Role of regulatory T cells in immune tolerance

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Von

Lee Kim SWEE

Aus Isorno (TI) Schweiz

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Auf Antrag von Prof. A. Rolink

Prof. E. Palmer

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Prof. Dr. Eberhard Parlow

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2. Abbreviations

Ab	antibody
Ag	antigen
AIRE	autoimmune gene regulator
APC	antigen presenting cell
ATOC	adult thymic organ culture
B6	C57/BL6
BCR	B cell receptor
BDF1	$(C57/BL6 \times DBA/2)_{F1}$
BM	bone marrow
BMT	bone marrow transplantation
cAMP	cyclic adenosine monophosphate
CLP	common lymphoid progenitor
CMP	common myeloid progenitor
DC	dendritic cell
DL1	delta-like 1
DN	double negative
DNA	deoxyribonucleic acid
DP	double positive
FLT3	Fms-like tyrosine kinase 3
FLT3L	FLT3L ligand
Foxp3	forkhead box P3
FTOC	fetal thymic organ culture
GvHD	graft versus host disease
HA	haemagglutinin protein of PR8 influenza virus
HSC	hematopoietic stem cell
IBD	inflammatory Bowel disease
IFNγ	interferon gamma
Ig	immunoglobulin
IL	interleukin
K.O.	knockout
LN	lymph node
LPS	lipopolysaccharide
mAb	monoclonal antibody
MHC	major histocompatibility complex
N.D.	not determined
NK	natural killer
NKT	NK T cell
NOD	non-obese diabetic
NTreg	naturally occurring regulatory T cell
NTX	non-thymectomized
Ova	ovalbumin
PAMP	pathogen associated molecular pattern
pGE	promiscuous gene expression
PRR	pattern recognition receptor
pTα	preT α
RA	retinoic acid
Rag	recombination activating genes

RNA	ribonucleic acid
SP	single positive
TBI	total body irradiation
TCD	T cell depletion
TCR	T cell receptor
TD	T dependant
TEC	thymic epithelial cell
cTEC	cortical TEC
mTEC	medullary TEC
TGFβ	tumor growth factor
TI	T independant
TLR	Toll-like receptor
TNF	tumor necrosis factor
Treg	regulatory T cell
TSA	tissu-specific antigen
TX	thymectomized

3. Summary

In this present work, we investigated the role of dendritic cells (DC) and regulatory T cells (Treg) in immune tolerance as well as their potential in the treatment of autoimmune or graft versus host (GvH) diseases.

In the first study, we show that double transgenic mice expressing influenza hemagglutinin (HA) under the control of the CD11c promoter and a T cell receptor (TCR) specific for HA develop systemic auto-immunity and die prematurely. In double transgenic mice, negative selection of the transgenic TCR expressing T cells in the thymus is practically complete, however significant number of activated T cells expressing the TCR clonotype can be found in the periphery. By breeding double transgenic mice on a rag2-deficient background, we could demonstrate that T cells escape negative selection by expression of an endogenous TCR α chain.

In clear contrast, our collaborators demonstrated that expression of HA under the control of the medullary thymic epithelial cell (mTEC)-specific autoimmune regulator (AIRE) promoter together with HA-specific TCR, results in the development of HA-specific naturally occurring Treg (NTreg) cells. AIRE-HA x TCR-HA are healthy and absolutely tolerant to HA. Altogether, these data strongly suggest that the outcome of antigen encounter in the thymus or the periphery critically depends on the identity of the antigen presenting cells.

In addition, we analysed the potential of transferred HA-specific Treg cells to interfere with spontaneous autoimmunity in CD11c-HA x TCR-HA double

transgenic mice. We conclude that transfer of HA-specific Treg into newborns rescues double transgenic mice from early death but does not prevent or delay the onset of the systemic autoimmunity.

In a second study, we showed that fms-like tyrosine kinase ligand (FLT3L) treatment, already known to increase DC number, indirectly leads to expansion of peripheral NTreg. The increased number of NTreg is due to proliferation of pre-existing NTreg, likely due to favoured interactions with the increased number of DC. We investigated the therapeutic potential of FLT3L and could show that FLT3L treatment had no effect on normal immune response but could prevent death induced by acute GvHD. These data re-enforce the relevancy of FLT3L treatment in transplantation or autoimmune settings by its ability to increase both the number of immature tolerizing DC and NTreg.

Finally, in addition to immune tolerance, we investigated the so-called phenomenon of auto-reconstitution already known since a long time.
We generated bone marrow (BM) chimeras by reconstituting lethally irradiated C57BL/6 mice with either syngeneic rag2-deficient or CD3ε-deficient BM neither of which is capable of generating T cells. We show that in the absence of donor-derived thymopoiesis, host-derived T cells can reconstitute 35% of the normal T cell pool. By comparing thymectomized versus non-thymectomized host, we show that host-derived T cells, comprised a major subpopulation of *de novo* generated, thymus-derived, polyclonal cells. Host-

derived thymocytes regenerated from conventional DN1-2 prothymocytes and their differentiation recapitulated normal thymic ontogeny. Thus, host-derived T cells might provide a first line of defence against infections during recovery from lymphopenia after BMT. This conclusion is supported by the fact that host-derived T cells were fully functional.

4. Introduction

The function of the immune system is to protect individual from pathogens while maintaining unresponsiveness against self or non-infectious agents.

In vertebrates, the immune system is composed of innate and acquired immune system, arising from bone marrow-resident haematopoietic stem cells (HSC). The innate immune system is one of the first lines of defence against pathogens; it is based on the recognition and sensing of pathogens associated molecular patterns (PAMP) through germline encoded pathogens recognition receptors (PRR) followed by activation.

The adaptive immune system is composed of T and B cells expressing clonally rearranged receptors, which proliferate and fight infections upon antigen encounter. After pathogens clearance, most of the antigen-specific T and B cells undergo apoptosis while few of them enter a memory pool, this ensures a quicker and stronger reaction in the case of secondary infection, a process called memory.

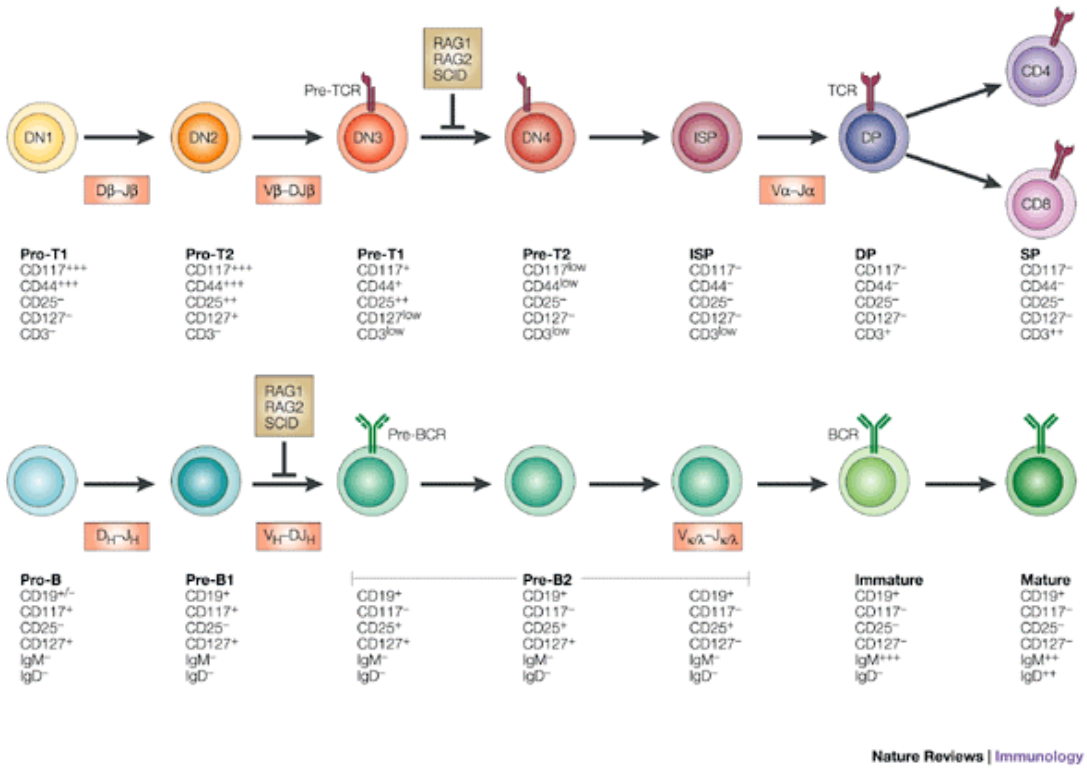
Rearrangement of T and B cell receptors is completely random and rearrangement products can be potentially self-reactive, therefore it is a vital requirement for the immune system to eliminate or control auto-reactive T and B cell clones in a process called tolerance. There are two different stages of tolerance, *central tolerance* defines mechanisms of clonal deletion or receptor editing of developing T or B cell whereas *peripheral tolerance* refers to the control of deletion auto-reactive lymphocytes that escaped central tolerance (Janeway, Travers, Walport & Shlomchik, Immunobiology 5th).

Haematopoiesis - T cell development

Like every system, the haematopoietic system relies on stem cells (HSC) with self-renewal capacity. HSC are located in the bone marrow and throughout life give rise to transient amplifying, committed and finally differentiated blood cells. For a long time, haematopoietic development was though to be dichotomic, composed of common myeloid progenitors (CMP) and common lymphoid progenitor (CLP) with mutually exclusive myeloid or lymphoid development potential [1,2]. Even though such cells can be indeed isolated, it seems more and more clear that this model should be re-considered. There are increasing amount of data, which demonstrate that there is no exclusive decision regarding myeloid or lymphoid fate but rather a progressive loss of differentiation potential influenced by cell intrinsic and extrinsic stimuli [3,4].

The first steps of haematopoiesis take place in the bone marrow and all cells of the blood except T cells are generated there. T cell development takes place in the thymus [5], an organ composed of an inner cortex and an outer medulla. The exact identity and homogeneity of thymus-settling cells are still unclear, however it is not an HSC. The essential role of notch in T versus B cell fate commitment in the thymus has been clearly established, it has been demonstrated that in absence of notch signal, B cell development occurs in the thymus [6], indicating that the bone marrow cell colonizing the thymus has still multi-potent development potential [4]. This is further supported by the fact that early thymocytes are still able to generate NK, myeloid and dendritic cells [4].

Development stages of thymocytes was first phenotypically fractionated according to CD4 and CD8 co-receptors expression which discriminate, from less to more mature, double negative cells (DN), double positive cells (DP) and finally CD4 or CD8 single positive cells [7]. The DN population is still heterogeneous and was further subdivided using CD25 and CD44 defining the DN1 (CD44+++CD25-), DN2 (CD44+++CD25++), DN3 (CD44+CD25++) and DN4 (CD44lowCD25-) [8-11].



Scheme 1. Comparison between T and B cell development. Rod Ceredig & Ton Rolink, NRI 2002 [12].

It is important to note that these two markers are not enough to precisely identify T cell precursors, for example, dendritic cells, NK and NKT cells fall in the DN gate according to CD4 and CD8 expression. The use of lineage cocktail is sometimes maid to distinguish T cell progenitors from CD4-CD8- differentiated non-T cells [13-15]. However it is not an ideal solution because of

the incomplete lineage-specificity of some markers. Alternatively the level of c-kit might be useful to distinguish pro-T cell [11].

Sorted DN1 and DN2 still show significant myeloid potential [4], suggesting that cells become fully committed at DN3 stage were V-DJ rearrangement of β chain begins [16]. There is a need of an in-frame rearranged β chain to continue beyond DN3 stage, a process called β-selection; the rearranged β-chain is expressed together with pTα [17] and delivers a ligand-independent signal to allow the cells to develop further and to stop further rearrangements in a mechanism called β allelic exclusion [18]. The rearrangement of the α chain takes place at DN4/DP stage [19]. If this rearrangement is in-frame and resulting αβ TCR expressed at cell surface, cell undergo positive and negative selection processes. There is no allelic exclusion for α chain, meaning that thymocytes can potentially proceed to rearrangement at both α locus [20,21], a process sometimes referred as receptor editing [22].

Positive selection ensures that TCR together with either of CD4 or CD8 co-receptors is able to recognize the complex formed by MHC class I or II and self-peptide with low affinity/avidity [23-28]. This is a ligand-dependant selection and thymocytes, which do not receive appropriate signals, undergo apoptosis by neglect.

