., 2007).

3.2.5.5 The role of endothelial cells in acute and chronic rejection

The endothelium had been seen for a long time as a simple passive layer within the vessel wall. Now it is known to be engaged in physiologic and pathophysiologic immune processes,

such as atherosclerosis, autoimmune diseases, inflammation, and allograft rejection (Briscoe et al., 1998b; Gimbrone, Jr. et al., 2000; Salmi et al., 1993; Wick et al., 1997). It is of major importance in maintaining normal vessel wall function; it controls thrombus building, leukocyte adhesion, the proliferation of vascular smooth muscle cells, and regulates the vessel tonus.

EC build the inner lining of graft vessels and are therefore the first graft cells encountered by host lymphocytes in solid organ transplantation. By promoting both the recruitment and the activation of alloreactive T-cells they participate in the process of allograft rejection (Valantine, 2003; Wick et al., 1997). Furthermore, they are exposed to multiple events that provoke EC injury which could subsequently initiate local vascular events, known as CTV.

After activation, T-cells are recruited to the peripheral tissue and thereby have to transmigrate through monolayers of vascular EC. The recruitment of activated T-cells from the blood to the site of inflammation is a multistep process regulated by leukocyte/EC interaction (Butcher and Picker, 1996). It starts with the rolling of T-lymphocytes along the endothelium, which is mediated by EC-expressed selectins, such as E- and P-selectin interacting with L-selectin expressed on leukocytes (Rao et al., 2007; Schon et al., 2002). Afterwards, leukocytes use integrins, such as LFA-1, MAC-1, and VLA-4, to interact with EC-expressed ligands including ICAM-1 and VCAM-1 to achieve adherence (Butcher and Picker, 1996; Nelson and Krensky, 2001). The expression of integrins on leukocytes can be regulated in part by EC-secreted chemokines, whereas their expression seems to be mainly dependent on IFN γ produced by T-cells (Kobayashi et al., 2003). Interaction with VCAM-1 and ICAM-1 on graft EC leads to the entry of activated T-cells into the allograft by transendothelial cell migration (Campbell et al., 1998). It has been shown that T-cell transmigration through the EC barrier changed their surface markers and their ability to migrate. Furthermore, the avidity for the allogeneic stimulators became higher which influenced the effector function of T-cells infiltrating into the graft (Denton et al., 1999).

However, resting EC express MHC class I molecules and low levels of costimulatory molecules, such as CD80 (B7-1), on their surface. In the presence of inflammatory cytokines, for instance IFN γ , MHC class II expression can be induced and the expression of MHC class I molecules may be upregulated, whereas CD80 expression is not changed (Marelli-Berg et al., 2000; Pober et al., 1997; Rose, 1998; Russell et al., 1994d). This phenomenon enables EC *in vitro* to act as APC which has been demonstrated via isolated mouse EC presenting an alloantigen triggering alloreactive CD8⁺ T-cells to become potent cytotoxic and IFN γ -secreting effector cells but not CD4⁺ T-cells (Kreisel et al., 2002a; Ma and Pober, 1998).

Stimulation of CD8⁺ T-cells has also been induced after culture with resting endothelium (Kreisel et al., 2002a). This direct activation is B7-dependent since T-cell proliferation was inhibited by CTLA4-Ig which blocks CD28 costimulation (Kreisel et al., 2002a). Besides, human resting CD8⁺ T-cells could also be directly activated by alloantigen presenting EC *in vitro* (Epperson and Pober, 1994). CD4⁺ T-cells proliferated only when cultured with IFN γ treated allogeneic EC since thereby the expression of MHC class II molecules was induced (Pober et al., 1997). Kreisel et al. additionally provided *in vivo* evidence of priming naive alloreactive CD8⁺ T-cells through non-hematopoietic allograft cells leading to acute rejection (Kreisel et al., 2002b). In this chimeric transplantation model alloantigens were expressed on graft EC but not on graft-derived hematopoietic cells. These facts suggest that EC may have the potential to stimulate naive allogeneic T-cells, particularly when no other alloactivation is present. Taken together, these data clearly show that vascular EC can act as APC to allogeneic CD8⁺ T-cells and can trigger allograft rejection via CD8⁺ direct allorecognition. This is of main significance considering that EC are present for the life span of the allograft and thus contribute to the progress of chronic rejection. Whether nonhematopoietic cells, such as vascular EC, possess the ability to activate alloreactive CD4⁺ T-cells, has been controversially discussed for a long time. An *in vivo* study by Kreisel et al. finally displayed that vascular endothelium could not activate CD4⁺ T-cells via direct allorecognition, even when EC were cytokine-activated and MHC class II molecules expression was induced. In this study, hearts that express MHC class II and hearts which lack MHC class II on hematopoietic cells were transplanted. Both types were acutely rejected, but with significant delay in the latter case, regardless of the expression of MHC class II on graft EC (Kreisel et al., 2004).

Further data from a minor histocompatibility disparate model showed that murine lung EC, loaded with peptide, could induce proliferation of CD8⁺ T-cells but they lose their immunogenicity following IFN γ treatment and become tolerogenic towards CD8⁺ T-cells *in vitro*. They further failed to induce a proliferative response in CD4⁺ T-cells, although EC expressed CD80 molecules and expression of MHC class II molecule was induced, and MHC class I upregulated (Marelli-Berg et al., 2001). In contrast, activated and peptide-pulsed EC were indeed killed more efficiently by effector CD8⁺ T cells (Marelli-Berg et al., 2000).

Furthermore, several *in vitro* and *in vivo* studies confirmed that cognate interaction between naive T-cells and resting EC, considered as non-professional APC, leads to CD8⁺ T-cell tolerance (Limmer et al., 2000; Marelli-Berg et al., 2000; Perez et al., 1998). Also cross-presentation of oral antigens or antigens from apoptotic tumour cells by LSEC lead to specific CD8⁺ T-cell tolerance (Berg et al., 2006; Limmer et al., 2005). The ability of EC to

cross-present antigens in the context of transplantation has been demonstrated by a study of Bagai et al. showing that cultured murine aortic EC are able to process and present MHC-I-restricted antigen (Bagai et al., 2005a).

In addition, a recent *in vitro* study displayed CD4⁺CD25⁺Foxp3⁺ regulatory T-cell induction by EC leading to the inhibition of T-cell proliferation. This process was independent of CD80 costimulation but dependent on PD-L1 (Krupnick et al., 2005).

However, only a few studies assessed the role of EC-CTL interaction *in vivo*, and whether EC tolerize or activate naive T-cells is a matter of further investigations. Although, the diverse settings of the different studies, such as dissimilar sources and activation states of EC, different affinities of T-cells and the use of nominal versus alloantigen, may be explanations for the partially contradictory results, one could summarize and conclude as follows: First, in the context of alloantigens (major histocompatibility antigen), CD8⁺ T-cells have been shown to become activated via resting EC *in vitro* and *in vivo*, whereas CD4⁺ T-cells become activated only via activated EC *in vitro* but not *in vivo*. Second, in the context of a nominal antigen (minor histocompatibility antigen), EC can induce CD8⁺ T-cell proliferation *in vitro*, whereas activated EC fail to induce CD8⁺ and CD4⁺ T-cell *in vitro* and *in vivo*, and there is indeed rather induction of T-cell tolerance.

However, the fact that EC constitutively express MHC-I molecules and MHC-II molecules expression is inducible, make them susceptible to cellular and humoral host immune reactions. They are target cells for activated alloreactive CTL during GVHD which is characterized by the circulation of a large number of mhAg-specific CTL (Biedermann et al., 2002; Mutis et al., 1999). Alloantigen-specific CD8⁺ T-cells directly recognize peptide-donor-MHC molecules complexes on EC and kill them. Apoptosis of EC induced by cytotoxic CD8⁺ T-cells is a key initiating event in the development of CTV. It leads to the contact to the subendothelial matrix and thus, to the enhancement of the inflammatory response. At sites of EC injury mononuclear leukocytes are recruited. Because of their production of biologically relevant molecules, they favour the accumulation and proliferation of vascular smooth muscle cells. This leads to neointima formation and at a later time point to ischemic graft loss. It has been shown, that it is mainly a perforin-dependent process since perforin-deficient CTL failed to destroy EC in culture. Interestingly, the deficiency in Fas/FasL had only a minor impact (Krupnick et al., 2002). Furthermore, Valujskikh et al. could provide evidence, that EC have the ability to process antigenic proteins derived from exogenous sources and present them to recipient CD8⁺ T-cells on recipient MHC I molecules. T-cells that recognized cross-presented antigen mediated skin-graft rejection (Valujskikh et al., 2002a). *In vitro* studies by Marelli-

Berg et al. demonstrated the ability of CD8⁺ cytotoxic T-cells to lyse cytokine-treated EC presenting a mhAg (Marelli-Berg et al., 2000; Marelli-Berg et al., 2001). And, in a heart transplantation mouse model of CTV with minor histocompatibility disparity, perforin knockout recipient mice as well as granzyme B knockout recipients, had indeed comparable levels of T-cell infiltration but less endothelial apoptosis and therefore less fibroproliferative changes (Choy et al., 2004b; Choy et al., 2004a). Apoptotic EC were also shown in coronary arteries of human transplants mounting early signs of CTV (Dong et al., 1996b; Dong et al., 1996a) confirming T-cell induced endothelial injury being a central event in the development of CTV.

Moreover, activated EC play a major role in the coagulation process supporting coagulation and platelet aggregation. In addition, vascular endothelial growth factor (VEGF) and platelet-derived growth factor (PDGF) are proinflammatory factors in the transplantation setting and help to develop CTV (Nykanen et al., 2006). This is of significant importance since they precipitate endothelial cell injury, which induces platelet adhesion and the release of growth factors and leads to the subsequent development of CTV.

Taken together, these data indicate the importance of EC in the development of acute and chronic rejections. And there is increasing evidence implying that EC are key players in the process of chronic rejection.

3.2.6 Therapy of allograft rejection

Immune responses directed against allografts are mainly orchestrated by T-cells that become activated after recognizing alloantigens and appropriate costimulatory signals. Since stimulation through TCR without costimulation leads to T-cell anergy (Schwartz, 1990), the blockade of T-cell costimulation provides an attractive target for therapies aimed at limiting acute rejection. In addition to the use of drugs, such as cyclosporine which reduces the T-cell response, or other macrolide immunosuppressants, such as rapamycin, the use of reagents that block costimulatory signals show promising results in preventing the acute rejection response (Khoury et al., 1999). Furthermore, the use of rapamycin together with anti-CD40L therapy promotes long-term allograft survival whereas cyclosporine, tacrolimus, and IL-2R monoclonal antibody therapy abolish the outcome of CD40L-block (Sho et al., 2002).

3.2.7 Tolerance induction in transplantation

Although the presence of effective immunosuppressive drugs prevents acute rejection, many of the standard drugs inhibit T-cell signalling and therefore inhibit the active process of tolerance induction, which is the treatment of choice in allograft rejection and GVHD.

Currently, different ways to induce specific T-cell tolerance are assessed, including co-stimulatory blockade, introduction of chimersim, the use of CD4⁺CD25⁺Foxp3⁺ regulatory T-cells, and the combination of those.

However, one of the best characterized and most important costimulatory pathways is the CD28/CTLA4:CD80/CD86 pathway (Salomon and Bluestone, 2001). These costimulatory molecules belong to the B7 family. CD28 is constitutively expressed on T-cells whereas CD86 molecules are expressed at low levels by resting professional APC. CD80 and CD86 are both upregulated on activated professional APC. Their interaction induces full T-cells activation including cytokine production (Rothstein and Sayegh, 2003) while the interaction of CTLA-4, a molecule similar to CD28 but only expressed after cellular activation, delivers a negative signal, resulting in T-cell inhibition (Sharpe and Freeman, 2002). However, it has been demonstrated that the blockade of this pathway by CTLA-4Ig, induced donor specific tolerance in several experimental models (Lin et al., 1993; Pearson et al., 1994; Pearson et al., 1996; Sho et al., 2002). Deficiency in CD80/CD86 even resulted in xenograft survival (Hosiawa et al., 2005).

A further target is the costimulatory CD40L:CD40 pathway. It consists of the CD40 and the CD40L molecules that belong to the TNFR and TNF super families, respectively. CD40 is a cell surface molecule that is expressed on several cell types including most professional APC (Grewal and Flavell, 1998). CD40 is also expressed on EC and therefore mediates their activation responses (Hollenbaugh et al., 1995). CD40L is expressed on activated T-cells, as well as on other cell types, such as activated B-cells and DC (Grewal and Flavell, 1998), and activates expression of adhesion molecules and cytokines in EC (Hollenbaugh et al., 1995; Karmann et al., 1995). However, it has been demonstrated that CD40 signalling is important in T-cell-mediated immunity where it induces the expression of co-stimulatory molecules and cytokines, such as CD80, CD86, ICAM-1, IL-1, and IL-12 on APC (van Essen et al., 1995), and upregulates MHC I and MHC II expression to turn the APC a more effective APC. Furthermore, CD40L:CD40 interaction activates antigen-specific T-cells to proliferate and secrete cytokines (Grewal et al., 1995; Grewal et al., 1996). The lack of CD40L in mice infected with lymphocytic choriomeningitis virus (LCMV) did not influence the virus-specific CD8⁺ T-cell response but the virus-specific CD4⁺ T-cell response was rigorously compromised (Whitmire et al., 1999). Blockade of both pathways can prevent allograft rejection (Kirk et al., 1997). Nevertheless, although promising results are seen in many experimental models, the blockade of these pathways does not completely control rejection (Guo et al., 2001; Jones et al., 2000). This indicates that not all alloreactive T-cells depend on

these costimulatory signals. For example, primed allospecific T-cells or alloreactive CD4⁺ memory T-cells act independently and circumvent CD40:CD40L costimulation (Chen et al., 2004; Valujskikh et al., 2002b).

A further pathway consisting of molecules belonging to the B7 family is the PD-1:PD-L1/PD-L2 pathway. PD-L1 and PD-L2 are expressed by a broader range of cells. Additionally to its expression on professional APC, PD-L1 is also expressed by vascular EC where it is upregulated in response to IFN γ and TNF (Eppihimer et al., 2002; Rodig et al., 2003). It is involved in the suppression of the T-cell cytokine synthesis which is proportional to the primary stimulus (Mazanet and Hughes, 2002). In contrast to PD-L1, PD-L2 is restricted to hematopoietic APC, such as DC and macrophages (Latchman et al., 2001). Both are ligands of PD-1 which transduces inhibitory signals and is induced on peripheral T-cells, B-cells, and myeloid cells upon activation (Agata et al., 1996). However, it has been shown that expression of PD-L1 is important for peripheral control of autoreactive T-cells (Keir et al., 2006). In a model where CD8⁺ T-cell myocarditis could be induced by transfer of OT-I CTL into mice which express membrane-anchored Ova exclusively in cardiac myocytes, IFN γ -induced PD-L1 on cardiac EC played a major role in preventing inflammatory injury to the myocardium (Grabie et al., 2007). Further studies have assessed the role of PD-1 and its ligand in murine cardiac allograft rejection and have shown that treatment with blocking anti-PD-1 antibody aggravate acute cardiac allograft rejection. Agonists, used with immunosuppressive drugs, improved acute cardiac rejection and CTV (Ito et al., 2005; Ozkaynak et al., 2002). In a very recent study PD-1:PDL1 interaction acquired transplantation tolerance. Indeed, using blocking antibodies against PDL1 demonstrated that recipient but not donor PDL1 is necessary to acquire tolerance induction and maintenance after CTLA-4lg therapy (Tanaka et al., 2007). These data provide evidence for the major role of PD-1:PDL1 interaction in peripheral tolerance induction after transplantation by limiting the expansion of alloreactive T-cells. Taken together, it is the integration of positive and negative costimulatory signals that finally decides on the outcome of a T-cell response.

Following transplantation of a solid organ allograft donor microchimerism can occur. Such rare cases of donor microchimerism are associated with long-term acceptance of the organ, therefore microchimerism is thought to play an active role in the induction of unresponsiveness (Ehl et al., 1998a; Cosimi and Sachs, 2004; Millan et al., 2002; Monaco, 2002; Wood and Sachs, 1996). However, chimerism has been shown to be a tool to induce specific transplantation tolerance (Claas, 2004). This has been confirmed in a study by Bonilla et al, demonstrating that microchimerism induced active clonal deletion of donor cell-specific

T-cells (Bonilla et al., 2006), and identifying the induction of chimerism as a specific and effective way to prevent transplant rejection.

Furthermore, the combination of chimerism with the adoptive transfer of CD4⁺CD25⁺Foxp3⁺ regulatory T-lymphocytes prevented acute and chronic allograft rejection in a skin and heart transplantation mouse model *in vivo* (Joffre et al., 2008). The combination of both methods may be a way to reduce the toxicity of previous irradiation in BM transplantation and may be a promising protocol acceptable in clinical settings.

Despite hopeful results for the therapy in acute rejection and the fact that acute rejection can be prevented by immunosuppressive drugs, chronic rejection still occurs. While the number of patients that survive up to one year after transplantation continues to increase, the percentage of long term survival has not changed significantly over the past 20 years (Taylor et al., 2005). There is indeed a current lack of effective preventive therapies for chronic rejection. On the one hand, actual therapies may not be able to ameliorate the problems of chronic rejection because they target the wrong mechanisms. On the other hand, since the fundamental basis of chronic rejection is not well understood, it is obvious that the current drugs do not solve the problem. A more targeted immunosuppressive treatment may be a step in this direction. Therefore, the diffuse nature of chronic vascular rejection has to become clearer. Any plan that can limit immune or nonimmune injury to the vessels should help hinder graft vasculopathy.

Consequently, the mechanism underlying acute and chronic rejection, particularly the detailed interaction between donor and host APC and recipient leucocytes, has to be investigated, with the aim to reach a more specific therapy to prevent allograft rejection.

3.3 A transgenic model to investigate the cognate interaction of CD8⁺ T-cells with vascular endothelial cells expressing a minor histocompatibility antigen

Transgenic techniques are powerful tools to set up models with microbial antigens present in specific tissues and therefore facilitate the targeting of antigens to, for example, vascular EC. Antigen transgenic mice combined with the use of TCR transgenic animals have been shown to provide essential insight into the basic principle of immunology, such as autoimmunity (Ohashi et al., 1991; von Herrath and Oldstone, 1996), and tumour immunity (Morgan et al., 1998; Speiser et al., 1997). To study the role of EC in presenting a mAg *in vivo*, two different lines of transgenic mice that express bacterial β -gal exclusively on the vascular endothelium exist. In VWF-LacZ mice the expression of β -gal in EC is restricted to the heart and the brain (Aird et al., 1995; Aird et al., 1997). In Tie2-LacZ mice, a portion of the promoter and first intron of the gene encoding *tie2*, the receptor for the vascular differentiation factor angiopoietin-1, drives the expression of β -gal antigen in EC in all organs (Schlaeger et al., 1997). In the studies hereafter, Tie2-LacZ mice, backcrossed to the C57BL/6 background were used. In Tie2-LacZ mice, cells remain in their physiological setting and transgene expression avoids inflammation, therefore they are ideal in order to investigate antigen presentation by EC *in vivo* and to gain insight into the basic mechanisms initiating chronic vascular rejection. Furthermore, the use of recent transgenic models, facilitating the specific ablation of CD11c-positive DC in lymphoid organs (CD11c-DTR-mice) (Jung et al., 2002), and the use of naturally occurring mutants, exhibiting a mutated H2-K^b molecule that precludes H2-K^b-restricted presentation of peptides (B6.C-H2^{bm1}-mice), allowed dissecting the role of professional versus non-professional APC. Additionally, *in vivo* analysis of antigen-specific interaction between EC and CD8⁺ T-cells has been eased by using high affinity β -gal specific TCR transgenic CD8⁺ T-cells (Bg1-cells). To assess the cognate interaction of EC with activated CTL in a transplantation model, where Tie2-LacZ and control C57BL/6 hearts were heterotopically transplanted into C57BL/6 recipients, the repetitive priming of recipients with DC presenting an H-2K^b-restricted peptide derived from β -gal, induced activation of β -gal specific CD8⁺ T-cells for more than two weeks and enabled the investigation of EC interaction with activated CD8⁺ T-cell in a chronic setting.

4 Aims of the study

The significance of chronic inflammatory processes in the pathogenesis of cardiovascular diseases is widely known. Atherosclerosis and autoimmune myocarditis are prototypic diseases demonstrating the unfavourable effect of constant immune activation within the cardiovascular system. Likewise, chronic rejection of solid organ transplants is characterized by a constant immune activation within the blood vessels of the transplanted organ. Despite the success in the treatment of acute rejection with immunosuppressive drugs, late graft rejection of a major organ (heart, liver, and kidney) occurs at a rate of 3-5% per year post transplantation (Cecka, 1999; Keck et al., 1999). Constant inflammation during chronic rejection results in a persistent activation of both helper and cytotoxic T-cells. It is likely that these inflammatory processes are driven through chronic antigen presentation within the blood vessel wall, by both professional APC (DC, macrophages and B cells) and non-professional APC (e.g. EC). The detailed characterization of the interaction of CD8⁺ T-cells with professional and non-professional APC would be a major issue helping to delineate the exact mechanisms underlying vascular rejection of solid organ transplants.

EC act as the major interface between blood and tissue. In the context of transplantation they fulfil important functions, including regulation of host leukocyte trafficking into the graft parenchyma, activation of the recipient lymphocytes, transmission of signals to other vascular cells, and angiogenesis reactions that develop during the healing process. Moreover, the vascular endothelium is a dynamic structure that contributes to the inflammatory response by the synthesis and display of various surface receptors and soluble molecules. Therefore, we hypothesized that the direct interaction of EC with T-cells is a critical factor for the activation or possible tolerization of T-cells.

A first specific aim of our study was to analyze the interaction of EC with CD8⁺ T-cells in Tie2-LacZ mice *in vivo*. Using BM chimeric mice, we determined whether there is a direct interaction of β -gal specific CD8⁺ T-cells with β -gal-expressing EC or whether β -gal peptides are cross-presented by BM derived APC.

In the second part of this study, we wanted to develop an experimental system that facilitates *in vivo* analysis on the cognate interaction of EC with activated CTL in a heterotopic heart transplantation model. Such a model should enable dissection of the immunologic mechanisms underlying the vascular rejection of Tie2-LacZ hearts transplanted

in C57BL/6 recipients. Furthermore, the role of persistent MCMV infection of transplant recipients for the acceptance of heart grafts was explored.

We expect to provide a detailed study of the factors influencing the decision making process between T-cell activation versus T-cell tolerization in the context of chronic vascular rejection of solid organ grafts. In the long-term, we want to analyze the factors that influence EC-CTL interaction. These insights into the basic immunological mechanisms underlying chronic inflammatory processes may permit the evaluation of treatment strategies that could help to specifically tone-down the harmful consequences of chronic immune activation in chronic vascular rejection.

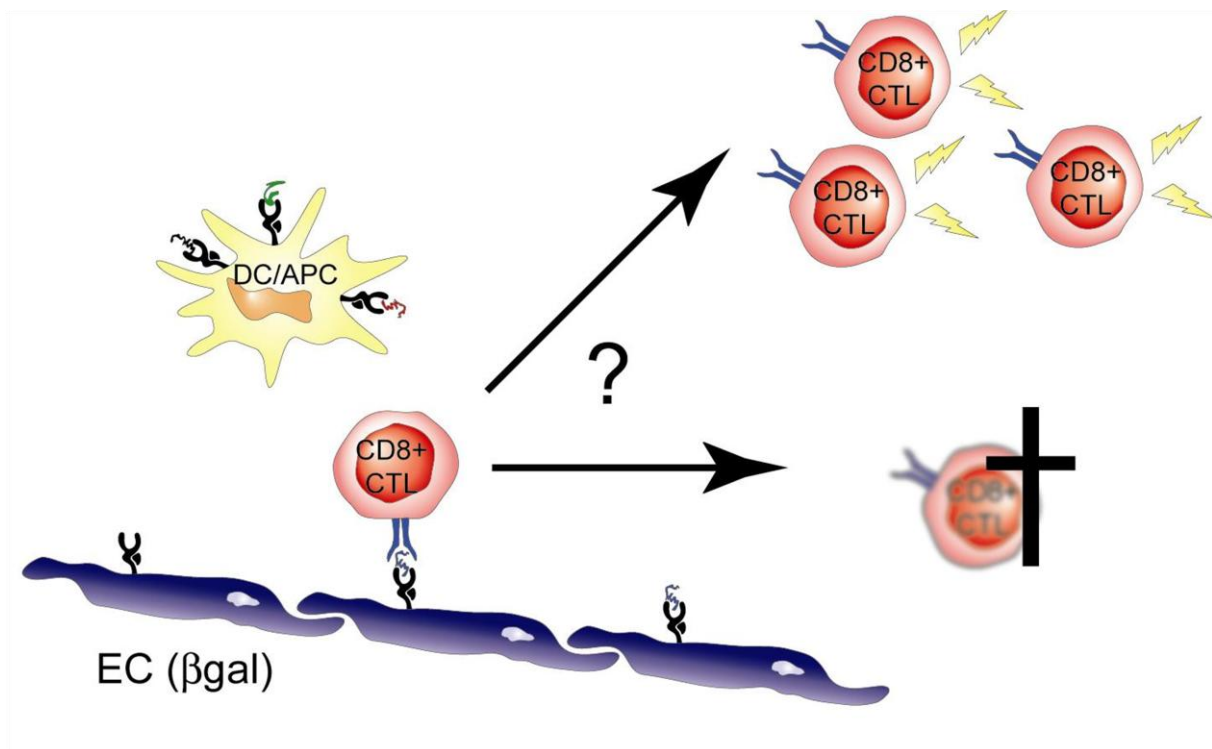


Figure 4: Do vascular EC that present a minor histocompatibility antigen activate or tolerize naive CD8⁺ T-cells?

5 Results

5.1 Immunologic ignorance of vascular endothelial cells expressing minor histocompatibility antigen

Beatrice Bolinger^{1*}, Philippe Krebs^{1*}, Yinghua Tian³, Daniel Engeler¹, Elke Scandella¹,
Simone Miller¹, Douglas C. Palmer², Nicholas P. Restifo², Pierre-Alain Clavien³ and
Burkhard Ludewig¹

¹Research Department, Cantonal Hospital St. Gallen, CH-9007 St. Gallen, Switzerland

²National Cancer Institute (NCI), National Institutes of Health (NIH), Bethesda, Maryland,
USA

³Departement of Visceral Surgery, University Hospital Zurich, CH-8091 Zurich, Switzerland

* These authors contributed equally to this work

Published in: Blood, 2008, May 1; 111 (9): 4588-95

5.1.1 Abstract

Endothelial cells (EC) presenting minor histocompatibility antigen (mhAg) are major target cells for alloreactive effector CD8⁺ T-cells during chronic transplant rejection and graft-versus-host disease (GVHD). The contribution of EC to T-cell activation, however, is still a controversial issue. In this study, we have assessed the antigen presenting capacity of EC in vivo using a transgenic mouse model with beta-galactosidase (β -gal) expression confined to the vascular endothelium (Tie2-LacZ mice). In a GVHD-like setting with adoptive transfer of β -gal-specific T-cell receptor transgenic T-cells, β -gal expression by EC was neither sufficient to activate nor to tolerize CD8⁺ T-cells. Likewise, transplantation of fully vascularized heart or liver grafts from Tie2-LacZ mice into non-transgenic recipients did not suffice to activate β -gal-specific CD8⁺ T-cells, indicating that CD8⁺ T-cell responses against mhAg cannot be initiated by EC. Moreover, we could show that spontaneous activation of β -gal-specific CD8⁺ T-cells in Tie2-LacZ mice was exclusively dependent on CD11c⁺ dendritic cells (DC) demonstrating that mhAg presented by EC remain immunologically ignored unless presentation by DC is granted.

5.1.2 Introduction

Endothelial cells act as the major interface between blood and tissues. Forming the inner lining of blood vessels, they are uniquely positioned between circulating lymphocytes and the periphery and thereby regulate the trafficking of T-lymphocytes from the bloodstream to sites of infection and inflammation. Following transplantation of vascularized organs, EC are the first graft cells encountered by activated host lymphocytes and are therefore primary targets of alloreactive CTL (Briscoe et al., 1998a; Libby and Pober, 2001). Since donor EC persist in vascularized organ transplants, they may contribute to chronic immune stimulation and thereby fuel the process of chronic rejection. Such late graft failure is a major problem in transplantation medicine that frequently necessitates retransplantation (Weis and von Scheidt, 1997). Furthermore, EC are important target cells for activated alloreactive CTL during graft versus host disease (GVHD) (Biedermann et al., 2002) which is characterized by large numbers of circulating minor histocompatibility antigen (mhAg)-specific CTL (Mutis et al., 1999).

EC can act as antigen presenting cells (APC) to CD8⁺ T-cells both via the direct pathway (i.e. recognition of allo-MHC:peptide complexes) or via the indirect pathway involving cross-presentation of exogenous antigens (Valujskikh et al., 2002a; Bagai et al., 2005b; Limmer et al., 2000). *In vitro* studies have demonstrated that both human (Epperson and Pober, 1994) and murine (Kreisel et al., 2002a) EC can activate resting allogeneic CD8⁺ T-cells suggesting that EC critically contribute to the initial stimulation of alloreactive T-lymphocytes (Briscoe et al., 1998a). Moreover, EC exhibit important functions of professional APC including expression of MHC class II and costimulatory molecules (Rose, 1998) and cross-presentation of mhAg (Valujskikh et al., 2002a; Limmer et al., 2000). The notion that EC may under particular circumstances act as professional APC has been supported by the finding that non-hematopoietic cells within vascularized grafts - presumably EC - are able to initiate CTL responses that mediate allograft rejection (Kreisel et al., 2002b).

There are, however, a number of reports challenging the view that EC may act as immune activators. Murine lung EC, for example, have been shown to negatively regulate CD8⁺ T-cell function (Marelli-Berg et al., 2000). Furthermore, liver sinusoidal EC can induce CD8⁺ T-cell tolerance to soluble (Limmer et al., 2000; Limmer et al., 2005) or tumour-derived antigens (Berg et al., 2006). A third possible form of EC-CTL interaction is that of immunological ignorance. Indeed, *aly/aly* mice lacking secondary lymphoid organs fail to reject vascularized organ transplants, even in an allogeneic setting (Lakkis et al., 2000) suggesting that the

environment of organized lymphoid tissues is critical for primary activation of T-cell responses. To a large extent the above-mentioned contradictory findings can be explained by the use of *in vitro* co-culture systems or the lack of an appropriate *in vivo* model with truly EC-restricted antigen presentation. An experimental *in vivo* system with expression of well-defined antigens exclusively in vascular EC may therefore be helpful to solve the question whether antigen presentation by vascular EC can lead to activation or tolerization of antigen-specific CD8⁺ T-cells.

The use of antigen-transgenic mice combined with the power of TCR transgenic animals has provided important insight into the basic principles of autoimmunity (Ohashi et al., 1991; von Herrath and Oldstone, 1996), and tumour immunity (Morgan et al., 1998; Speiser et al., 1997). Recently, similar systems have been exploited to analyze T-cell responses in different allograft transplantation (Kreisel et al., 2004; Valujskikh et al., 2002a; Valujskikh et al., 2006; Ford et al., 2007) and GVHD models (Ehl et al., 1998b). However, despite significant advances in our understanding of the antigen presenting function of non-hematopoietic cells during allograft reactions (Kreisel et al., 2004) or the importance of T-cell frequencies for solid organ graft rejection (Ford et al., 2007; He et al., 2004b), the precise role of mhAg presentation by EC has remained elusive. We have used here Tie2-LacZ mice to model mhAg presentation by EC. In these mice, the *tie2* promoter drives the expression of the beta-galactosidase (β -gal) antigen in EC in all tissues (Schlaeger et al., 1997). *In vivo* analysis of antigen-specific interaction between EC and CD8⁺ T-cell has been facilitated by using high affinity β -gal-specific TCR transgenic CD8⁺ T-cells (Bg1 cells). Adoptive transfer of Bg1 CD8⁺ T-cells into Tie2-LacZ mice revealed that mhAg presentation by EC did not suffice to activate or to tolerize CD8⁺ T-cells. Furthermore, β -gal expression by EC in heterotopically transplanted Tie2-LacZ hearts or orthotopically transplanted Tie2-LacZ livers did not result in CD8⁺ T-cell activation in naive recipients. Finally, generation of bone marrow (BM) chimeric mice that facilitated selective ablation of CD11c-positive dendritic cells (DC) revealed that EC-associated mhAg has to be cross-presented by DC in order to elicit CD8⁺ T-cell activation.