Negative selection is the mechanism by which thymocytes with a too high affinity/avidity for self-peptide/MHC complex are deleted from the repertoire and is also called T cell central tolerance [23,29].

For a long time, it was hard to understand how self-specific thymocytes, which react against tissu-specific antigen (TSA, e.g. insulin) can be deleted in the

thymus. However recently it has been suggested that central tolerance toward TSA could be achieved via two major mechanisms. The first one is the active transport of TSA by dendritic cells from the periphery to the thymus or simple passive transport via blood circulation [30]. The second one is the expression of TSA by thymic epithelial cells (TEC) them-selves, a mechanism known as promiscuous gene expression (pGE, reviewed in [31]). Up to now, only one molecular determinant has been identified for pGE, which is the autoimmune regulator or AIRE protein [32,33]. AIRE is mainly expressed in a minor subpopulation of mTEC [34] and gene deficiency in mice or human leads to multi-organ autoimmune disease due to partial lack of pGE. How AIRE is involved in tolerance toward TSA is still not completely understood. It can be either by deleting TSA-specific thymocytes from the repertoire via direct presentation of antigen by TEC or cross-presentation by dendritic cells or alternatively AIRE-dependent tolerance might be mediated by TSA-specific Treg differentiation by AIRE+mTEC [35].

Thymus-dependent immune tolerance and naturally occurring regulatory T cells development

The role of the thymus in the development of cells displaying dominant regulatory activity was suggested by several independent experiments in the 1980's. It has been demonstrated that when quail wing buds are transplanted on chicken embryo, the graft is rejected within 1 or 2 weeks after birth. However, the graft is accepted if embryonic quail thymus (still empty form quail haematopoietic progenitors) is co-transplanted [36]. This suggests that

although chicken thymus is exporting cells capable of graft rejection, cells capable of dominant tolerance develop in grafted quail thymus.

Similarly, in mouse it has been shown that ablation of the thymus between day 3 and 7 after birth results in the development of organ-specific autoimmune diseases [37]. From these findings it was concluded that in this time-window a T cell subset with crucial role in self-tolerance had to be generated. Ten years later, the same authors could show that regulatory activity correlated with CD25 expression. They demonstrated that transfer of CD4+CD25- cells in athymic nude mice led to the induction of organ-specific autoimmunity. By co-transferring CD4+CD25+ cells, the induction of the disease could be prevented [38]. Thereafter these cells were named regulatory T cells (Treg).

Treg encompass a T cell population, which prevents harmful immune responses against self and foreign [38,39]. Among Treg cells, the so-called naturally occurring CD4 regulatory T cells (NTreg) are the best characterized. This lineage is defined by the expression of the transcription factor Foxp3 [40-42]. A natural mutation or conditional deletion of Foxp3 leads to lethal autoimmune disease, due to absence of functional NTreg, indicating their non-redundant role played in self tolerance and the crucial importance of Foxp3 [43-46]. NTreg develop in the thymus but it now also well accepted that naïve T cell can convert to NTreg in the periphery (discussed later).

There are two different models of NTreg development in the thymus (discussed in [47]). The first model states that NTreg development is dictated by TCR affinity/avidity for self-peptide/MHC class II complex, thymocyte would differentiate into NTreg if TCR has an affinity/avidity, which is just below

negative selection threshold [48]. A corollary of this is that NTreg repertoire should be biased toward self. Most of the data supporting this hypothesis come from studies of double mutant mice were a transgenic TCR is expressed together with its ligand in the thymus. Notably, it has been shown that thymocyte differentiate into NTreg only when the agonist ligand is expressed [49]. Moreover NTreg do not develop when thymocytes express a transgenic TCR with lower affinity for the agonist ligand, suggesting a threshold-hypothesis.

In the second model, NTreg development is independent of TCR affinity/avidity, thymocyte would develop into the NTreg lineage either stochastically or upon selection on particular niche of APC. In such a model, TCR repertoire would not be biased toward self but rather have an overlapping repertoire with effectors cells.

Large-scale repertoire analysis of effectors versus regulatory T cells TCR should give precious indication on mechanism of NTreg development however it remains challenging because of the infinite number of rearrangements and α/β combinations.

Repertoire studies have been performed on mice having a fixed transgenic β chain. Authors reported 20% overlap between conventional and regulatory cell repertoire and by cloning TCR's sampled in both population and addressing their self-specificity, they concluded that NTreg repertoire was biased toward self [50,51]. The major critisms concerning these data were that authors performed their analysis of TCR's with highest frequency and ignoring low-frequency TCR's. To restrict the repertoire further, others have used transgenic mice with fixed β chain and a mini α locus [52]. In this work, authors

carried out more detailed repertoire analysis including all TCR's above a certain frequency threshold. They similarly addressed self-specificity by cloning TCR's and concluded that they was NTreg were not more self-specific than conventional cells. It is reasonable to think that the discrepancy between these two studies relies in the size of the studied repertoire and whether NTreg are self-specific or not will certainly remains a matter of debate in the field.

Immune response and peripheral tolerance

The efficiency of the immune system relies on the sharp distinction between the need for an immune response or not. As already discussed, this is mainly achieved by the sensing of pathogens presence via expression of a battery of PRR [53]. In normal conditions dendritic cells are poor T cell stimulators because they express low levels of MHC class II and co-stimulatory molecules. Upon ligand binding to PPR for example, dendritic cells up-regulate MHC class II, co-stimulatory molecules and express inflammatory cytokines. In a mature state, they can prime T cell toward the appropriate response. In this regard, dendritic cells have a central role in the balance between immune response or not (tolerance) [54-56].

Negative selection of self-specific thymocytes is not perfect, either due to low expression of self-antigen in the thymus or expression of two different TCR's for example [20]. Therefore there is a need for the immune system to control self-reactive clones in the periphery.

Clonal deletion. It is accepted that deletion of self-reactive clone not only takes place in the thymus but also in the periphery. Self-reactive T cells, which encounter antigen on immature DC are believed to undergo apoptosis. By targeting antigen expression to DC either using Ag-conjugated mAb [57] or by transgenesis [58], it has been shown that antigen specific mature T cells were deleted from the repertoire due to antigen encounter on immature DC.

Regulatory T cells. As mentioned in the previous section, the term regulatory T cell does not refer to a homogenous T cell subset but rather regroups T cell subset with "immunomodulatory" functions. In this section I will only discuss about Foxp3+ NTreg. NTreg play an obvious non-redundant role in the establishment of tolerance, given the severity of Foxp3 deficient mice, however the mechanisms by which they control self-specific cells are less clear.

Potentially NTreg could interfere with DC or T cells at all the steps of an immune response, namely naive T cell priming, proliferation, differentiation and migration. The following modes of suppressions were reported: inhibition of IL-2 production by conventional cells, down-regulation of co-stimulatory molecules or induction of tryptophan catabolism in DC, secretion of immunomodulatory cytokines (TGFβ, IL-10) or Perforin and granzyme and more recently delivery of suppressive molecules (cAMP) via gap junctions [59,60].

It is reasonable to hypothesize that activation of NTreg is antigen-specific whereas once activated NTreg can suppress in a bystander fashion [61].

Interestingly, published data reported the influence of the presence of PRR ligand in the mechanisms of suppression. Authors reported that NTreg were loosing their suppressive function in the presence of LPS. This was not due to a direct sensing by NTreg cells but rather secretion of inflammatory cytokine by DC upon LPS binding, which rendered conventional T cells insensitive to suppression [62,63]. Based on these findings it was hypothesized that under physiological conditions conventional T cells are under constant suppression, irrespective of their specificity and that in the presence of pathogens suppression is shut down.

Extrathymic NTreg differentiation. NTreg are known to develop in the thymus, however conventional cells can also convert to NTreg cells in the periphery.

The resulting autoimmune disease after neonatal thymectomy suggests that such mechanisms are not sufficient to fulfil NTreg-mediated tolerance but give more flexibility to the immune system and more importantly clinical perspectives by manipulation of T cell tolerance.

The instrumental role of dendritic cells in the conversion of naïve cell into Treg lineage has been demonstrated by many groups [64-67] and some molecular requirements of conversion mechanism have already been discovered, notably in oral tolerance. Two different groups independently showed that secondary lymphoid organs of the gut harbour a specialized subset of dendritic cell, which is particularly efficient in converting naïve cells to NTreg [66,67]. This process could be inhibited by addition of inhibitors of retinal deshydrogenases or blocking anti-TGFβ demonstrating that the conversion was dependent on both retinoic acid (RA) and TGFβ. The interpretation of these results was that RA-receptors ligation could inhibit the transcriptional

activity of AP-1, a dimeric transcription factor downstream of CD28 co-stimulatory signalling and that AP-1 might interfere with stable expression of Foxp3 [68].

This later statement once again underlines the crucial importance of maturation state of DC regarding tolerance or immune response.

5. Thesis projects

The aim of the work was to study the direct and indirect mechanisms of Treg and DC-mediated central and peripheral tolerance.

In the first project, we addressed the role of DC in central and peripheral tolerance using a double transgenic system.

The “TCR HA” mouse is a transgenic line, which expresses a T cell receptor specific for hemagglutinin protein (HA) of influenza virus PR8. This TCR was originally cloned from a T cell hybridoma derived from a T helper clone specific for the HA peptide 111-119 presented by I-E^d MHC class II molecules [69].

The “CD11c HA “ transgenic mouse expresses a membrane-bound form of HA under the DC-specific CD11c promoter.

By crossing these two transgenic mice, we investigated:

a) The role of DC in thymic clonal deletion and Treg differentiation.
b) The role of DC in peripheral tolerance and autoimmunity.
c) The therapeutic potential of Treg transfer in the treatment of systemic autoimmunity.

In the second project, we proposed to study the function of dendritic cells in the homeostasis of NTreg cells.

By using a mouse model were DC populations are either dramatically increased or decreased, we were able to investigate the role of DC's in setting the size of the peripheral NTreg niche.

6.1

Systemic autoimmunity caused by dual TCR-expressing T cells

Maurus Curti[1], Lee Kim Swee[1], Fritz Melchers[2], Jan Andersson[1] and Antonius G. Rolink[1]

[1.]Department of Clinical and Biological Sciences (DKBW), Division of Molecular Immunology, Center for Biomedicine, University of Basel, Mattenstrasse 28, 4058 Basel, Switzerland.
[2] Max Planck Institute for Infectious Biology, Berlin, Germany

Key words: Dendritic cells, Autoimmunity, Double TCR expressing T cells, negative selection.

Correspondence to: Antonius G. Rolink
DKBW, Division of Molecular Imunology
Center for Biomedicine, University of Basel
Mattenstrasse 28
CH-4058 Basel, Switzerland
Phone: +41-61-2671631 Fax: +41-61-6953070
e-mail: antonius.rolink@unibas.ch

Abbreviation:

HA: PR8 influenza hemagglutinin protein

Abstract

T cells escaping negative selection in the thymus can cause autoimmune diseases. Here we show that double transgenic mice expressing influenza hemagglutinin (HA) under the control of the CD11c promoter and a TCR specific for HA develop systemic auto-immunity. Increased mortality, lymphocyte infiltrations in multiple organs, high incidence of arthritis and development of immune-complex glomerulonephritis a well as high titres of IgG autoantibodies in the serum characterize this disease. In single HA-transgenic mice negative selection of HA-specific B cells is almost complete, as no HA-specific antibodies can be elicited by influenza virus stimulation. Although in double transgenic mice, negative selection of the transgenic TCR expressing T cells in the thymus is practically complete, significant numbers of activated T cells expressing the TCR clonotype can be found in the periphery. A large part of these T cells express a non-transgenic TCR α protein, indicating that they have escaped negative selection by expression of a second TCR α chain. In line with this conclusion is the finding that RAG-2 deficient double transgenic mice have no detectable peripheral T cells and therefore, obviously, do not show any sign of autoimmunity. Additionally, we have analysed the potential of transferred HA-specific regulatory T cells to interfere with autoimmunity. Transfer of HA-specific Tregs into newborns rescued the double transgenic mice from early death but did not prevent or delay the onset of the systemic autoimmunity.

Introduction

The ability to discriminate between self and non-self is a hallmark of the immune system and ensures that the individual is tolerant to his body's own components yet able to respond to pathogens. The thymus plays a central role in the establishment of tolerance within the T cell compartment. Thus double positive thymocytes expressing a TCR with high affinity for self-antigen are normally negatively selected. Negative selection is caused by bone marrow derived antigen presenting cells cross-presenting self-antigens that are not synthesized within the thymus (1). Moreover, medullary thymic epithelial cells can also cause negative selection since they may express gene products which are otherwise only expressed in a tissue specific manner (2). This promiscuous gene expression is at least to a large extent regulated by the transcription factor AIRE (3). The fate of thymocytes undergoing negative selection is either deletion (apoptosis) or functional inactivation (anergy).

Although the process of negative selection in the thymus is rather efficient, T cells with auto-reactive receptors may escape deletion/inactivation by various mechanisms, such as down modulation of TCR or alterations in co-receptor expression levels (4-6). Nevertheless, autoimmunity caused by T cells escaping negative selection is rare, since their potential auto-aggression is further controlled by peripheral mechanisms (7).

Nemazee (8) and Weigert (9, 10),more then a decade ago, showed that autoreactive B cells may be rescued from deletion by a process they named "receptor editing". Thus, cross-linking of the B cell receptor on immature bone

marrow B cells resulted in the up-regulation of RAG gene expression, thereby allowing the cells to undergo secondary gene rearrangements, especially at the light chain locus (11, 12). These secondary rearrangements may then result in the generation of B cells with a different, non-autoreactive receptor, which might rescue these cells from deletion. However, we have previously shown that such secondary light chain rearrangements also occurs when pre B cells are unable to form a complete BCR or when the pairing of the light chain with the muH chain is inefficient (13, 14).

Recently, it was shown, that receptor editing does not necessarily result in the complete disapearance of the autoreactive Ig light chain (15). Thus Gerdes and Wabl (15) showed that a monoclonal mouse generated by transfer of mature B cell nuclei carried two productively rearranged Ig light chain alleles. Both light chains could combine with the one productively rearranged Ig heavy chain so as to form one non-autoreactive and unother, autoreactive BCR. These findings suggest that B cells expressing an autoreactive receptor might be rescued from negative selection by co-expressing a non-autoreactive receptor. In fact, similar findings have been reported earlier using Ig transgenic mice, expressing autoreactive receptors (16). These dual BCR- expressing B cells might in part be responsible for the autoreactive antibodies found in the serum of normal individuals. Moreover a potential role for these B cells in the generation of “B cell” autoimmune-diseases could well be envisaged.

In T cells, both the organization and the large size of the TCR α locus is very conducive to receptor editing. However, whether autoreactive T cells can be rescued from negative selection by receptor editing is not yet clear. Using a

very elegant TCR α “knock-in” approach, Buch et al. recently showed that thymocytes expressing a TCR with specificity for the HY antigen cannot be rescued from deletion by receptor editing (17). However, in their experimental system, thymocytes could only be rescued by secondary rearrangements at the “knock-in” allele, since the mice used for these studies carried a TCR α null allele on the other chromosome. Therefore, the possibility of autoreactive thymocytes escaping negative selection by the expression of a second TCR was not addressed in this study.

Several groups have indicated that allelic exclusion at the TCR α chain locus is incomplete (18-20) thereby resulting in the generation of T cells that express two TCR’s. Indeed, during thymocyte selection, DP cells expressing two TCR have been shown to down-regulate expression of the potentially auto-reactive TCR (21). It has been suggested that dual TCR-expressing T cells might be involved in the development of autoimmune-diseases. In fact, it has been shown that T cells expressing an autoreactive receptor can be rescued from negative selection by the expression of a second, non-autoreactive TCR [19]. However, these T cells are tolerant to self, but can be activated via their non-autoreactive TCR and thus develop into self-reactive effector cells.

Here, we have analyzed the potential of dendritic cells (DC) to induce B and T cell autoimmunity and tolerance. Transgenic mice expressing PR8 influenza hemagglutinin (HA) under the control of a mouse CD11c promoter were generated to guide the expression of this experimental autoantigen to CD11c-expressing DC’s. Immunization of these CD11c-HA transgenic mice with complete PR8 virus resulted in a strong humoral anti-viral response.

However, unlike wild type mice, no antibodies to HA could be detected, indicating that the B cell compartment in these transgenic animals was tolerant to HA. In order to test the induction of T cell tolerance, CD11c-HA mice were crossed with mice expressing a transgenic α/β TCR specific for peptide 111-119 of the HA from PR8 influenza virus (22). Although negative selection in the thymus of these double transgenic mice is practically complete, all mice develop systemic autoimmune disease. This disease is characterized by leukocyte infiltrations in many organs, high IgG titres of autoantibodies, spontaneous germinal centre formation and antibodies depositions in the kidney. Most dramatically, over 90% of these mice die before 12 weeks of age. This disease appears to be caused by transgenic TCR expressing $CD4^+$ T cells that have escaped negative selection through the concomitant expression of an endogenous TCR α gene product.

Moreover, we investigated the therapeutic potential of transferred HA-specific Treg in this system. We show here that Treg transfer rescues double transgenic mice from premature death but neither prevents nor delays overall autoimmunity.

Results

CD11c-HA mice do mount a humoral influenza-virus specific, but not anti-HA specific response upon immunization with PR8 virus

Mice expressing HA under the control of the CD11c promoter were immunized with PR8 virus in order to test whether they were able to mount an IgG anti-HA response. Normal, non-transgenic BALB/c mice were used as positive controls. In both groups of mice the highest IgG anti-PR8 titers were found 21 days after immunization. However, the titer of anti-PR8 anitibody in CD11c-HA mice was 3-7 fold lower than in BALB/c controls (Table1). IgG anti-HA titers differ however much more dramatically. In immunized BALB/c mice, high titers of anti-HA antibodies were detectable. In fact, in BALB/c mice, the anti-PR8 antibody response is almost exclusively directed to HA (Table1). In immunized CD11c-HA mice the anti-HA titers are very low and comparable to those found in non-immunized mice (Table1). Thus the anti-PR8 response observed in CD11c-HA is directed against viral components other then HA. We conclude that the B cell compartment in CD11c-HA mice appears to be tolerant to HA.

Mice (number of animals)	IgG anti-HA titer	IgG anti-PR8 titer
Control BALB/c (12)	1/5000 – 1/7000	1/5000 – 1/7000
CD11c-HA transgenic BALB/c (9)	1/100 – 1/300	1/1000 – 1/2000

Table 1.

Primary IgG antibody responses in normal and HA transgenic BALB/c mice after 21 days of i.v. immunization with 1000 haemagglutinating units of UV-inactivated PR8 influenza virus.

Pathological manifestations of systemic autoimmunity in

(CD11c-HA x TCR-HA)F_1 mice

In order to test the potential of dendritic cells to induce T cell tolerance, CD11c-HA mice were crossed with mice expressing a transgenic $\tilde{\alpha}\beta$ TCR (22) derived from a CD4 T cell clone recognizing the peptide 111-119 of PR8-HA in the context I-E^d (23). We expected that the HA expressing DC's could induce a complete arrest of all the T cells expressing the anti-HA TCR. Severe arrest of T cell development in the thymus was observed, however, to our surprise, all double transgenic mice developed a systemic autoimmune disease while none of the single transgenic mice showed signs of such disease. Thus, around 10% of double transgenic mice die before weaning (before 3 weeks of age). Those that survive longer, show dramatic growth retardation compared with single transgenic littermates. Moreover, all double transgenic mice suffer from diarrhea and have ruffled fur. As shown in Fig.1 more then 90% of these mice die before 14 weeks of age. From week 5 of age and onwards, the vast majority of double transgenic mice show swelling of their paws, often starting with the front paws, suggesting the development of arthritis.

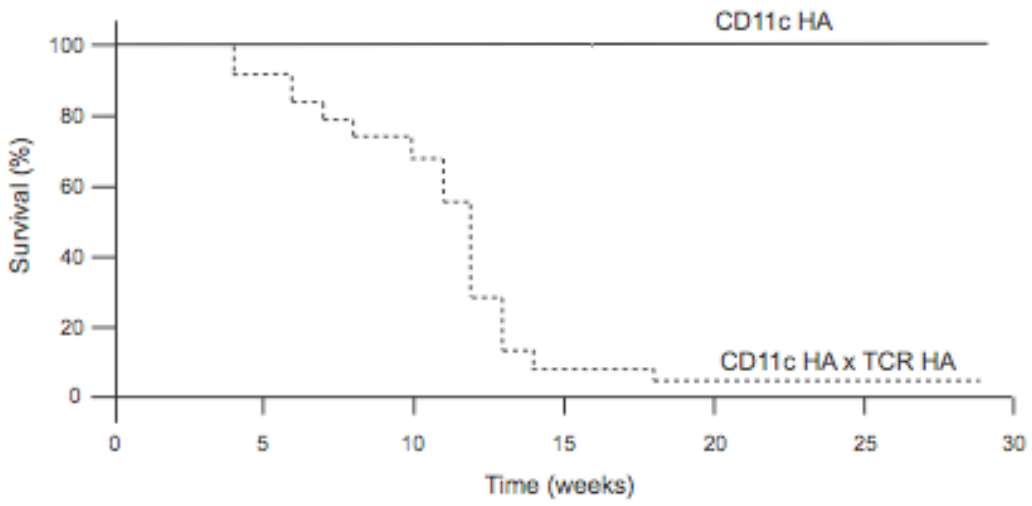


Figure 1: Premature death selectively among CD11c-HA – TCR-HA double transgenic mice

The number of surviving CD11c-HA single and CD11c-HA – TCR-HA double transgenic mice was recorded twice a week. The groups consisted of 12 single transgenic and 38 double transgenic mice.

Histological analysis revealed leukocyte infiltrations in multiple organs, especially those that harbor relatively large numbers of dendritic cells such as lung (Fig.2A), liver (Fig.2B) and heart (Fig.2C). Indirect immunofluorescence analysis of these sections showed that T, B and dendritic cells were present in these infiltrates (data not shown).

In order to test whether the observed paw swelling in these double transgenic mice was the result of arthritis, cryo-sections were prepared and stained with safranin O. As shown in Fig. 2D, cartilage erosion and pannus formation was detectable in the joints of these paws. Moreover, leukocyte infiltrations were also observed. Thus, (CD11c-HA x TCR-HA)F_1 also spontaneously develop arthritis.

Macroscopic analysis revealed that double transgenic mice also develop severe splenomegaly. Thus, 300 – 400 million nucleated cells could be recovered from these spleens whereas spleens of single transgenic animals contained the normal range of 60 – 80 million nucleated cells as found in non-transgenic BALB/c mice. FACS analysis of double transgenic spleen cells revealed that about one third of the nucleated cells were Ter119 positive cells and therefore most likely erythroid precursors, while another third were Gr.1/CD11b double positive cells, most likely representing myeloid precursor cells. Thus, the observed splenomegaly seems to be caused by extramedullary hematopoiesis, a phenomenon occurring in various hematological abnormalities, including severe autoimmunity.

Indirect immunofluorescence analyses of spleen or lymph node sections from double transgenic mice showed the presence of large numbers of germinal centers (Fig.2E). This observation prompted us to investigate, whether the serum of these mice contained autoantibodies. As summarized in Fig.2G, all (CD11c-HA x TCR-HA)F_1 mice had high titres of serum IgG anti-nuclear autoantibodies whereas these autoantibodies could never be detected in single transgenic mice.

High titres of autoantibodies can lead to the formation of large quantities of immune complexes, which, in cases of inefficient clearing, may be deposited in the kidneys and thus result in the development of immune complex glomerulonephritis. In order to determine whether (CD11c-HA x TCR-HA)F_1 mice had Ig depositions in their kidneys, cryo-sections were stained with FITC labeled anti mouse Ig antibodies. As shown in fig. 2F, Ig deposits were

detectable in the glomerulæ of these double transgenic mice, suggesting that they also may develop glomerulonephritis.

Overall, our findings demonstrate that (CD11c-HA x TCR-HA)F_1 mice develop severe and systemic autoimmune disease.