5.1.3 Materials and Methods

Mice

Male and female C57BL/6 mice were obtained from Charles River (Sulzfeld, Germany). Tie2-LacZ mice (Schlaeger et al., 1997) had been backcrossed with C57BL/6 for at least 14 times. B6.C-H2^{bm1} mice were provided by Christian Kurts (University of Bonn, Germany). Bg1 mice were produced with TCR cassette vectors generously provided by Dr Diane Mathis (Brigham and Women's Hospital, Boston, MA). RNA was isolated from a β -gal₉₆₋₁₀₃-specific CD8⁺ T-cell clone, generated by limiting dilution, using silica matrix columns (Qiagen, Valencia, CA). Known TCR α and β constant region sequences were used to perform 5' rapid amplification of cDNA ends (Invitrogen, Carlsbad, CA), and TCR sequences were then cloned into pCR4TOPO TA cloning sequencing vectors (Invitrogen). The TCR α and β transcripts were sequenced using an ABI Prism (Perkin-Elmer, Wellesley, MA), and these sequences were compared with available sequences to develop genomic cloning polymerase chain reaction (PCR) primers. These cloning primers provide amplification of the variable domains consisting of 10–20 bp upstream of the start codon through 200–300 bp of intronic sequence downstream of the junctional regions, thereby preserving splice donor/acceptor sites. The α and β genomic variable domains were PCR-amplified (Perkin-Elmer) and TA-cloned into a sequencing vector (Invitrogen). The genomic variable domains were sequenced (V α 1/J α TA13/C α and V β 7S1/J β 2S4/C β 2) and subcloned into the TCR cassette vectors. The α and β cassette vectors were coinjected into fertilized C57BL/6 embryos (SAIC, Frederick, MD) and founders were obtained. The resulting mice, named Bg1, were maintained as heterozygotes, as a high rate of lymphoma in homozygotes reduced their life span. Heterozygotes were bred to B6.SJL mice and transgene expression was monitored by staining of blood cells with anti-V β 7 by flow cytometry. Bg1 mice were further crossed with C57BL/6 mice expressing the congenic marker Thy 1.1. Mice expressing the human high affinity diphtheria toxin receptor (DTR) under the control of the CD11c promoter (Jung et al., 2002) were provided by Steffen Jung (The Weizmann Institute of Science, Rehovot, Israel). The presence of the β -gal and DTR transgenes was determined by PCR from genomic DNA; the presence of the H2-K^{bm1} molecule was determined by flow cytometry of blood lymphocytes using the 5F1 antibody (Sherman and Randolph, 1981). All animals were kept under conventional conditions in individually ventilated cages and fed with normal chow diet. Experiments were carried out with age (6-8 weeks) and sex-matched animals. Experiments were performed in accordance with Swiss Cantonal and Federal legislations.

Viruses and peptides

Recombinant MCMV expressing the β -gal protein under the transcriptional control of the human CMV *ie1/ie2* promoter-enhancer (MCMV-LacZ RM427 (Manning and Mocarski, 1988)) was kindly provided by Prof. E. S. Mocarski (Stanford University, San Francisco). MCMV-LacZ was propagated and titrated on NIH 3T3 cells (ECACC, UK) and injected intravenously at a dose of 2×10^6 pfu. β -gal₉₆₋₁₀₃ (DAPIYTNV) (Overwijk et al., 1997), β -gal₄₉₇₋₅₀₄ (ICPMYARV) (Oukka et al., 1996) and MCMV M45₉₈₅₋₉₉₃ (HGIRNASFI) (Gold et al., 2002) peptides were purchased from Neosystem (Strasbourg, France).

Generation of bone marrow chimeric mice

Recipient mice were lethally irradiated with 900 rads from a linear accelerator (clinics of radio-oncology, Cantonal Hospital St. Gallen) and intravenously injected 1 day later with 2×10^7 of the indicated donor BM cells. Chimeric mice were maintained on antibiotic water containing Sulfadoxin and Trimethoprim (Borgal, Veterinaria AG, Zurich, Switzerland) for the following 3 weeks. Recipient mice carrying the K^{bm1} mutation, received CD4⁺- and CD8⁺-T-cell-depleted BM and were further depleted of NK 1.1⁺ cells by i.p. injection of 20 μ l anti-asialo GM1 antibody (Wako Pure Chemical Industries, Ltd.) on the day before and in weekly intervals for 6 wk following irradiation. Mice were used for experiments 8 to 10 weeks after bone marrow reconstitution.

Antibodies and flow cytometry

Anti-CD8-FITC, anti-CD4-PerCP, anti-V β 7-FITC, anti-CD90.1-PE, anti-CD44-PE, and anti-IFN γ -PE were obtained from BD PharMingen (Basel, Switzerland). Anti-CD8-APC was obtained from Biolegend (LuBioScience GmbH, Lucerne, Switzerland). Anti-CD62L-PE was obtained from ImmunoTools (Friesoythe, Germany). For flow cytometry, single cell suspensions were generated from the indicated organs and 1×10^6 cells were incubated with the indicated mAb at 4°C for 20 min. For PBL samples, erythrocytes were lysed with FACS Lysing Solution (BD PharMingen). Cells were analyzed with a FACScalibur flow cytometer using the CellQuest software (BD Biosciences). The cells were analyzed by flow cytometry, gating on viable leukocytes using 7-aminoactinomycin D (Sigma).

Construction of tetrameric MHC class I-peptide complexes and flow cytometry

MHC class I monomers complexed with β -gal (H-2K^b) or M45 peptides (H-2D^b) were produced as previously described (Altman et al., 1996) and tetramerized by addition of streptavidin-PE (Molecular Probes, Eugene, OR). At the indicated time points following infection, organs were removed and single cell suspensions were prepared. Aliquots of 5×10^6 cells or 300 μ l of blood were stained using 50 μ l of a solution containing tetrameric class I-peptide complexes at 37°C for 10 min followed by staining with anti-CD8-FITC (BD Pharmingen) at 4°C for 20 min. Absolute cell counts were determined by counting leukocytes in an improved Neubauer chamber.

Chromium release assay

EL-4 cells pulsed with peptide or without peptide (negative control) were used as target cells in a standard ^{51}Cr release assay. Cells were labelled with 200 μCi ^{51}Cr (EGT Chemie, Tägerig, Switzerland) for 1 h at 37°C. A total of 10^4 target cells/well were incubated for 5 h in 96-well round bottom plates with 3-fold serial dilutions of effector cells. Splenocytes from MCMV-LacZ infected mice that were restimulated with the indicated peptides for 5 days were tested for their cytolytic activity. Spontaneous chromium release was always below 15%.

CFSE labelling of TCR transgenic T cells and adoptive transfer

Single cell suspensions from spleens of Bg1 mice were subjected to hypotonic red blood cell lysis and stained with CFSE (Molecular Probes, Leiden, The Netherland). A maximum concentration of 2.5×10^7 cells/ml were incubated in 5 μM CFSE in PBS for 10 min at 37°C. Cells were washed twice with ice-cold BSS and resuspended in BSS at a concentration of 1.5×10^7 splenocytes/ml. Recipient C57BL/6, Tie2-LacZ and the different subsets of chimeric mice were injected i.v. with 1.5×10^7 Bg1-Thy 1.1⁺ splenocytes in 500 μ l BSS.

Immunohistology

Freshly removed organs were immersed in HBSS and snap-frozen in liquid nitrogen. Frozen tissue sections were cut in a cryostat and fixed in acetone for 10 minutes. Sections were incubated with antibodies against β -gal (MP Biomedicals), CD8 (clone YTS169.4.2) followed by goat anti-rat Ig (Caltag Labs) and alkaline phosphatase-labelled donkey anti-goat Ig (Jackson ImmunoResearch Labs). Alkaline phosphatase was visualized by using AS-BI

phosphate/New Fuchsin, and sections were counterstained with hemalum, and images were acquired using a Leica DM R microscope equipped with a Leica DC300 FX camera. Digital images were processed using Adobe Photoshop.

Surgical procedure for liver transplantation

Donor procedure, back-table preparation, and recipient procedure were performed as described previously with minor modifications (Tian et al., 2002). Briefly, all vessels and ligaments of the liver were dissected in the donor after midline laparotomy. *In situ* perfusion of the liver was performed, using cold (4°C) Ringer's solution. Subsequently, the liver was separated from its retroperitoneal attachments and removed. The graft was stored in cold (4°C) Ringer's solution for 60 minutes until implantation into the recipient. Following hepatectomy of the native liver in the recipient, the donor liver was implanted in an orthotopic position. The anhepatic time in the recipient was consistently kept below 20 minutes. The portal vein was reconstructed and the liver was reperfused after completing the anastomosis between the suprahepatic inferior vena cava of the recipient and donor. Arterial recirculation was established by an end-to-side anastomosis between the recipient aorta and an aortic segment attached to the hepatic artery of the graft. A single subcutaneous injection of 5 mg cefazolin provided antibiotic prophylaxis.

Heterotopic heart transplantation

Heterotopic vascularized cardiac transplantation was performed according to the method described by Corry et al. (Corry et al., 1973). Donor hearts were explanted from either male Tie2-LacZ or male C57BL/6 mice. The donor heart was removed from the chest after intracaval injection of 1 ml of heparin (100 U/ml), rinsed with NaCl 0.9% and placed on ice. After isolation of the recipient's abdominal aorta and inferior vena cava, the donor ascending aorta and pulmonary artery were joined end-to-side to the recipient's aorta and vena cava, respectively, using 10-0 nylon running suture. The abdomen was closed with individual running sutures to musculofascial layer and skins. The recipient mouse was then warmed for a few hours during recovery from anesthesia and had free access to water and food. The function of the transplanted heart was assessed daily by abdominal palpation.

Statistical data analysis

To evaluate statistically significant differences, the unpaired two-tailed Student's test was used. p values smaller than 0.05 were considered statistically significant. Statistical data analysis was performed using GraphPad Prism version 4.03 for Windows (GraphPad Software Inc., San Diego California USA).

5.1.4 Results

5.1.4.1 CD8 T-cell tolerance in Tie2-LacZ mice

Currently, a number of transgenic mouse lines are available that exhibit EC-restricted transgene expression: von Willebrandt Factor-LacZ (Aird et al., 1995) and thrombomodulin-LacZ (Weiler-Guettler et al., 1996) mice which both show patchy transgene distribution in some arteries, and tie2-H-2Kb mice (Limmer et al., 2005) which express the H2-K^b molecule as a transgene. In this study, Tie2-LacZ mice (Schlaeger et al., 1997) backcrossed to the C57BL/6 background have been used because of the uniform β -gal Ag expression in EC of all organs. It is noteworthy that the intensity of β -gal expression in Tie2-LacZ mice is most pronounced in small and large arteries, but clearly detectable in venous and capillary EC (Figure 5 and Figure S 1). Furthermore, expression levels of β -gal mRNA in various organs were comparable (Figure S 2) indicating that these mice are well-suited to study EC-CD8⁺ T-cells interaction *in vivo*. Indeed, in a previous study, Rothermel et al. (Rothermel et al., 2004) have used Tie2-LacZ mice on the FVB genetic background to assess CD4⁺ and CD8⁺ T-cell responsiveness under conditions of persisting Ag expression in EC. Whereas this previous investigation suggested that Tie2-LacZ mice can mount CD8⁺ T-cell responses against the EC-restricted β -gal antigen (Rothermel et al., 2004), the results obtained in the present study indicate that CD8⁺ T-cells in Tie2-LacZ mice are tolerant to the β -gal antigen. This is shown by the fact that Tie2-LacZ mice failed to mount β -gal-specific CTL responses following infection with β -gal-recombinant mouse cytomegalovirus (MCMV-LacZ), whereas CD8⁺ T-cell responses against the viral M45 epitope were not influenced by the EC-specific transgene expression (Figure 5 B and C).

We next addressed whether the apparent β gal-specific CD8⁺ T-cell tolerance in Tie2-LacZ mice is mediated by thymic negative selection or by peripheral tolerizing mechanisms. To this end, Tie2-LacZ mice were crossed with TCR transgenic Bg1 mice which possess CD8⁺ T-cells that recognize the H2-K^b-restricted β -gal₉₆₋₁₀₃ epitope (Overwijk et al., 1997). 60 - 70% of the Bg1 CD8⁺ T-cells bind H2-K^b/ β -gal₉₆₋₁₀₃ tetramers (Figure 6) and Bg1 CD8⁺

T-cells possess a high functional avidity for the β -gal₉₆₋₁₀₃ epitope as shown by target cell recognition and proliferation assays (Figure S 4). In the thymus of Tie2-LacZ×Bg1 mice, the numbers of transgenic V β 7 chain-positive and tetramer-binding CD8⁺ cells were reduced to 40% (Figure 6), suggesting that central tolerance led to partial deletion of β -gal-specific T-cells. Interestingly, in peripheral lymphoid organs such as the spleen, the numbers of V β 7-positive and β -gal₉₆₋₁₀₃ tetramer-binding CD8⁺ T cells was further reduced from 60-70% in Bg1 to less than 10% in Tie2-LacZ×Bg1 mice. Thus, at a first glance, it appears that EC-specific antigen expression in Tie2-LacZ mice precipitated both central and peripheral tolerance.

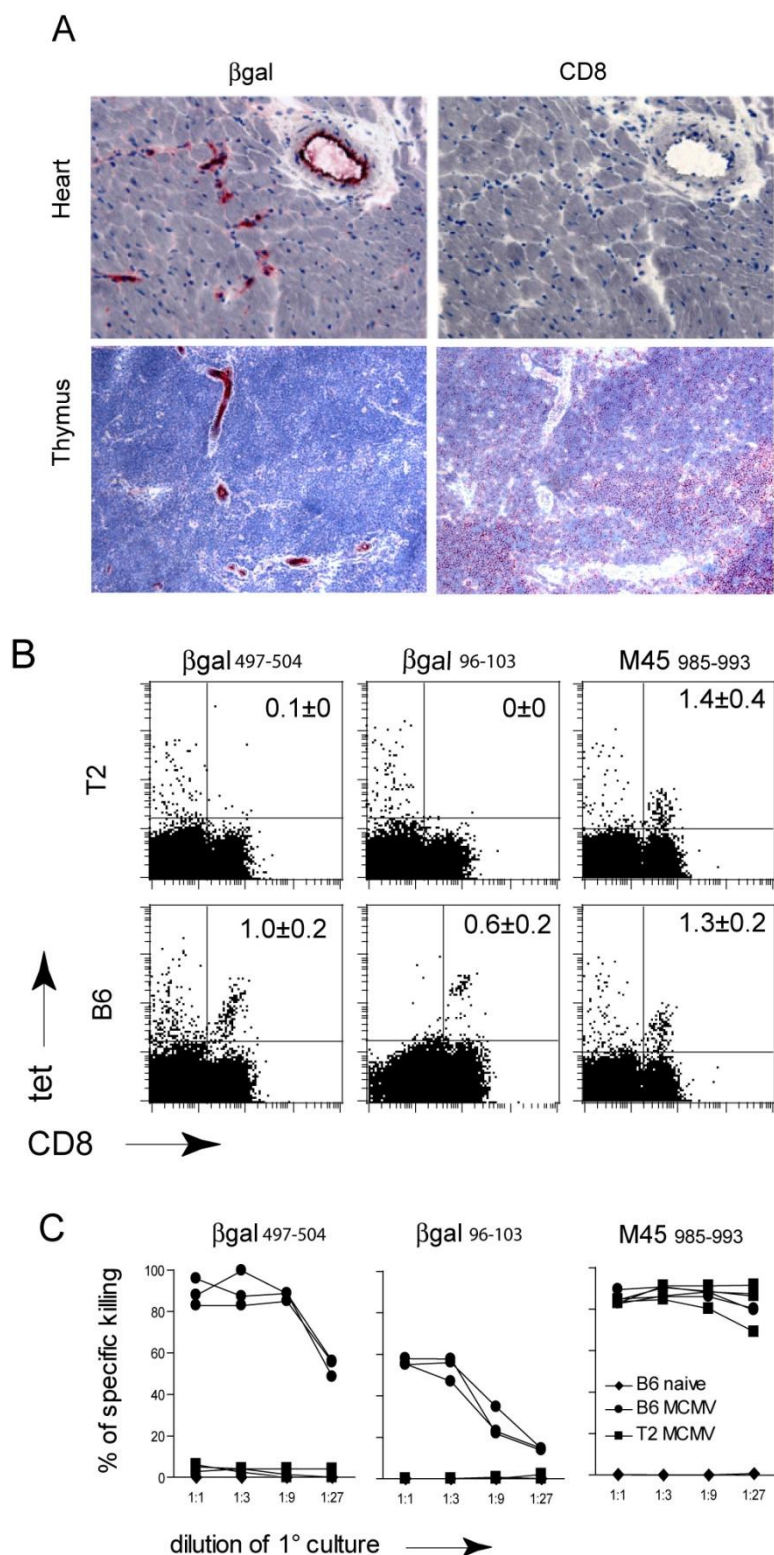


Figure 5: CD8⁺ T-cell reactivity in Tie2-LacZ mice. (A) Heart and thymus sections of naïve Tie2-LacZ mice were stained for β -gal and CD8. (B-C) C57BL/6 (B6) and Tie2-LacZ (T2) mice were infected i.v. with 10^6 pfu MCMV-LacZ. (B) Tetramer analysis for the indicated β -gal and MCMV-derived M45 epitopes was performed on day 6 post infection. Mean percentage of tetramer-positive cells within the CD8 compartment are indicated (\pm SEM; n=3-4). (C) Lysis of peptide-pulsed EL-4 cells by MCMV-LacZ-induced CTL. On day 6 post infection, splenocytes from the indicated mouse strains were restimulated in vitro for 5 days with β -gal₄₉₇₋₅₀₄, β -gal₉₆₋₁₀₃ or M45₉₈₅₋₉₉₃ peptide and tested in a standard chromium release assay.

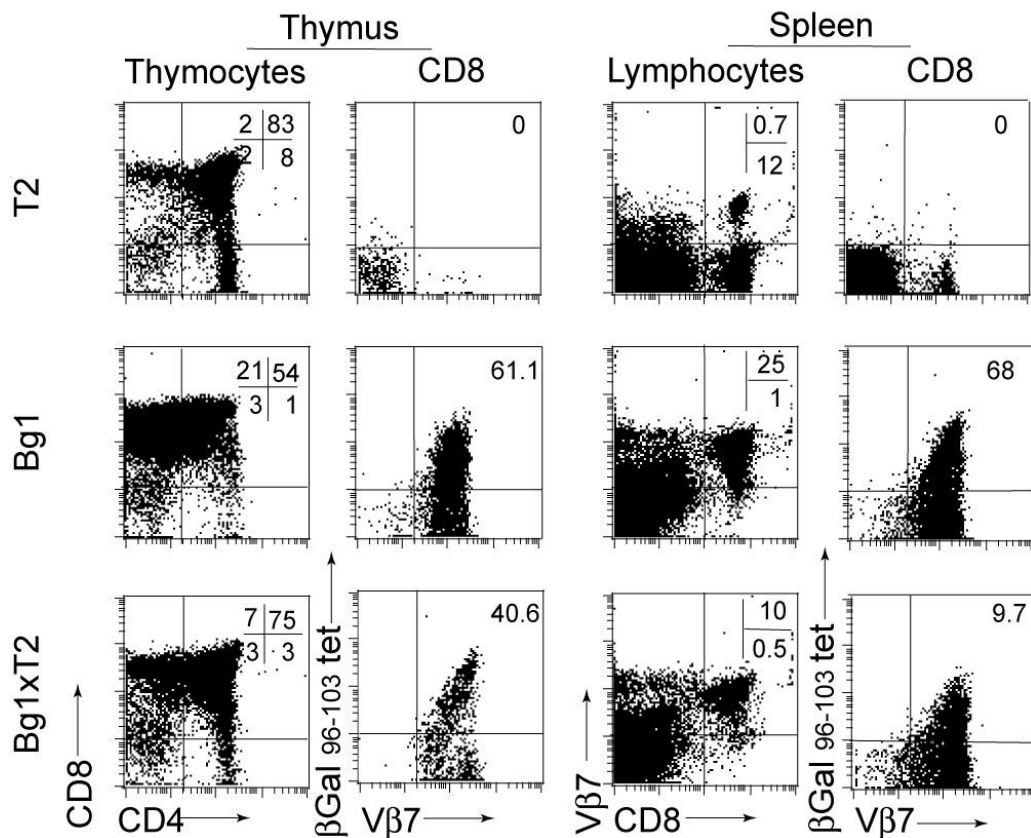


Figure 6: CD8⁺ T-cell tolerance in Tie2-LacZ mice. Tie2-LacZ mice were crossed with TCR transgenic Bg1 mice. Thymocytes from Tie2-LacZ (T2, upper row), Bg1 (middle row), and Bg1×Tie2-LacZ mice (Bg1×T2, lower row) were stained for CD4 and CD8 expression. The expression of the transgenic Vβ7 chain and binding of the H2-K^b-βgal₉₆₋₁₀₃ tetramer was determined by gating on CD8 T-cells. Values in the upper right quadrant indicate mean frequencies of CD4/CD8 positive cells in thymocytes or percentage of antigen-specific cells in single CD8 positive thymocytes, respectively (T2, n=2; Bg1, n=3; Bg1×T2 n=7). Splenocytes were assessed for Vβ7 and CD8 expression. Expression of βgal₉₆₋₁₀₃-specific cells was determined by Vβ7 and H2-K^b-βgal₉₆₋₁₀₃ tetramer staining, gating on CD8 T-cells (T2, n=3; Bg1, n=5; Bg1×T2 n=9).

5.1.4.2 EC-independent peripheral CD8⁺ T-cell tolerance

Peripheral CD8⁺ tolerance can be induced via different cell types including circulating hematopoietic cells expressing mhAg (Ehl et al., 1998a; Bonilla et al., 2006), BM-derived APC cross-presenting antigen derived from parenchymal tissues (Kurts et al., 1997; Kurts et al., 1998), or particular subsets of EC that, as well, possess the ability to cross-present circulating antigens (Limmer et al., 2000; Limmer et al., 2005). In order to assess truly EC-mediated peripheral tolerance induction, we established first a highly sensitive *in vivo* restimulation assay to detect very low amounts of circulating Bg1 cells. To this end, graded numbers of sorted CD8⁺Thy1.1⁺ cells from naive Bg1 mice were transferred into Thy1.2-positive Tie2-LacZ and C57BL/6 mice. Six days following adoptive transfer, mice were infected with MCMV-LacZ and the expansion of Bg1 cells was assessed six days later. As shown in Figure 7A, β -gal-specific CD8⁺ T-cells expanded in C57BL/6 but not in Tie2-LacZ mice, confirming that Bg1 cells encounter their antigen in Tie2-LacZ mice outside of the thymus and that this interaction leads to their deletion. However, reconstituting Tie2-LacZ mice with C57BL/6 BM revealed that CD8⁺ T-cell tolerance in Tie2-LacZ mice was solely dependent on β -gal expression within the BM (Figure 7B and C). These data indicate that expression of a mhAg by EC alone is neither sufficient to directly tolerize CD8⁺ T-cells nor is this antigen available to BM-derived APC in a way that would lead to CD8⁺ T-cell tolerance.

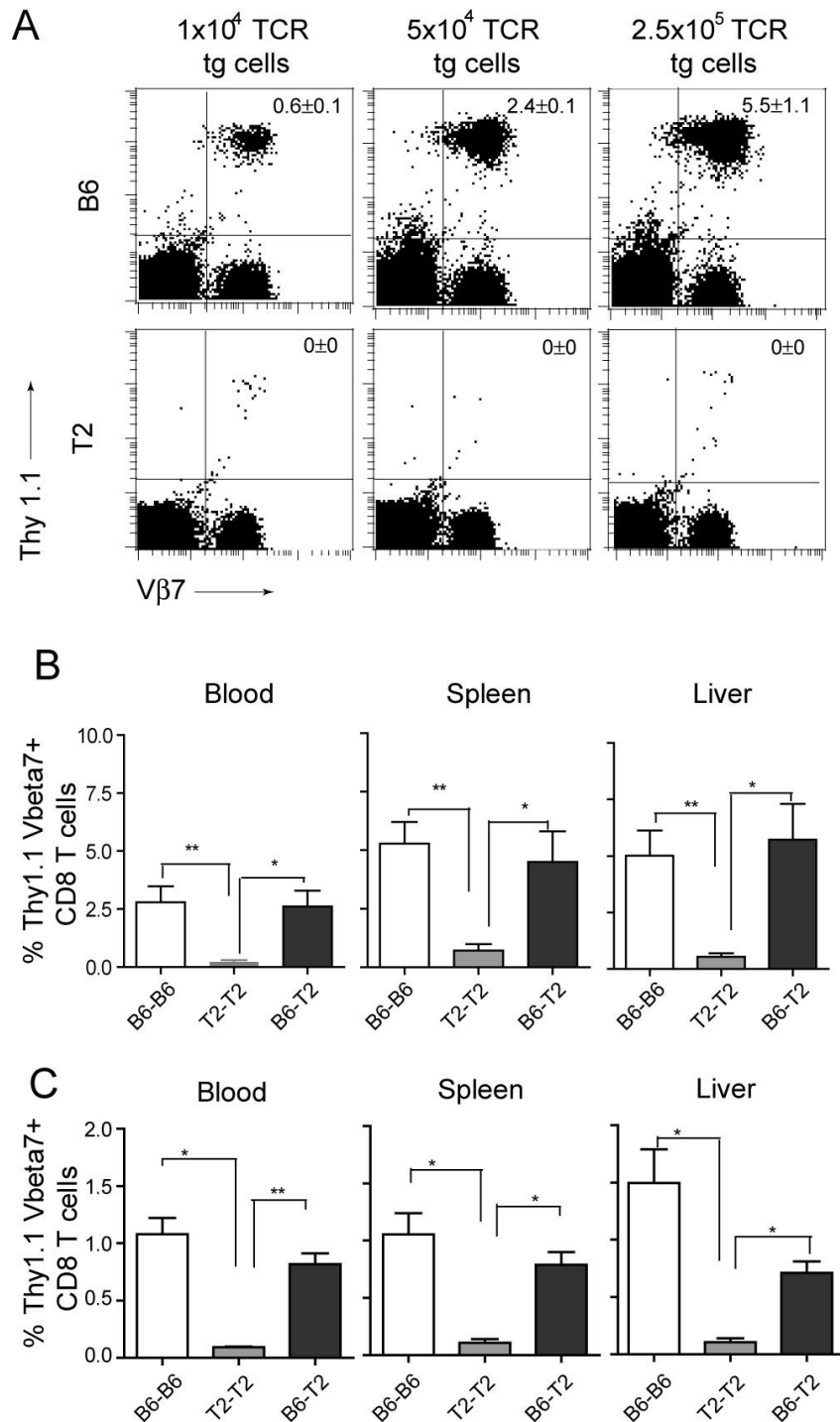


Figure 7: Loss of adoptively transferred Bg1 CD8⁺ T-cells in Tie2-LacZ mice is not dependent on β -gal expression by EC. (A) Graded numbers of CD8⁺ Bg1 cells expressing the congenic marker Thy1.1 were adoptively transferred into Thy1.2⁺ C57BL/6 or Tie2-LacZ recipient mice. Six days later, mice were challenged with 2×10^6 pfu MCMV-LacZ and the proliferation of Bg1 CD8⁺ T-cells was determined on day six following immunization by staining for CD8, Thy 1.1 and the transgenic V β 7 chain. Representative data from one out of 2 independent experiments are shown. (B, C) Adoptive transfer of Bg1 CD8⁺ T-cells in bone marrow chimeric mice. 5×10^4 (B) or 10^5 (C) TCR transgenic Thy1.1⁺ Bg1 cells were adoptively transferred into the indicated Thy1.2⁺ bone marrow chimeric mice. Nine days (B) or 30 days (C) later, mice were challenged with 2×10^6 pfu MCMV-LacZ and proliferation of Bg1 CD8⁺ T-cells was determined on day six following MCMV-LacZ challenge in the indicated organs. Values represent mean percentage (\pm SEM) of Thy1.1⁺V β 7⁺ cells within the CD8 T-cell compartment.

5.1.4.3 EC fail to directly activate naive CD8⁺ T-cells in vivo

Deletional tolerization of CD8⁺ T-cells via activation-induced cell-death is usually associated with a transient period of T-cell activation and proliferation (Ehl et al., 1998b; Kyburz et al., 1993). Furthermore, it is possible that EC in Tie2-LacZ mice might directly interact with CD8⁺ T-cells in a way that leads to T-cell activation and/or proliferation. In order to assess a potential spontaneous T-cell activation by EC in a GVHD-like model situation, 3×10^6 CFSE-labelled TCR transgenic CD8⁺ T-cells were adoptively transferred into Tie2-LacZ mice and T-cell activation was monitored as CD44 upregulation on proliferating Bg1 cells (Figure 8A). Quantification of β gal-dependent T-cell proliferation was achieved by adoptive transfer of CFSE-labelled, Thy 1.1-positive CD8⁺ Bg1 cells into either C57BL/6 or Tie2-LacZ mice (Figure 8B). This sensitive read-out system was then used to assess whether antigen presentation by EC alone is sufficient to mediate CD8⁺ T-cell activation or whether bone marrow-derived APC, in particular DC, contribute to the observed initial CD8⁺ T-cell triggering.

An array of BM chimeric mice was generated using different combinations between C57BL/6 (B6) and Tie2-LacZ (T2) controls, Tie2-LacZ mice on the C57BL/6^{bm1} background (T2bm1) exhibiting a mutated H2-K^b molecule that precludes H2-K^b-restricted presentation, and CD11c-DTR mice (Jung et al., 2002) which facilitate the specific ablation of CD11c-positive DC in lymphoid organs. As expected, Bg1 cells were not activated in B6→B6 chimeras (Figure 9A), whereas transgenic CD8⁺ T cells proliferated in T2→T2 chimeric mice (Figure 9B). Proliferation of Bg1 cells in B6→T2 chimeras indicated that BM-derived non-transgenic APC had activated the transgenic T cells (Figure 9C). This interpretation is supported by the fact that Bg1 proliferation was aborted in T2bm1→T2 chimeras where direct and cross-presentation via BM-derived APC is abolished and only EC can present the β -gal epitope (Figure 9D). Experiments with T2bm1→T2bm1 chimeras confirmed that Bg1 cells do not proliferate in the absence of the appropriate H2 restriction element (Figure 9E). Bg1 activation could be restored in B6→T2bm1 chimeras confirming that indeed BM-derived APC are crucial in this setting (Figure 9F). Finally, reconstitution of T2bm1 mice with BM from CD11c-DTR mice together with diphtheria toxin-mediated ablation of DC showed that the proliferation of Bg1 cells depended strictly on the cross-presentation of β -gal antigen by DC (Figure 9G). Taken together, these results indicate that β gal-presenting vascular EC remain immunologically ignored by CD8⁺ T-cells and that

activation and proliferation of CD8⁺ T-cells recognizing the mhAg in Tie2-LacZ mice is solely dependent on cross-presentation of Ag by BM-derived DC.