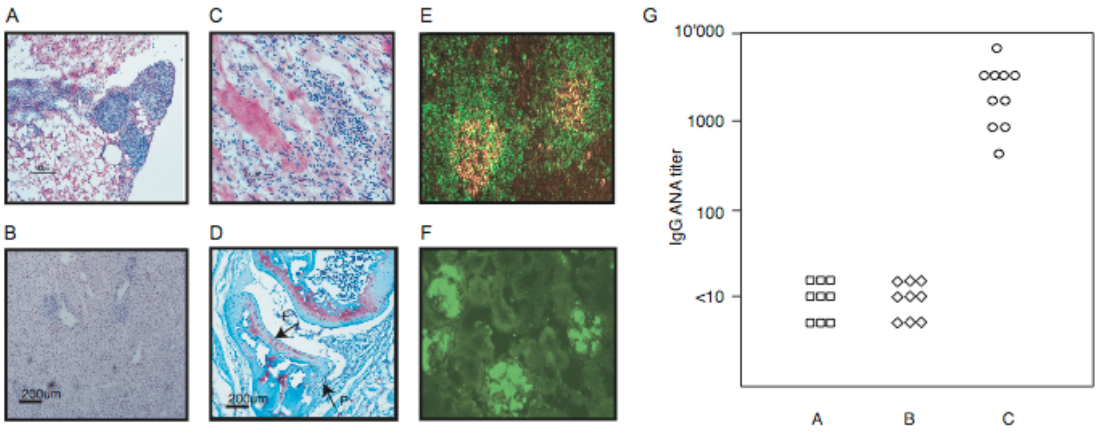


Figure 2: Lymphocytic infiltrations in lung (A), liver (B) and heart (C) and arthritis (D) germinal centers (E) and immune complex glomerulonephritis (F) in CD11c-HA – TCR-HA double transgenic mice.

A-C Hematoxylin-eosin staining of 4□M cryosections of lung, liver and heart of a 8 week old CD11c-HA – TCR-HA double transgenic mouse. **D** Hematoxylin-eosin-safranin O staining of a 4□M cryosection of a decalcified front paw of a 8 week old CD11c-HA – TCR-HA double transgenic mouse. E indicates cartilage erosion and P indicates pannus formation. **E** 4□M cryosection of a 6 weeks old CD11c-HA – TCR-HA double transgenic mouse stained with anti-IgM^{Fitc} and biotinylated Peanut agglutinin revealed by TexasRed labelled streptavidin. **F** 4□M cryosection of a kidney of a 10 weeks old CD11c-HA – TCR-HA double transgenic mouse stained with anti mouse Ig^{Fitc}. **G** IgG antinuclear antibodies in the serum of 8 weeks old TCR-HA (A), CD11c-HA (B) and CD11c-HA – TCR-HA double transgenic mice (C). Each symbol represents an individual mouse. The titer is defined as the highest serum dilution still giving a positive nuclear staining in the indirect-immunofluorescence test.

Activated T cells expressing the transgenic TCR are present in the periphery of (CD11c-HA x TCR-HA)F_1 mice

Our findings that (CD11c-HA x TCR-HA)F_1 double transgenic but not the single transgenic mice develop systemic autoimmunity suggests that T cells expressing the transgenic TCR escape negative selection and become activated by recognizing the HA peptide on dendritic cells thereby, initiating disease. In order to test this hypothesis, T cell development in the thymus and the composition of the peripheral T cell compartment of (CD11c-HA x TCR-HA)F_1 mice were analyzed. TCR-HA single transgenic mice were used as controls. Our findings with the single TCR-HA transgenic mice confirm the previous findings of others (22), namely that in the thymus, there was strong positive selection of CD4 single positive cells (Fig.3A). Within the CD4SP, almost half of the cells express the HA-specific transgenic TCR (6.5+) (Fig.3C), as also previously observed (22).

The thymi of single TCR-HA transgenic mice contained about 10^8 cells. In marked contrast, only 5 -10 x 10^6 thymocytes were obtained from (CD11c-HA x TCR-HA)F_1 double transgenic mice. This low cell yield is, to a large extent, a reflection of effective, but not complete negative selection resulting in a reduced DP compartment found in these thymi (Fig.3B). Since these mice suffer from severe autoimmunity, the reduced size of the DP compartment could also be the result of stress.

In (CD11c-HA x TCR-HA) double transgenic F_1 mice, practically all CD4SP thymocytes expressed the transgenic TCR α chain however cells expressing the complete transgenic TCR were almost completely absent (Fig.3D). This

finding indicates, that negative selection of TCR-HA expressing cells is very efficient in the thymus of (CD11c-HA x TCR-HA)F_1 mice.

In some double transgenic system, the expression of both a transgenic TCR together with its antigen in the thymus results in the development of antigen speciific Treg (24, 25). Therefore, we wondered whether the few HA-specific CD4SP cells were Foxp3+. However as shown in figure 3F, most of the HA-specific CD4SP were Foxp3-, suggesting that dendritic cells induce negative selection rather then development of Treg cells.

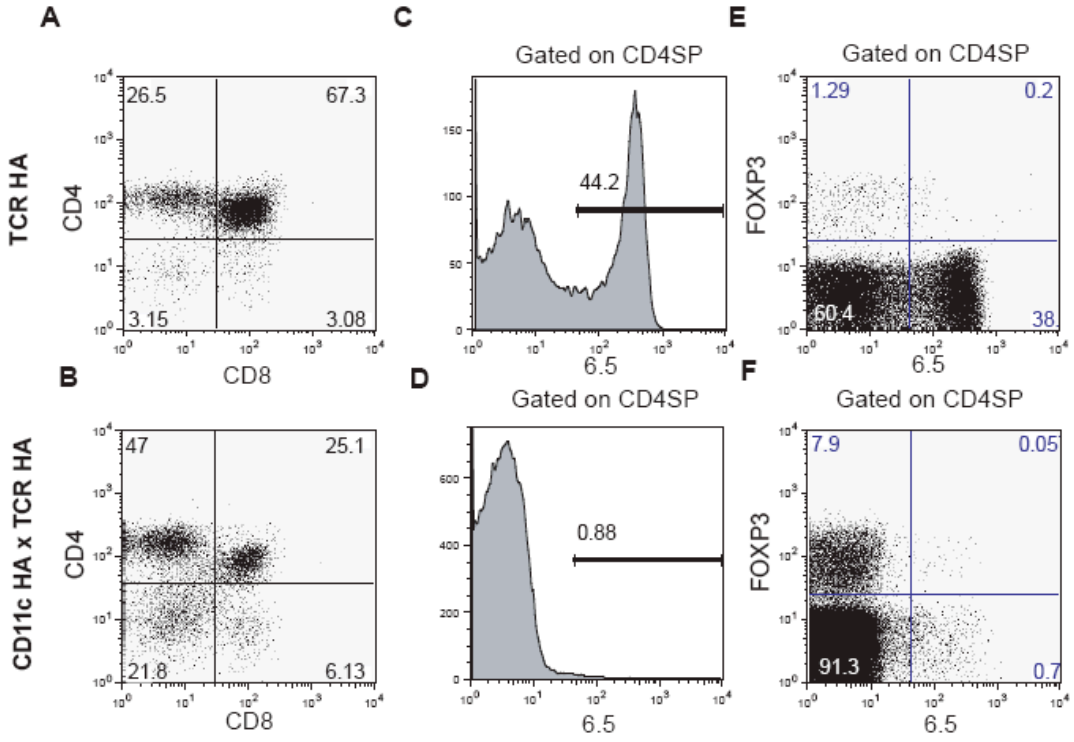


Figure 3. Central tolerance in CD11c HA x TCR HA mice.

A & B, CD4 vs CD8 profile of TCR HA or CD11c HA x TCR HA thymocytes. **C & D**, Expression of HA-specific TCR (6.5+) within CD4SP of TCR HA or CD11c HA x TCR HA thymocytes. **E & F**, Foxp3 vs 6.5 profile of TCR HA or CD11c HA x TCR HA CD4SP thymocytes.

In order to determine whether TCR-HA expressing T cells were present in the periphery of (CD11c-HA x TCR-HA)F_1 mice, lymph node cells were stained with 6.5 and CD4. As shown in Fig.4A about half of the CD4 positive lymph node T cells of TCR-HA single transgenic mice were 6.5 positive. Moreover, the majority (70%) of these were naïve T cells since they express CD62L. Only low numbers of these were CD62L negative (21%) and practically none expressed the early activation marker CD69. In marked contrast, only 10 – 15% of lymph node CD4 cells from (CD11c-HA x TCR-HA)F_1 mice were 6.5 positive (Fig.4C). The vast majority of 6.5^+ CD4 cells were $CD69^+$ and did not express CD62L i.e. they were activated T cells. Additionnaly, the number of IFNγ-secreting CD4 T cells upon PMA/ionomycine stimulation was much higher in double transgenic compared to single transgenic mice (Fig 4E and F). Thus, although negative section of TCR-HA expressing T cells in the thymus of (CD11c-HA x TCR-HA)F_1 mice is very efficient, some cells nevertheless escape this selection, and thus might be the cause of the systemic autoimmunity.

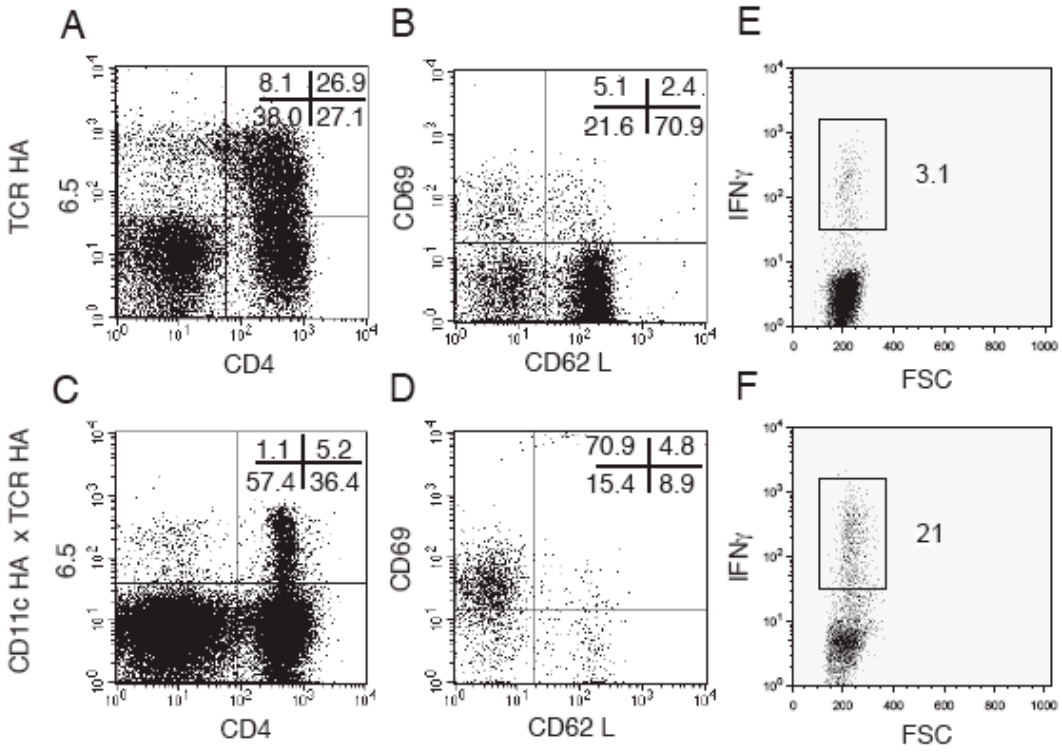


Figure 4: FACS analysis of lymph-node cells from a TCR-HA single (A & B) and a CD11c-HA – TCR-HA double transgenic mouse (C & D)

Lymph-node cells from a 6 weeks old TCR-HA single (top) and a CD11c-HA – TCR-HA double transgenic mouse (bottom) were stained with CD4, clonotype specific 6.5, CD62L and CD69. **A & C**: CD4-6.5 staining. **C & D**: CD62L-CD69 staining gated on CD4+6.5+ double positive cells. **E & F**, IFNγ secretion upon PMA/ionomycine stimulation. Dot plots show IFNγ intracellular staining of CD4 T from spleen of TCR HA (E) or TCR HA x CD11c HA (F).