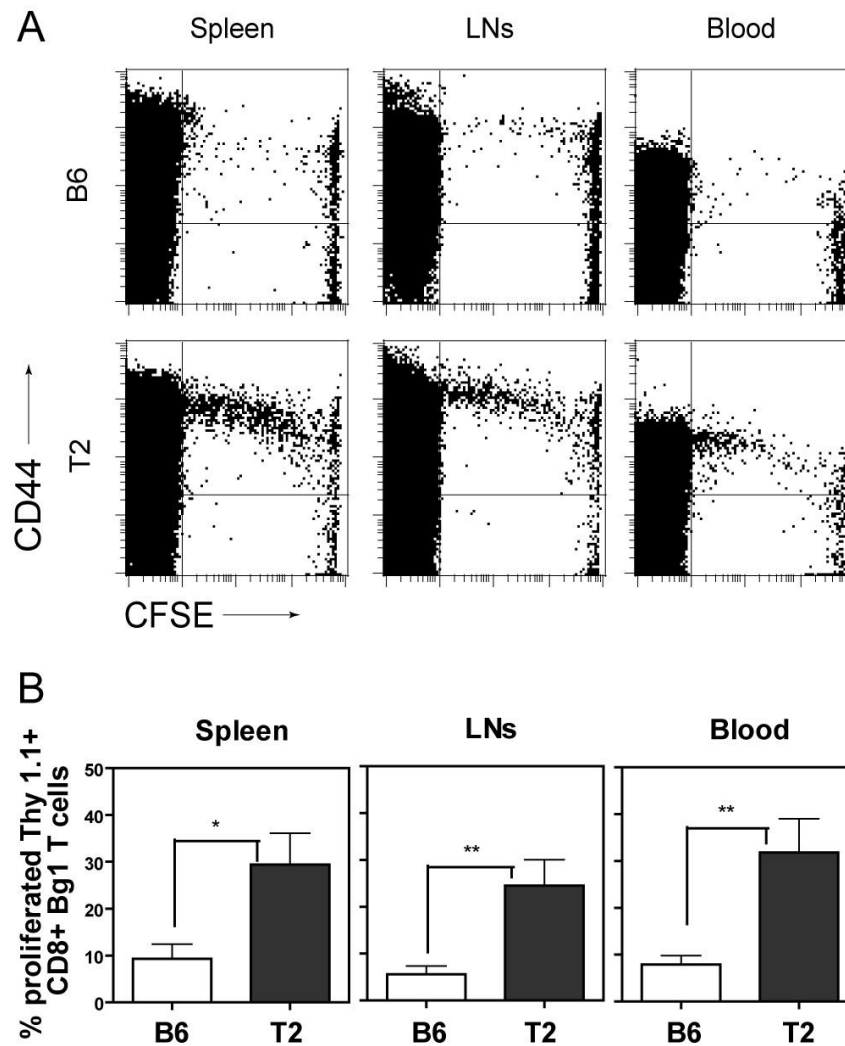


Figure 8: Activation of Bg1 CD8⁺ T-cells in Tie2-LacZ mice. (A) A total of 1.5×10^7 CFSE-labelled splenocytes (corresponding to 3×10^6 CD8⁺ TCR transgenic T-cells) from Bg1 mice were adoptively transferred into C57BL/6 or Tie2-LacZ mice. Mice were sacrificed on day four following transfer and cells from blood, spleen and LNs were analyzed by flow cytometry for CFSE dilution and CD44 upregulation on CD8⁺ lymphocytes. FACS-plots from one representative out of two independent experiments are shown. (B) Quantification of Bg1 T-cell proliferation. 1.5×10^7 CFSE-labelled Bg1 Thy1.1⁺ splenocytes were injected into C57BL/6 or naïve Tie2-LacZ mice. Mice were killed on day four following transfer and cells from blood, spleen and LNs were analyzed by flow cytometry. Values represent mean percentage (\pm SEM, n=7, pooled data from two independent experiments) of proliferating CD8⁺Thy1.1⁺ cells (*, p<0.05; **, p<0.005; ***, p<0.001).

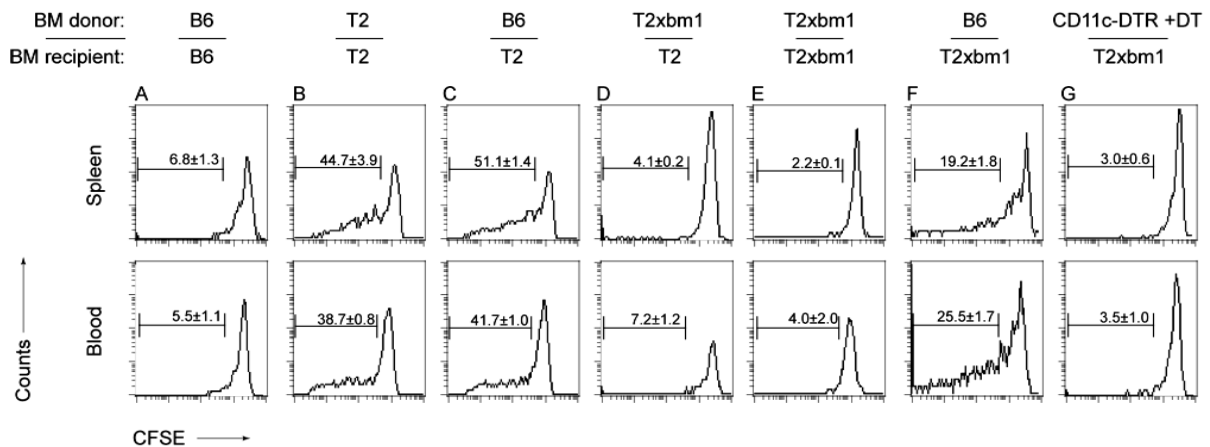


Figure 9: In vivo proliferation of Bg1 CD8⁺ T-cells in bone marrow chimeric mice. 1.5×10^7 CFSE-labelled splenocytes (corresponding to 3×10^6 CD8⁺ TCR transgenic T-cells) expressing the congenic marker Thy1.1 were adoptively transferred into the indicated Thy1.2⁺ bone marrow chimeras: (A) C57BL/6 → C57BL/6, (B) Tie2-LacZ → Tie2-LacZ, (C) C57BL/6 → Tie2-LacZ, (D) Tie2-LacZ×B6.C-H2^{bm1} → Tie2-LacZ, (E) Tie2-LacZ×B6.C-H2^{bm1} → Tie2-LacZ×B6.C-H2^{bm1}, (F) C57BL/6 → Tie2-LacZ×B6.C-H2^{bm1}, and (G) CD11cDTR → Tie2-LacZ×B6.C-H2^{bm1}. CD11c-DTR bone marrow recipients had been injected intraperitoneally with 4 ng/g body weight diphtheria toxin which led to a 95 to 98 % depletion of CD11c⁺ cells for > 48 h. Mice were sacrificed on day four following adoptive transfer and cells from blood, and spleens were analyzed by flow cytometry. Values in the histograms represent mean percentage (± SEM, n=5-7, pooled data from three independent experiments) of proliferating CD8⁺Thy1.1⁺ T-cells.

5.1.4.4 Immunologic ignorance of antigen expressing EC in vascularized organ transplants

In order to assess whether the above findings, obtained in a GVHD-like setting, also reflect EC-CTL interaction within vascularized organ grafts, a series of heart and liver transplantations were performed. Heterotopically transplanted Tie2-LacZ hearts were well-accepted in C57BL/6 recipients and spontaneous CD8⁺ T-cells responses against both the β -gal₄₉₇₋₅₀₃ (Figure 10A) and the β -gal₉₆₋₁₀₃ epitope (not shown) could not be detected. To analyze with higher sensitivity the effect of β -gal expression on vascular EC, we employed the adoptive transfer system of CFSE-labelled Bg1 cells. Two weeks following heterotopic heart transplantation (Figure 10B and D) or orthotopic liver transplantation (Figure 10C and E), CFSE-labelled Bg1 CD8⁺ T cells were adoptively transferred either into recipients that had received C57BL/6 (Figure 10B and C) or transgenic Tie2-LacZ organs (Figure 10D and E). This analysis revealed no significant differences in Bg1 CD8⁺ T-cell activation between recipients of Tie2-LacZ and C57BL/6 control organs indicating that EC within the transplanted organs had not primed naive CD8⁺ T-cells.

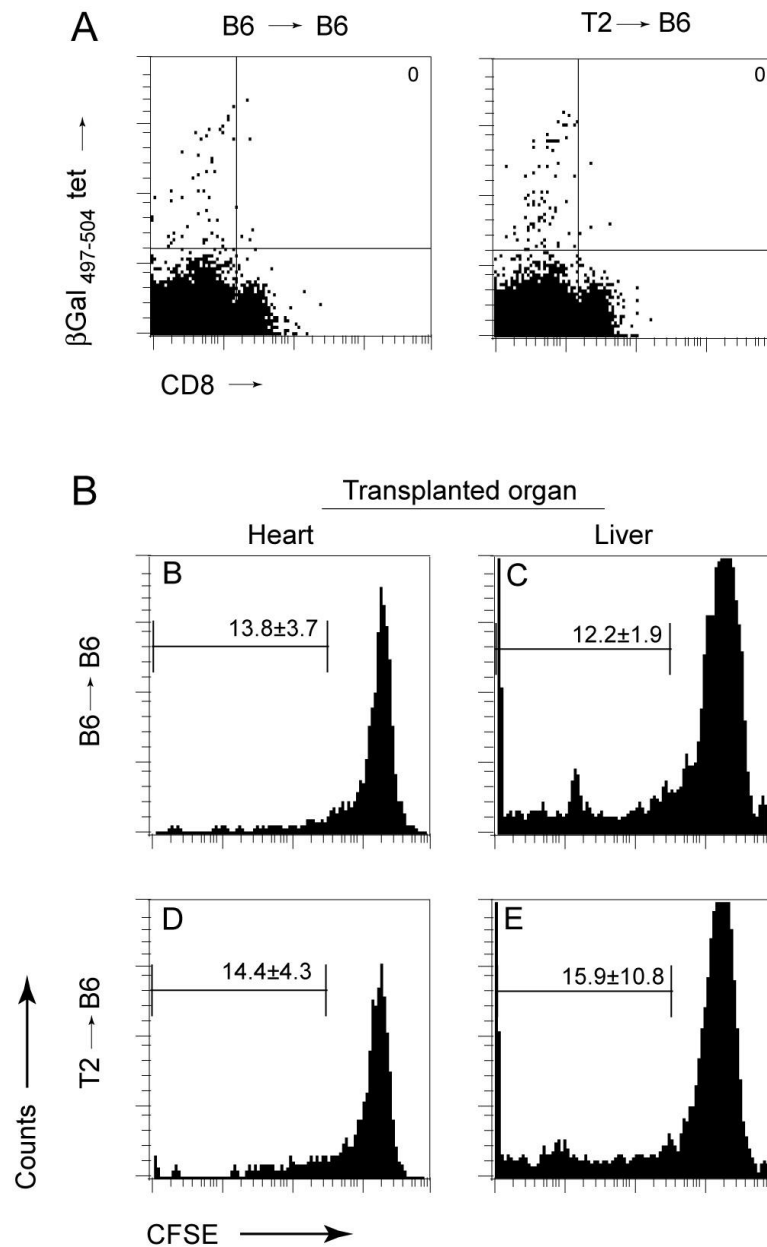


Figure 10: Lack of CD8⁺ cell activation in naive recipients of Tie2-LacZ vascularized organ grafts. (A) Spontaneous β-gal-specific CD8⁺ T-cell reactivity measured by tetramer analysis. C57BL/6 recipients received either C57BL/6 (B6 → B6) or Tie2-LacZ (T2 → B6) hearts and the presence of β-gal₄₉₇₋₅₀₄-specific CD8⁺ T-cells in blood was assessed by flow cytometry on day 20 post transplantation. (B-E) Activation of CD8⁺ Bg1 T-cells after adoptive transfer of 1.5×10^7 CFSE-labelled Bg1 splenocytes in C57BL/6 recipients on day 10 post transplantation. (B, D) Heterotopic heart transplantation with donor organs from C57BL/6 (n=7) (B) and Tie2-LacZ (n=8) (D) mice. Orthotopic liver transplantation with donor organs from C57BL/6 (n=5) (B) and Tie2-LacZ (n=4) (D) mice. Mice were sacrificed on day four following adoptive transfer and cells from blood were analyzed by flow cytometry. Values in the histograms represent mean percentage (\pm SEM) of proliferating CD8⁺Thy1.1⁺ cells.

5.1.5 Discussion

In this study, we have used antigen-transgenic mice with uniform mhAg expression in EC in combination with CD8⁺ T-cell TCR transgenics to demonstrate that mhAg presentation by EC does neither precipitate T-cell activation nor tolerization. The lack of any tolerizing effect of prolonged EC-CD8⁺ T-cell interaction is unexpected because non-activated, mhAg presenting EC in Tie2-LacZ provide "signal 1", i.e. antigen, in the absence of "signal 2", i.e. costimulation. Thus, the EC-associated antigen in Tie-LacZ mice that is expressed in a widespread and easily accessible fashion should lead to CD8⁺ T-cell tolerance, in particular, in the absence of "danger signals" (Matzinger, 1994). One could argue that EC possess an impaired capacity to present immunodominant peptides (Kummer et al., 2005) and therefore fail to interact with CD8⁺ T-cells. In Tie2-LacZ mice, however, EC can become target cells of CTL that have been appropriately activated via DC priming *in vivo* thereby leading to typical vascular rejection of organ grafts (Engeler et al., manuscript in preparation). Furthermore, it is unlikely that the antigen expression levels in EC of Tie2-LacZ are too low to allow for productive EC-CD8⁺ T-cell interaction because sufficient EC-associated antigen is present in Tie2-LacZ mice for indirect (cross-) presentation by BM-derived DC. It is therefore possible that studies describing activation and subsequent tolerization of CD8⁺ T-cells, for example by particular subsets of EC such as liver sinusoidal EC (Limmer et al., 2000; Limmer et al., 2005; Berg et al., 2006) may not have considered the contribution of professional APC such as DC and other BM-derived APC.

Indeed, the complexity of the multi-cellular processes involved in EC-mediated antigen presentation *in vivo* requires careful consideration of possible confounding factors. Rothermel et al. (Rothermel et al., 2004) have suggested that immune recognition of EC is context-dependent with antigen expressed in hearts of Tie2-LacZ mice being immunologically ignored whereas EC presenting β -gal antigen in skin are immunogenic and thus elicit T-cell responses capable of rejecting skin grafts. The results of our study clearly confirm the notion that direct presentation of mhAg by EC is accompanied by immunologic ignorance. However, in the context of mhAg presentation in transplant vasculopathy and GVHD, DC are probably the most important cell population that cross-presents the antigen in an immunogenic fashion.

Our study revealed further details that could confound the analysis of T-cell activation/tolerization in Tie2-LacZ mice: β gal-specific CD8⁺ T-cells were effectively tolerized in non-irradiated Tie2-LacZ and in T2→T2 bone marrow chimeric mice. We conclude from these findings that cells within the bone marrow, but not professional APC that

descend from bone marrow precursors exert a tolerizing stimulus in Tie2-LacZ mice. Hematopoietic stem cells express the angiopoietin 1 receptor Tie2 (Arai et al., 2004) and it is therefore likely that the complete *tie2*-promoter that has been used in Tie2-LacZ (Schlaeger et al., 1997) is active in hematopoietic cells. Since circulating lymphocytes presenting mhAg efficiently tolerize naive CD8⁺ T-cells (Ehl et al., 1998b; Bonilla et al., 2006), it is reasonable to assume that naive CD8⁺ T-cells travelling through the BM can receive tolerizing stimuli within this compartment. Using Tie2-LacZ BM chimeras, it will be feasible to further characterize those cells within the bone marrow that are highly efficient in inducing tolerance to mhAg.

Taken together, this study identifies the initial priming of mhAg-specific CD8⁺ T-cells via DC as a critical step in the generation of alloimmune responses. Therefore, it appears to be crucial that therapeutic intervention should aim at preventing or at least reducing the initial T-cell activation against mhAg. Indeed, blockade of essential costimulatory pathways such as CD40-CD40L (Larsen et al., 1996; Ford et al., 2007) or CD28-CD80/86 (Salomon and Bluestone, 2001) interaction during initial DC-mediated CD8⁺ T-cell stimulation bear a high potential for clinical application. It may well be that a combination of costimulatory blockade before and during priming of EC-specific CD8⁺ cell responses together with the induction of regulatory T-cells (Salomon and Bluestone, 2001) will help to protect EC from injury following transplantation of vascularized organs or during GVHD.

5.1.6 Supplementary data

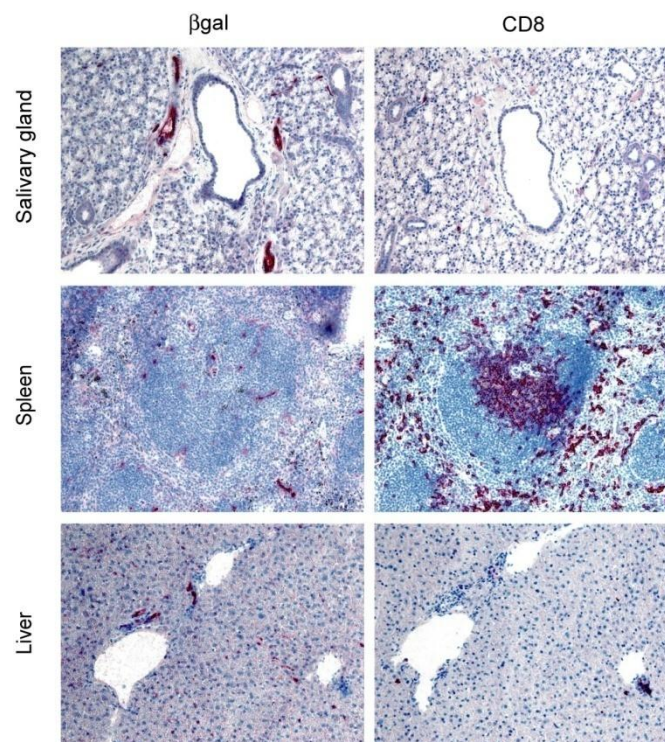


Figure S 1: β gal and CD8 staining of salivary gland, spleen and liver sections of naïve Tie2-LacZ mice. Freshly removed organs were immersed in HBSS and snap-frozen in liquid nitrogen. Frozen tissue sections were cut in a cryostat and fixed in acetone for 10 min. Sections were incubated with antibodies against β gal (MP Biomedicals,), CD8 (clone YTS169.4.2) followed by goat anti-rat Ig (Caltag Labs) and alkaline phosphatase-labelled donkey anti-goat Ig (Jackson ImmunoResearch Labs). Alkaline phosphatase was visualized by using AS-BI phosphate / New Fuchsin, and sections were counterstained with hemalum, and pictures were acquired using a Leica DM R microscope.

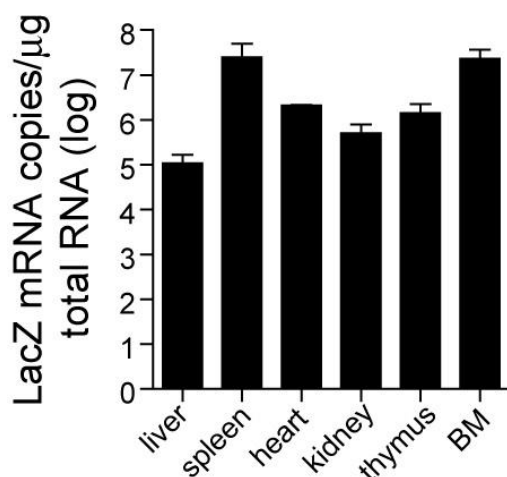


Figure S 2: LacZ mRNA copy numbers in liver, spleen, heart, kidney, thymus and BM of naïve Tie2-LacZ mice. Organs were homogenized in Trizol (Sigma) using a MagNA Lysor instrument (Roche Diagnostics). RNA was isolated by isopropanol precipitation, washed with ethanol 70% and resuspended in DEPC-water. RNA (10 μ g) was subjected to RT-PCR analysis. For RT-PCR the high capacity cDNA archive Kit from Applied Biosystem (ABI PRISM, Warrington, United Kingdom) was used according to the specifications of the manufacturer to generate cDNA from RNA samples. Quantitative real-time PCR was performed using a LightCycler (Roche Diagnostics) and the LightCycler FastStart DNA Master^{PLUS} HybProbe reaction mix (Roche Diagnostics) following the manufacturer's protocol. Data analysis was performed with LightCycler Software 3 (Roche Diagnostics). Oligonucleotides were purchased from Microsynth (Balgach, Switzerland). The following oligonucleotides from LacZ sequences were used as primers for quantitative real-time PCR: 5'-GCGTGGATGAAGACCAGC-3' and 5'-CGAAGCCGCCCTGTAAAC-3'. The following oligonucleotides were used as probes: 5' CAGTCTTGGCGGTTTCGCTAA 3' (probe 1) and 5' TACTGGCAGGCGTTTCGTCAG 3' (probe 2). Probe 1 carried a 3' FAM reporter and probe 2 was Cy5 labelled at the 5' end. Thermal cycling started with HotStarTaq activation during 15 min at 95°C. Thereafter 50 cycles of amplification were run consisting of 15 s at 95°C, 20 s 60°C and 20 s of 72°C. A negative control, containing reagents only, and serial dilutions of plasmid containing the specific LacZ sequence were included in each run to generate a standard curve. The concentrations of the plasmid dilutions were: 280000, 28000, 2800, 280 and 28 copies per reaction. LacZ mRNA concentration in the unknown samples was calculated by the ABI Prism Software using the data from the standard curve. Each sample was measured twice and the average concentration was used. Final copy numbers were calculated per μ g total RNA.

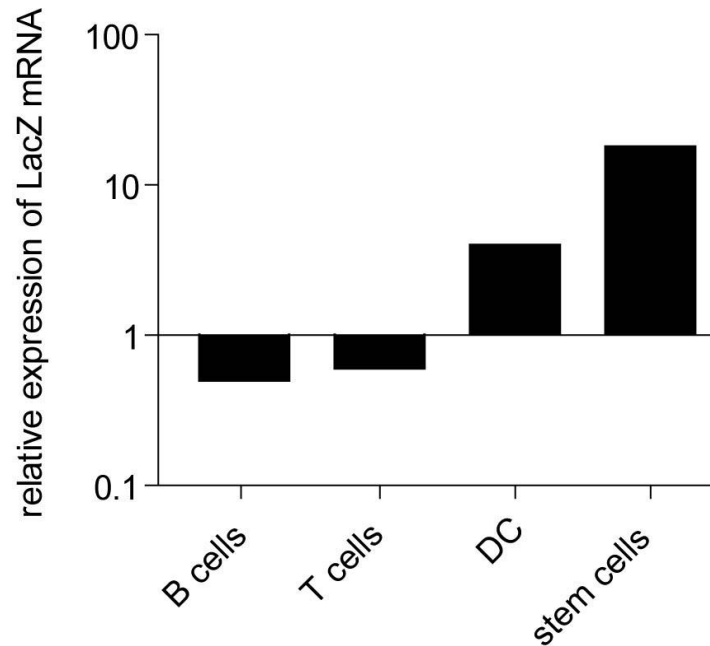


Figure S 3: Expression of LacZ transcripts in sorted bone marrow cells. For cell sorting, the FACS Aria (BD Biosciences) was used. Erythrocyte-depleted BM cells were stained in IMDM 2% FBS with saturating concentrations of anti-TCR, anti-CD3 ϵ , anti-CD11b, anti-CD11c, anti-CD19, anti-CD117, and anti-Ly6A/E (SCA-1). Following a 30 min incubation at 4°C, cells were washed in PBS 2% FBS and resuspended in PBS, filtered through a 20 μ m diameter nylon mesh, and resuspended at about 20×10^6 /mL in filtered PBS 2% FBS prior to sorting. Reanalysis of sorted cells indicated purity >95%. Real-time RT-PCR for the quantification of LacZ expression in the sorted BM cells was performed essentially as described in Figure S2 with the following modifications. Expression of the TATA-binding protein (TBP) was used for normalization. The following primers were used for amplification of TBP: TBPfw CCTTCACCAATGACTCCTATGAC, TBPrev CAAGTTTACAGCCAAGATTCAC. Amplification program for the LightCycler RT-PCR was 95°C 15 min; 50 cycles – 95°C 10 sec, 58°C 10 sec, 72°C 20 sec. The level of expression between samples derived from the different cell populations was calculated by the comparative C_T method ($\Delta\Delta C_T$) with expression in total bone marrow cells set as reference.

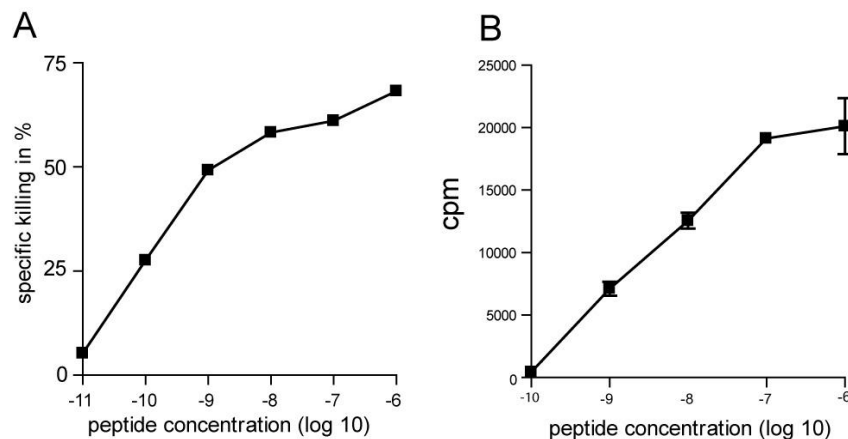


Figure S 4: Functional avidity of TCR transgenic Bg1 CD8⁺ T-cells. (A) EL-4 cells pulsed with different concentration of the β gal₉₆₋₁₀₃ (DAPIYTNV) peptide or without peptide (negative control) were used as target cells in a standard ⁵¹Cr release assay. Cells were labelled with 200 μ Ci ⁵¹Cr (EGT Chemie, Tägerig, Switzerland) for 1 h at 37°C. A total of 10^4 target cells/well were incubated for 5h in 96-well round bottom plates with 3-fold serial dilutions of effector cells. CTL from naïve Bg1 mice that were restimulated with β gal₉₆₋₁₀₃ (DAPIYTNV) for three days and were tested at a effector:target ratio of 10:1. (B) MACS-purified CD8⁺ splenocytes from Bg1 mice were cocultured with β gal₉₆₋₁₀₃ (DAPIYTNV) peptide presenting DC. DC were generated from bone marrow of C57BL/6 mice using GM-CSF containing medium as described previously (Ludewig et al., 1998, J. Virol. 72:3812). DC were pulsed at the indicated concentration of the β gal₉₆₋₁₀₃ (DAPIYTNV) peptide or left

unloaded as a negative control and plated onto a 96-well plate in RPMI 1640 (Sigma) supplemented with 5% FCS, 25mM L-glutamine, and 100U/ml penicillin with 100 μ g streptomycin sulfate. Responder to stimulator ratio of the displayed data was 30:1. Cells were incubated at 37°C for 60 hours. During the last 12 hours of coculture, 1 μ Ci of [³H] thymidine (MP Biomedical, EGT Chemie, Tägerig, Switzerland) was added to each well. To measure incorporation into DNA, the plates were frozen, thawed, harvested, and counted on a beta liquid scintillation counter (TRI-CARB, Packard). The mean of triplicates (\pm SD) was calculated, background values from cultures with unpulsed DC were subtracted.

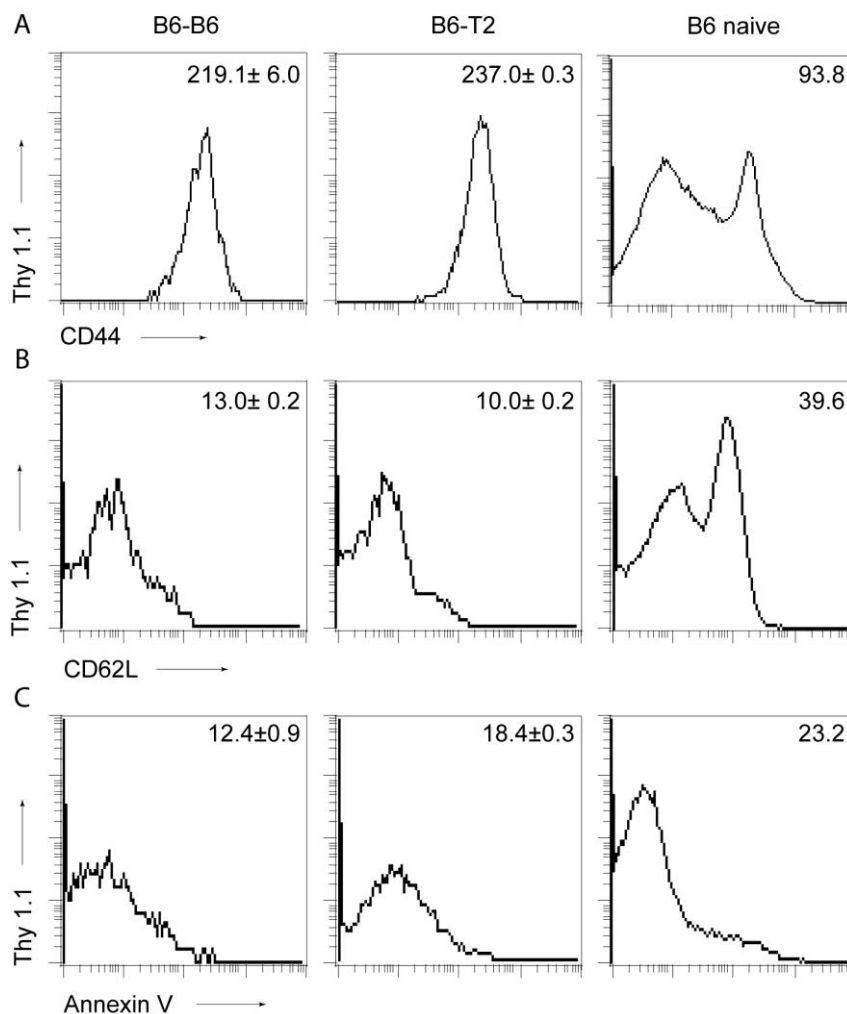


Figure S 5: Phenotype of MCMV-LacZ-activated Bg1 TCR transgenic T cells in B6→B6 and B6→T2 chimeras. The indicated bone marrow chimeric mice received 10⁵ CD8⁺Thy1.1⁺ cells from naive Bg1 mice. Nine days following adoptive transfer, mice were infected with MCMV-LacZ and the phenotype of the activated Bg1 cells was assessed six days later. Bg1 T cells activation was measured as upregulation of CD44 (A), and down-regulation of CD62L (B). Progression of activation-induced cell death was measured as Annexin V upregulation (C). Expression of the respective markers on naive CD8⁺ T-cells from B6 mice is shown as controls. Mean fluorescence intensity is indicated (n=2-3).

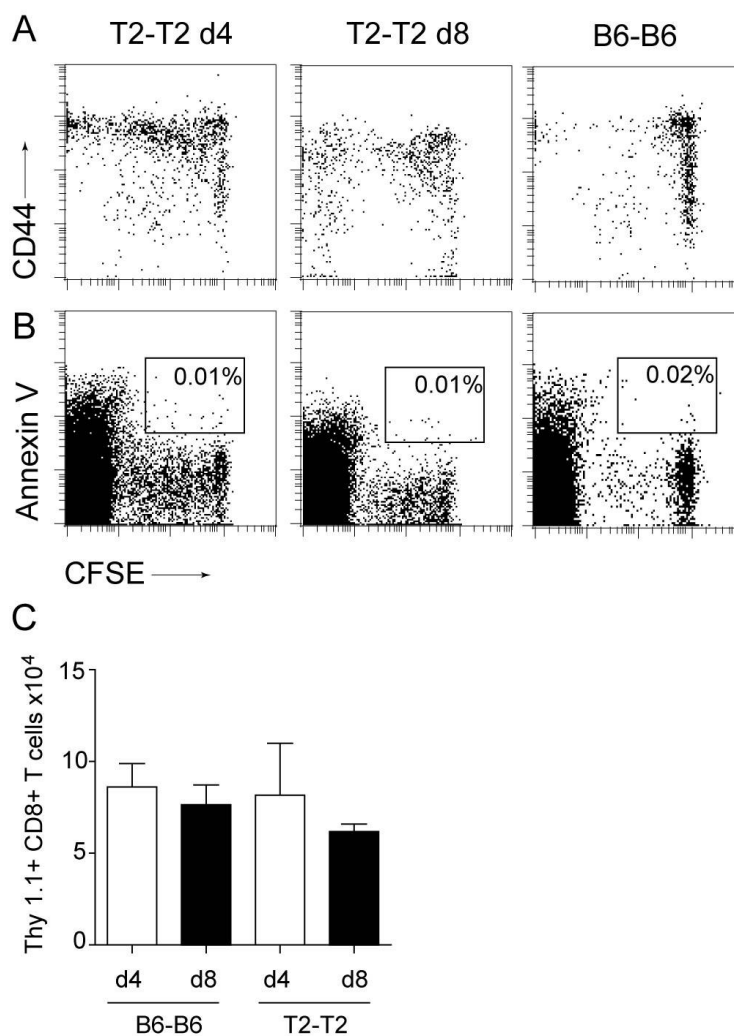


Figure S 6: Fate of Bg1 TCR transgenic T cells in T2→T2 chimeric mice. 1.5×10^7 CFSE-labelled splenocytes (corresponding to 3×10^6 CD8⁺ TCR transgenic T cells) from Bg1 mice were adoptively transferred into T2→T2 bone marrow chimeras. Mice were sacrificed on days 4 and 8 following transfer and dividing Bg1 cells from spleens were analyzed by flow cytometry for CD44 (A), and Annexin V (B) upregulation. Total numbers of transgenic Bg1 cells in spleens of recipient T2→T2 and B6→B6 mice were determined (C) at the indicated time points.

5.1.7 Acknowledgments

Burkhard Ludewig designed the study and wrote the paper; Beatrice Bolinger performed research and wrote the paper; Pierre-Alain Clavien designed the experimental procedure of liver transplantation, Nicholas P. Restifo and Douglas C. Palmer provided mice. Elke Scandella, Philippe Krebs, Daniel Engeler, Yinghua Tian and Simone Miller performed research.

The authors declare no potential conflict of interest.

The project received support from the Swiss National Science Foundation, the Velux Foundation (Zürich), and the Canton of St. Gallen.

We would like to thank Silvia Behnke and Andre Fitsche for help with immunohistochemistry.

5.2 Dendritic cell-activated, endothelial cell-specific CTL recognizing a minor histocompatibility antigen rapidly induce transplant vasculopathy

Daniel Engeler^{1*}, Beatrice Bolinger^{1*}, Philippe Krebs¹, Simone Miller¹, Matthias Hoffmann², Pierre-Alain Clavien³, Yinghua Tian³, and Burkhard Ludewig¹

¹Research Department, Cantonal Hospital St. Gallen, CH-9007 St. Gallen, Switzerland

²Department of Visceral Surgery, Hannover Medical School, D-30625 Hannover, Germany

³Department of Visceral Surgery, University Hospital Zurich, CH-8091 Zurich, Switzerland

* These authors contributed equally to this work

5.2.1 Abstract

Background: Occlusive coronary artery disease is the major cause for cardiac allograft rejection. Endothelial cells (EC) are the first graft cells encountered by host lymphocytes and are therefore primary targets of activated cytotoxic T-lymphocytes (CTL). This study determined whether CTL-mediated injury of EC exclusively presenting a minor histocompatibility antigen (mhAg) would suffice to precipitate transplant vasculopathy (TV).

Methods: The interaction of CTL with antigen-presenting EC *in vivo* has been examined here using a transgenic mouse model with beta-galactosidase (β -gal) expression confined to the vascular endothelium (Tie2-LacZ mice). Cardiac grafts from Tie2-LacZ mice were transplanted heterotopically into C57BL/6 recipients. EC-specific CTL were activated *in vivo* either by priming with β -gal peptide-pulsed dendritic cells (DCs) or by infection with β -gal-recombinant mouse cytomegalovirus (MCMV-LacZ).

Results: In the absence of β -gal-specific effector CTL, Tie2-LacZ heart grafts remained immunologically ignored for more than 100 days post transplantation. Repetitive priming with β -gal peptide-pulsed DC elicited severe vascular inflammation in transplanted Tie2-LacZ hearts with neointima formation and vascular occlusion. Activation of EC-specific CTL by infection with MCMV-LacZ caused less severe vascular inflammation in Tie2-LacZ hearts presumably due to the timely limited activation of β -gal-specific CTL under these conditions.

Conclusions: EC injury mediated by activated CTL recognizing a mhAg specifically expressed on EC is sufficient to elicit TV. Prolonged antigen presentation within secondary lymphoid organs - most likely by DCs - appears to be a key factor for the development of chronic vascular rejection.

5.2.2 Introduction

Despite advances in immunosuppressive therapies for acute allograft rejection, successful long-term outcome of transplanted solid organs is still hampered by late graft failure (Keck et al., 1999; Cecka, 1999). Chronic graft rejection is caused, in large part, by host-anti-graft immune responses that target cells of the graft vasculature thereby precipitating transplant vasculopathy (TV) (Libby and Pober, 2001). The most prominent feature of TV is the remodelling of graft vessels caused by neointima formation and progressive luminal narrowing (Mitchell and Libby, 2007). Since the progressive TV-associated loss of lumen affects mainly conduit arteries, this disease entity can also be referred to as "graft arteriosclerosis" (Tellides and Pober, 2007). Endothelial cells (EC) are the first graft cells encountered by the host immune system and it is thus most likely that a complex series of immune-mediated EC injuries initiate and drive the process of chronic vascular rejection (Valantine, 2003). It is therefore important to delineate the specifics of the inciting EC injury in well-defined model systems.

Among the different immune effector molecules that have been implicated in the pathogenesis of TV, IFN- γ appears to be a particularly important factor. This is supported not only by the fact that IFN- γ transcription in endomyocardial biopsies of human heart grafts is upregulated prior the development of TV (Russell et al., 1994a), but also by several experimental studies showing that IFN- γ is instrumental for the development of TV (Russell et al., 1994d; Nagano et al., 1997). Studies of Russell and colleagues indicated that cardiac TV in a MHC-disparate setting is largely driven by T-cells (Russell et al., 1994b) and that humoral alloresponses can contribute to the chronic inflammatory process (Russell et al., 1994c; Russell et al., 1997). It is important to note that these and other experimental studies on cardiac TV (Armstrong et al., 1997a; Armstrong et al., 1997b) had to employ immunosuppressive treatment to down-modulate a full-blown alloimmune response. Evidently, such a setting precludes comprehensive functional analyses of T-cells involved in the disease process.

A major aim of the present study was to develop an experimental system that facilitates *in vivo* studies on the cognate interaction of EC with activated CTL in a heterotopic heart transplantation model. To this end, we employed a transgenic mouse with EC-specific β -galactosidase (β -gal) expression under the control of the Tie2 promoter (Tie2-LacZ mice) (Schlaeger et al., 1997). These mice had been generated on the FVB/NJ background (H2q haplotype) thus hampering thorough immunological analysis of EC-CTL interaction

(Rothermel et al., 2004). Following backcrossing to the C57BL/6 background, we have shown in a recent study (Bolinger et al., 2008) that β -gal expression by EC in heterotopically transplanted Tie2-LacZ hearts or orthotopically transplanted Tie2-LacZ livers did not result in activation of naive CD8⁺ T-cells. Furthermore, generation of bone marrow (BM) chimeric mice that facilitated selective ablation of CD11c-positive dendritic cells (DC) revealed that EC-associated minor histocompatibility antigen (mhAg) has to be cross-presented by DC in order to elicit CD8⁺ T-cell activation. Having thus established that naive CTL immunologically ignore vascular EC expressing a mhAg, we examined in the present study whether β -gal-specific effector CTL are able to mediate significant EC injury and whether this particular interaction would suffice to precipitate TV in transplanted Tie2-LacZ hearts. We found that priming with β -gal peptide-pulsed DC induced vascular inflammatory disease with neointima formation and vascular occlusion. Less pronounced activation of EC-specific CTL by infection with β -gal-recombinant mouse cytomegalovirus (MCMV-LacZ) led to reduced vascular inflammation in Tie2-LacZ hearts suggesting that it is the DC-mediated, prolonged presentation of mhAg within secondary lymphoid organs that drives activation of EC-specific CTL fostering thereby the development of chronic vascular rejection.

5.2.3 Materials and Methods

Mice

Male and female C57BL/6 mice were obtained from Charles River (Sulzfeld, Germany). Tie2-LacZ mice (Schlaeger et al., 1997) had been crossed to the C57BL/6 background for at least 14 times. The presence of the β -gal transgene was determined by PCR from genomic DNA. Mice were thymectomized at 6-7 weeks of age as described (Aichele et al., 1995) and used 4 weeks after thymectomy. All animals were kept under conventional conditions in individually ventilated cages and fed with normal chow diet. Experiments were carried out with age (6-10 weeks) and sex-matched animals. Experiments were performed in accordance with Swiss Cantonal and Federal legislations.

Viruses and peptides

Recombinant MCMV expressing the β -gal protein under the transcriptional control of the human CMV *ie1/ie2* promoter-enhancer (MCMV-LacZ RM427 (Manning and Mocarski, 1988)) was kindly provided by Prof. E. S. Mocarski (Stanford University, San Francisco). MCMV-LacZ was propagated and titrated on NIH 3T3 cells (ECACC, UK) and injected

intravenously at a dose of 2×10^6 pfu. β -gal₉₆₋₁₀₃ (DAPIYTNV) (Overwijk et al., 1997), β -gal₄₉₇₋₅₀₄ (ICPMYARV) (Oukka et al., 1996) and MCMV M45₉₈₅₋₉₉₃ (HGIRNASFI) (Gold et al., 2002) peptides were purchased from Neosystem (Strasbourg, France).

Antibodies and flow cytometry

Anti-CD8-FITC and anti-IFN γ -PE were obtained from BD PharMingen (Basel, Switzerland). For flow cytometry, single cell suspensions were generated from the indicated organs, and 1×10^6 cells were incubated with the indicated mAb at 4°C for 20 min. For PBL samples, erythrocytes were lysed with FACS Lysing Solution (BD PharMingen). Cells were analyzed with a FACScalibur flow cytometer using the CellQuest software (BD Biosciences). Cells were analyzed by flow cytometry, gating on viable leukocytes using 7-aminoactinomycin D (Sigma).

Construction of tetrameric MHC class I-peptide complexes and flow cytometry

MHC class I monomers complexed with β -gal (H-2K^b) or M45 peptides (H-2D^b) were produced as previously described (Altman et al., 1996) and tetramerized by addition of streptavidin-PE (Molecular Probes, Eugene, OR). At the indicated time points following infection, organs were removed and single cell suspensions were prepared. Aliquots of 5×10^6 cells or 300 μ l of blood were stained using 50 μ l of a solution containing tetrameric class I-peptide complexes at 37°C for 10 minutes followed by staining with anti-CD8-FITC (BD Pharmingen) at 4°C for 20 min. Absolute cell counts were determined by counting leukocytes in an improved Neubauer chamber.

DC preparation and injection

DC were generated from C57BL/6 bone marrow as described previously (Ludewig et al., 1998a). Before injection, cells were loaded with the β gal₄₉₇₋₅₀₄ peptide for 1 h at 37°C, washed three times with ice-cold BSS and resuspended in BSS at a concentration of 4×10^5 DC/ml. DC were injected in 500 μ l BSS i.v. on days 0, 2, 10, and 12.

Immunohistology

Freshly removed organs were immersed in HBSS and snap-frozen in liquid nitrogen. Frozen tissue sections were cut in a cryostat and fixed in acetone for 10 minutes. Sections were incubated with antibodies against β -gal (MP Biomedicals.), CD8 (clone YTS169.4.2), or

F4/80 (Biomedicals AG, clone BM8) followed by goat anti-rat Ig (Caltag Labs) and alkaline phosphatase-labelled donkey anti-goat Ig (Jackson ImmunoResearch Labs). Alkaline phosphatase was visualized by using AS-BI phosphate/New Fuchsin, and sections were counterstained with hemalum. Hematoxylin-eosin, and elastica-Van Giesson (EVG) stainings were done following standard procedures. Images were acquired using a Leica DM R microscope equipped with a Leica DC300 FX camera. Digital images were processed using Adobe Photoshop.

Heterotopic heart transplantation

Heterotopic vascularised cardiac transplantation was performed according to the method described by Corry et al. (Corry et al., 1973). Donor hearts were explanted from either male Tie2-LacZ or male C57BL/6 mice. The donor heart was removed from the chest after intracaval injection of 1 ml of heparin (100 U/ml), rinsed with NaCl 0.9% and placed on ice. After isolation of the recipient's abdominal aorta and inferior vena cava, the donor ascending aorta and pulmonary artery were joined end-to-side to the recipient's aorta and vena cava, respectively, using 10-0 nylon running suture. The abdomen was closed with individual running sutures to musculo-fascial layer and skin. The recipient mouse was then warmed for a few hours during recovery from anaesthesia and had free access to water and food. The function of the transplanted heart was assessed daily by abdominal palpation.

Histological scoring of chronic vascular transplant rejection

Based on histological changes observed in chronic allograft rejection, we established a modified scoring system to analyze different degrees of vascular transplant rejection based on criteria previously published (Hirozane et al., 1995; Russell et al., 1994d) using a scale from 0 to 3 for the different pathological alterations. The degrees for intimal thickening of coronary arteries (0 = <10%; 1 = 10 to <50%; 2 = 50 to <90%; 3 = 90-100% luminal occlusion in at least one artery/section), perivascular fibrosis (0 = no changes; 1 = minor fibrotic changes around 1 to 3 arteries/section; 2 = vast fibrotic changes around 1 to 3 arteries/section; 3 = vast fibrotic changes around more than 3 arteries/section), and perivascular and vascular inflammatory infiltrates (0 = no inflammatory infiltrates; 1 = few inflammatory infiltrates around 1 to 3 arteries/section; 2 = vast inflammatory infiltrations around 1 to 3 arteries/section; 3 = vast inflammatory infiltrates in more than 3 arteries/section) were

determined. Six to eight sections from each heart were evaluated by two independent observers blinded for the tested specimen.

Statistical data analysis

To evaluate statistically significant differences, the unpaired two-tailed Student's test or the nonparametric Kruskal-Wallis test were used. p values smaller than 0.05 were considered statistically significant. Statistical data analysis was performed using GraphPad Prism version 4.03 for Windows (GraphPad Software Inc., San Diego California USA).

5.2.4 Results

5.2.4.1 β -gal-specific immune responsiveness in heart transplant recipients

We have shown previously that EC-associated mhAg in Tie2-LacZ hearts transplanted into naive C57BL/6 recipients remains immunologically ignored during the first 2-3 weeks post transplantation (Bolinger et al., 2008). To determine whether EC in transplanted Tie2-LacZ hearts remain intact over a longer period of time, we performed two independent series of transplantations of Tie2-LacZ (T2→B6, n=5) or C57BL/6 (B6→B6, n=6) hearts in C57BL/6 recipients. Daily palpation revealed that Tie2-LacZ hearts were well-accepted even over a period of 100 days. In situ analysis on day 100 post transplantation showed that inflammatory reactions both in T2→B6 and B6→B6 hearts were transplantation procedure-associated with thrombus organization in the left ventricle. Importantly, hearts from both groups lacked signs of vascular rejection (Figure 11). Furthermore, β -gal-expression in coronary arteries of T2→B6 heart was preserved (Figure 11B, lower right panel) indicating that the graft endothelium of Tie2-LacZ had not been replaced by recipient EC.

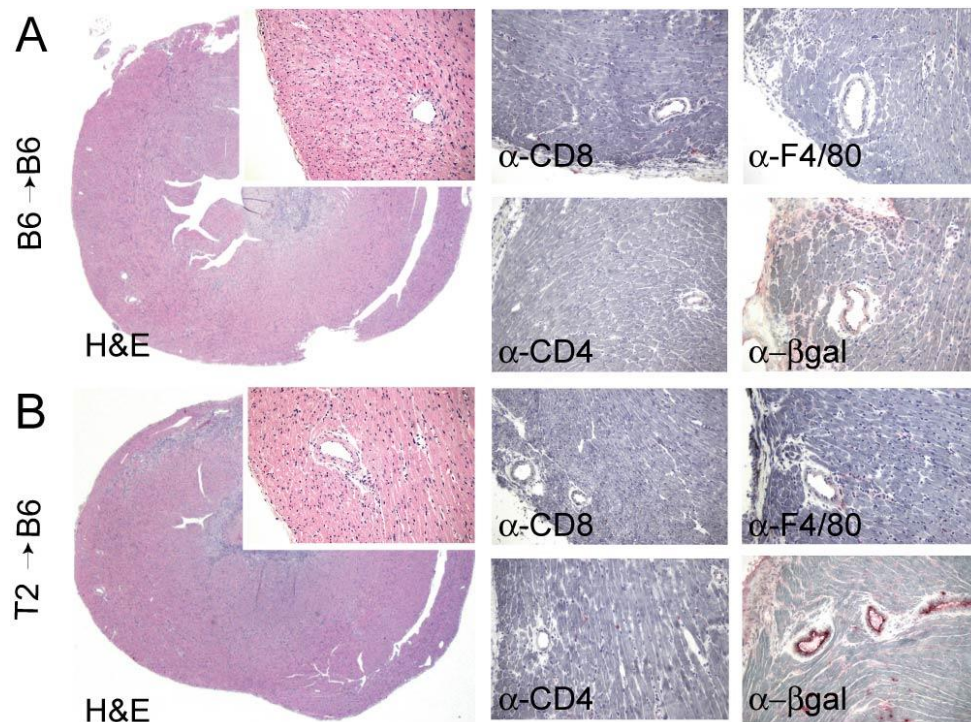


Figure 11: In situ analysis of heterotopically transplanted (A) C57BL/6 (B6→B6) and (B) Tie2-LacZ (T2→B6) hearts on day 100 post transplantation. Hematoxylin-eosin (HE), α -CD8, α -CD4, and α -F4/80 stainings are indicated. Original magnification: $\times 200$ (HE overview: $\times 16$).

Next, we assessed whether the persistent presence of a mhAg in the vasculature of a heart graft affected CTL reactivity. To this end, C57BL/6 recipients of transgenic and control hearts were challenged with β -gal peptide-pulsed DC. In this setting, three different scenarios can be envisioned: (i) elevated CTL reactivity in T2→B6 recipients due to preactivation of CTL, (ii) no or lowered CTL reactivity as a consequence of tolerizing stimuli, and (iii) no alteration in CTL reactivity because of immunologic ignorance. DC-immunization elicited a comparable expansion of β -gal-specific CD8⁺ T-cells both in recipients of Tie2-LacZ and C57BL/6 control hearts (Figure 12A and B) supporting the notion that mhAg presentation by cardiac EC did neither activate nor tolerize naive CTL. To further substantiate this finding and to exclude that recent thymic emigrants had replenished a pool of β -gal-reactive CTL that were tolerized due to a low-level exposure to their cognate antigen in recipients of Tie2-LacZ hearts, transgenic hearts were transplanted into mice that had been thymectomized 4 weeks before heart transplantation. As shown in Figure 12C, induction of β -gal-specific CTL in thymectomized recipients of Tie2-LacZ hearts was not affected by the replenishment of the peripheral T-cell pool from the thymus. Taken together, these data provide further evidence that a mhAg antigen expressed by vascular EC remains immunologically ignored.

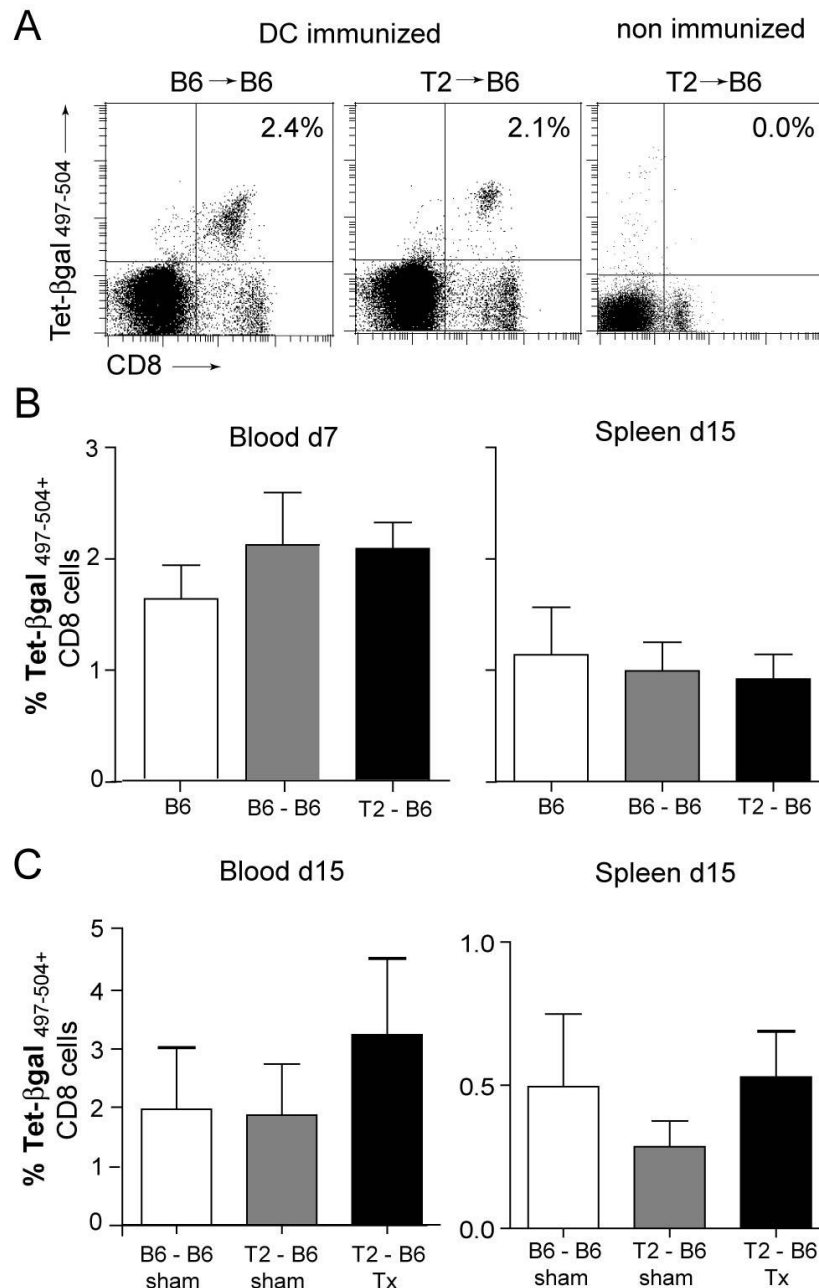


Figure 12: CTL reactivity in recipients of C57BL/6 (B6→B6) or Tie2-LacZ (T2→B6) heart transplants following DC immunization. (A, B) Four weeks post transplantation; mice were repetitively immunized with 2×10^5 β gal peptide-pulsed DC. (A) Representative FACS histograms from blood of B6→B6 and T2→B6 mice on day 7, or untreated B6 recipients of a Tie2-LacZ heart on day 30 post transplantation. Values in the upper right quadrant represent percentage of tetramer-positive CD8⁺ T-cells. (B) Percentage of β gal₄₉₇₋₅₀₄-specific CD8⁺ T-cells in blood on day 7 post immunization (left panel), and in spleens on day 15 post immunization (right panel) (mean \pm SEM; n=4-7 animals per group). (C) CD8⁺ T cell responses in thymectomized mice following DC immunization. Male C57BL/6 mice were thymectomized, and received two weeks following thymectomy either C57BL/6 or Tie2-LacZ hearts. Four weeks post transplantation, mice were immunized with DC as in (A and B). Percentage of β gal₄₉₇₋₅₀₄-specific CD8⁺ T-cells in blood (left panel), and in spleens on day 15 post immunization (right panel) (mean \pm SEM; n=3 animals per group) Statistical analysis using the nonparametric Kruskal-Wallis test indicated no significant difference between the different groups ($p > 0.05$ for B and C).

5.2.4.2 CTL-induced vascular rejection after DC immunization

In the next set of experiments, we assessed whether EC in transplanted Tie2-LacZ hearts may serve as target cells for activated CTL. Recipient mice were challenged with β -gal₄₉₇₋₅₀₄ peptide-loaded DC starting 4 weeks post transplantation. Two injections of β -gal peptide-pulsed DC (days 0 and 2), were sufficient to generate significant CTL responses on day 7 (Figure 12) and to precipitate significant inflammatory reactions around coronary arteries of T2→B6 hearts, whereas the vasculature of B6→B6 hearts did not show signs of inflammation (Figure 13A). Based on our previous experience with DC-based immunization against tumours (Ochsenbein et al., 1999; Ludewig et al., 2004) and the requirement of prolonged DC-mediated antigen presentation to precipitate autoimmune disease (Ludewig et al., 1998b; Ludewig et al., 2001), immunization of transplant recipients was repeated on days 10 and 12, and hearts were evaluated for signs of vascular rejection on day 15 following the first DC application. Histological analysis revealed typical signs of chronic vascular rejection including intimal thickening combined with perivascular infiltrations of CD8⁺ T cells and F4/80-positive macrophages, perivascular fibrosis, and vascular occlusion (Figure 13B). Since these pathological alterations were prominent in T2→B6 hearts, we developed a scoring system based on criteria published by Russell et al. (Russell et al., 1994d) and by Hirozane et al. (Hirozane et al., 1995). T2→B6 hearts from DC-immunized recipients showed significantly more perivascular infiltration (Figure 14A), intimal thickening (Figure 14B), and perivascular fibrosis (Figure 14C) compared to B6→B6 hearts. The overall vascular score of T2→B6 hearts (Figure 14D) clearly indicates that this read-out is well-suited to monitor the vigour of vascular rejection in this setting.

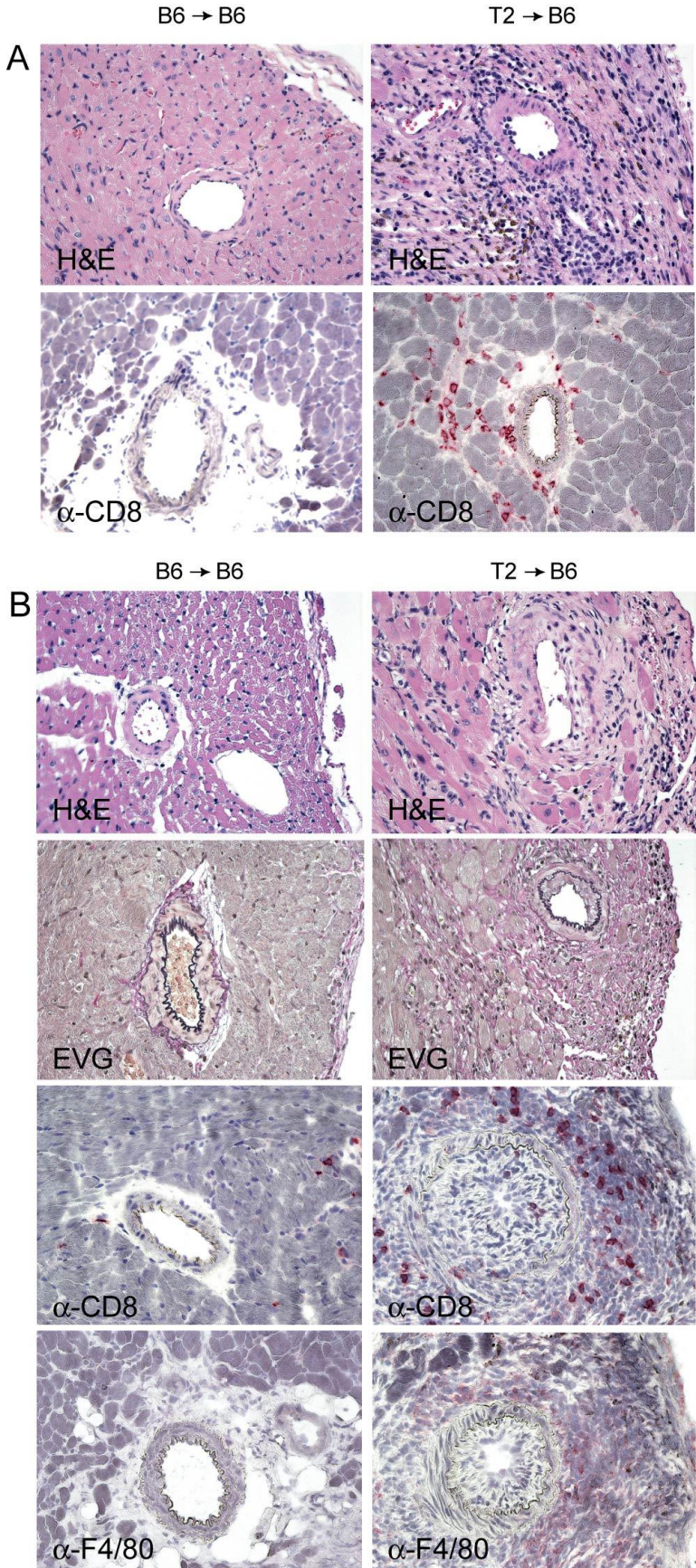


Figure 13: In situ analysis of transplanted Tie2-LacZ hearts following repetitive DC immunization on days 7 (A) and 15 (B) post immunization. Hematoxylin-eosin (HE), elastic-van Giesson (EVG), α -CD8, α -CD4, and α -F4/80 stainings are indicated. Original magnification: $\times 200$.

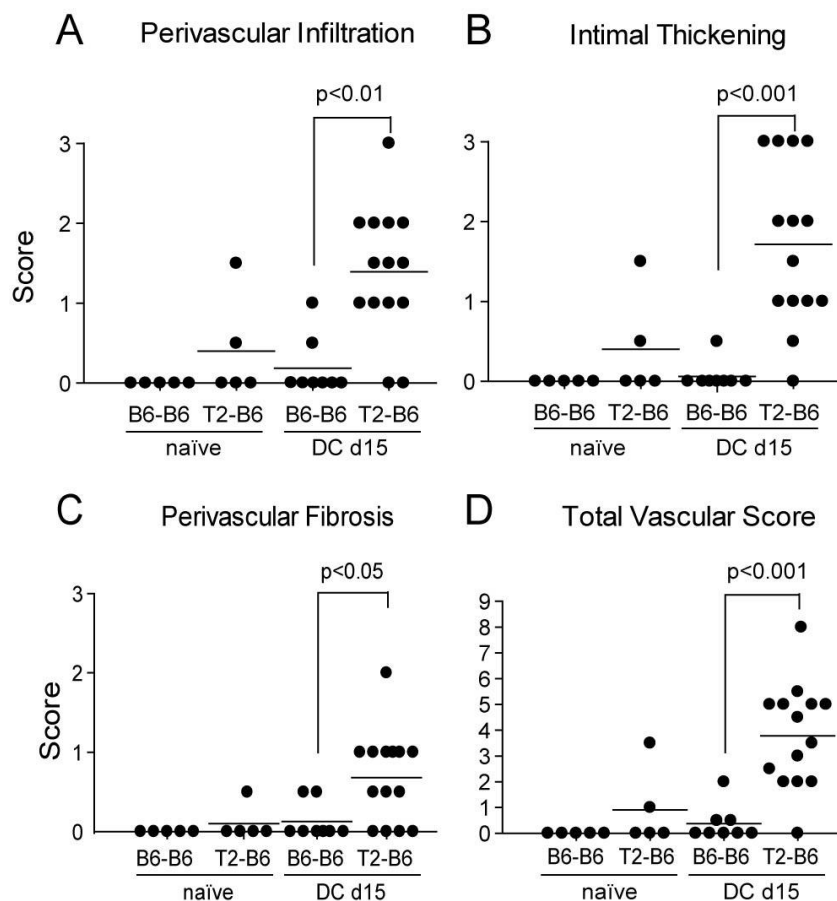


Figure 14: Quantification of vascular rejection following DC immunization. (A) Perivascular infiltration, (B) intimal thickening, (C) perivascular fibrosis, was determined on heart sections on day 15 post DC immunization. (D) Total of the vascular rejection indices. Values represent scores of individual mice, horizontal bars represent mean values.