TCR-HA expressing peripheral CD4 T cells in (CD11c-HA x TCR-HA)F_1 mice express an endogenous TCR α chain

The transgenic TCR of TCR-HA mice is formed by the combination of α- and β-chains containing Vβ8 and Vα4. As shown above, allelic exclusion at the endogenous β locus is to all intents and purposes complete in these TCR transgenic mice. It has however been shown, that autoreactive T cells may escape negative selection by the expression of a second TCR α chain [17, 18, 19]. In order to test whether such a mechanism is also operative in our

(CD11c-HA x TCR-HA) F_1 mice, we stained lymph node cells with CD4, 6.5 and an antibody specific for the non-transgenic Vα2 family, expressed by 10-15% of T cells in normal Balb/c mice. A representative example of such a staining is shown in Fig.5. Thus, about 20% of the CD4, 6.5 double positive T cells in (CD11c-HA x TCR-HA)F_1 mice expressed a TCR α chain using the Vα2 family member. This finding demonstrates that in addition to the Vα4 transgene, some 6.5^+ T cells express on their surface a second, endogenous, TCR α chain. Note also the slightly reduced 6.5 expression on the Vα2+ cells (Fig 5, upper right quandrant). Therefore, autoreactive T cells in (CD11c-HA x TCR-HA)F_1 mice might indeed escape negative selection by expressing a second TCR α chain.

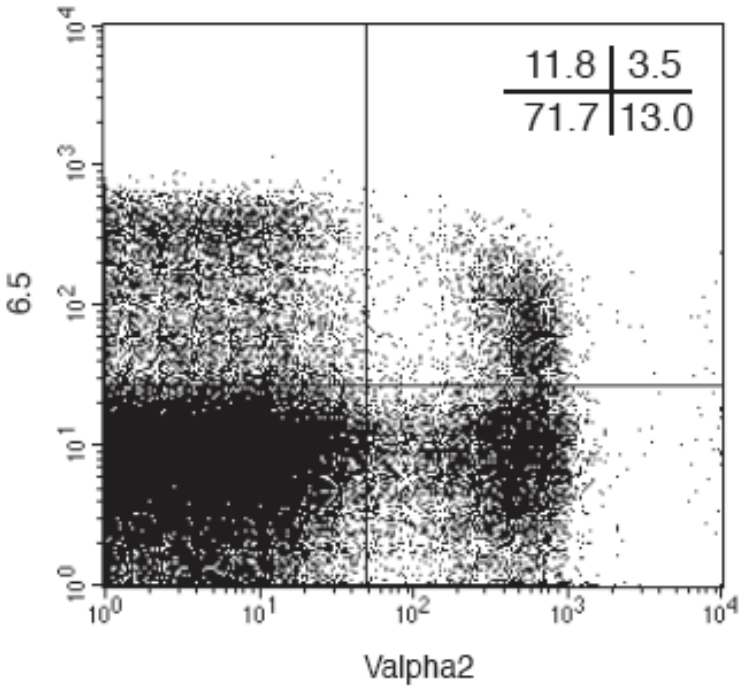


Figure 5: CD4+6.5+ double positive lymph-node cells from CD11c-HA – TCR-HA double transgenic mice do express a second TCR chain

Lymph-node cells from 6 weeks old CD11c-HA – TCR-HA double transgenic mouse were stained with CD4, clonotype specific 6.5 and clontype unrelated anti Vα2. The dot plot represents the 6.5 – Vα2 staining on gated CD4 positive cells.

No signs of systemic autoimmunity in (CD11c-HA x TCR-HA) rearrangement deficient, RAG2$^{-/-}$ F_1 mice

To test the hypothesis that the observed systemic autoimmunity in (CD11c-HA x TCR-HA)F_1 mice might be caused by autoreactive T cells that have used a second TCR αchain to escape negative selection, we generated (CD11c-HA x TCR-HA)F_1 mice on a RAG2 deficient background.

In marked contrast to (CD11c-HA x TCR-HA)F_1 mice on a RAG2 proficient background, none of these RAG2 deficient, double transgenic mice developed diarrhea, nor did they develop paw swelling. Moreover, over 95% of the double transgenic mice on a RAG2 deficient background survived for more then 3 months. Also, histological analysis of organs from RAG2 deficient double transgenic mice showed no signs of lymphocyte infiltrations in lung, liver or heart. In conclusion, RAG2 deficient (CD11c-HA x TCR-HA)F_1 mice do not develop systemic autoimmunity.

In order to test whether the absence of disease correlated with complete negative selection in the thymus and no T cells in the periphery ™FACS analysis was performed. TCR-HA single transgenic mice on a RAG2 deficient background were used as controls. The recovery of thymocytes from both types of mice was 50 – 70 x10^6. However, CD4, CD8 and 6.5 staining revealed a marked phenotypic difference between them. Thus, a strong positive selection of CD4 single positive thymocytes was found in RAG2 deficient TCR-HA single transgenic mice (Fig.6A). Moreover, CD4 dull cells (representing CD4/CD8 double positive cells) expressed low to intermediate levels of 6.5 whereas CD4 high cells (representing CD4 single positive cells)

expressed high levels of 6.5 (Fig.6B). In the thymus of RAG2 deficient (CD11c-HA x TCR-HA)F_1 mice, only double negative and double positive thymocytes were found (Fig.6C). Moreover, low level expression of 6.5 was observed on double negative cells whereas CD4 cells did not express 6.5 (Fig.6D). Thus, negative selection of TCR-HA T cells is complete in the thymus of RAG2 deficient (CD11c-HA x TCR-HA)F_1 mice. Moreover, no TCR-HA T cells were found in the periphery of these mice (Fig.6F), while about 15% of the cells in the spleen of RAG2 deficient TCR-HA single transgenic mice expressed both CD4 and 6.5 (Fig.6E). From these findings, we conclude that the systemic autoimmunity in RAG2 proficient (CD11c-HA x TCR-HA)F_1 mice is caused by autoreactive T cells, which have escaped negative selection through the concomitant expression of a second TCR αchain.

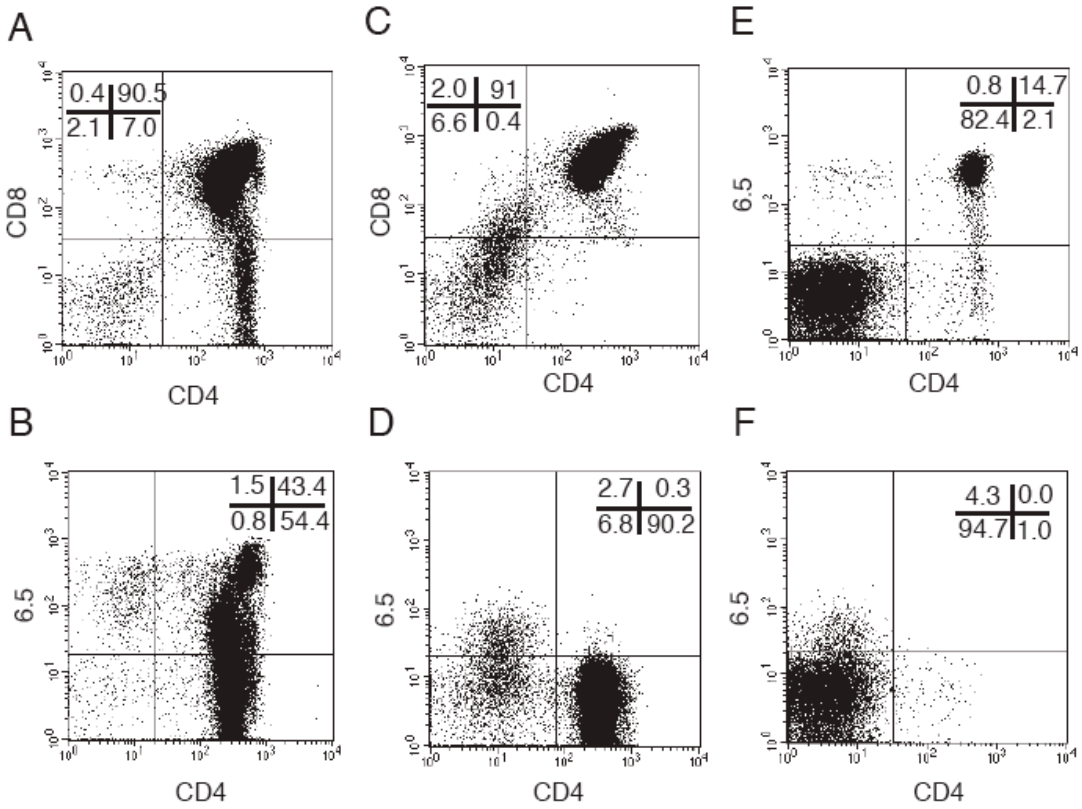


Figure 6: FACS analysis of thymus and spleen of TCR-HA single and CD11c-HA – TCR-HA double transgenic mouse on a RAG2 deficient background.

Thymocytes derived from 6 weeks old RAG2 deficient TCR-HA single transgenic (A & B) and CD11c-HA – TCR-HA double transgenic mice (C & D) were stained with CD4, CD8 and clonotype specific 6.5. A, C: CD4-CD8 staining. B, D: CD4-6.5 staining. Spleen cells derived from 6 weeks old RAG2 deficient TCR-HA single transgenic (E) and CD11c-HA – TCR-HA double transgenic mice (F) were stained with CD4 and 6.5.

Partial rescue from CD11c HA x TCR HA mice premature death but not autoimmunity by transfer of HA specific Treg from Ig HA x TCR HA

We hypothesize that if systemic autoimmunity in CD11c-HA x TCR-HA was due to lack of peripheral T cell tolerance toward HA, disease could be treated by transfer of HA-speficic regulatory T cells. In Igκ-HA transgenic mice, HA expression is driven by kappa light chain promoter (26). These mice have

originally been generated to target HA expression to B cells but it appeared that HA expression was broader than expected due to promoter leakiness (27). At mRNA level, expression was reported in B cells as well as cTEC and mTEC. Igk-HA x TCR-HA double mice are tolerant to HA and do not suffer from autoimmune disease. In addition, it has been demonstrated that sorted CD4+6.5+ cells from Igκ-HA x TCR-HA display in vitro and in vivo HA-specific regulatory activity.

We wondered whether transfer of sorted cells would ameliorate the outcome of spontaneous autoimmune disease in CD11c-HA x TCR-HA mice. We reasoned that injecting cells as early as possible would increase the chances to establish tolerance and we therefore decided to inject newborn CD11c-HA x TCR-HA mice. As control, we injected either effector HA-specific CD4 T cells from TCR-HA mice or CD25+ CD4+ Treg from WT mice with an expected polyclonal repertoire. As shown in figure 7A, only injection of HA-specific Treg cells could partially prevent premature death of CD11c-HA x TCR-HA animals, whereas injection of effector HA-specific T cells were actually accelerating death rate. However, transfer of HA-specific Treg could not prevent autoimmune symptoms, treated CD11c-HA x TCR-HA mice still suffered from arthritis and had similar auto-antibody titres as non-treated animals (Fig 7B). In contrast, injecting HA-specific Treg cells 3 weeks after birth of CD11c-HA x TCR-HA mice could not prevent premature death of the mice suggesting that they inefficiently improve outcome of ongoing disease (data not shown). In conclusion, transfer of HA-specific Treg in CD11c HA x TCR HA animals improved autoimmune symptoms responsible for early death but did not prevent overall auto-immunity.