5.2.4.3 Virus-induced vascular pathology in transplanted Tie2-LacZ hearts

Epidemiologic and experimental data indicate that human cytomegalovirus (HCMV) represents an important viral pathogen that elicits arterial inflammation and may thereby exacerbate TV (Valantine, 2004; Potena and Valantine, 2007). It has been suggested that CMV can alter the susceptibility of EC to CTL-inflicted injury by fostering upregulation of adhesion and MHC molecules (Sedmak et al., 1994; van Dorp et al., 1989) thereby rendering infected EC better targets for antiviral CTL. To assess the impact of murine CMV (MCMV) infection on chronic vascular rejection in the Tie2-LacZ heart transplant setting, we infected B6→B6 and T2→B6 recipients with β -gal-recombinant MCMV (MCMV-LacZ). Peak expansion of β -gal-specific CTL on day 6 post infection (Figure 15A, left panel), was lower compared to DC-based immunization (Figure 12). Furthermore, MCMV-induced CTL responses showed a pronounced contraction to less than 0.5% β -gal-specific CTL in the CD8⁺ T-cell compartment on day 14 post infection (Figure 15, right panel). Immunohistologic

analysis of heart sections on day 14 following MCMV-LacZ infection revealed a significantly higher amount of perivascular inflammatory infiltrates (Figure 15B and C, left panel) in T2→B6 compared to B6→B6 hearts. These infiltrates were mainly CD8⁺ T-cells (Figure 15B, right panel). Interestingly, intimal thickening and perivascular fibrosis were not detectable (Figure 15B and C) indicating that the MCMV-LacZ-driven CTL response was sufficient to direct activated CTLs to antigen-expressing blood vessels, but that this activation did not suffice to generate the full pathology of vascular rejection.

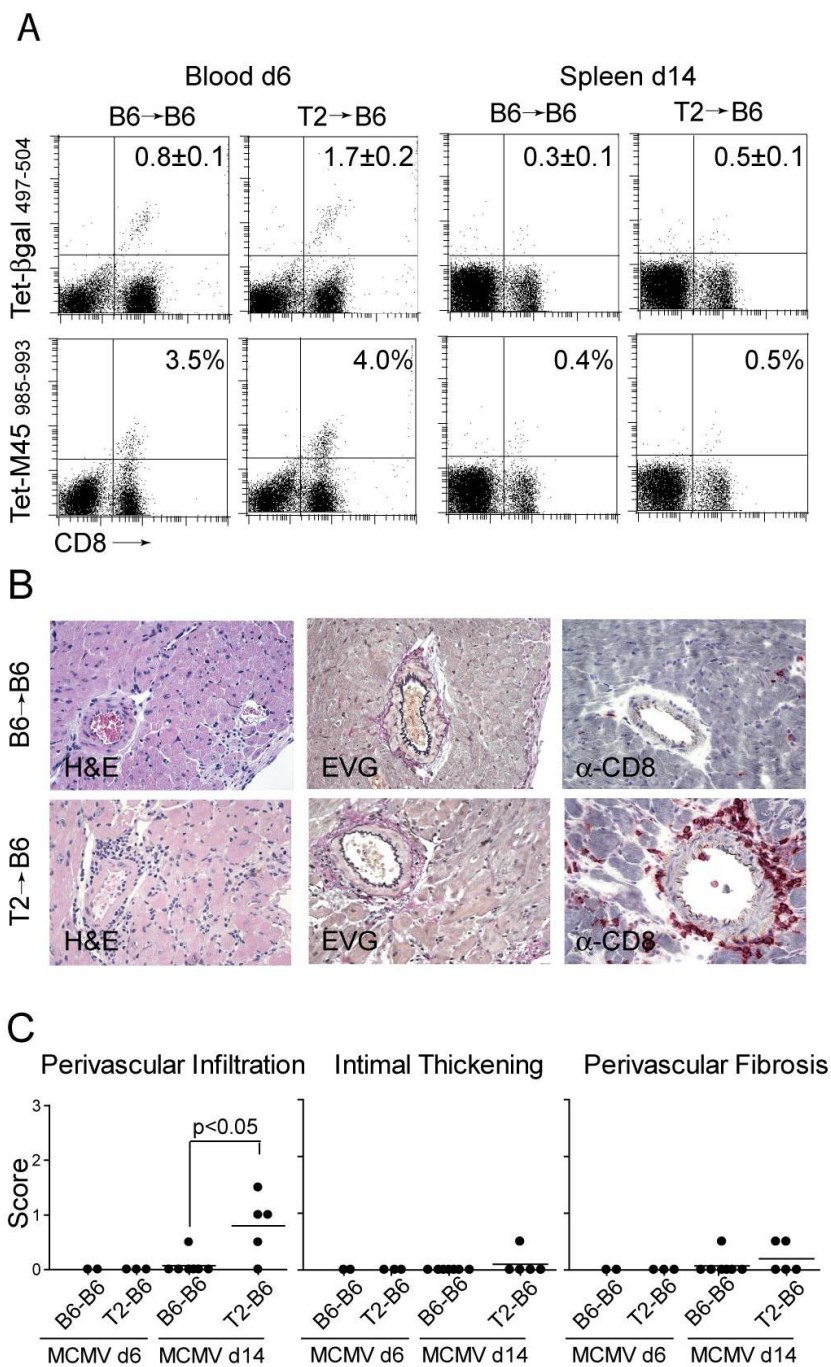


Figure 15: Immune reactivity and vascular pathology following MCMV-LacZ infection. C57BL/6 recipients of Tie2-LacZ (T2→B6) or C57BL/6 (B6→B6) hearts were infected with 2×10^6 pfu MCMV-LacZ 4 weeks after transplantation. (A) CTL activation in blood and spleens was determined by tetramer analysis for the indicated β -gal and MCMV-derived M45 epitopes on days 6 and 14, respectively. Mean percentage of tetramer-positive cells within the CD8 compartment are indicated (\pm SEM; n=3-4). (B) In situ analysis of heterotopically transplanted C57BL/6 (B6→B6) and Tie2-LacZ (T2→B6) hearts on day 14 post infection. Hematoxylin-eosin (HE), elastic-van Giesson (EVG), and α -CD8 stainings are shown. Original magnification: $\times 200$. (C) Quantification of vascular rejection following MCMV-LacZ infection measured as perivascular infiltration, intimal thickening, and perivascular fibrosis on day 14 post MCMV-LacZ infection. Values represent scores of individual mice, horizontal bars represent mean values.

5.2.5 Discussion

It has been suggested that TV underlying chronic graft failure represents a delayed-type hypersensitivity response directed against cells of the vascular wall (Libby et al., 1989). The present study further supports this notion by providing a mechanistic link between direct T-cell-mediated EC injury and subsequent pathological remodelling of coronary arteries. In a model situation where activated CTL recognizing an EC-specific mhAg cause vascular inflammation, prolonged antigen presentation by DC within secondary lymphoid organs was necessary and sufficient for the development of chronic vascular rejection.

Within vascularised organ transplants, donor EC can persist and may therefore contribute to chronic alloimmune stimulation. Under particular circumstances, EC may even initiate CTL responses that mediate allograft rejection (Kreisel et al., 2002b), and EC can be recognized by CD8⁺ T-cells through the indirect antigen presentation pathway involving cross-presentation of exogenous antigens (Valujskikh et al., 2002a; Bagai et al., 2005b; Limmer et al., 2000). These previous studies have depicted EC-CTL interaction as an almost exclusive encounter of two cell types. However, in a recent study, we have shown, using Tie2LacZ mice in combination with CD8⁺ T-cell TCR transgenics, that EC presenting a mhAg remain immunologically ignored by naïve CTL and that EC-associated mhAg has to be cross-presented by DC in order to elicit CD8⁺ T-cell activation (Bolinger et al., 2008). The results of the present study support the notion that DC represents a crucial intermediary cell in the interaction between EC and CTL. β -gal peptide-pulsed DC that were adoptively transferred into recipients of Tie2-LacZ heart efficiently expanded EC-specific CTL. Importantly, prolonged antigen presentation by DC was mandatory for the development of TV in this experimental system.

Infection with CMV is an important risk factor for TV in human transplant recipients (Valantine, 2004; Potena and Valantine, 2007), most likely through its ability to directly infect cells of the vascular wall (Wu et al., 1992). Furthermore, experimental studies have indicated that MCMV infection can exacerbate allograft rejection (Carlquist et al., 1993). The results from the present study are in line with this interpretation since antiviral T cell responses directed against the β -gal antigen within the vasculature favoured the development of vascular inflammation. However, in the absence of concomitant strong alloimmune responses, MCMV-induced anti-EC CTL did not suffice to elicit the full pathology of chronic vascular rejection. It is likely that a significant acceleration of TV by CMV infection is based on a combination of various factors including (i) the particular vascular tropism of the virus

resulting in virus-induced T-cell responses against persisting viral antigens within the vasculature, and (ii) bystander cytokine-mediated augmentation of allo-T-cell responses. Likewise, atherosclerotic lesion formation in hypercholesterolemic mice can be accelerated through MCMV-induced bystander cytokines (Vliegen et al., 2004; Vliegen et al., 2005) and by the specific activation of T-cells reactive against viral antigens that persist in the vasculature (Krebs et al., 2007).

Taken together, using a novel mouse model of CTL-mediated EC injury in heterotopic heart transplantation, we could demonstrate that activated CTL recognizing a mhAg specifically expressed on EC can elicit TV. Furthermore, this interaction is facilitated by DC as intermediary cells which promote prolonged antigen presentation within secondary lymphoid organs. Thus, therapeutic intervention should target DC-mediated T-cell activation, for example through blockade of critical costimulatory circuits to protect EC from CTL-mediated injury following transplantation of vascularised organs.

5.2.6 Acknowledgments

We would like to thank Silvia Behnke and Andre Fitsche for help with immunohistochemistry.

6 Discussion

Numerous factors contribute to the development of CTV. In addition to non-immunological aspects, a number of cell-cell interactions play a key role in its initiation and maintenance. It is thus important to provide appropriate models with well-defined conditions for the dissection of the different features.

6.1 Can EC activate naive CD8⁺ T-cells?

The detailed interaction of EC with CD8⁺ T-cells has been investigated in several *in vitro* and *in vivo* studies. Most *in vitro* studies were based on allogeneic differences between EC and CD8⁺ T-cells (Kreisel et al., 2002a; Ma and Pober, 1998; Epperson and Pober, 1994; Bagai et al., 2005a; Pober et al., 1997). Only a few studies have assessed the role of EC presenting a mhAg to CD8⁺ T-cells (Marelli-Berg et al., 2000; Marelli-Berg et al., 2001). Many *in vivo* studies have been performed using combinations with allogeneic differences between host and donor (Kreisel et al., 2002a; Kreisel et al., 2002b; Valujskikh et al., 2002a) or have studied the role of specific EC populations, such as the liver sinusoidal EC (LSEC), which cross-present antigen (Berg et al., 2006; Limmer et al., 2000; Limmer et al., 2005). However, in these experimental settings cross-presentation by professional APC, the role of mhAg, or the direct interaction of EC with CD8⁺ T-cells could not be examined.

Therefore, our transgenic mouse, with the expression of the well-defined model antigen β -gal in the vascular endothelium (Tie2-LacZ mice) combined with the use of TCR transgenic mice (Bg1mice) with CD8⁺ T-cells specific for a β -gal epitope, represents a suitable model for the investigation of the cognate interaction of vascular EC with CD8⁺ T-cells. It is the first model that allows studying the mhAg-specific cell-cell interaction *in vivo*. Furthermore, by the generation of BM chimeric mice, facilitating the ablation of CD11c-positive BM-derived DC (CD11c-DTR mice), antigen presentation by other cells than EC could be considered and analyzed.

In this work, we demonstrated that mhAg presentation by EC does neither lead to CD8⁺ T-cell activation nor tolerization. This was shown by the fact that adoptively transferred β -gal-specific CD8⁺ T-cells ignored mhAg expressed on the vascular endothelium. However, antigen expressed on vascular EC was cross-presented by BM-derived DC and induced activation of adoptively transferred antigen-specific CD8⁺ T-cells.

There are several explanations for the observed immunologic ignorance. First, it could be that levels of antigen expression in EC of Tie2-LacZ mice are too low. It has been shown that

low antigen dose lead to T-cell tolerance (Kurts et al., 1999). However, this is implausible in our study since there is sufficient EC-associated antigen present for enabling the indirect cross-presentation by BM-derived DC. Second, there is the possibility that EC possess an impaired capacity to present immunodominant peptides (Kummer et al., 2005) and therefore fail to interact with CD8⁺ T-cells. However, it seems more reasonable that it is the location and the availability of antigen which determine whether an immune response is induced (Zinkernagel et al., 1997). Antigen has to be presented in organized lymphoid tissue to induce an immune response since *aly/aly* mice, which lack secondary lymphoid organs, failed to reject vascularised organ transplants (Lakkis et al., 2000). In other words, antigens that never reach organized lymphoid tissue or only for a too short period of time or in too low quantities are not able to activate T-cells. Staying outside lymphoid tissue is even a trick used by some viruses, for example the papilloma virus that induces skin warts, to escape immune surveillance (Zinkernagel et al., 1997). A further example of antigen ignorance is a transgenic mouse model with LCMV glycoprotein (GP) expressed in islet cells of the pancreas under the rat insulin promoter (RIP). These mice do not spontaneously develop diabetes. However, rapidly after infection with LCMV that induces a potent CTL response, they become diabetic (Ohashi et al., 1991). Only after specific activation T-cells are able to migrate to the periphery where target cells present the antigen. This underscores the necessity of the antigen being transported and presented within secondary lymphoid organs. Unless antigen does not reach organised lymphoid tissue, potentially reactive T-cells may be present but become neither activated nor actively tolerized.

In our study, EC present a mhAg and therefore provide “signal 1”, i.e., antigen, in the absence of “signal 2”, i.e., costimulation which should result in tolerization of the antigen-specific CD8⁺ T-cells. It is reasonable to assume that cell-cell contact between EC and CD8⁺ T-cells was not possible or not within the right environment to enable productive interaction between EC and naive CTL. Nevertheless, BM-derived DC cross-presented mhAg and thus induced CD8⁺ T-cell activation and proliferation, identifying DC as potent APC that present antigen to CD8⁺ T-cells within secondary lymphoid organs.

We explain the controversial outcome as compared to previous studies investigating the capacity of EC to induce a CD8⁺ T-response by their *in vitro* settings, where cell-cell contact is guaranteed (Marelli-Berg et al., 2000; Marelli-Berg et al., 2001), but which do not correspond to the *in vivo* situation where cell-cell contact represents a major challenge. A further explanation is the use of allogeneic disparities between host and donor leading to a different starting-point with higher antigen amount and higher precursor frequency (Kreisel et

al., 2002a; Kreisel et al., 2002b; Valujskikh et al., 2002a). A further reason is the difference in antigen amount presented by EC. In the study performed by Limmer et al. antigen was supplied systemically to the liver. This huge amount of antigen was then cross-presented by LSEC and led to the tolerization of antigen-specific CD8⁺ T-cells (Limmer et al., 2000). It would be of major interest whether the threshold amount of antigen necessary for the induction of CD8⁺ T-cell tolerance, instead of ignorance as seen in a study by Kurts et al. (Kurts et al., 1999) could be achieved supplying Tie2-LacZ mice with such high amounts of antigen. To address this question, we are going to inject soluble β -gal antigen i.v. into Tie2-LacZ mice, thereafter TCR transgenic CD8⁺ T-cells are transferred and CD8⁺ T-cell activation analyzed. However, in the experimental settings by Limmer and Berg et al. (Berg et al., 2006; Limmer et al., 2000; Limmer et al., 2005) antigen cross-presentation by professional APC, as seen in our study, could not be excluded and therefore may be a further reason for the different outcome.

In our study, steady-state BM-derived DC cross-presenting mAg induced CD8⁺ T-cell activation. Although they present antigen in the absence of “danger” or any inflammatory reaction, they did not induce T-cell tolerization. However, it is possible that some of the transferred cells became activated during *ex vivo* preparation before adoptive transfer. But since proliferation in wild-type C57BL/6 mice was not observed, this possibility can be ruled out. A better explanation for the independence of costimulatory signals could be the precursor frequency of CD8⁺ T-cells. Precursor frequency has been shown to play a particular role that impacts T-cell proliferation, differentiation, and requirement for costimulation. Donor-reactive T-cells primed at a low frequency exhibited an increased requirement for CD28- and CD40L-mediated costimulation, whereas donor-reactive T-cells primed above a certain threshold cannot become tolerized by the blockade of costimulatory pathways (Ford et al., 2007) and thus overcome peripheral tolerance mechanisms. Furthermore, it has been demonstrated that vigorous allograft rejection occurs despite the absence of “danger” (Bingaman et al., 2000).

However, in our model CD8⁺ T-cells became indeed activated and proliferated but they did not acquire full effector functions. This phenomenon could be a result of the different requirements for a T-cell to proliferate and expand or to become a cytotoxic T-cell. (Hernandez et al., 2002; Valitutti et al., 1996). Taken together, our results indicate that cell-cell contact between vascular EC and CD8⁺ T-cells did not occur or in an inadequate manner, whereas contact between DC and CD8⁺ T-cells was strong and long enough to trigger CD8⁺ T-cell activation and proliferation.

Tie2-LacZ hearts and livers transplanted into C57BL/6 mice have been well accepted. The presentation of a mhAg did not lead to T-cell activation. Moreover, Tie2-LacZ hearts resided in the recipients for over 100 days without any signs of CTV. This is in contrast to the study of Hirozane et al. where presentation of mhAg (more than one) lead to the development of CTV until day 70 (Hirozane et al., 1995). In our study, transferred β -gal specific CD8⁺ T-cells have neither been activated nor tolerized by mhAg presentation via EC of the transplanted organs. mhAg on EC has been ignored. This confirms the results seen in Tie2-LacZ mice. Furthermore, the mhAg amount was too low to be cross-presented by BM-derived DC (Kurts et al., 1999).

However, β -gal expression in Tie2-LacZ mice refers to a single mhAg only. In the study by Valujskikh et al. the male antigen H-Y represents more than one single mhAg and cross-presentation of the H-Y antigens by host EC was enough to mediate skin graft rejection. This estimate could also be considered in our setting. Transplantation of male Tie2-LacZ hearts into female C57BL/6 recipients would augment mhAg number and amount. Thus, we could address the question whether increase in mhAg number influences CD8⁺ T-cell activation and whether EC can become target cells of specific CTL.

Overall, CD8⁺ T-cell activation or CD8⁺ T-cell induced rejection of transplanted organs has only been observed in *in vivo* transplantation models with allogeneic differences between host and donor (Kreisel et al., 2002a; Kreisel et al., 2002b). This indicates that the high amount of antigen together with the high precursor frequency provides a stimulus strong enough to overcome the standard rules for T-cell priming and thus to activate CD8⁺ T-cells. In the case of a mhAg, we argue that these requirements are not fulfilled and CD8⁺ T-cells therefore ignore mhAg presented by vascular EC (Figure 16).

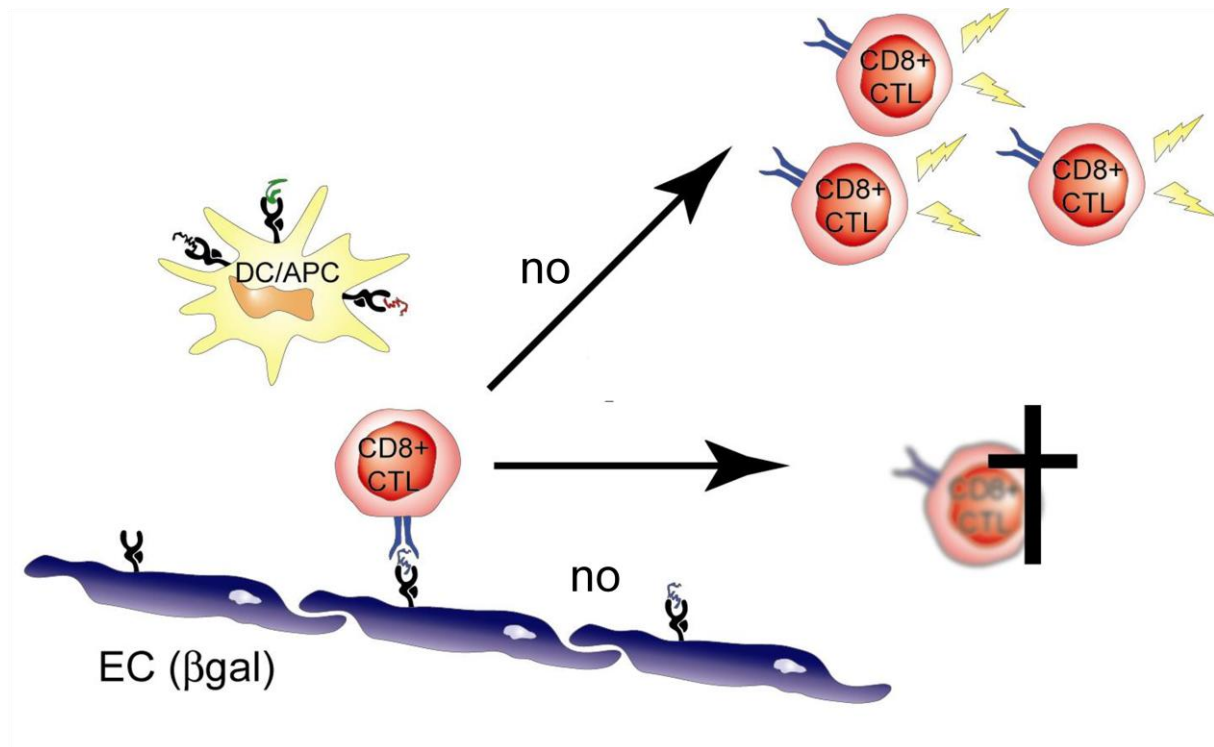


Figure 16: Minor histocompatibility antigen presented by vascular EC is ignored by naive CD8⁺ T-cells. CD8⁺ T-cells do not become activated nor tolerized.

6.2 Can EC become target cells of activated CTL?

To find out whether antigen-specific CTL mediate injury to EC presenting a mhAg and whether these injuries are sufficient to induce TV, we heterotopically transplanted Tie2-LacZ hearts into C57BL/6 recipients. In order to activate β -gal specific CD8⁺ T-cells over a longer period of time, β -gal peptide-loaded, *in vitro* cultured DC were repetitively injected. Thus, a strong β -gal specific CD8⁺ T-cell response could still be measured on day 15 and the detailed interaction of EC with β -gal-specific CTL was assessed. Induction of a β -gal specific CTL response elicited severe vascular inflammation in transplanted Tie2-LacZ hearts. Furthermore, neointima formation and vascular occlusion could be observed leading thereby to the development of chronic vascular rejection. Infection with MCMV-LacZ was characterized by a less pronounced activation and a shorter time of β -gal-specific CTL responses and therefore led to reduced vascular inflammation in Tie2-LacZ hearts.

These results suggest that once activated, β -gal specific CD8⁺ T-cells easily migrate to the periphery where they make cognate interactions with peptide/MHC complexes expressed on vascular EC, thereby engaging their effector machinery and leading to specific destruction of the EC. This indicates that the second antigen contact has less stringent costimulatory requirements. Furthermore, these findings identify EC presenting mhAg as target cells of specific activated CTL (Figure 17). Nevertheless, this issue is crucial in the context of GVHD

which is characterized by the presence of activated mAg-specific CTL capable of EC destruction and thereby leading to CTV development (Mutis et al., 1999). Furthermore, EC injury has been shown to influence the outcome of CTV.

However, we show that it is yet again the long-term antigen presentation and thus the long-term CTL activation within secondary lymphoid organs which plays a key role in the induction of chronic vascular rejection. Short-time activation of CTL, as seen in MCMV-LacZ infection, is clearly less efficient in inducing specific CTV. Despite the fact that MCMV-LacZ induces a strong specific and overall immune stimulation, we argue that it is the lack of chronic activation that leads to a less pronounced outcome of vascular inflammation, neointima thickening, and vessel occlusion. Additionally, this is even more evident when lesions of Tie2-LacZ hearts were compared with lesions in wild-type hearts. This phenomenon could be explained by the fact that MCMV preferentially infects and replicates in vascular EC (Jarvis and Nelson, 2002), together with the general immune stimulation it leads to cell infiltration and mild inflammation around the vessels in C57BL/6 mice. The use of TCR transgenic β -gal specific CD8⁺ T-cells in combination with MCMV-LacZ could be an approach to render the system more specific and enable the investigation of the influence of viral infections. This is of major interest since, as a consequence of immunosuppressive treatment in transplantation patients, viral reactivation may occur and accumulating evidence suggests that such virus reactivations resulting in chronic viral infection play a major role in the development of CTV. Beside herpes viruses, such as CMV, viral genomes including adenovirus have been found to correlate with the outcome of acute and chronic rejection (Potena et al., 2003). By the constant presence of antigen, chronic viral infections lead to the continuous stimulation of the immune system. The permanent release of pro-inflammatory cytokines favours local inflammation and allograft injury leading to CTV. Nonetheless, it has been shown that antiviral therapy in transplant patients leads to a prolonged graft life (Hodson et al., 2005; Potena et al., 2006). Furthermore, chronic immune reactivity against persisting antigen in the vasculature exacerbates atherosclerotic lesion formation (Krebs et al., 2007). However, there is evidence that the development of CTV is influenced by the simultaneous co-existence of a chronic viral infection. Therefore, MCMV-LacZ infection together with the transfer of β -gal-specific CD8⁺ and/or CD4⁺ T-cells will help to measure the influence of specific immune reactivity against a persisting antigen in the vasculature. Nevertheless, since MCMV-LacZ infection did not display a long-term activation of β -gal-specific CTL in our model, we are going to further use the β -gal recombinant and replication deficient adenovirus (Adeno-LacZ). After Adeno-LacZ infection a long-term β -gal-specific CTL response could be

observed. Infection of C57BL/6 mice with 2×10^9 pfu Adeno-LacZ induces a β -gal-specific CTL response with its peak for the β -gal₄₉₇₋₅₀₄ epitope on day 14 measured by MHC class I tetramer staining, whereas the CTL response for a second β -gal epitope, the β -gal₉₆₋₁₀₃, increased over time and displayed more than 30% specific CD8⁺ T-cells on day 100 identifying Adeno-LacZ as an ideal virus to test chronic immune stimulation in our transplantation model.

Taken together our results corroborate studies performed with major histocompatibility disparities between donor and host, except that mhAg-specific CTL had to be generated by vaccination in our model. This is because of the lower antigen amount and particularly because of the lower precursor frequency of T-cells specific for a mhAg compared to alloreactive T-cells (Lindahl and Wilson, 1977; Suchin et al., 2001). Nonetheless, CTV has been proposed to represent a delayed-type hypersensitivity response against cells of the vascular wall (Libby et al., 1989; Mitchell and Libby, 2007). The present findings support this view by showing the link between direct T-cell-mediated EC injury and subsequent pathological remodelling of coronary arteries. However, since EC are target cells for activated CTL they clearly drive the process of chronic vascular rejection by their immune-mediated injuries (Valantine, 2003). Furthermore, EC persist in organ transplants and are thereby able to serve as a place for chronic inflammation, injuries, and subsequent chronic vascular rejection.

However, one criticism that could be raised concerning our model is the use of a single mhAg which does not represent the “real” situation where donor and host differ in more than one mhAg and principally display major histocompatibility antigen differences. This lack could be bypassed, as already mentioned, by the transplantation of a male Tie2-LacZ heart into a female C57BL/6 recipient. Whether this simple change could lead to spontaneous T cell activation has to be investigated. Although it has already been shown that a male heart transplanted into female recipient did not lead to rejection (He et al., 2004a), the cumulating effect of a mhAg and the H-Y antigens has never been assessed. Moreover, the use of TCR transgenic β -gal specific CD8⁺ T-cells in combination with MCMV-LacZ infection could additionally augment the precursor frequency of specific CD8⁺ T-cells which has been shown to accelerate the pace and severity of vasculopathy (He et al., 2004a). Yet, it was the restriction to a single mhAg presented by EC that made it possible to independently study the effect of mhAg expression on EC and its role in the development of CTV. Furthermore, the lack of any immunosuppressive therapy, either by immunosuppressive drugs or via co-stimulatory blockade, made it an ideal model for studying cell-cell interaction in chronic

vascular rejection. Indeed, we could clearly demonstrate that activated CTL recognizing a mhAg expressed on EC were able to induce TV. Furthermore, by showing the importance of DC as intermediary cells promoting prolonged antigen presentation within secondary lymphoid organs, we propose that therapeutic intervention should clearly target DC-mediated T-cell activation in order to prevent CTL-mediated injury of EC following transplantation.

Still, our findings concerning MCMV-LacZ infection clearly show that virus clearance or injury healing are critical steps contributing to the development of CTV (Valantine, 2003). Taken together, chronic graft rejection is a complex disorder in which numerous known and unknown factors participate and it is in the end the overall sum of stimuli leading to the disease (Caforio et al., 2004; Goodman and Mohanakumar, 2003).

In order to obtain more mechanistic insights, we plan to further investigate the role of IFN γ in the development of TV since it has been shown to have a number of actions on EC, such as up-regulation of MHC class I molecules. Transplantation of hearts from Tie2-LacZ mice lacking the IFN γ -receptor should display whether the release of IFN γ by T-lymphocytes accelerate TV development induced by β -gal-specific CTL through its effect on the activation status of EC.

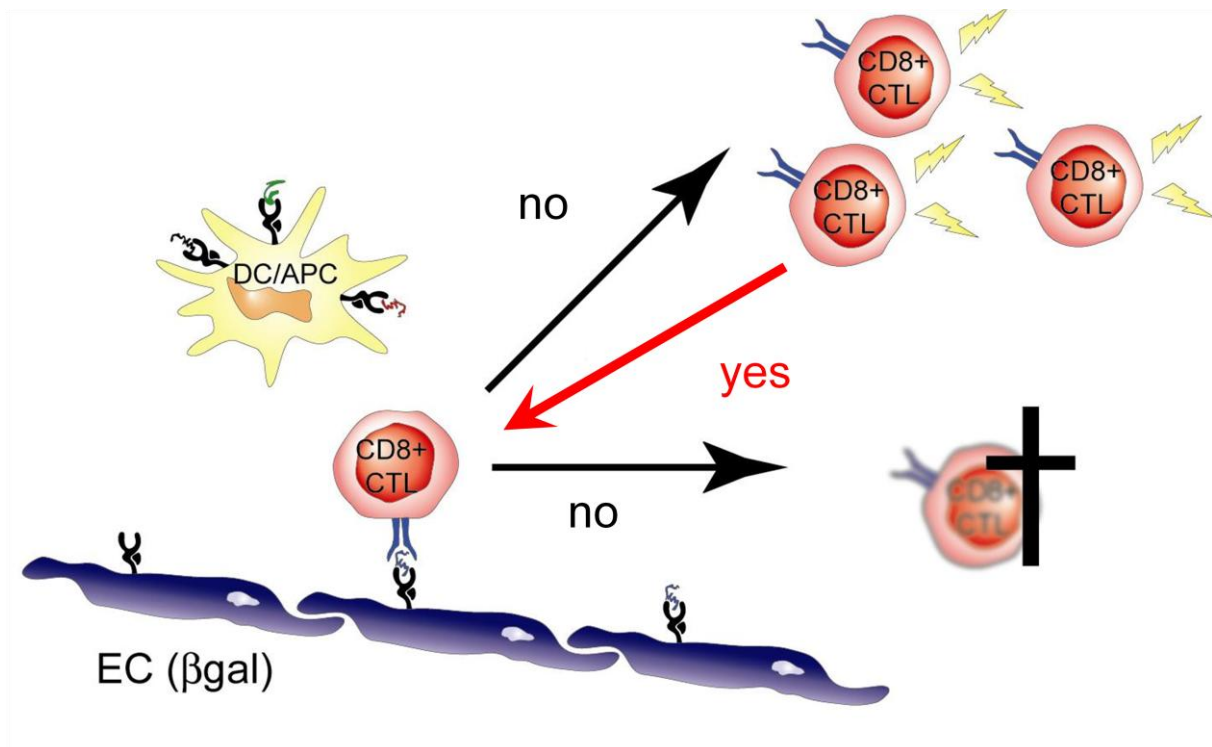


Figure 17: Activated CD8⁺ T-cells recognize minor histocompatibility antigen expressed on EC, thus EC become target cells of antigen-specific CTL.

6.3 CD4⁺ T-cell help

It is known that both CD4⁺ and CD8⁺ T-cells contribute to allograft rejection. The central importance of CD4⁺ T-cells in the initiation of allograft rejection has been illustrated in multiple studies and there is no doubt that hematopoietic cells of donor and host origin, such as DC and macrophages, are potent activators of alloreactive CD4⁺ T-cells. Furthermore, rejection of a solid organ mismatched for minor antigens is also dependent on CD4⁺ and CD8⁺ T-cells (Rosenberg and Singer, 1992; Filatenkov et al., 2005). Heart transplants with mhAg disparities are rapidly rejected, but only if they received both specific CD8⁺ and CD4⁺ T-cells before transplantation (Filatenkov et al., 2005). Moreover, it has long been recognized that CD4⁺ T-cell help is essential for CD8⁺ T-cells, in particular for responses to some bacterial and viral infections (Bevan, 2004). However, it is the CD4⁺ T-cell help that renders the APC competent to efficiently stimulate naive CD8⁺ T-cells via CD40L-CD40 ligation (Ridge et al., 1998). Thus, the expression of B7 costimulatory molecules on DC (Yang and Wilson, 1996) is increased and the production of IL-12, which significantly augments CD8⁺ T-cell responses, is stimulated (Filatenkov et al., 2005; Cella et al., 1996). This is of major importance since it is the DC that delivers the signals which decide whether there is T-cell immunity or tolerance (Steinman, 2003). CD4⁺ T-cells further assist CD8⁺ T-cells by providing IL-2. Moreover Hernandez et al. have shown that high numbers of *in vitro* activated specific CD4⁺ T-cells transferred into a transgenic mouse with antigen expressed in the pancreas could transform the tolerizing signal, resulting in CD8⁺ T-cell proliferation but not effector function, into a signal leading to the differentiation of CD8⁺ T-cells into effector cells (Hernandez et al., 2002).

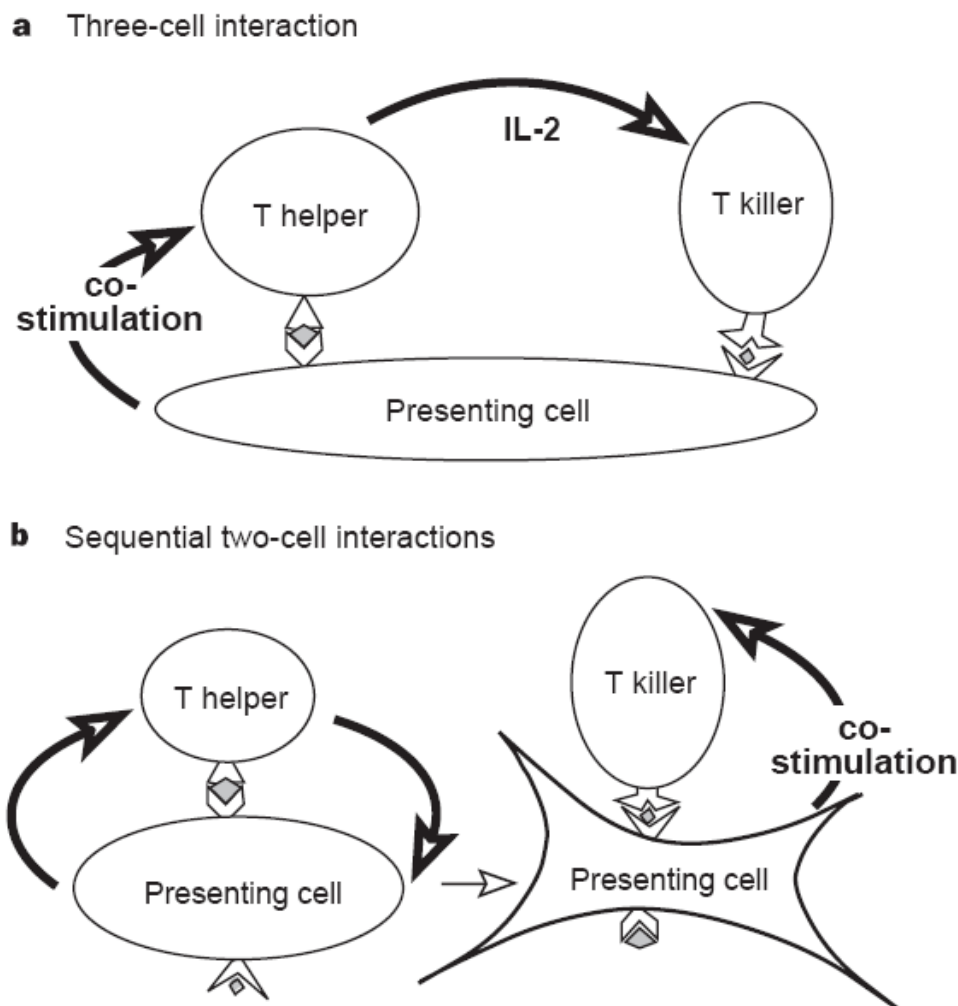


Figure 18: Two models of help delivery to CTL. (a) The passive model: DC present antigen to T helper cells and CTL. Costimulatory signals are only provided to T helper cells. (b) The dynamic model: DC offer costimulation to both. They initially stimulate T helper cells, which, in turn, stimulates and “conditions” DC to differentiate to a state where they can directly costimulate CTL (Ridge et al., 1998).

However, in our study the transfer of β -gal-specific $CD8^+$ T-cells into Tie2-LacZ mice lead to activation and proliferation but not effector function of $CD8^+$ T-cells, indicating that proliferative potential and the gain of effector function are independent events (Hernandez et al., 2002). It would be of major interest whether activated $CD4^+$ T-cells could render DC competent to induce a potent CTL response in our mouse model. Therefore, we are going to investigate the influence of β -gal-specific $CD4^+$ T-cells in this setting. Mice expressing a β -gal-specific TCR on $CD4^+$ T-cells (Bg2 mice) have been kindly provided by Dr. Nicolas Restifo. The properties of this novel TCR transgenics are currently investigated. Once the basic characteristics of the Bg2 TCR have been recorded, it will be investigated whether adoptively transferred TCR transgenic $CD4^+$ T-cells are activated by presenting DC *in vivo*. If yes, are they able to transform the $CD8^+$ T-cell response in order that $CD8^+$ T-cells reach effector function? If no, is there nevertheless a change in the $CD8^+$ T-cell response due to the

release of cytokines such as IL-2? To address this question preactivated β -gal-specific CD4⁺ T-cells (Bg2) together with CD8⁺ T-cells will be transferred into Tie2-LacZ mice and CD8⁺ T-cell response will be assessed. A positive outcome of CD4⁺ T-cell help would have significant consequences in transplantations where both CD4⁺ and CD8⁺ T-cells become activated. This would lead to specific CTL activation, the induction of EC injury, and subsequently to the development of CTV.

However, the transfer of CD4⁺ T-cells could also be of major interest after the transplantation of a Tie2-LacZ heart into a C57BL/6 recipient. Naive and preactivated CD4⁺ T-cells together with CD8⁺ T-cells could be adoptively transferred into transplanted mice and the development of CTV could be analyzed. The combined injection with DC is not necessary since DC cultivated *ex vivo* are fully activated and therefore able to induce a CTL response, as well as the injection of MCMV-LacZ which leads to fully activated CTL.

Nevertheless, knowing that EC cannot directly activate CD8⁺ T-cells, the requirements that render a DC a fully activated and competent APC are worth to be assessed. Therefore, the investigation of CD4⁺ T-cell help in the process of chronic vascular rejection will further explain the detailed cell-cell interaction during the development of TV and thus reveal new strategies for the prevention of late graft rejection.

6.4 CD4⁺CD25⁺Foxp3⁺ regulatory T-cells

Isolated and activated vascular EC have been shown to stimulate allogeneic CD4⁺CD25⁺Foxp3⁺ Treg cells, although they are known to be unable to induce cell division in allogeneic CD4⁺ T-cells. Treg cells clearly inhibited allogeneic T-cell proliferation *in vitro* and *in vivo* (Kreisel et al., 2004; Krupnick et al., 2005). However, the *in vivo* induction of CD4⁺CD25⁺Foxp3⁺ Treg cells has not been shown and therefore the contribution of hematopoietic APC in the induction of Treg cells could not be excluded. This aspect must be considered since it has been demonstrated that Treg cells become activated via the interaction of antigen presenting DC in secondary lymphoid organs (Sakaguchi, 2004). Moreover, spleen has clearly been shown to play an imperative role in the maintaining of tolerance towards heart allografts induced by CD4⁺CD25⁺Foxp3⁺ Treg cells (Chosa et al., 2007) confirming the significance of secondary lymphoid organs for T-cell priming. To dissect the role of professional APC versus non-hematopoietic APC, such as EC, in the induction of CD4⁺CD25⁺Foxp3⁺ Treg cells further *in vivo* studies are necessary. We are going to assess Treg cell stimulation in Tie2-LacZ mice where mAg expression is confined to the vascular

endothelium by FACS analysis. In order to exclude antigen presentation by BM-derived cells, BM-chimeric mice could be generated and the induction of Treg cells analyzed.

CD4⁺CD25⁺Foxp3⁺ Treg cells have further been demonstrated to regulate alloreactive T-cell responses and the acute rejection of single class II MHC-disparate heart allografts (Schenk et al., 2005). Moreover, in a very recent study the use of *ex vivo* expanded CD4⁺CD25⁺Foxp3⁺ T regulatory cells has been suggested as therapeutics in combination with irradiation to treat and prevent GVHD in BM transplantation (Joffre et al., 2008). However, only after knowing the exact mechanisms of how Treg cells become activated, in particular after organ transplantation, new strategies using Treg cells to induce specific tolerance towards the graft transplant can be established. Therefore, the detailed role of Treg cells in solid graft transplantation and the comprehension of the active suppressive mechanisms which induce long-term tolerance may help to generate new therapies and has thus to be further assessed.

In several rodent transplantation studies, it has been shown that alloantigen-specific and suppressive CD4⁺CD25⁺ Treg cells accumulated within the graft and controlled effector cells by their regulatory mechanism (Cobbold et al., 2004; Graca et al., 2002). Therefore we suggest that there must be an interaction between Treg cells, effector cells and APC leading to tolerance induction. To assess the detailed role of Treg cells in transplantation, transgenic DEREK mice will be used (Lahl et al., 2007). DEREK mice express a diphtheria toxin receptor (DTR)-enhanced GFP fusion protein under the control of the *foxp3* locus which allows both the detection of Foxp3⁺ regulatory T cells and their inducible depletion via DT injection. Therefore we are going to transplant Tie2-LacZ hearts into DEREK recipients. In a first set of experiments the induction of CD4⁺CD25⁺Foxp3⁺ Treg cells will be studied by FACS analysis and immune histochemistry of heart slides. To find out whether CD4⁺CD25⁺Foxp3⁺ regulatory T cells participate in tolerance towards Tie2-LacZ hearts in C57BL/6 recipients we are going to deplete Treg cells by the injection of DT. Depending on the outcome, the interaction between Treg cells and DC as well as effector T cells will be further analyzed. With the help of transgenic mice, facilitating the depletion of CD11c⁺ DC, and via the transfer of β -gal-specific CD4⁺ T-cells further insights into the detailed interplay between those cells will be achieved. We will additionally investigate the role of IFN γ via the transplantation of Tie2-LacZ hearts lacking the IFN γ -receptor since it has been demonstrated that IFN γ plays a key role in allograft tolerance based on Treg cells (Thebault et al., 2007). However, the options given by the well established transplantation model and the numerous available transgenic mice are almost without limits. Nevertheless, a further step is the

establishment of solid organ transplantation leading to physiologically functional organs. In order to fulfil this requirement we are going to orthotopically transplant kidney grafts. Chronic rejection is the most prevalent reason of renal transplant failure as well. In most cases the histopathology is not specific, such as fibrointima thickening of arteries, interstitial fibrosis, and tubular atrophy, but characterized by glomerulopathy and multilayering of the peritubular capillaries. However, a main advantage of renal transplantations is the easiness of measuring the loss of function by controlling the creatinine clearance and certainly the proximity to the conditions in humans since a host kidney is replaced by a donor kidney that takes over its physiological role.

7 Appendix

7.1 References

1. Abbas, A.K. and Sharpe, A.H. (2005). Dendritic cells give and take away. *Nat. Immunol.* *6*, 227-228.
2. Agata, Y., Kawasaki, A., Nishimura, H., Ishida, Y., Tsubata, T., Yagita, H., and Honjo, T. (1996). Expression of the PD-1 antigen on the surface of stimulated mouse T and B lymphocytes. *Int. Immunol.* *8*, 765-772.
3. Aichele, P., Brduscha-Riem, K., Zinkernagel, R.M., Hengartner, H., and Pircher, H. (1995). T cell priming versus T cell tolerance induced by synthetic peptides. *J. Exp. Med.* *182*, 261-266.
4. Aird, W.C., Edelberg, J.M., Weiler-Guettler, H., Simmons, W.W., Smith, T.W., and Rosenberg, R.D. (1997). Vascular bed-specific expression of an endothelial cell gene is programmed by the tissue microenvironment. *J. Cell Biol.* *138*, 1117-1124.
5. Aird, W.C., Jahroudi, N., Weiler Guettler, H., Rayburn, H.B., and Rosenberg, R.D. (1995). Human von Willebrand factor gene sequences target expression to a subpopulation of endothelial cells in transgenic mice. *Proc. Natl. Acad. Sci. U. S. A.* *92*, 4567-4571.
6. Alexander-Miller, M.A., Burke, K., Koszinowski, U.H., Hansen, T.H., and Connolly, J.M. (1993). Alloreactive cytotoxic T lymphocytes generated in the presence of viral-derived peptides show exquisite peptide and MHC specificity. *J. Immunol.* *151*, 1-10.
7. Altman, J.D., Moss, P.A.H., Goulder, P.J.R., Barouch, D.H., McHeyzer-Williams, M.G., Bell, J.I., McMichael, A.J., and Davis, M.M. (1996). Phenotypic analysis of antigen-specific T lymphocytes. *Science* *274*, 94-96.
8. Aluvihare, V.R., Kallikourdis, M., and Betz, A.G. (2004). Regulatory T cells mediate maternal tolerance to the fetus. *Nat. Immunol.* *5*, 266-271.
9. Anderson, M.S. (2002). Autoimmune endocrine disease. *Curr. Opin. Immunol.* *14*, 760-764.
10. Arai, F., Hirao, A., Ohmura, M., Sato, H., Matsuoka, S., Takubo, K., Ito, K., Koh, G.Y., and Suda, T. (2004). Tie2/angiopoietin-1 signaling regulates hematopoietic stem cell quiescence in the bone marrow niche. *Cell.* *118*, 149-161.
11. Ardehali, A., Laks, H., Drinkwater, D.C., Ziv, E., and Drake, T.A. (1995). Vascular cell adhesion molecule-1 is induced on vascular endothelia and medial smooth muscle cells in experimental cardiac allograft vasculopathy. *Circulation* *92*, 450-456.
12. Armstrong, A.T., Strauch, A.R., Starling, R.C., Sedmak, D.D., and Orosz, C.G. (1997a). Morphometric analysis of neointimal formation in murine cardiac allografts. *Transplantation.* *63*, 941-947.
13. Armstrong, A.T., Strauch, A.R., Starling, R.C., Sedmak, D.D., and Orosz, C.G. (1997b). Morphometric analysis of neointimal formation in murine cardiac allografts: II. Rate and location of lesion development. *Transplantation.* *64*, 322-328.
14. Asseman, C., Mauze, S., Leach, M.W., Coffman, R.L., and Powrie, F. (1999). An essential role for interleukin 10 in the function of regulatory T cells that inhibit intestinal inflammation. *J. Exp. Med.* *190*, 995-1004.

15. Bagai,R., Valujskikh,A., Canaday,D.H., Bailey,E., Lalli,P.N., Harding,C.V., and Heeger,P.S. (2005a). Mouse endothelial cells cross-present lymphocyte-derived antigen on class I MHC via a. *J. Immunol.* *174*, 7711-7715.
16. Bagai,R., Valujskikh,A., Canaday,D.H., Bailey,E., Lalli,P.N., Harding,C.V., and Heeger,P.S. (2005b). Mouse endothelial cells cross-present lymphocyte-derived antigen on class I MHC via a TAP1- and proteasome-dependent pathway. *J. Immunol.* *174*, 7711-7715.
17. Banchereau,J., Briere,F., Caux,C., Davoust,J., Lebecque,S., Liu,Y.J., Pulendran,B., and Palucka,K. (2000). Immunobiology of dendritic cells. *Annu. Rev. Immunol.* *18*, 767-811.
18. Banchereau,J. and Steinman,R.M. (1998). Dendritic cells and the control of immunity. *Nature* *392*, 245-252.
19. Belkaid,Y., Blank,R.B., and Suffia,I. (2006). Natural regulatory T cells and parasites: a common quest for host homeostasis. *Immunol. Rev.* *212*, 287-300.
20. Bennett,S.R., Carbone,F.R., Karamalis,F., Flavell,R.A., Miller,J.F., and Heath,W.R. (1998). Help for cytotoxic-T-cell responses is mediated by CD40 signalling. *Nature* *393*, 478-480.
21. Bensinger,S.J., Bandeira,A., Jordan,M.S., Caton,A.J., and Laufer,T.M. (2001). Major histocompatibility complex class II-positive cortical epithelium mediates the selection of CD4(+)25(+) immunoregulatory T cells. *J. Exp. Med.* *194*, 427-438.
22. Berg,M., Wingender,G., Djandji,D., Hegenbarth,S., Momburg,F., Hammerling,G., Limmer,A., and Knolle,P. (2006). Cross-presentation of antigens from apoptotic tumor cells by liver sinusoidal endothelial cells leads to tumor-specific CD8+ T cell tolerance. *Eur. J. Immunol.* *36*, 2960-2970.
23. Bevan,M.J. (1984). High Determinant Density May Explain the Phenomenon of Alloreactivity. *Immunology Today* *5*, 128-130.
24. Bevan,M.J. (2004). Helping the CD8(+) T-cell response. *Nat. Rev. Immunol.* *4*, 595-602.
25. Beyer,M. and Schultze,J.L. (2006). Regulatory T cells in cancer. *Blood* *108*, 804-811.
26. Biedermann,B.C. and Pober,J.S. (1998). Human endothelial cells induce and regulate cytolytic T cell differentiation. *J. Immunol.* *161*, 4679-4687.
27. Biedermann,B.C. and Pober,J.S. (1999). Human vascular endothelial cells favor clonal expansion of unusual alloreactive CTL. *J. Immunol.* *162*, 7022-7030.
28. Biedermann,B.C., Sahner,S., Gregor,M., Tsakiris,D.A., Jeanneret,C., Pober,J.S., and Gratwohl,A. (2002). Endothelial injury mediated by cytotoxic T lymphocytes and loss of microvessels in chronic graft versus host disease. *Lancet* *359*, 2078-2083.
29. Billingham,R.E., Brent,L., and Medawar,P.B. (1953). Actively acquired tolerance of foreign cells. *Nature* *172*, 603-609.
30. Bingaman,A.W., Ha,J., Waitze,S.Y., Durham,M.M., Cho,H.R., Tucker-Burden,C., Hendrix,R., Cowan,S.R., Pearson,T.C., and Larsen,C.P. (2000). Vigorous allograft rejection in the absence of danger. *J. Immunol.* *164*, 3065-3071.
31. Black,C.A. (1999). Delayed type hypersensitivity: current theories with an historic perspective. *Dermatol. Online. J.* *5*, 7.
32. Bluestone,J.A., Jameson,S., Miller,S., and Dick,R. (1992). Peptide-induced conformational changes in class I heavy chains alter major histocompatibility complex recognition. *J. Exp. Med.* *176*, 1757-1761.

33. Boehme, K.W., Guerrero, M., and Compton, T. (2006). Human cytomegalovirus envelope glycoproteins B and H are necessary for TLR2 activation in permissive cells. *J. Immunol.* *177*, 7094-7102.
34. Bolinger, B., Krebs, P., Tian, Y., Engeler, D., Scandella, E., Miller, S., Palmer, D. C., Restifo, N. P., Clavien, P. A., and Ludewig, B. Immunological ignorance of vascular endothelial cells expressing minor histocompatibility antigen. *Blood* . 2008.
Ref Type: In Press
35. Bonilla, W.V., Geuking, M.B., Aichele, P., Ludewig, B., Hengartner, H., and Zinkernagel, R.M. (2006). Microchimerism maintains deletion of the donor cell-specific CD8+ T cell repertoire. *J. Clin. Invest.* *116*, 156-162.
36. Briscoe, D.M., Alexander, S.I., and Lichtman, A.H. (1998a). Interactions between T lymphocytes and endothelial cells in allograft rejection. *Curr. Opin. Immunol.* *10*, 525-531.
37. Briscoe, D.M., Alexander, S.I., and Lichtman, A.H. (1998b). Interactions between T lymphocytes and endothelial cells in allograft rejection. *Curr. Opin. Immunol.* *10*, 525-531.
38. Burnet, F.M. (1957). A modification of Jerne's theory of antibody production using the concept of clonal selection. *Australian Journal of Science* *20*, 67-69.
39. Burnet, F. M and Fenner. The production of antibodies. second edition. 1949. *Macmillan*.
Ref Type: Serial (Book, Monograph)
40. Butcher, E.C. and Picker, L.J. (1996). Lymphocyte homing and homeostasis. *Science* *272*, 60-66.
41. Caforio, A.L., Tona, F., Fortina, A.B., Angelini, A., Piaserico, S., Gambino, A., Feltrin, G., Ramondo, A., Valente, M., Illiceto, S., Thiene, G., and Gerosa, G. (2004). Immune and nonimmune predictors of cardiac allograft vasculopathy onset and severity: multivariate risk factor analysis and role of immunosuppression. *Am. J. Transplant.* *4*, 962-970.
42. Campbell, J.J., Hedrick, J., Zlotnik, A., Siani, M.A., Thompson, D.A., and Butcher, E.C. (1998). Chemokines and the arrest of lymphocytes rolling under flow conditions. *Science* *279*, 381-384.
43. Carlquist, J.F., Edelman, L., Bennion, D.W., and Anderson, J.L. (1999). Cytomegalovirus induction of interleukin-6 in lung fibroblasts occurs independently of active infection and involves a G protein and the transcription factor, NF-kappaB. *J. Infect. Dis.* *179*, 1094-1100.
44. Carlquist, J.F., Shelby, J., Shao, Y.L., Greenwood, J.H., Hammond, M.E., and Anderson, J.L. (1993). Accelerated rejection of murine cardiac allografts by murine cytomegalovirus-infected recipients. Lack of haplotype specificity. *J. Clin. Invest* *91*, 2602-2608.
45. Cecka, J.M. (1999). The UNOS Scientific Renal Transplant Registry. *Clin. Transpl.* *:1-21.*, 1-21.
46. Cella, M., Scheidegger, D., Palmer, Lehmann, K., Lane, P., Lanzavecchia, A., and Alber, G. (1996). Ligation of CD40 on dendritic cells triggers production of high levels of interleukin-12 and enhances T cell stimulatory capacity: T-T help via APC activation. *J. Exp. Med.* *184*, 747-752.
47. Chattopadhyay, S., Theobald, M., Biggs, J., and Sherman, L.A. (1994). Conformational differences in major histocompatibility complex-peptide complexes can result in alloreactivity. *J. Exp. Med.* *179*, 213-219.
48. Chen, Y., Heeger, P.S., and Valujskikh, A. (2004). In vivo helper functions of alloreactive memory CD4+ T cells remain intact despite donor-specific transfusion and anti-CD40 ligand therapy. *J. Immunol.* *172*, 5456-5466.

49. Chosa,E., Hara,M., Watanabe,A., Matsuzaki,Y., Nakamura,K., Hamano,K., Wood,K.J., and Onitsuka,T. (2007). Spleen plays an important role in maintaining tolerance after removal of the vascularized heart graft. *Transplantation* 83, 1226-1233.
50. Choy,J.C., Hung,V.H., Hunter,A.L., Cheung,P.K., Motyka,B., Goping,I.S., Sawchuk,T., Bleackley,R.C., Podor,T.J., McManus,B.M., and Granville,D.J. (2004a). Granzyme B induces smooth muscle cell apoptosis in the absence of perforin: involvement of extracellular matrix degradation. *Arterioscler. Thromb. Vasc. Biol.* 24, 2245-2250.
51. Choy,J.C., Kerjner,A., Wong,B.W., McManus,B.M., and Granville,D.J. (2004b). Perforin mediates endothelial cell death and resultant transplant vascular disease in cardiac allografts. *Am. J. Pathol.* 165, 127-133.
52. Claas,F. (2004). Chimerism as a tool to induce clinical transplantation tolerance. *Curr. Opin. Immunol.* 16, 578-583.
53. Cobbold,S.P., Castejon,R., Adams,E., Zelenika,D., Graca,L., Humm,S., and Waldmann,H. (2004). Induction of foxP3+ regulatory T cells in the periphery of T cell receptor transgenic mice tolerized to transplants. *J. Immunol.* 172, 6003-6010.
54. Compton,T., Kurt-Jones,E.A., Boehme,K.W., Belko,J., Latz,E., Golenbock,D.T., and Finberg,R.W. (2003). Human cytomegalovirus activates inflammatory cytokine responses via CD14 and Toll-like receptor 2. *J. Virol.* 77, 4588-4596.
55. Corry,R.J., Winn,H.J., and Russell,P.S. (1973). Primarily vascularized allografts of hearts in mice. The role of H-2D, H-2K and non-H-2 antigens in rejection. *Transplantation* 16, 343-350.
56. Cosimi,A.B. and Sachs,D.H. (2004). Mixed chimerism and transplantation tolerance. *Transplantation* 77, 943-946.
57. Denton,M.D., Geehan,C.S., Alexander,S.I., Sayegh,M.H., and Briscoe,D.M. (1999). Endothelial cells modify the costimulatory capacity of transmigrating leukocytes and promote CD28-mediated CD4(+) T cell alloactivation. *J. Exp. Med.* 190, 555-566.
58. Dong,C., Redenbach,D., Wood,S., Battistini,B., Wilson,J.E., and McManus,B.M. (1996a). The pathogenesis of cardiac allograft vasculopathy. *Curr. Opin. Cardiol.* 11, 183-190.
59. Dong,C., Wilson,J.E., Winters,G.L., and McManus,B.M. (1996b). Human transplant coronary artery disease: pathological evidence for Fas-mediated apoptotic cytotoxicity in allograft arteriopathy. *Lab Invest* 74, 921-931.
60. Ehl,S., Aichele,P., Ramseier,H., Barchet,W., Hombach,J., Pircher,H., Hengartner,H., and Zinkernagel,R.M. (1998a). Antigen persistence and time of T-cell tolerization determine the efficacy of tolerization protocols for prevention of skin graft rejection [In Process Citation]. *Nat. Med.* 4, 1015-1019.
61. Ehl,S., Hombach,J., Aichele,P., Rulicke,T., Odermatt,B., Hengartner,H., Zinkernagel,R.M., and Pircher,H. (1998b). Viral and bacterial infections interfere with peripheral tolerance induction and activate CD8+ T cells to cause immunopathology. *J. Exp. Med.* 187, 763-774.
62. Epperson,D.E. and Pober,J.S. (1994). Antigen-presenting function of human endothelial cells. Direct activation of resting CD8 T cells. *J. Immunol.* 153, 5402-5412.

63. Eppihimer,M.J., Gunn,J., Freeman,G.J., Greenfield,E.A., Chernova,T., Erickson,J., and Leonard,J.P. (2002). Expression and regulation of the PD-L1 immunoinhibitory molecule on microvascular endothelial cells. *Microcirculation*. 9, 133-145.
64. Fahlen,L., Read,S., Gorelik,L., Hurst,S.D., Coffman,R.L., Flavell,R.A., and Powrie,F. (2005). T cells that cannot respond to TGF-beta escape control by CD4(+)CD25(+) regulatory T cells. *J. Exp. Med.* 201, 737-746.
65. Felix,N.J. and Allen,P.M. (2007). Specificity of T-cell alloreactivity. *Nat. Rev. Immunol.* 7, 942-953.
66. Felix,N.J., Donermeyer,D.L., Horvath,S., Walters,J.J., Gross,M.L., Suri,A., and Allen,P.M. (2007). Alloreactive T cells respond specifically to multiple distinct peptide-MHC complexes. *Nat. Immunol.* 8, 388-397.
67. Filatenkov,A.A., Jacovetty,E.L., Fischer,U.B., Curtsinger,J.M., Mescher,M.F., and Ingulli,E. (2005). CD4 T cell-dependent conditioning of dendritic cells to produce IL-12 results in CD8-mediated graft rejection and avoidance of tolerance. *J. Immunol.* 174, 6909-6917.
68. Fischbein,M.P., Yun,J., Laks,H., Irie,Y., Fishbein,M.C., Bonavida,B., and Ardehali,A. (2002). Role of CD8+ lymphocytes in chronic rejection of transplanted hearts. *J. Thorac. Cardiovasc. Surg.* 123, 803-809.
69. Fisson,S., Darrasse-Jeze,G., Litvinova,E., Septier,F., Klatzmann,D., Liblau,R., and Salomon,B.L. (2003). Continuous activation of autoreactive CD4+ CD25+ regulatory T cells in the steady state. *J. Exp. Med.* 198, 737-746.
70. Ford,M.L., Koehn,B.H., Wagener,M.E., Jiang,W., Gangappa,S., Pearson,T.C., and Larsen,C.P. (2007). Antigen-specific precursor frequency impacts T cell proliferation, differentiation, and requirement for costimulation. *J. Exp. Med.* 204, 299-309.
71. Gallegos,A.M. and Bevan,M.J. (2004). Central tolerance to tissue-specific antigens mediated by direct and indirect antigen presentation. *J. Exp. Med.* 200, 1039-1049.
72. Gimbrone,M.A., Jr., Topper,J.N., Nagel,T., Anderson,K.R., and Garcia-Cardena,G. (2000). Endothelial dysfunction, hemodynamic forces, and atherogenesis. *Ann. N. Y. Acad. Sci.* 902, 230-239.
73. Gold,M.C., Munks,M.W., Wagner,M., Koszinowski,U.H., Hill,A.B., and Fling,S.P. (2002). The murine cytomegalovirus immunomodulatory gene m152 prevents recognition of infected cells by M45-specific CTL but does not alter the immunodominance of the M45-specific CD8 T cell response in vivo. *J. Immunol.* 169, 359-365.
74. Goodman,J. and Mohanakumar,T. (2003). Chronic rejection: failure of immune regulation. *Front Biosci.* 8, s838-s844.
75. Goulmy,E., Gratama,J.W., Blokland,E., Zwaan,F.E., and van Rood,J.J. (1983). A minor transplantation antigen detected by MHC-restricted cytotoxic T lymphocytes during graft-versus-host disease. *Nature* 302, 159-161.
76. Grabie,N., Gotsman,I., DaCosta,R., Pang,H., Stavrakis,G., Butte,M.J., Keir,M.E., Freeman,G.J., Sharpe,A.H., and Lichtman,A.H. (2007). Endothelial programmed death-1 ligand 1 (PD-L1) regulates CD8+ T-cell mediated injury in the heart. *Circulation* 116, 2062-2071.
77. Graca,L., Cobbold,S.P., and Waldmann,H. (2002). Identification of regulatory T cells in tolerated allografts. *J. Exp. Med.* 195, 1641-1646.