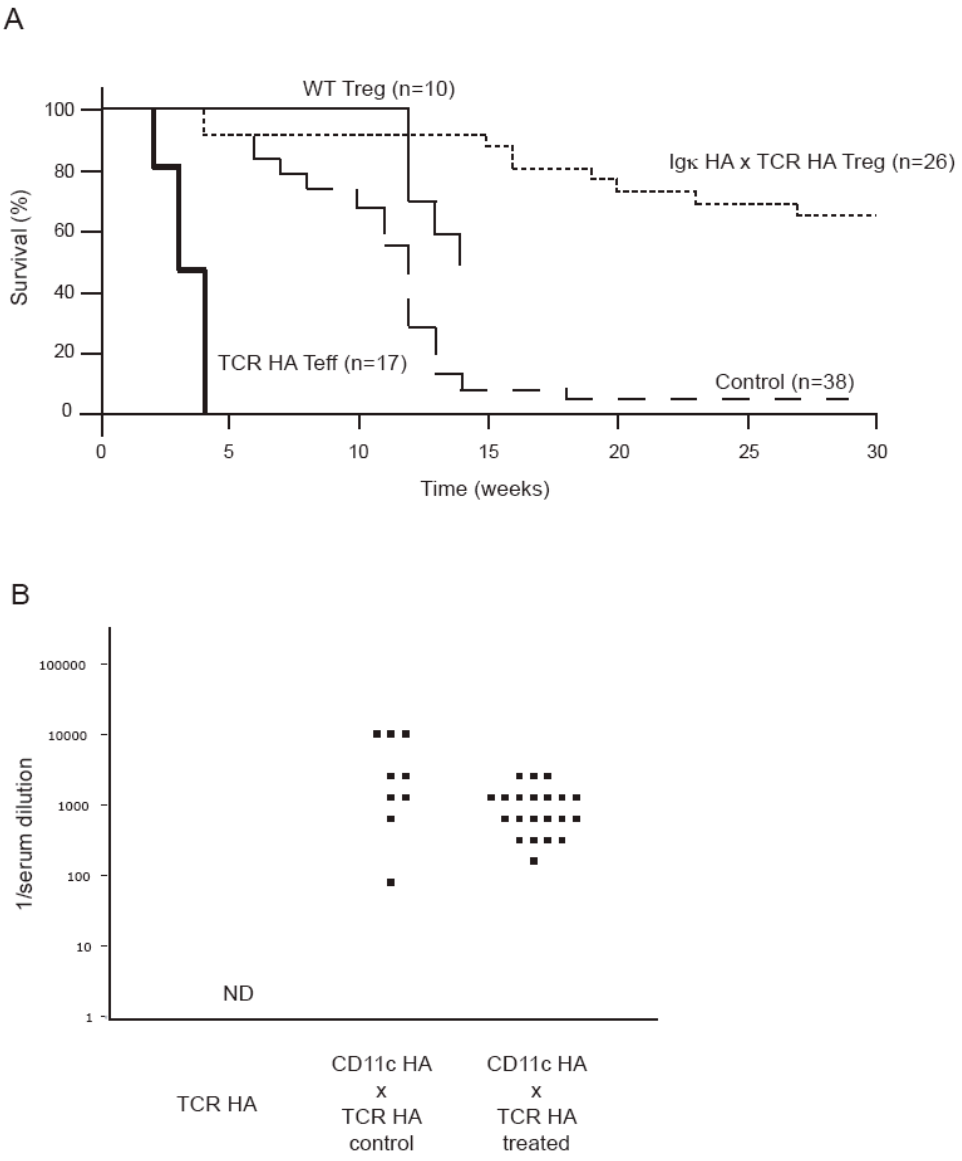


Figure 7: Treatment of CD11c HA x TCR HA mice.

A, Newborn CD11c HA x TCR HA mice were injected with either HA-specific cells from Igk HA x TCR HA mice (75'000-370'000 sorted cells), HA-specific cells from TCR HA mice (200'000-300'000 sorted cells) or CD4+CD25+ from BalB/c WT mice (75'000-120'000 sorted cells). The number of surviving CD11c-HA single and CD11c-HA – TCR-HA double transgenic mice was recorded twice a week.**B**, Autoantibody titres in TCR HA, CD11c HA x TCR HA control or treated with HA-specific Treg. (The titer is defined as the highest serum dilution still giving a positive nuclear staining in the indirect-immunofluorescence test).

Discussion

In this paper we described a spontaneous, lethal systemic autoimmune disease which develops in TCR-HA transgenic mice when expression of the HA antigen was controlled by the CD11c promoter. The disease could be avoided when the expression of a second, endogenously rearranged TCR α chain cannot occur in RAG-deficient (CD11c-HA x TCR-HA) F_1 mice. Therefore, TCR-HA expressing T cells that escaped negative selection in the thymus by the expression of second TCR α chain seems to cause this disease. In WT animals, it was estimated that about 30% of T cells could express two different TCR's, due to rearrangement of two α chains and the absence of α allelic exclusion (18). Some of these cells might escape negative selection by similar mechanisms described here and could potentially lead to autoimmunity, e.g. by bystander activation through the non self-reactive TCR. However, there are controversies among data regarding this issue (28) and systematic usage of TCR transgenic mice is a clear limitation for the relevancy of these data under physiologic conditions.

We discussed the reasons why, despite efficient negative selection HA-specific T cells were present in the periphery, however the way these cells get activated remains obscure and we can only speculate about this "spontaneous activation".

Currently it is believed that T cells need three different signals to get activated i) a strong TCR signal, which ensures specificity of the response ii) co-stimulatory signals delivered by mature APC, which ensures the need of an

immune response in presence of pathogens or endogenous “danger” signals and iii) signals priming naïve cells to Th1, Th2 or Th17 phenotype (29). When T cells receive only a strong TCR stimulation without any others signals, they are believed to undergo apoptosis (30, 31). Among others, this mechanism ensures that self-reactive T cells that escaped negative selection will not initiate autoimmune response when antigen is encountered in the periphery. There are numbers of publications supporting this model, most of them using transgenic TCR mice. We have no clear experimental evidences to explain why HA-specific T cells get activated in CD11c-HA x TCR-HA mice whereas T cells are deleted in similar conditions in other systems however we can come with several hypothesises.

Firstly, it might be due to spontaneous or abnormal activation of DC by expression of HA itself. Influenza viruses are known to be mitogenic with high variations between strains and it has also been shown that purified HA protein have mitogenic potential as well (32, 33). Further more, it has been demonstrated that HA from measles virus was activating TLR2 signalling (34), therefore it might be possible that HA from PR8 is inducing direct DC maturation as a TLR ligand although we do not have any data supporting this hypothesis.

Secondly, it might be due to the pathogen status of the mice, which can also be a cause of dendritic maturation. Mice were bred in specific-pathogen-free conditions and not germ-free conditions and therefore occasional minor local infections could induce maturation of DC. No matter the cause of DC maturation, in this system autoimmune response might be very quickly initiated for two reasons i) as discussed before, every CD4 T cells can

potentially express HA-specific TCR at the cell surface and ii) once activated, T cells express CD40L and can activate more DC upon antigen encounter (35), creating an activation circle (DC activate T cells, T cells activate DC via CD40 and so on).

Thirdly, we cannot exclude that the CD11c transgene inserted in a gene or a regulatory element disturbing expression of gene(s) playing a role in the maintenance of immunological tolerance. However, it seems rather unlikely because of the absence of a phenotype in CD11c-HA single transgenic mice.

Fourthly, the anti-HA immune response might be due to lack of control by HA-specific Treg cells. We have indeed demonstrated that HA-specific Treg are found in very low percentages in CD11c-HA x TCR-HA mice. Therefore in the absence of sufficient number of HA-specific Treg, effector cells could get activated and induce autoimmune response.

There is currently considerable research ongoing on Treg manipulation to cure autoimmune disease and allergies, as well as to induce transplantation tolerance. We have investigated here their potential to establish peripheral tolerance in transfer settings. Our data demonstrate that injection of HA-specific Treg into CD11c-HA x TCR-HA mice shortly after birth was improving their survival but could not prevent autoimmunity. Unfortunately we were not able to monitor injected cells proliferation, migration and survival because we were lacking congenic marker to distinguish donor from host cells. It was therefore not possible to dissect mechanism of Treg mediated protection.

However, it seems likely that premature death of double transgenic mice is due to IBD. Therefore we favour the hypothesis that transferred Treg ameliorate IBD symptoms as it was shown by others already (36).

Although Treg treatment improved survival of CD11c-HA x TCR-HA mice it is important to underline that it failed to establish peripheral tolerance. The fact that the thymus of CD11c-HA x TCR-HA mice constantly produces auto-reactive T cells whereas we performed a single Treg injection let us think that there might be an imbalanced ratio between effector and regulatory cells, which could explain the persistence of autoimmune symptoms. We did not perform repetitive injections of Treg in order to keep a fair effector/regulatory ratio, however the therapeutic success of such a treatment is questionable.

It seems that when thymus is chronically exporting self-reactive T cells, the resulting autoimmunity is very hard to control. E.g. AIRE deficient mice suffer from multiple signs of autoimmunity likely due to defect in negative selection of tissue-specific antigens (TSA) reactive T cells and maybe absence of TSA-specific Treg. Grafting AIRE deficient thymus on nude mouse recapitulates AIRE deficient phenotype. It has been shown that even co-transplantation of 4 wild-type thymi, which are expected to produce a “normal” Treg compartment, cannot completely rescue autoimmunity (37). In other words, it suggests that Treg fail to control self-reactive T cells when they are constantly produced by the thymus. We can postulate, that Treg compartment can only control a limited frequency of self-reactive T cells above which autoimmunity can manifest. Not tolerant is dominant in this experimental setting.

Overall it seems that there are not much backup in mechanisms controlling tolerance. Example of single mutations leading to autoimmunity are not rare, examples are listed in table 2. This suggests that when a mechanism of central or peripheral tolerance is defective, immune system has difficulties to cope with it and in the worse cases it leads to death of the mice.

It also illustrates the difficulty to keep the balance between tolerance and immune response.

Gene	Human disease	Mouse mutant or knockout	Mechenism of autoimmunity
AIRE	APS-1	K.O.	Decreased expression of TSA. Defect in negative selection of TSA specific T cells
CTLA4	Association with Graves' disease type 1 diabetes and others	K.O.	Failure of T cell anergy and reduced activation threshold of self-reactive T cells
FOXP3	IPEX (scurfy)	K.O. and mutations	Decreased generation of regulatorgy T cells
FAS, FASL	ALPS	Lpr/lpr; gld/gld mutants	Failure of apoptotic death of self-reactive B and T cells
C4 complement protein	Associated with SLE	K.O.	Defective clearance of immune complexes and possible failure of B cell tolrance

Table 2. Siimple genetic traits linked with autimmunity. Adapated from (38).

Materials and methods

Mice

BALB/c, BALB/c RAG2 deficient, Igk HA BALB/c (26)

mice and BALB/c mice expressing a transgenic ãb TCR specific for peptide 111 – 119 of HA (22) were bred under SPF conditions in our animal unit. For the generation of transgenic mice expressing HA under control of the CD11c promoter, the complete coding region of the HA gene of influenza virus A/PR8/34 was PCR amplified from the cDNA clone pGEM-4-HA (kindly provided by Dr. A. Caton, Wistar Institute, Philadelphia, PA). The HA PCR product was inserted as a blunt-end product into the EcoRI site of rabbit b-globin gene of the CD11c-promoter vector as previously described (39), (the CD11c-promoter vector was kindly provided by Dr. T.Brocker, University of Munich, Germany). The plasmid DNA was linearized by XhoI and NotI digestion and injected into fertilized oozytes from BALB/c mice. HA-transgenic mice were selected by PCR analysis using mouse tail DNA as a template and the following HA-specific primers: 5'-TCCCTCAGCTCCTCATAGTC-3' and 5'- GAAAGGACTCTGGATTTCCATG-3'. Of the nine different founder mice, five expressed the HA transgene as determined by RT-PCR and by FACS analysis using a rat anti HA monoclonal antibody (kindly provided by Dr. A. Caton, Wistar Institute, Philadelphia, PA). All 5 founder mice expressed the HA in a CD11c specific manner. The

CD11c-HA founder mouse 22 was used in the experiments described in this manuscript.

Antibodies and flow cytometric analysis

FITC-, PE-, APC- or biotin-conjugated mAb specific for CD4 (L3T4), CD8a(53-6.7), CD69, CD62L, Vβ8 (F23.1) and Vα2 (B20.1) were purchased from BD Biosciences (Allschwil, Switzerland). Streptavidin, conjugated to PE, APC or PE/Cy7 were also purchased from BD Biosiences. Anti-Foxp3 (FJK-16s) and anti-IFNg (XMG1.2) were purchased from ebioscience. The rat anti-TCR-HA clonotype specific mAb 6.5 (22) was purified from the hybridoma supernatant and labeled with biotin in our lab by standard methods. Flow cytometry was performed using a ^{TM}FACS Calibur (BD Biosiences) and data were analyzed using the ^{TM}Cell Quest Pro Software (BD Biosiences).

Histology

Fresh organs were embedded in OCT-compound (Sakura, Zoetermeer, NL), snap frozen and 5mm sections were cut with a cryostat. Sections were air dried for 60 min and then stained with hematoxylin-eosin according to standard methods. To determine bone/cartilage destruction, paws were fixed for 24hrs in 4% paraformaldehyde. After washing for 60 min in PBS and subsequently 60 min H_2O the specimens were decalcified for 3 – 5 days in water containing 0.15M EDTA and 70mM HCL. After extensive washing in water the paws were embedded in OCT-compound, snap frozen and 5-8mm cryosections were prepared. Cryosections were stained with Safranin O according to standard methods.