78. Grewal, I.S. and Flavell, R.A. (1998). CD40 and CD154 in cell-mediated immunity. *Annu. Rev. Immunol.* *16*, 111-135.
79. Grewal, I.S., Foellmer, H.G., Grewal, K.D., Xu, J.C., Hardardottir, F., Baron, J.L., Janeway, C.A., and Flavell, R.A. (1996). Requirement for CD40 ligand in costimulation induction, T cell activation, and experimental allergic encephalomyelitis. *Science* *273*, 1864-1867.
80. Grewal, I.S., Xu, J., and Flavell, R.A. (1995). Impairment of antigen-specific T-cell priming in mice lacking CD40 ligand. *Nature* *378*, 617-620.
81. Guermonprez, P., Valladeau, J., Zitvogel, L., Thery, C., and Amigorena, S. (2002). Antigen presentation and T cell stimulation by dendritic cells. *Annu. Rev. Immunol.* *20*, 621-667.
82. Guimezanes, A., Barrett-Wilt, G.A., Gulden-Thompson, P., Shabanowitz, J., Engelhard, V.H., Hunt, D.F., and Schmitt-Verhulst, A.M. (2001). Identification of endogenous peptides recognized by in vivo or in vitro generated alloreactive cytotoxic T lymphocytes: distinct characteristics correlated with CD8 dependence. *Eur. J. Immunol.* *31*, 421-432.
83. Guo, Z., Meng, L., Kim, O., Wang, J., Hart, J., He, G., Alegre, M.L., Thistlethwaite, J.R., Jr., Pearson, T.C., Larsen, C.P., and Newell, K.A. (2001). CD8 T cell-mediated rejection of intestinal allografts is resistant to inhibition of the CD40/CD154 costimulatory pathway. *Transplantation* *71*, 1351-1354.
84. He, C., Schenk, S., Zhang, Q., Valujskikh, A., Bayer, J., Fairchild, R.L., and Heeger, P.S. (2004a). Effects of T cell frequency and graft size on transplant outcome in mice. *J. Immunol.* *172*, 240-247.
85. He, C., Schenk, S., Zhang, Q., Valujskikh, A., Bayer, J., Fairchild, R.L., and Heeger, P.S. (2004b). Effects of T cell frequency and graft size on transplant outcome in mice. *J. Immunol.* *172*, 240-247.
86. Heath, W.R., Kane, K.P., Mescher, M.F., and Sherman, L.A. (1991). Alloreactive T cells discriminate among a diverse set of endogenous peptides. *Proc. Natl. Acad. Sci. U. S. A* *88*, 5101-5105.
87. Heath, W.R. and Sherman, L.A. (1991). Cell-type-specific recognition of allogeneic cells by alloreactive cytotoxic T cells: a consequence of peptide-dependent allorecognition. *Eur. J. Immunol.* *21*, 153-159.
88. Hernandez, J., Aung, S., Marquardt, K., and Sherman, L.A. (2002). Uncoupling of proliferative potential and gain of effector function by CD8(+) T cells responding to self-antigens. *J. Exp. Med.* *196*, 323-333.
89. Hernandez-Fuentes, M.P., Baker, R.J., and Lechler, R.I. (1999). The alloresponse. *Rev. Immunogenet.* *1*, 282-296.
90. Herrera, O.B., Golshayan, D., Tibbott, R., Salcido, O.F., James, M.J., Marelli-Berg, F.M., and Lechler, R.I. (2004). A novel pathway of alloantigen presentation by dendritic cells. *J. Immunol.* *173*, 4828-4837.
91. Hillebrands, J.L., Klatter, F.A., van den Hurk, B.M., Popa, E.R., Nieuwenhuis, P., and Rozing, J. (2001). Origin of neointimal endothelium and alpha-actin-positive smooth muscle cells in transplant arteriosclerosis. *J. Clin. Invest* *107*, 1411-1422.
92. Hirozane, T., Matsumori, A., Furukawa, Y., and Sasayama, S. (1995). Experimental graft coronary artery disease in a murine heterotopic cardiac transplant model. *Circulation* *91*, 386-392.
93. Hodson, E.M., Jones, C.A., Webster, A.C., Strippoli, G.F., Barclay, P.G., Kable, K., Vimalachandra, D., and Craig, J.C. (2005). Antiviral medications to prevent cytomegalovirus disease and early death in recipients of solid-organ transplants: a systematic review of randomised controlled trials. *Lancet* *365*, 2105-2115.

94. Hollenbaugh,D., Mischel-Petty,N., Edwards,C.P., Simon,J.C., Denfeld,R.W., Kiener,P.A., and Aruffo,A. (1995). Expression of functional CD40 by vascular endothelial cells. *J. Exp. Med.* *182*, 33-40.
95. Hornick,P.I., Mason,P.D., Baker,R.J., Hernandez-Fuentes,M., Frasca,L., Lombardi,G., Taylor,K., Weng,L., Rose,M.L., Yacoub,M.H., Batchelor,R., and Lechler,R.I. (2000). Significant frequencies of T cells with indirect anti-donor specificity in heart graft recipients with chronic rejection. *Circulation* *101*, 2405-2410.
96. Horowitz,M.M., Gale,R.P., Sondel,P.M., Goldman,J.M., Kersey,J., Kolb,H.J., Rimm,A.A., Ringden,O., Rozman,C., Speck,B., and . (1990). Graft-versus-leukemia reactions after bone marrow transplantation. *Blood* *75*, 555-562.
97. Hosiawa,K.A., Wang,H., DeVries,M.E., Garcia,B., Liu,W., Zhou,D., Akram,A., Jiang,J., Sun,H., Cameron,M.J., Zhong,R., and Kelvin,D.J. (2005). CD80/CD86 costimulation regulates acute vascular rejection. *J. Immunol.* *175*, 6197-6204.
98. Hruban,R.H., Beschoner,W.E., Baumgartner,W.A., Augustine,S.M., Ren,H., Reitz,B.A., and Hutchins,G.M. (1990). Accelerated arteriosclerosis in heart transplant recipients is associated with a T-lymphocyte-mediated endothelialitis. *Am. J. Pathol.* *137*, 871-882.
99. Hsieh,C.S., Liang,Y., Tyznik,A.J., Self,S.G., Liggitt,D., and Rudensky,A.Y. (2004). Recognition of the peripheral self by naturally arising CD25+ CD4+ T cell receptors. *Immunity.* *21*, 267-277.
100. Isobe,M., Kosuge,H., and Suzuki,J. (2006). T cell costimulation in the development of cardiac allograft vasculopathy: potential targets for therapeutic interventions. *Arterioscler. Thromb. Vasc. Biol.* *26*, 1447-1456.
101. Ito,T., Ueno,T., Clarkson,M.R., Yuan,X., Jurewicz,M.M., Yagita,H., Azuma,M., Sharpe,A.H., Auchincloss,H., Jr., Sayegh,M.H., and Najafian,N. (2005). Analysis of the role of negative T cell costimulatory pathways in CD4 and CD8 T cell-mediated alloimmune responses in vivo. *J. Immunol.* *174*, 6648-6656.
102. Jarvis,M.A. and Nelson,J.A. (2002). Human cytomegalovirus persistence and latency in endothelial cells and macrophages. *Curr. Opin. Microbiol.* *5*, 403-407.
103. Joffre,O., Gorsse,N., Romagnoli,P., Hudrisier,D., and van Meerwijk,J.P. (2004). Induction of antigen-specific tolerance to bone marrow allografts with CD4+CD25+ T lymphocytes. *Blood* *103*, 4216-4221.
104. Joffre,O., Santolaria,T., Calise,D., Al Saati,T., Hudrisier,D., Romagnoli,P., and van Meerwijk,J.P. (2008). Prevention of acute and chronic allograft rejection with CD4+CD25+Foxp3+ regulatory T lymphocytes. *Nat. Med.* *14*, 88-92.
105. Jones,N.D., Van Maurik,A., Hara,M., Spriewald,B.M., Witzke,O., Morris,P.J., and Wood,K.J. (2000). CD40-CD40 ligand-independent activation of CD8+ T cells can trigger allograft rejection. *J. Immunol.* *165*, 1111-1118.
106. Julius,B.K., Attenhofer Jost,C.H., Sutsch,G., Brunner,H.P., Kuenzli,A., Vogt,P.R., Turina,M., Hess,O.M., and Kiowski,W. (2000). Incidence, progression and functional significance of cardiac allograft vasculopathy after heart transplantation. *Transplantation* *69*, 847-853.
107. Jung,S., Unutmaz,D., Wong,P., Sano,G., De los,S.K., Sparwasser,T., Wu,S., Vuthoori,S., Ko,K., Zavala,F., Pamer,E.G., Littman,D.R., and Lang,R.A. (2002). In vivo depletion of CD11c(+) dendritic

- cells abrogates priming of CD8(+) T cells by exogenous cell-associated antigens. *Immunity*. 17, 211-220.
108. Kappler,J.W., Roehm,N., and Marrack,P. (1987). T cell tolerance by clonal elimination in the thymus. *Cell* 49, 273-280.
109. Karmann,K., Hughes,C.C., Schechner,J., Fanslow,W.C., and Pober,J.S. (1995). CD40 on human endothelial cells: inducibility by cytokines and functional regulation of adhesion molecule expression. *Proc. Natl. Acad. Sci. U. S. A.* 92, 4342-4346.
110. Keck,B.M., Bennett,L.E., Rosendale,J., Daily,O.P., Novick,R.J., and Hosenpud,J.D. (1999). Worldwide thoracic organ transplantation: a report from the UNOS/ISHLT International Registry for Thoracic Organ Transplantation. *Clin. Transpl.* :35-49., 35-49.
111. Keir,M.E., Liang,S.C., Guleria,I., Latchman,Y.E., Qipo,A., Albacker,L.A., Koulmanda,M., Freeman,G.J., Sayegh,M.H., and Sharpe,A.H. (2006). Tissue expression of PD-L1 mediates peripheral T cell tolerance. *J. Exp. Med.* 203, 883-895.
112. Kemna,M.S., Valantine,H.A., Hunt,S.A., Schroeder,J.S., Chen,Y.D., and Reaven,G.M. (1994). Metabolic risk factors for atherosclerosis in heart transplant recipients. *Am. Heart J.* 128, 68-72.
113. Khoury,S., Sayegh,M.H., and Turka,L.A. (1999). Blocking costimulatory signals to induce transplantation tolerance and prevent autoimmune disease. *Int. Rev. Immunol.* 18, 185-199.
114. Kirk,A.D., Harlan,D.M., Armstrong,N.N., Davis,T.A., Dong,Y., Gray,G.S., Hong,X., Thomas,D., Fechner,J.H., Jr., and Knechtle,S.J. (1997). CTLA4-Ig and anti-CD40 ligand prevent renal allograft rejection in primates. *Proc. Natl. Acad. Sci. U. S. A* 94, 8789-8794.
115. Kobayashi,H., Koga,S., Novick,A.C., Toma,H., and Fairchild,R.L. (2003). T-cell mediated induction of allogeneic endothelial cell chemokine expression. *Transplantation* 75, 529-536.
116. Koffron,A.J., Hummel,M., Patterson,B.K., Yan,S., Kaufman,D.B., Fryer,J.P., Stuart,F.P., and Abecassis,M.I. (1998). Cellular localization of latent murine cytomegalovirus. *J. Virol.* 72, 95-103.
117. Krebs,P., Scandella,E., Bolinger,B., Engeler,D., Miller,S., and Ludewig,B. (2007). Chronic immune reactivity against persisting microbial antigen in the vasculature exacerbates atherosclerotic lesion formation. *Arterioscler. Thromb. Vasc. Biol.* 27, 2206-2213.
118. Kreisel,D., Krasinskas,A.M., Krupnick,A.S., Gelman,A.E., Balsara,K.R., Popma,S.H., Riha,M., Rosengard,A.M., Turka,L.A., and Rosengard,B.R. (2004). Vascular endothelium does not activate CD4+ direct allorecognition in graft rejection. *J. Immunol.* 173, 3027-3034.
119. Kreisel,D., Krupnick,A.S., Balsara,K.R., Riha,M., Gelman,A.E., Popma,S.H., Szeto,W.Y., Turka,L.A., and Rosengard,B.R. (2002a). Mouse vascular endothelium activates CD8+ T lymphocytes in a B7-dependent fashion. *J. Immunol.* 169, 6154-6161.
120. Kreisel,D., Krupnick,A.S., Gelman,A.E., Engels,F.H., Popma,S.H., Krasinskas,A.M., Balsara,K.R., Szeto,W.Y., Turka,L.A., and Rosengard,B.R. (2002b). Non-hematopoietic allograft cells directly activate CD8+ T cells and trigger acute rejection: an alternative mechanism of allorecognition. *Nat. Med.* 8, 233-239.
121. Krupnick,A.S., Gelman,A.E., Barchet,W., Richardson,S., Kreisel,F.H., Turka,L.A., Colonna,M., Patterson,G.A., and Kreisel,D. (2005). Murine vascular endothelium activates and induces the generation of allogeneic CD4+25+Foxp3+ regulatory T cells. *J. Immunol.* 175, 6265-6270.

122. Krupnick,A.S., Kreisel,D., Popma,S.H., Balsara,K.R., Szeto,W.Y., Krasinskas,A.M., Riha,M., Wells,A.D., Turka,L.A., and Rosengard,B.R. (2002). Mechanism of T cell-mediated endothelial apoptosis. *Transplantation*. *74*, 871-876.
123. Kummer,M., Lev,A., Reiter,Y., and Biedermann,B.C. (2005). Vascular endothelial cells have impaired capacity to present immunodominant, antigenic peptides: a mechanism of cell type-specific immune escape. *J. Immunol*. *174*, 1947-1953.
124. Kurts,C., Heath,W.R., Kosaka,H., Miller,J.F., and Carbone,F.R. (1998). The peripheral deletion of autoreactive CD8+ T cells induced by cross- presentation of self-antigens involves signaling through CD95 (Fas, Apo- 1). *J. Exp. Med*. *188*, 415-420.
125. Kurts,C., Kosaka,H., Carbone,F.R., Miller,J.F., and Heath,W.R. (1997). Class I-restricted cross-presentation of exogenous self-antigens leads to deletion of autoreactive CD8(+) T cells. *J. Exp. Med*. *186*, 239-245.
126. Kurts,C., Sutherland,R.M., Davey,G., Li,M., Lew,A.M., Blanas,E., Carbone,F.R., Miller,J.F., and Heath,W.R. (1999). CD8 T cell ignorance or tolerance to islet antigens depends on antigen dose. *Proc. Natl. Acad. Sci. U. S. A*. *96*, 12703-12707.
127. Kyburz,D., Aichele,P., Speiser,D.E., Hengartner,H., Zinkernagel,R.M., and Pircher,H. (1993). T cell immunity after a viral infection versus T cell tolerance induced by soluble viral peptides. *Eur. J. Immunol*. *23*, 1956-1962.
128. Kyewski,B. and Derbinski,J. (2004). Self-representation in the thymus: an extended view. *Nat. Rev. Immunol*. *4*, 688-698.
129. Lahl,K., Loddenkemper,C., Drouin,C., Freyer,J., Arnason,J., Eberl,G., Hamann,A., Wagner,H., Huehn,J., and Sparwasser,T. (2007). Selective depletion of Foxp3+ regulatory T cells induces a scurfy-like disease. *J. Exp. Med*. *204*, 57-63.
130. Lakkis,F.G., Arakelov,A., Konieczny,B.T., and Inoue,Y. (2000). Immunologic 'ignorance' of vascularized organ transplants in the absence of secondary lymphoid tissue. *Nat. Med*. *6*, 686-688.
131. Larsen,C.P., Elwood,E.T., Alexander,D.Z., Ritchie,S.C., Hendrix,R., Tucker-Burden,C., Cho,H.R., Aruffo,A., Hollenbaugh,D., Linsley,P.S., Winn,K.J., and Pearson,T.C. (1996). Long-term acceptance of skin and cardiac allografts after blocking CD40 and CD28 pathways. *Nature* *381*, 434-438.
132. Lassila,O., Vainio,O., and Matzinger,P. (1988). Can B cells turn on virgin T cells? *Nature* *334*, 253-255.
133. Latchman,Y., Wood,C.R., Chernova,T., Chaudhary,D., Borde,M., Chernova,I., Iwai,Y., Long,A.J., Brown,J.A., Nunes,R., Greenfield,E.A., Bourque,K., Boussiotis,V.A., Carter,L.L., Carreno,B.M., Malenkovich,N., Nishimura,H., Okazaki,T., Honjo,T., Sharpe,A.H., and Freeman,G.J. (2001). PD-L2 is a second ligand for PD-1 and inhibits T cell activation. *Nat. Immunol*. *2*, 261-268.
134. Lees,J.R., Charbonneau,B., Swanson,A.K., Jensen,R., Zhang,J., Matusik,R., and Ratliff,T.L. (2006). Deletion is neither sufficient nor necessary for the induction of peripheral tolerance in mature CD8+ T cells. *Immunology* *117*, 248-261.
135. Libby,P. and Pober,J.S. (2001). Chronic rejection. *Immunity*. *14*, 387-397.
136. Libby,P., Salomon,R.N., Payne,D.D., Schoen,F.J., and Pober,J.S. (1989). Functions of vascular wall cells related to development of transplantation-associated coronary arteriosclerosis. *Transplant. Proc*. *21*, 3677-3684.

137. Limmer,A., Ohl,J., Kurts,C., Ljunggren,H.G., Reiss,Y., Groettrup,M., Momburg,F., Arnold,B., and Knolle,P.A. (2000). Efficient presentation of exogenous antigen by liver endothelial cells to CD8+ T cells results in antigen-specific T-cell tolerance. *Nat. Med.* 6, 1348-1354.
138. Limmer,A., Ohl,J., Wingender,G., Berg,M., Jungerkes,F., Schumak,B., Djandji,D., Scholz,K., Klevenz,A., Hegenbarth,S., Momburg,F., Hammerling,G.J., Arnold,B., and Knolle,P.A. (2005). Cross-presentation of oral antigens by liver sinusoidal endothelial cells leads to CD8 T cell tolerance. *Eur. J. Immunol.* 35, 2970-2981.
139. Lin,H., Bolling,S.F., Linsley,P.S., Wei,R.Q., Gordon,D., Thompson,C.B., and Turka,L.A. (1993). Long-term acceptance of major histocompatibility complex mismatched cardiac allografts induced by CTLA4Ig plus donor-specific transfusion. *J. Exp. Med.* 178, 1801-1806.
140. Lindahl,K.F. and Wilson,D.B. (1977). Histocompatibility antigen-activated cytotoxic T lymphocytes. II. Estimates of the frequency and specificity of precursors. *J. Exp. Med.* 145, 508-522.
141. Lowry, R. P. DTH-induced tissue injury. Tilney, N. L., Strom, T. B., and Paul, L. E. 487-502. 1996. In *Transplantation Biology: Cellular and Molecular Aspects*, Philadelphia: Lippincott Raven. Ref Type: Serial (Book,Monograph)
142. Ludewig,B., Ehl,S., Karrer,U., Odermatt,B., Hengartner,H., and Zinkernagel,R.M. (1998a). Dendritic cells efficiently induce protective antiviral immunity. *J. Virol.* 72, 3812-3818.
143. Ludewig,B., Junt,T., Hengartner,H., and Zinkernagel,R.M. (2001). Dendritic cells in autoimmune diseases. *Curr. Opin. Immunol.* 13, 657-662.
144. Ludewig,B., Krebs,P., Junt,T., Metters,H., Ford,N.J., Anderson,R.M., and Bocharov,G. (2004). Determining control parameters for dendritic cell-cytotoxic T lymphocyte interaction. *Eur. J. Immunol.* 34, 2407-2418.
145. Ludewig,B., Odermatt,B., Landmann,S., Hengartner,H., and Zinkernagel,R.M. (1998b). Dendritic cells induce autoimmune diabetes and maintain disease via de novo formation of local lymphoid tissue. *J. Exp. Med.* 188, 1493-1501.
146. Ma,W. and Pober,J.S. (1998). Human endothelial cells effectively costimulate cytokine production by, but not differentiation of, naive CD4+ T cells. *J. Immunol.* 161, 2158-2167.
147. Manning,W.C. and Mocarski,E.S. (1988). Insertional mutagenesis of the murine cytomegalovirus genome: one prominent alpha gene (ie2) is dispensable for growth. *Virology* 167, 477-484.
148. Marelli-Berg,F.M., Scott,D., Bartok,I., Peek,E., Dyson,J., and Lechler,R.I. (2000). Activated murine endothelial cells have reduced immunogenicity for CD8+ T cells: a mechanism of immunoregulation? *J. Immunol.* 165, 4182-4189.
149. Marelli-Berg,F.M., Scott,D., Bartok,I., Peek,E., Dyson,J., and Lechler,R.I. (2001). Antigen presentation by murine endothelial cells. *Transplant. Proc.* 33, 315-316.
150. Matzinger,P. (1994). Tolerance, danger, and the extended family. *Annu. Rev. Immunol.* 12:991-1045, 991-1045.
151. Matzinger,P. and Bevan,M.J. (1977). Hypothesis: why do so many lymphocytes respond to major histocompatibility antigens? *Cell Immunol.* 29, 1-5.
152. Mazanet,M.M. and Hughes,C.C. (2002). B7-H1 is expressed by human endothelial cells and suppresses T cell cytokine synthesis. *J. Immunol.* 169, 3581-3588.

153. Mazza,C., Auphan-Anezin,N., Gregoire,C., Guimezanes,A., Kellenberger,C., Roussel,A., Kearney,A., van der Merwe,P.A., Schmitt-Verhulst,A.M., and Malissen,B. (2007). How much can a T-cell antigen receptor adapt to structurally distinct antigenic peptides? *EMBO J.* 26, 1972-1983.
154. Medzhitov,R. and Janeway,C.A., Jr. (1998). Innate immune recognition and control of adaptive immune responses. *Semin. Immunol.* 10, 351-353.
155. Mendiratta,S.K., Kovalik,J.P., Hong,S., Singh,N., Martin,W.D., and Van Kaer,L. (1999). Peptide dependency of alloreactive CD4+ T cell responses. *Int. Immunol.* 11, 351-360.
156. Millan,M.T., Shizuru,J.A., Hoffmann,P., Dejbakhsh-Jones,S., Scandling,J.D., Grumet,F.C., Tan,J.C., Salvatierra,O., Hoppe,R.T., and Strober,S. (2002). Mixed chimerism and immunosuppressive drug withdrawal after HLA-mismatched kidney and hematopoietic progenitor transplantation. *Transplantation* 73, 1386-1391.
157. Mitchell,R.N. and Libby,P. (2007). Vascular remodeling in transplant vasculopathy. *Circ. Res.* 100, 967-978.
158. Monaco,A.P. (2002). Tolerance and chimerism: separate and unequal concepts. *Transplant. Proc.* 34, 1991-1997.
159. Morgan,D.J., Kreuwel,H.T., Fleck,S., Levitsky,H.I., Pardoll,D.M., and Sherman,L.A. (1998). Activation of low avidity CTL specific for a self epitope results in tumor rejection but not autoimmunity. *J. Immunol.* 160, 643-651.
160. Munder,M., Mallo,M., Eichmann,K., and Modolell,M. (1998). Murine macrophages secrete interferon gamma upon combined stimulation with interleukin (IL)-12 and IL-18: A novel pathway of autocrine macrophage activation. *J. Exp. Med.* 187, 2103-2108.
161. Mutis,T., Gillespie,G., Schrama,E., Falkenburg,J.H., Moss,P., and Goulmy,E. (1999). Tetrameric HLA class I-minor histocompatibility antigen peptide complexes demonstrate minor histocompatibility antigen-specific cytotoxic T lymphocytes in patients with graft-versus-host disease. *Nat. Med.* 5, 839-842.
162. Nagano,H., Libby,P., Taylor,M.K., Hasegawa,S., Stinn,J.L., Becker,G., Tilney,N.L., and Mitchell,R.N. (1998). Coronary arteriosclerosis after T-cell-mediated injury in transplanted mouse hearts: role of interferon-gamma. *Am. J. Pathol.* 152, 1187-1197.
163. Nagano,H., Mitchell,R.N., Taylor,M.K., Hasegawa,S., Tilney,N.L., and Libby,P. (1997). Interferon-gamma deficiency prevents coronary arteriosclerosis but not myocardial rejection in transplanted mouse hearts. *J. Clin. Invest* 100, 550-557.
164. Nankivell,B.J., Borrows,R.J., Fung,C.L., O'Connell,P.J., Allen,R.D., and Chapman,J.R. (2003). The natural history of chronic allograft nephropathy. *N. Engl. J. Med.* 349, 2326-2333.
165. Nelson,P.J. and Krensky,A.M. (2001). Chemokines, chemokine receptors, and allograft rejection. *Immunity.* 14, 377-386.
166. Nykanen,A.I., Tikkanen,J.M., Krebs,R., Keranen,M.A., Sihvola,R.K., Sandelin,H., Tuuminen,R., Raisky,O., Koskinen,P.K., and Lemstrom,K.B. (2006). Angiogenic growth factors in cardiac allograft rejection. *Transplantation* 82, S22-S24.
167. Ochsenbein,A.F., Klenerman,P., Karrer,U., Ludewig,B., Pericin,M., Hengartner,H., and Zinkernagel,R.M. (1999). Immune surveillance against a solid tumor fails because of immunological ignorance. *Proc. Natl. Acad. Sci. U. S. A.* 96, 2233-2238.

168. Ohashi,P.S., Oehen,S., Buerki,K., Pircher,H., Ohashi,C.T., Odermatt,B., Malissen,B., Zinkernagel,R.M., and Hengartner,H. (1991). Ablation of "tolerance" and induction of diabetes by virus infection in viral antigen transgenic mice. *Cell* 65, 305-317.
169. Oukka,M., Cohen-Tannoudji,M., Tanaka,Y., Babinet,C., and Kosmatopoulos,K. (1996). Medullary thymic epithelial cells induce tolerance to intracellular proteins. *J. Immunol.* 156, 968-975.
170. Overwijk,W.W., Surman,D.R., Tsung,K., and Restifo,N.P. (1997). Identification of a Kb-restricted CTL epitope of beta-galactosidase: potential use in development of immunization protocols for "self" antigens. *Methods.* 12, 117-123.
171. Ozkaynak,E., Wang,L., Goodearl,A., McDonald,K., Qin,S., O'Keefe,T., Duong,T., Smith,T., Gutierrez-Ramos,J.C., Rottman,J.B., Coyle,A.J., and Hancock,W.W. (2002). Programmed death-1 targeting can promote allograft survival. *J. Immunol.* 169, 6546-6553.
172. Pearson,T.C., Alexander,D.Z., Hendrix,R., Elwood,E.T., Linsley,P.S., Winn,K.J., and Larsen,C.P. (1996). CTLA4-Ig plus bone marrow induces long-term allograft survival and donor specific unresponsiveness in the murine model. Evidence for hematopoietic chimerism. *Transplantation* 61, 997-1004.
173. Pearson,T.C., Alexander,D.Z., Winn,K.J., Linsley,P.S., Lowry,R.P., and Larsen,C.P. (1994). Transplantation tolerance induced by CTLA4-Ig. *Transplantation* 57, 1701-1706.
174. Perez,V.L., Henault,L., and Lichtman,A.H. (1998). Endothelial antigen presentation: stimulation of previously activated but not naive TCR-transgenic mouse T cells. *Cell Immunol.* 189, 31-40.
175. Piccirillo,C.A. and Shevach,E.M. (2004). Naturally-occurring CD4+CD25+ immunoregulatory T cells: central players in the arena of peripheral tolerance. *Semin. Immunol.* 16, 81-88.
176. Pober,J.S., Ma,W., Biedermann,B., and Libby,P. (1997). Vascular cells have limited capacities to activate and differentiate T cells: implications for transplant vascular sclerosis. *Transpl. Immunol.* 5, 251-254.
177. Pober,J.S., Orosz,C.G., Rose,M.L., and Savage,C.O. (1996). Can graft endothelial cells initiate a host anti-graft immune response? *Transplantation* 61, 343-349.
178. Potena,L., Grigioni,F., Ortolani,P., Magnani,G., Marrozzini,C., Falchetti,E., Barbieri,A., Bacchi-Reggiani,L., Lazzarotto,T., Marzocchi,A., Magelli,C., Landini,M.P., and Branzi,A. (2003). Relevance of cytomegalovirus infection and coronary-artery remodeling in the first year after heart transplantation: a prospective three-dimensional intravascular ultrasound study. *Transplantation* 75, 839-843.
179. Potena,L., Holweg,C.T., Chin,C., Luikart,H., Weisshaar,D., Narasimhan,B., Fearon,W.F., Lewis,D.B., Cooke,J.P., Mocarski,E.S., and Valantine,H.A. (2006). Acute rejection and cardiac allograft vascular disease is reduced by suppression of subclinical cytomegalovirus infection. *Transplantation* 82, 398-405.
180. Potena,L. and Valantine,H.A. (2007). Cytomegalovirus-associated allograft rejection in heart transplant patients. *Curr. Opin. Infect. Dis.* 20, 425-431.
181. Probst,H.C., Lagnel,J., Kollias,G., and van den,B.M. (2003). Inducible transgenic mice reveal resting dendritic cells as potent inducers of CD8+ T cell tolerance. *Immunity.* 18, 713-720.
182. Probst,H.C., McCoy,K., Okazaki,T., Honjo,T., and van den,B.M. (2005). Resting dendritic cells induce peripheral CD8+ T cell tolerance through PD-1 and CTLA-4. *Nat. Immunol.* 6, 280-286.

183. Pulendran,B., Palucka,K., and Banchereau,J. (2001). Sensing pathogens and tuning immune responses. *Science* 293, 253-256.
184. Rao,R.M., Yang,L., Garcia-Cardena,G., and Lusinskas,F.W. (2007). Endothelial-dependent mechanisms of leukocyte recruitment to the vascular wall. *Circ. Res.* 101, 234-247.
185. Reiser,J.B., Darnault,C., Gregoire,C., Mosser,T., Mazza,G., Kearney,A., van der Merwe,P.A., Fontecilla-Camps,J.C., Housset,D., and Malissen,B. (2003). CDR3 loop flexibility contributes to the degeneracy of TCR recognition. *Nat. Immunol.* 4, 241-247.
186. Ridge,J.P., Di Rosa,F., and Matzinger,P. (1998). A conditioned dendritic cell can be a temporal bridge between a CD4+ T- helper and a T-killer cell. *Nature* 393, 474-478.
187. Rifle,G., Mousson,C., and Herve,P. (2006). Endothelial cells in organ transplantation: Friends or foes? *Transplantation* 82, S4-S5.
188. Roake,J.A., Rao,A.S., Morris,P.J., Larsen,C.P., Hankins,D.F., and Austyn,J.M. (1995). Dendritic cell loss from nonlymphoid tissues after systemic administration of lipopolysaccharide, tumor necrosis factor, and interleukin 1. *J. Exp. Med.* 181, 2237-2247.
189. Rodig,N., Ryan,T., Allen,J.A., Pang,H., Grabie,N., Chernova,T., Greenfield,E.A., Liang,S.C., Sharpe,A.H., Lichtman,A.H., and Freeman,G.J. (2003). Endothelial expression of PD-L1 and PD-L2 down-regulates CD8+ T cell activation and cytotoxicity. *Eur. J. Immunol.* 33, 3117-3126.
190. Rose,M.L. (1998). Endothelial cells as antigen-presenting cells: role in human transplant rejection. *Cell Mol. Life Sci.* 54, 965-978.
191. Rosenberg,A.S. and Singer,A. (1992). Cellular basis of skin allograft rejection: an in vivo model of immune-mediated tissue destruction. *Annu. Rev. Immunol.* 10, 333-358.
192. Rothermel,A.L., Wang,Y., Schechner,J., Mook-Kanamori,B., Aird,W.C., Pober,J.S., Tellides,G., and Johnson,D.R. (2004). Endothelial cells present antigens in vivo. *BMC. Immunol.* 5:5., 5.
193. Rothstein,D.M. and Sayegh,M.H. (2003). T-cell costimulatory pathways in allograft rejection and tolerance. *Immunol. Rev.* 196, 85-108.
194. Rouse,B.T., Sarangi,P.P., and Suvas,S. (2006). Regulatory T cells in virus infections. *Immunol. Rev.* 212, 272-286.
195. Russell,P.S., Chase,C.M., and Colvin,R.B. (1995). Coronary atherosclerosis in transplanted mouse hearts. IV Effects of treatment with monoclonal antibodies to intercellular adhesion molecule-1 and leukocyte function-associated antigen-1. *Transplantation* 60, 724-729.
196. Russell,P.S., Chase,C.M., and Colvin,R.B. (1997). Alloantibody- and T cell-mediated immunity in the pathogenesis of transplant arteriosclerosis: lack of progression to sclerotic lesions in B cell-deficient mice. *Transplantation* 64, 1531-1536.
197. Russell,P.S., Chase,C.M., Winn,H.J., and Colvin,R.B. (1994a). Coronary atherosclerosis in transplanted mouse hearts. I. Time course and immunogenetic and immunopathological considerations. *Am. J. Pathol.* 144, 260-274.
198. Russell,P.S., Chase,C.M., Winn,H.J., and Colvin,R.B. (1994b). Coronary atherosclerosis in transplanted mouse hearts. I. Time course and immunogenetic and immunopathological considerations. *Am. J. Pathol.* 144, 260-274.
199. Russell,P.S., Chase,C.M., Winn,H.J., and Colvin,R.B. (1994c). Coronary atherosclerosis in transplanted mouse hearts. II. Importance of humoral immunity. *J. Immunol.* 152, 5135-5141.

200. Russell,P.S., Chase,C.M., Winn,H.J., and Colvin,R.B. (1994d). Coronary atherosclerosis in transplanted mouse hearts. III. Effects of recipient treatment with a monoclonal antibody to interferon-gamma. *Transplantation* 57, 1367-1371.
201. Sakaguchi,S. (2004). Naturally arising CD4+ regulatory t cells for immunologic self-tolerance and negative control of immune responses. *Annu. Rev. Immunol.* 22, 531-562.
202. Sakaguchi,S., Ono,M., Setoguchi,R., Yagi,H., Hori,S., Fehervari,Z., Shimizu,J., Takahashi,T., and Nomura,T. (2006a). Foxp3+ CD25+ CD4+ natural regulatory T cells in dominant self-tolerance and autoimmune disease. *Immunol. Rev.* 212, 8-27.
203. Sakaguchi,S., Ono,M., Setoguchi,R., Yagi,H., Hori,S., Fehervari,Z., Shimizu,J., Takahashi,T., and Nomura,T. (2006b). Foxp3+ CD25+ CD4+ natural regulatory T cells in dominant self-tolerance and autoimmune disease. *Immunol. Rev.* 212, 8-27.
204. Sallusto,F., Palermo,B., Lenig,D., Miettinen,M., Matikainen,S., Julkunen,I., Forster,R., Burgstahler,R., Lipp,M., and Lanzavecchia,A. (1999). Distinct patterns and kinetics of chemokine production regulate dendritic cell function. *Eur. J. Immunol.* 29, 1617-1625.
205. Salmi,M., Kalimo,K., and Jalkanen,S. (1993). Induction and function of vascular adhesion protein-1 at sites of inflammation. *J. Exp. Med.* 178, 2255-2260.
206. Salomon,B. and Bluestone,J.A. (2001). Complexities of CD28/B7: CTLA-4 costimulatory pathways in autoimmunity and transplantation. *Annu. Rev. Immunol.* 19, 225-252.
207. Salomon,R.N., Hughes,C.C., Schoen,F.J., Payne,D.D., Pober,J.S., and Libby,P. (1991). Human coronary transplantation-associated arteriosclerosis. Evidence for a chronic immune reaction to activated graft endothelial cells. *Am. J. Pathol.* 138, 791-798.
208. Samy,E.T., Parker,L.A., Sharp,C.P., and Tung,K.S. (2005). Continuous control of autoimmune disease by antigen-dependent polyclonal CD4+CD25+ regulatory T cells in the regional lymph node. *J. Exp. Med.* 202, 771-781.
209. Schenk,S., Kish,D.D., He,C., El Sawy,T., Chiffolleau,E., Chen,C., Wu,Z., Sandner,S., Gorbachev,A.V., Fukamachi,K., Heeger,P.S., Sayegh,M.H., Turka,L.A., and Fairchild,R.L. (2005). Alloreactive T cell responses and acute rejection of single class II MHC-disparate heart allografts are under strict regulation by CD4+ CD25+ T cells. *J. Immunol.* 174, 3741-3748.
210. Schlaeger,T.M., Bartunkova,S., Lawitts,J.A., Teichmann,G., Risau,W., Deutsch,U., and Sato,T.N. (1997). Uniform vascular-endothelial-cell-specific gene expression in both embryonic and adult transgenic mice. *Proc. Natl. Acad. Sci. U. S. A.* 94, 3058-3063.
211. Schon,M.P., Krahn,T., Schon,M., Rodriguez,M.L., Antonicek,H., Schultz,J.E., Ludwig,R.J., Zollner,T.M., Bischoff,E., Bremm,K.D., Schramm,M., Henninger,K., Kaufmann,R., Gollnick,H.P., Parker,C.M., and Boehncke,W.H. (2002). Efomycine M, a new specific inhibitor of selectin, impairs leukocyte adhesion and alleviates cutaneous inflammation. *Nat. Med.* 8, 366-372.
212. Schwartz,R.H. (1990). A cell culture model for T lymphocyte clonal anergy. *Science* 248, 1349-1356.
213. Sedmak,D.D., Knight,D.A., Vook,N.C., and Waldman,J.W. (1994). Divergent patterns of ELAM-1, ICAM-1, and VCAM-1 expression on cytomegalovirus-infected endothelial cells. *Transplantation.* 58, 1379-1385.
214. Sharpe,A.H. and Freeman,G.J. (2002). The B7-CD28 superfamily. *Nat. Rev. Immunol.* 2, 116-126.

215. Sherman,L.A. and Randolph,C.P. (1981). Monoclonal anti-H-2Kb antibodies detect serological differences between H-2Kb mutants. *Immunogenetics*. *12*, 183-186.
216. Shimizu,K., Sugiyama,S., Aikawa,M., Fukumoto,Y., Rabkin,E., Libby,P., and Mitchell,R.N. (2001). Host bone-marrow cells are a source of donor intimal smooth- muscle-like cells in murine aortic transplant arteriopathy. *Nat. Med.* *7*, 738-741.
217. Shirwin, H. Chronic allograft rejection. *Transplant* *68*, 715-726. 1995.
Ref Type: Serial (Book,Monograph)
218. Sho,M., Sandner,S.E., Najafian,N., Salama,A.D., Dong,V., Yamada,A., Kishimoto,K., Harada,H., Schmitt,I., and Sayegh,M.H. (2002). New insights into the interactions between T-cell costimulatory blockade and conventional immunosuppressive drugs. *Ann. Surg.* *236*, 667-675.
219. Soule,J.L., Streblow,D.N., Andoh,T.F., Kreklywich,C.N., and Orloff,S.L. (2006). Cytomegalovirus accelerates chronic allograft nephropathy in a rat renal transplant model with associated provocative chemokine profiles. *Transplant. Proc.* *38*, 3214-3220.
220. Speiser,D.E., Lees,R.K., Hengartner,H., Zinkernagel,R.M., and MacDonald,H.R. (1989). Positive and negative selection of T cell receptor V beta domains controlled by distinct cell populations in the thymus. *J. Exp. Med.* *170*, 2165-2170.
221. Speiser,D.E., Miranda,R., Zakarian,A., Bachmann,M.F., McKall-Faienza,K., Odermatt,B., Hanahan,D., Zinkernagel,R.M., and Ohashi,P.S. (1997). Self antigens expressed by solid tumors do not efficiently stimulate naive or activated T cells: implications for immunotherapy. *J. Exp. Med.* *186*, 645-653.
222. Steinman,R.M. (1991). The dendritic cell system and its role in immunogenicity. *Annu. Rev. Immunol.* *9*, 271-296.
223. Steinman,R.M. (2003). The control of immunity and tolerance by dendritic cell. *Pathol. Biol. (Paris)* *51*, 59-60.
224. Steinman,R.M. and Cohn,Z.A. (1973). Identification of a novel cell type in peripheral lymphoid organs of mice. I. Morphology, quantitation, tissue distribution. *J. Exp. Med.* *137*, 1142-1162.
225. Steinman,R.M., Hawiger,D., Liu,K., Bonifaz,L., Bonnyay,D., Mahnke,K., Iyoda,T., Ravetch,J., Dhodapkar,M., Inaba,K., and Nussenzweig,M. (2003a). Dendritic cell function in vivo during the steady state: a role in peripheral tolerance. *Ann. N. Y. Acad. Sci.* *987*, 15-25.
226. Steinman,R.M., Hawiger,D., and Nussenzweig,M.C. (2003b). Tolerogenic dendritic cells. *Annu. Rev. Immunol.* *21*, 685-711.
227. Suchin,E.J., Langmuir,P.B., Palmer,E., Sayegh,M.H., Wells,A.D., and Turka,L.A. (2001). Quantifying the frequency of alloreactive T cells in vivo: new answers to an old question. *J. Immunol.* *166*, 973-981.
228. Sun,H., Subbotin,V., Woodward,J., Valdivia,L., Fung,J.J., and Rao,A.S. (2001). Role of MHC class I and CD8(+) T cells in the pathogenesis of chronic rejection. *Transplant. Proc.* *33*, 319.
229. Suzuki,J., Cole,S.E., Batirel,S., Kosuge,H., Shimizu,K., Isobe,M., Libby,P., and Mitchell,R.N. (2003). Tumor necrosis factor receptor -1 and -2 double deficiency reduces graft arterial disease in murine cardiac allografts. *Am. J. Transplant.* *3*, 968-976.
230. Tallquist,M.D., Yun,T.J., and Pease,L.R. (1996). A single T cell receptor recognizes structurally distinct MHC/peptide complexes with high specificity. *J. Exp. Med.* *184*, 1017-1026.
231. Tanaka,K., Albin,M.J., Yuan,X., Yamaura,K., Habicht,A., Murayama,T., Grimm,M., Waaga,A.M., Ueno,T., Padera,R.F., Yagita,H., Azuma,M., Shin,T., Blazar,B.R., Rothstein,D.M., Sayegh,M.H., and

- Najafian,N. (2007). PDL1 is required for peripheral transplantation tolerance and protection from chronic allograft rejection. *J. Immunol.* *179*, 5204-5210.
232. Taylor,D.O., Edwards,L.B., Boucek,M.M., Trulock,E.P., Deng,M.C., Keck,B.M., and Hertz,M.I. (2005). Registry of the International Society for Heart and Lung Transplantation: twenty-second official adult heart transplant report--2005. *J. Heart Lung Transplant.* *24*, 945-955.
233. Tellides,G. and Pober,J.S. (2007). Interferon-gamma axis in graft arteriosclerosis. *Circ. Res.* *100*, 622-632.
234. Thebault,P., Condamine,T., Heslan,M., Hill,M., Bernard,I., Saoudi,A., Josien,R., Anegon,I., Cuturi,M.C., and Chiffolleau,E. (2007). Role of IFN γ in allograft tolerance mediated by CD4+CD25+ regulatory T cells by induction of IDO in endothelial cells. *Am. J. Transplant.* *7*, 2472-2482.
235. Tian,Y., Rudiger,H.A., Jochum,W., and Clavien,P.A. (2002). Comparison of arterialized and nonarterialized orthotopic liver transplantation in mice: prowess or relevant model? *Transplantation* *74*, 1242-1246.
236. Tu,W., Potena,L., Stepick-Biek,P., Liu,L., Dionis,K.Y., Luikart,H., Fearon,W.F., Holmes,T.H., Chin,C., Cooke,J.P., Valantine,H.A., Mocarski,E.S., and Lewis,D.B. (2006). T-cell immunity to subclinical cytomegalovirus infection reduces cardiac allograft disease. *Circulation* *114*, 1608-1615.
237. Valantine,H.A. (2003). Cardiac allograft vasculopathy: central role of endothelial injury leading to transplant "atheroma". *Transplantation.* *76*, 891-899.
238. Valantine,H.A. (2004). The role of viruses in cardiac allograft vasculopathy. *Am. J. Transplant.* *4*, 169-177.
239. Valitutti,S., Muller,S., Dessing,M., and Lanzavecchia,A. (1996). Different responses are elicited in cytotoxic T lymphocytes by different levels of T cell receptor occupancy. *J. Exp. Med.* *183*, 1917-1921.
240. Valujskikh,A., Lantz,O., Celli,S., Matzinger,P., and Heeger,P.S. (2002a). Cross-primed CD8(+) T cells mediate graft rejection via a distinct effector pathway. *Nat. Immunol.* *3*, 844-851.
241. Valujskikh,A., Pantenburg,B., and Heeger,P.S. (2002b). Primed allospecific T cells prevent the effects of costimulatory blockade on prolonged cardiac allograft survival in mice. *Am. J. Transplant.* *2*, 501-509.
242. Valujskikh,A., Zhang,Q., and Heeger,P.S. (2006). CD8 T cells specific for a donor-derived, self-restricted transplant antigen are nonpathogenic bystanders after vascularized heart transplantation in mice. *J. Immunol.* *176*, 2190-2196.
243. van Dorp,W.T., Jonges,E., Bruggeman,C.A., Daha,M.R., van Es,L.A., and Der Woude,F.J. (1989). Direct induction of MHC class I, but not class II, expression on endothelial cells by cytomegalovirus infection. *Transplantation.* *48*, 469-472.
244. van Essen,D., Kikutani,H., and Gray,D. (1995). CD40 ligand-transduced co-stimulation of T cells in the development of helper function. *Nature* *378*, 620-623.
245. Vassalli,G., Gallino,A., Weis,M., von Scheidt,W., Kappenberger,L., von Segesser,L.K., and Goy,J.J. (2003). Alloimmunity and nonimmunologic risk factors in cardiac allograft vasculopathy. *Eur. Heart J.* *24*, 1180-1188.
246. Villasenor,J., Benoist,C., and Mathis,D. (2005). AIRE and APECED: molecular insights into an autoimmune disease. *Immunol. Rev.* *204*, 156-164.

247. Vliegen,I., Duijvestijn,A., Grauls,G., Hergreen,S., Bruggeman,C., and Stassen,F. (2004). Cytomegalovirus infection aggravates atherogenesis in apoE knockout mice by both local and systemic immune activation. *Microbes. Infect.* 6, 17-24.
248. Vliegen,I., Hergreen,S.B., Grauls,G.E., Bruggeman,C.A., and Stassen,F.R. (2005). Mouse cytomegalovirus antigenic immune stimulation is sufficient to aggravate atherosclerosis in hypercholesterolemic mice. *Atherosclerosis.* 181, 39-44.
249. von Herrath,M.G. and Oldstone,M.B. (1996). Virus-induced autoimmune disease. *Curr Opin Immunol* 8, 878-885.
250. Weber,D.A., Terrell,N.K., Zhang,Y., Strindberg,G., Martin,J., Rudensky,A., and Braunstein,N.S. (1995). Requirement for peptide in alloreactive CD4+ T cell recognition of class II MHC molecules. *J. Immunol.* 154, 5153-5164.
251. Weiler-Guettler,H., Aird,W.C., Husain,M., Rayburn,H., and Rosenberg,R.D. (1996). Targeting of transgene expression to the vascular endothelium of mice by homologous recombination at the thrombomodulin locus. *Circ. Res.* 78, 180-187.
252. Weis,M. and von Scheidt,W. (1997). Cardiac allograft vasculopathy: a review. *Circulation.* 96, 2069-2077.
253. Whitelegg,A.M., Oosten,L.E., Jordan,S., Kester,M., van Halteren,A.G., Madrigal,J.A., Goulmy,E., and Barber,L.D. (2005). Investigation of peptide involvement in T cell allorecognition using recombinant HLA class I multimers. *J. Immunol.* 175, 1706-1714.
254. Whitmire,J.K., Flavell,R.A., Grewal,I.S., Larsen,C.P., Pearson,T.C., and Ahmed,R. (1999). CD40-CD40 ligand costimulation is required for generating antiviral CD4 T cell responses but is dispensable for CD8 T cell responses. *J. Immunol.* 163, 3194-3201.
255. Wick,G., Romen,M., Amberger,A., Metzler,B., Mayr,M., Falkensammer,G., and Xu,Q. (1997). Atherosclerosis, autoimmunity, and vascular-associated lymphoid tissue. *FASEB J.* 11, 1199-1207.
256. Wood,K. and Sachs,D.H. (1996). Chimerism and transplantation tolerance: cause and effect. *Immunol. Today* 17, 584-587.
257. Wu,T.C., Hruban,R.H., Ambinder,R.F., Pizzorno,M., Cameron,D.E., Baumgartner,W.A., Reitz,B.A., Hayward,G.S., and Hutchins,G.M. (1992). Demonstration of cytomegalovirus nucleic acids in the coronary arteries of transplanted hearts. *Am. J. Pathol.* 140, 739-747.
258. Yang,Y. and Wilson,J.M. (1996). CD40 ligand-dependent T cell activation: requirement of B7-CD28 signaling through CD40. *Science* 273, 1862-1864.
259. Zehn,D. and Bevan,M.J. (2006). T cells with low avidity for a tissue-restricted antigen routinely evade central and peripheral tolerance and cause autoimmunity. *Immunity.* 25, 261-270.
260. Zinkernagel,R.M., Ehl,S., Aichele,P., Oehen,S., Kundig,T., and Hengartner,H. (1997). Antigen localisation regulates immune responses in a dose- and time- dependent fashion: a geographical view of immune reactivity. *Immunol. Rev.* 156:199-209, 199-209.

7.2 Figure legend

Figure 1: Immune activation and tolerance induced by DC.....	16
Figure 2: T-cell recognition of conventional and allogeneic complexes.....	19
Figure 3: Arterial remodelling in chronic vascular rejection (Libby and Pober, 2001).....	22
Figure 4: Do vascular EC that present a minor histocompatibility antigen activate or tolerize naive CD8 ⁺ T-cells?	36
Figure 5: CD8 ⁺ T cell reactivity in Tie2-LacZ mice.	47
Figure 6: CD8 ⁺ T cell tolerance in Tie2-LacZ mice.....	48
Figure 7: Loss of adoptively transferred Bg1 CD8 ⁺ T cells in Tie2-LacZ mice is not dependent on β-gal expression by EC.	50
Figure 8: Activation of Bg1 CD8 ⁺ T cells in Tie2-LacZ mice.	52
Figure 9: In vivo proliferation of Bg1 CD8 ⁺ T cells in bone marrow chimeric mice.....	53
Figure 10: Lack of CD8 ⁺ cell activation in naive recipients of Tie2-LacZ vascularized organ grafts.	54
Figure 11: In situ analysis of heterotopically transplanted (A) C57BL/6 (B6→B6) and (B) Tie2-LacZ (T2→B6) hearts on day 100 post transplantation.	69
Figure 12: CTL reactivity in recipients of C57BL/6 (B6→B6) or Tie2-LacZ (T2→B6) heart transplants following DC immunization.	70
Figure 13: In situ analysis of transplanted Tie2-LacZ hearts following repetitive DC immunization on days 7 (A) and 15 (B) post immunization.....	72
Figure 14: Quantification of vascular rejection following DC immunization.	73
Figure 15: Immune reactivity and vascular pathology following MCMV-LacZ infection.	75
Figure 16: Minor histocompatibility antigen presented by vascular EC is ignored by naive CD8 ⁺ T cells..	82
Figure 17: Activated CD8 ⁺ T cells recognize minor histocompatibility antigen expressed on EC, thus EC become target cells of antigen-specific CTL.....	85
Figure 18: Two models of help delivery to CTL.....	87
Figure S 1: βgal and CD8 staining of salivary gland, spleen and liver sections of naïve Tie2-LacZ mice.	57
Figure S 2: LacZ mRNA copy numbers in liver, spleen, heart, kidney, thymus and BM of naïve Tie2-LacZ mice.....	57
Figure S 3: Expression of LacZ transcripts in sorted bone marrow cells.	58
Figure S 4: Functional avidity of TCR transgenic Bg1 CD8 ⁺ T-cells.	58
Figure S 5: Phenotype of MCMV-LacZ-activated Bg1 TCR transgenic T cells in B6→B6 and B6→T2 chimeras.	59
Figure S 6: Fate of Bg1 TCR transgenic T cells in T2→T2 chimeric mice.	60

7.3 Abbreviations

β -gal	β -galactosidase
Ab	Antibody
Ag	Antigen
AIRE	Human autoimmune regulator protein
APC	Allophycocyanin
APC	Antigen-presenting cell
B6	C57BL/6
BM	Bone marrow
BSS	Balanced salt solution
CCR	Chemokine receptor, C-C subgroup
CD	Cluster of differentiation
CD40L	CD40 ligand
CD62L	CD62 L-Selectin
cDNA	Cloned deoxyribonucleic acid
CFSE	Carboxyfluorescein diacetatsuccinimidyl ester
CMV	Cytomegalovirus
CTL	Cytotoxic T-lymphocyte
CTLA4	Cytotoxic T-lymphocyte antigen 4
CTLA4-Ig	Cytotoxic T-lymphocyte antigen 4-ligand
CTV	Chronic transplant vasculopathy
DC	Dendritic cell
DEPC	Diethylene pyrocarbonate
DEREG	Depletion of regulatory T-cells
DMEM	Dulbecco's modified Eagle's medium
DNA	Deoxyribonucleic acid
DT	Diphtheria toxin
DTR	Diphtheria toxin receptor
EC	Endothelial cell
EL-4	Mouse thymome cell line
EVG	Elastic van Giesson
FACS	Fluorescence activated cell scanning/sorting
FBS	Fetal bovine serum

FCS	Fetal calf serum
FITC	Fluorescein isothiocyanat
Foxp3	Forkhead box protein 3
GFP	Green fluorescent protein
GM-CSF	Granulocyte/macrophage colony-stimulating factor
GP	Glycoprotein
GVHD	Graft versus host disease
HBSS	Hanks balanced salt solution
HCS	Hematopoietic stem cell
HCMV	Human cytomegalovirus
HLA	Human leukocyte antigen
i.p.	Intraperitoneal
i.v.	Intravenous
ICAM	Intercellular adhesion molecule
IFN	Interferon
Ig	Immunglobulin
IL	Interleukin
IMDM	Iscove's modified Dulbecco's medium
IP-10	Interferon-inducible protein 10
kDa	Kilodalton
LacZ	Gene encoding for the β -galactosidase protein
LCMV	Lymphocyte choriomeningitis virus
LFA	Lymphocyte-function-associated antigen 1
LN	Lymph node
LPS	Lipopolysaccharide
LSEC	Liver sinusoidal endothelial cell
LT	Lymphotoxin
mAb	Monoclonal antibody
MAC-1	Membrane-attack complex
MACS	Magnetic assisted cell sorting
MCMV	Murine cytomegalovirus
MCP-1	Monocyte chemoattractant protein 1
MEM	Minimal essential medium
mhAg	Minor histocompatibility antigen

MHC	Major histocompatibility complex
MIG	Monokine induced by interferon gamma
MIP-1 α	Macrophage inflammatory protein
MLN	Mesenteric lymph node
mTECs	Medullary thymic epithelial cells
NP	Nucleoprotein
PBL	Peripheral blood lymphocytes
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PD-1	Programmed death-1
PD-L	Programmed death-ligand
PDGF	Platelet-derived growth factor
PE	Phycoerythrin
PerCp	Peridinin Chlorophyll protein
pfu	Plaque forming unit
PMA	Phorbol myristate acetate
RANTES	Regulated on activation, normal T expressed and secreted
RCMV	Rat cytomegalovirus
RIP	Rat insulin promoter
RNA	Ribonucleic acid
RT-PCR	Reverse transcriptase polymerase chain reaction
SEM	Standard error of the mean
s.c.	Subcutaneous
SM	Smooth muscle
SMC	Smooth muscle cell
T2	Tie2-LacZ
TBP	TATA binding protein
TCR	T-cell receptor
TGF- β	Transforming growth factor beta
Th	T helper cell
TLR	Toll-like receptor
TNF	Tumor necrosis factor
TNFR	Tumor necrosis factor receptor
Treg	T regulatory cell

TSA	Tissue-specific antigens
TV	Transplant vasculopathy
Wt	Wild type
VEGF	Vascular endothelial growth factor
VLA-4	Very late antigen 4

7.4 Acknowledgments

In this section I would like to thank all the people who contributed in their different ways to my PhD thesis.

It is to Burkhard Ludewig and the whole LFA-crew that I owe great thanks for supporting me all the way from the very beginning of my PhD to the last page of this thesis. The positive ambience in the lab constantly motivated me and kept me going on. A special thank goes to **Burkhard**, with his confident and restless nature he motivated me to continue again and again. I thank him for helping me improving my skills for scientific discussions and arguments, and certainly for giving me a first introduction into mouse-handling. I acknowledge further his persistence concerning the teaching of the young PhD students, it was not always easy, but worth it in the end!

I would like to thank **Philippe** for introducing me to my projects and for all the help in planning and performing experiments, and certainly for the hard training at the Neudorf, improving our physical condition.

Elke danke ich für Ihre unendliche Geduld bei der Erklärung des FACs-Gerätes, der Realtime-PCR und der Einführung in die Arbeit mit Mäusen. Weiter möchte ich ihr für die Hilfe und die vielen Ratschläge während meiner ganzen Doktorarbeit danken.

Simone danke ich für Ihre praktische und moralische Unterstützung während grossen Experimenten. Weiter danke ich ihr für alle Typing-PCRs, ICS, Histoschnitte und Tipps beim Zurechtfinden im Labor, und für eine tolle Freundschaft auch ausserhalb des Labors!

Rita danke ich für ihre Frohnatur und die Unterstützung und Hilfe im Labor und in anderen schwierigen Situationen.

Dani danke ich für die gute Zusammenarbeit im Transplantationsprojekt.

Muchas gracias a **Luisa** por su ayuda en el laboratorio y para escucharme en las situaciones desesperadas y simplemente por ser una buena amiga.

Computer-**Roli** danke ich für seine prompte Hilfe bezüglich Computeraussetzer und natürlich für seine anregenden und immer richtigen Behauptungen in jeglichen Diskussionen, aber auch für das gemeinsame Durchhalten schwieriger Zeiten als Doktoranden.

Ein spezielles Dankeschön geht auch an **Vroni**, mit der es sogar Freude macht, das Pult zu teilen.

Furthermore, I would like to thank **Reinhard, Divine, Clara, Sonja, Lucas, Volker** and all the people who have ever worked for some time at the LFA, for helping with problems in the lab and for creating a friendly working environment.

Thanks a lot to **Harinda** for all his work and help in the mouse-house.

I would like to thank the thesis committee Prof. Dr. Ed Palmer and Prof. Dr. A. Rolink for taking the time to read and evaluate my thesis.

Natürlich danke ich auch meinen Freundinnen, die immer ein offenes Ohr für die Sorgen einer Doktorandin hatten. Ich danke ihnen aber auch für die vielen tollen Stunden, die wir zusammen verbracht haben und hoffentlich noch verbringen werden.

Weiter danke ich auch meinen Leichtathletik-Mädels die sich von mir nach langen Labortagen geduldig „zutexten“ liessen und immer wieder dafür sorgten, dass ich für mindestens zwei Stunden am Tag völlig abschalten konnte.

Ein ganz spezielles Dankeschön geht an meinen Freund Thomas, er hat mir in den schwierigsten Zeiten am meisten Unterstützung und Erholung geboten. Weiter hat er sich die Riesenmühe gemacht, meine Arbeit zu lesen. Vielen lieben Dank!

Und zuletzt möchte ich auch meinen Eltern und Geschwistern ganz herzlich für ihre Unterstützung danken. Nur mit ihrer Hilfe war es möglich, dass ich all meine Ziele erreichen konnte.

7.5 Bibliography

Krebs, P., E. Scandella, **B. Bolinger**, S. Miller, and B. Ludewig. 2007. Chronic immune reactivity against persisting microbial antigen in the vasculature exacerbates atherosclerotic lesion formation. *Arteriosclerosis, Thrombosis & Vascular Biol.* 27:2206-13.

Bolinger, B., P. Krebs, Y. Tian, D. Engeler, E. Scandella, S. Miller, D.C. Palmer, N.P. Restifo, P.-A. Clavien and B. Ludewig. 2008. Immunologic ignorance of vascular endothelial cells expressing minor histocompatibility antigen. *Blood* 2008 May 1; 111 (9): 4588-95.

Scandella, E., **B. Bolinger**, E. Lattmann, S. Miller, S. Favre, D.R. Littman, D. Finke, S. Luther, T.M. Junt, and B. Ludewig. 2008. Restoration of lymphoid organ integrity through interaction of lymphoid tissue inducer cells with stromal cells. *Nature Immunology* 2008 Jun; 9 (6): 667-75.

Lang, P.A., C. Contaldo, P. Georgiev, A.M. El-Badry, M. Recher, M. Kurrer, L. Cervantes-Barragan, **B. Bolinger**, B. Ludewig, D. Merkler, B. Odermatt, M. Bader, R. Graf, P.-A. Clavien, H. Hengartner, R.M. Zinkernagel, and K.S. Lang. Protraction of viral hepatitis by platelet-derived serotonin. *Nature Medicine* 2008 Jul; 14 (7): 756-761.

Kang, T., G. Oh, E. Scandella, **B. Bolinger**, B. Ludewig, A. Kovalenko, and D. Wallach. Cell death induction yet not non-apoptotic functions of caspase-8 is compromised in BAC transgenic mice with mutation of its initial self-processing site. *Journal of Immunology* 2008 Aug 15; 181 (4): 2522-32.

Daniel Engeler*, **Beatrice Bolinger***, Philippe Krebs, Simone Miller, Matthias Hoffmann, Pierre-Alain Clavien, Yinghua Tian, and Burkhard Ludewig. Dendritic cell-activated, endothelial cell-specific CTL recognizing a minor histocompatibility antigen rapidly induce transplant vasculopathy (submitted).

* First shared

Zinkernagel, M., P.Krebs, S.Miller, **B.Bolinger**, and B.Ludewig. Immunopathologic basis of lymphocytic choriomeningitis virus-induced chorioretinitis. *Journal of Virology* 2008 Oct 22 [E pub ahead of print].

7.6 Curriculum Vitae

Name Beatrice Barbara Bolinger
Date of Birth April 24, 1977
Nationality Swiss

Education

2004-2008 PhD thesis in the group of PD Dr. B. Ludewig, Labor-
forschungsabteilung, Kantonsspital St.Gallen
“Interaction of vascular endothelial cells with CD8⁺ T-cells in vivo”
2002 Diploma thesis in the group of Prof. Dr. W. Schaffner at the Institute of
Pathology, University of Basel
“Induction of apoptosis by phytoestrogens and plant extracts on
tumour cells: An in-vitro study on human cell cultures”
1997-2002 Studies in Pharmacy, University of Basel
1993-1996 Gymnasium Muttenz, Type C (mathematics)

Work experience

2002-2004 Pharmacist at Adler Apotheke, Liestal
2000-2002 Part time substitution as assistant pharmacist at Kirschgartenapotheke,
Basel
2000 Practical training at the pharmacy of Hôpital Riviera, Site du
Samaritain, Vevey
1999-2000 Practical training at Pharmacie de Chailly, Lausanne

Languages

German Mother tongue
English Fluent, written and oral
French Fluent, written and oral
Spanish Basic knowledge
Italian Basic knowledge