Immunofluorescence and detection of autoantibodies

Cryostat sections from fresh organs prepared as described above were fixed in acetone for 10 min and then air dried for 60 min. To determine the presence of germinal centers in spleen, sections were incubated with anti-IgM FITC (mAb M41) and biotinylated peanut agglutinin (PNA) (Vector, Burlingame, CA) for 30 min. After 20 min washing in PBS, PNA binding was revealed with texas red conjugated strepavidin (SouthernBiotech, Birmingham, AL). To determine Ig deposits in kidneys, sections were incubated with FITC labeled goat anti-mouse Ig (Jackson Immunoresearch, Milan Analytica, La Roche, CH). IgG autoantibodies against nuclear antigens were detected by an indirect immunofluorescence technique, using a FITC labeled goat ant-mouse IgG (Jackson Immunoresearch, Milan Analytica, La Roche, CH); cryosections of kidneys from Rag2 deficient mice were used as substrate.

Immunizations and detection of anti-PR8 and anti-HA antibodies

Mice were immunized i.v. with UV-inactivated inluenza virus PR8 at a dose of 1000 HAU. Thereafter mice were bled at regular intervals and IgG anti-PR8 antibodies determined by ELISA as previously described (26). IgG anti-HA antibodies were detected by FACS analyses using HA-transfected cell lines as a substrate and FITC-conjugated goat-anti-mouse IgG for detection. For both assays, titers of antibodies are expressed as the highest dilution resulting in readings twice over background.

PMA/ionomycine stimulation

Total lymphocytes were stimulated during 2h in Ionomycine (sigma, I9657) 1μg/ml and Phorbol-12-Myristate-13-acetate (PMA) 5ng/ml at 37°C. After 2h, Brefeldin A (Calbiochem, 524400) was added at 10μg/ml final concentration and cells were incubated 2 more hours. Finally cells were harvested and stained by standard intracellular staining procedure ( ).

Treg treatment

Treg cells were sorted as described using a FACS ARIA and injected in the liver of newborn mice.

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6.2

Selection of Foxp3+ regulatory T cells specific for self-antigen expressed and presented by Aire+ medullary thymic epithelial cells

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Selection of Foxp3$^+$ regulatory T cells specific for self antigen expressed and presented by Aire$^+$ medullary thymic epithelial cells

Katharina Aschenbrenner[1], Louise M D'Cruz[1,3], Elisabeth H Vollmann[1,3], Maria Hinterberger[1], Jan Emmerich[1], Lee Kim Swee[2], Antonius Rolink[2] & Ludger Klein[1]

The parameters specifying whether autoreactive CD4$^+$ thymocytes are deleted (recessive tolerance) or differentiate into regulatory T cells (dominant tolerance) remain unresolved. Dendritic cells directly delete thymocytes, partly through cross-presentation of peripheral antigens 'promiscuously' expressed in medullary thymic epithelial cells (mTECs) positive for the autoimmune regulator Aire. It is unclear if and how mTECs themselves act as antigen-presenting cells during tolerance induction. Here we found that an absence of major histocompatibility class II molecules on mTECs resulted in fewer polyclonal regulatory T cells. Furthermore, targeting of a model antigen to Aire$^+$ mTECs led to the generation of specific regulatory T cells independently of antigen transfer to dendritic cells. Thus, 'routing' of mTEC-derived self antigens may determine whether specific thymocytes are deleted or enter the regulatory T cell lineage.

The idea that the thymic epithelium is critical for the induction of dominant tolerance dates back to the 1980s[1]. The cell type mediating tolerance in those studies remained elusive; however, follow-up studies suggested a subset of CD4$^+$ T cells[2]. It is now widely accepted that 'natural' Foxp3$^+$CD25$^+$ regulatory T cells (T_{reg} cells), the best characterized mediators of dominant tolerance so far, originate in the thymus[3–5].

Early studies suggesting a thymic origin of T_{reg} cells[6,7] have been complemented by T cell receptor (TCR) sequence analyses demonstrating a considerable overlap between thymic and peripheral T_{reg} cell repertoires[8,9]. Findings in mice expressing transgenes encoding both TCR and cognate antigen have bolstered this idea by establishing that expression of self antigen in the thymic epithelium can be sufficient for the generation of antigen-specific T_{reg} cells. Those findings were often (incorrectly) interpreted to mean that recognition of the respective antigen in the context of major histocompatibility complex (MHC) class II on epithelial cells induces T_{reg} cell differentiation. The antigen-presenting cell (APC) that ultimately displayed thymic epithelium–derived antigen was not identified in any of the models. Furthermore, most of those studies did not allow for a distinction between cortical thymic epithelial cells (cTECs) and medullary thymic epithelial cells (mTECs) as a source of antigen[10–15], and only one report suggested involvement of exclusively mTEC-derived antigen[16]. Open issues pertaining to the exact nature of the antigen-expressing and/or antigen-presenting cells involved in T_{reg} cell development are intimately related to the issue of whether specification of T_{reg} cells is an early (cortical) or late (medullary) event during thymocyte development. Studies of polyclonal T cell repertoires have yielded conflicting results, some favoring a cortical specification[17] and others favoring a medullary specification[18] of T_{reg} cell fate.

A separate line of experiments has firmly established that mTECs contribute to T cell tolerance by 'promiscuously' expressing a wide range of otherwise tissue-specific antigens[19,20]. Mutations in the gene encoding the autoimmune regulator Aire, which in epithelial compartments of the thymus is expressed exclusively in the medulla, perturb 'promiscuous' expression of tissue-specific antigens and cause autoimmune polyendocrine syndrome in humans and general autoimmunity in mice[21]. Evidence suggests that tissue-specific antigens expressed in mTECs contribute to central tolerance in an essential way by inducing recessive tolerance (such as clonal deletion of autoreactive thymocytes)[22,23], whereas their eventual function in the induction of dominant tolerance remains speculative. Very limited information is available about the type of APC that ultimately presents mTEC-derived antigens to trigger deletion of antigen-specific thymocytes.

The importance of TEC-derived self antigens for the induction of both dominant and recessive modes of central tolerance is undisputed[24–26]. However, it remains unclear whether thymocyte deletion or the generation of T_{reg} cells resulting from antigen expression in mTECs or TECs in general is triggered in a TEC-autonomous way. This issue is of particular importance because intercellular transfer

[1]Research Institute of Molecular Pathology, 1030 Vienna, Austria. [2]Department of Clinical and Biological Sciences, Center for Biomedicine, University of Basel, CH-4051 Basel, Switzerland. [3]Present addresses: Division of Biological Sciences, University of California, San Diego, La Jolla, California 92093, USA (L.M.D.) and The CBR Institute for Biomedical Research, Department of Pathology, Brigham and Women's Hospital, Harvard Medical School, Boston, Massachusetts 02115, USA (E.H.V.). Correspondence should be addressed to L.K. (klein@imp.univie.ac.at).

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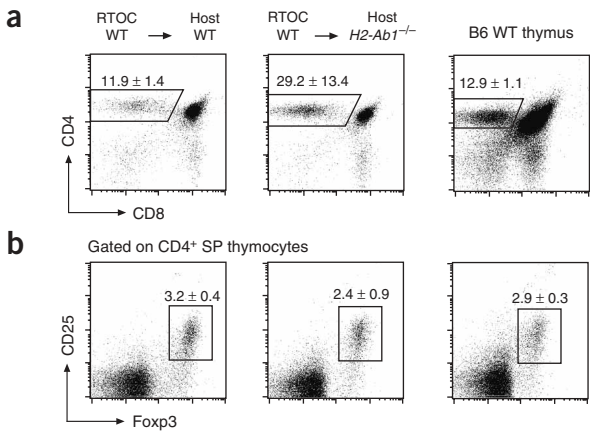

Figure 1 Selection of polyclonal Foxp3$^+$CD25$^+$ T_{reg} cells by MHC class II expressed exclusively on thymic epithelium. RTOCs were generated from CD45$^-$ thymic stromal cells prepared from E14 MHC class II–positive (wild-type) donors. After 48 h of *in vitro* culture, RTOCs were grafted under the kidney capsules of wild-type or MHC class II–deficient (*H2-Ab1*$^{-/-}$) recipients; 8–10 weeks later, single-cell suspensions of grafts were stained with antibodies specific for CD4, CD8, CD25 and intracellular Foxp3. (**a**) Thymocyte subsets in grafts (donors and hosts, above plots) and in thymi of C57BL/6 mice (B6). Wild-type host, $n = 3$; *H2-Ab1*$^{-/-}$ host, $n = 6$; C57BL/6 mice, $n = 5$. (**b**) Expression of surface CD25 and intracellular Foxp3 on gated CD4$^+$ SP thymocytes. Numbers above outlined areas indicate percent (± s.d.) CD4$^+$ SP cells (**a**) or Foxp3$^+$CD25$^+$ cells (**b**). Data are representative of two independent experiments.

of self antigens from epithelial to hematopoietic cells in the thymic microenvironment is a well documented phenomenon[27,28]. In fact, two reports have suggested a central function for thymic dendritic cells (DCs) in the establishment of both recessive and dominant CD4$^+$ T cell tolerance. First, deletion of TCR-transgenic CD4$^+$ thymocytes in response to a model antigen expressed in mTECs requires antigen transfer to hematopoietic cells, most likely DCs[29]. Second, *in vitro* evidence suggests that thymic DCs conditioned with thymic stromal lymphopoietin may promote the generation of T_{reg} cells in the human thymus[30]. Those findings collectively sparked the idea that thymic DCs may serve a dual, possibly mandatory, function in central CD4$^+$ T cell tolerance by inducing negative selection as well as T_{reg} cell generation; in contrast TECs may merely serve as an antigen reservoir. The ensuing mode of tolerance might then be dictated by the maturation state of the DCs[31]. In mechanistic terms, this model may offer a 'missing link' between thymic induction of 'natural' T_{reg} cells and peripheral, new generation of 'induced' T_{reg} cells through subimmunogenic delivery of cognate antigen[32,33]. An alternative view would hold that quantitative and/or qualitative features of intrathymic antigen recognition in the context of DCs versus TECs would favor deletion versus T_{reg} cell differentiation, respectively. We began our study here to clarify the functions of epithelial versus hematopoietic antigen presentation during the generation of T_{reg} cells in mouse thymus. Furthermore, we aimed to explore the potential contribution of mTECs to T_{reg} cell development, with particular emphasis on their potential functions as antigen reservoirs and/or as true APCs.

RESULTS

Selection of polyclonal T_{reg} cells by mTECs

To test whether MHC class II expressed exclusively on TECs was sufficient for the selection of polyclonal T_{reg} cells, we generated chimeric mice in which only TECs expressed MHC class II. To do this, we made use of the fact that reaggregate thymic organ cultures (RTOCs)[34] form a normal thymic microenvironment after engraftment under the kidney capsule[35]. Experiments addressing this with fetal thymic lobes at embryonic day 10 (E10) or treated with deoxyguanosine have been called into question because of the formal caveat that minute amounts of MHC class II–positive hematopoietic cells may have persisted in the graft[31]. To rigorously prevent contamination with 'carryover' hematopoietic cells, we prepared RTOCs from cell suspensions of MHC class II–positive E14 thymi after stringently depleting them of CD45$^+$ hematopoietic cells and transplanted the RTOCs into MHC class II–sufficient C57BL/6 recipient mice (WT→WT) or MHC class II–deficient C57BL/6 recipient mice (WT→*H2-Ab1*$^{-/-}$). Analysis of thymocyte subsets in the grafts after 8–10 weeks showed that selection of Foxp3$^+$CD25$^+$ T_{reg} cells occurred efficiently in WT→*H2-Ab1*$^{-/-}$ and WT→WT chimeras (**Fig. 1**). As these grafts did not differ in their overall cellularity, the somewhat lower frequency of Foxp3$^+$CD25$^+$ T_{regs} among CD4$^+$ single-positive (SP) cells in WT→*H2-Ab1*$^{-/-}$ grafts was more than compensated for in terms of absolute numbers by the much greater frequency of CD4$^+$ SP cells in the absence of MHC class II on hematopoietic cells (**Fig. 1a**). The extent to which CD4$^+$ SP numbers were higher was in good agreement with estimates that normally about 60% of CD4$^+$ SP cells are negatively selected after encounter with self antigen on thymic DCs[36].

Next we sought to delineate the contribution of cortical versus medullary TECs to the generation of polyclonal Foxp3$^+$ T_{reg} cells. We hypothesized that elimination of MHC class II expression on medullary TECs would demonstrate involvement of mTECs in T_{reg} cell selection. To test this idea, we again grafted RTOCs into *H2-Ab1*$^{-/-}$ mice. This time, the RTOCs contained a 1:1 mixture of CD45$^-$ cells from wild-type and *H2-Ab1*$^{-/-}$ E14 thymi. Flow cytometry of these mixed RTOCs at 8–10 weeks after grafting confirmed that bulk T cell development was efficiently supported and histological examination confirmed that the grafts were properly compartmentalized into cortical and medullary regions (data not shown). Notably, whereas the cortex contained a random mixture of MHC class II–positive and MHC class II–negative input cells, some medullary 'islands' formed that, because medullary regions arise clonally from individual precursors[35], were composed of either MHC class II–positive or MHC class II–negative cells (**Fig. 2a** and **Supplementary Fig. 1** online). An

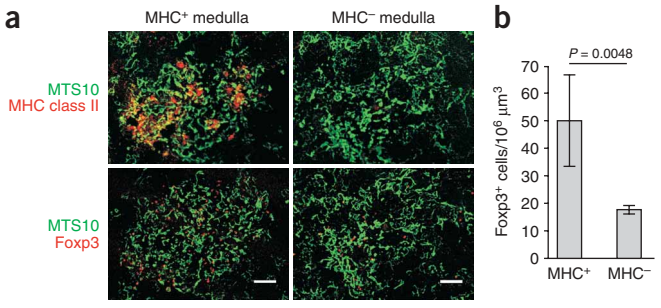

Figure 2 *In situ* detection of Foxp3$^+$ cells in MHC class II–positive or MHC class II–negative medullary 'islands' in mixed RTOCs. (**a**) Staining of serial cryosections through entire grafts for medullary areas (MTS10) and MHC class II to identify MHC class II–positive (MHC$^+$) or MHC class II–negative (MHC$^-$) medullary regions. Top row, MTS10 (green) and MHC class II (red); bottom row, MTS10 (green) and Foxp3 (red) in an adjacent section. Scale bars, 50 μm. (**b**) Quantification of Foxp3$^+$ cells in medullary 'islands'; two to four exclusively MHC class II–positive or MHC class II–negative medullary regions could be identified in each of a total of three mixed RTOCs.

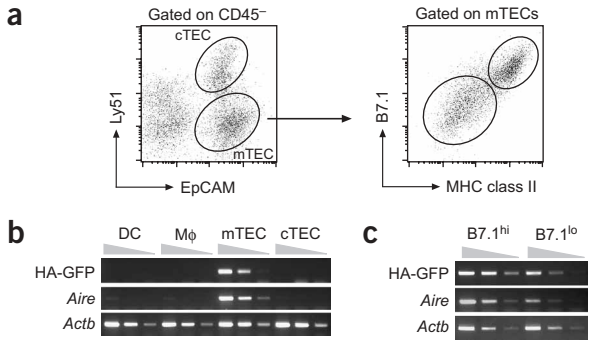

Figure 3 Medulla-specific expression of HA-GFP with bacterial artificial chromosome transgenesis. (**a**) Sorting gates (outlined ovals) used for the isolation of cTECs (CD45⁻EpCAM⁺Ly51⁺) and mTECs (CD45⁻EpCAM⁺Ly51⁻; left) as well as MHC class II–high B7.1^{hi} and MHC class II–low B7.1^{lo} mTECs (right). (**b**,**c**) RT-PCR analysis of the expression of transgenic HA-GFP and endogenous *Aire* in thymic stromal cell subsets (above lanes). Serial fivefold dilutions (wedges) of cDNA prepared from sorted cells were normalized to *Actb* transcripts (encoding β-actin) before PCR amplification of HA-GFP and *Aire* transcripts. Mϕ, macrophage. Data are representative of at least two experiments.

experimental limitation of our approach was that the clonally derived 'spheres' expanded over time and thus ultimately became confluent; a considerable fraction of medullae consisted of a 'patchwork' of MHC class II–positive and MHC class II–negative regions (data not shown). Therefore, we used serial sectioning to identify medullary regions that were composed exclusively of either MHC class II–positive or MHC class II–negative cells. We then quantified Foxp3$^+$ cells per volume unit in MHC class II–positive or MHC class II–negative medullae (**Fig. 2b**) and found significantly more Foxp3$^+$ T$_{reg}$ cells in MHC class II–positive medullary regions. These results collectively supported the idea that interactions of developing thymocytes with MHC class II on mTECs are critical for the development of polyclonal T$_{reg}$ cells.

Antigen-specific differentiation of T$_{reg}$ cells

The function of specific antigen remains unclear when studying polyclonal T$_{reg}$ cells. To address the function of mTEC-derived self antigen in the generation of antigen-specific T$_{reg}$ cells, we exploited the specific expression pattern of *Aire* to produce a model antigen exclusively in mTECs. We generated a bacterial artificial chromosome transgenic construct containing 50 kilobases of 5′ region and 122 kilobases of 3′ region flanking *Aire*, in which we replaced the *Aire* start codon with an open reading frame encoding a fusion protein of influenza hemagglutinin (HA) and green fluorescent protein (GFP). Preliminary characterization of two transgenic founder lines indicated they had identical phenotypes, and we chose one founder line (called 'AIRE-HA' here) for all further analyses. We analyzed the expression pattern of the transgene in highly purified thymic stromal cell populations by RT-PCR (**Fig. 3**). Expression of the HA-GFP cassette faithfully reflected that of endogenous *Aire*, with substantial quantities in mTECs and faint signals in DCs. Neither the transgene nor endogenous *Aire* was expressed in cTECs (**Fig. 3b**). In subfractionated MHC class II–high B7.1^{hi} and MHC class II–low B7.1^{lo} mTECs[37], transgene expression again closely mimicked that of endogenous *Aire*, with substantially more in MHC class II–high B7.1^{hi} mTECs (**Fig. 3c**). Attempts to directly exploit GFP fluorescence for analyses of expression by histology or flow cytometry were hampered by insufficient expression of the transgene (data not shown).

To visualize the fate of hemagglutinin-specific CD4$^+$ T cells developing in AIRE-HA thymi, we crossed AIRE-HA mice with 'TCR-HA' mice expressing a transgenic TCR that recognizes a peptide of amino acids 107–119 of influenza hemagglutinin (HA(107–119)) in the context of I-E^d (ref. 38). The thymic cellularity of AIRE-HA × TCR-HA mice was not significantly different from that of mice expressing only the TCR-HA transgene ($74.6 \times 10^6 \pm 25 \times 10^6$ ($n = 11$) versus $84 \times 10^6 \pm 19 \times 10^6$ ($n = 8$); $P = 0.58$), and the frequency of CD4$^+$ SP cells was essentially identical (**Fig. 4a**). In the CD4$^+$ SP population, the frequency of cells expressing the TCR-HA clonotype was lower in AIRE-HA × TCR-HA mice than in TCR-HA control mice ($13.4 \times 10^6 \pm 7.8 \times 10^6$ versus $33.4 \times 10^6 \pm 8.5 \times 10^6$). About half of those CD4$^+$ SP TCR-HA$^+$ cells expressed CD25, suggesting efficient differentiation into the T$_{reg}$ cell lineage (**Fig. 4b**). In accordance with that observation, 23.9% ± 6.8% ($3.5 \times 10^5 \pm 0.5 \times 10^5$) of CD4$^+$TCR-HA$^+$ cells in AIRE-HA × TCR-HA mice expressed Foxp3, whereas the frequency of CD4$^+$TCR-HA$^+$Foxp3$^+$ cells in TCR-HA mice was negligible (corresponding to $7 \times 10^2 \pm 5 \times 10^2$ cells; **Fig. 4c**). *In vitro* suppression assays showed that sorted CD4$^+$ SP TCR-HA$^+$CD25$^+$ thymocytes from AIRE-HA × TCR-HA mice efficiently inhibited the proliferation of naive CD4$^+$ TCR-HA$^+$ T cells obtained

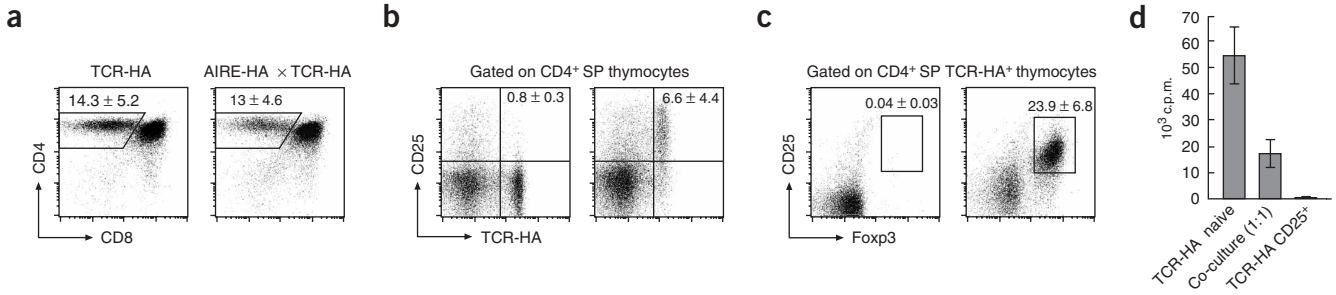

Figure 4 Generation of Foxp3$^+$CD25$^+$ T$_{reg}$ cells in AIRE-HA × TCR-HA mice. (**a**) Frequency of CD4$^+$ SP thymocytes in TCR-HA mice ($n = 8$) and AIRE-HA × TCR-HA mice ($n = 11$). (**b**) Frequency of CD25$^+$ cells in TCR-HA$^+$ CD4$^+$ SP populations. (**c**) Expression of Foxp3 in TCR-HA$^+$ cells, assessed by intracellular staining. Numbers above outlined areas (**a**,**c**) and in quadrants (**b**) indicate the average frequency (± s.d.) of CD4$^+$ SP thymocytes (**a**), of TCR-HA$^+$ CD25$^+$ cells among CD4$^+$ SP cells (**b**) or of Foxp3$^+$CD25$^+$ cells in the TCR-HA$^+$ population (**c**). Mouse strains, above top plots. (**d**) Proliferation of naive peripheral TCR-HA$^+$ CD4$^+$ T cells from TCR-HA mice deficient in recombination-activating gene and TCR-HA$^+$CD25$^+$ CD4$^+$ SP thymocytes from AIRE-HA × TCR-HA mice; cells were cultured alone or together in the presence of syngeneic splenocytes and HA(107–119) and proliferation was assessed by scintillation counting after a pulse with [^{3}H]thymidine for the last 20 h of a 96-hour incubation period. Data are representative of 8 TCR-HA mice and 11 AIREHA × TCR-HA mice (**a**–**c**) or are representative of three independent experiments (**d**).

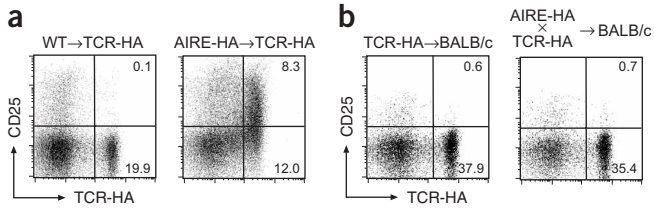

Figure 5 Expression of HA in AIRE-HA thymic epithelium but not expression in hematopoietic cells recapitulates the phenotype of AIRE-HA × TCR-HA mice. (**a**) Phenotype of CD4$^+$ SP thymocytes in wild-type or AIRE-HA thymi grafted under the kidney capsules of TCR-HA recipients, analyzed 8–12 weeks after transplantation of deoxyguanosine-treated E14 thymi. (**b**) Phenotype of CD4$^+$ SP thymocytes in BALB/c wild-type recipients of TCR-HA or AIRE-HA TCR-HA bone marrow. Numbers in quadrants indicate percent TCR-HA$^+$CD25$^+$ cells (top right) or TCR-HA$^+$CD25$^-$ cells (bottom right) among CD4$^+$ SP cells. Data are representative of two independent experiments with at least seven grafts each (**a**) or ten chimeras each in two independent experiments (**b**).

from TCR-HA mice deficient in recombination-activating gene 2 (**Fig. 4d**). The peripheral phenotype of AIRE-HA × TCR-HA mice reflected the situation in the thymus; a distinct TCR-HA$^+$CD25$^+$ subset was present among CD4$^+$ T cells (**Supplementary Fig. 2** online).

To formally demonstrate that mTEC-derived antigen was causing T_{reg} cell differentiation in this mouse model system, we grafted deoxyguanosine-treated thymi from E14 AIRE-HA fetuses into TCR-HA mice. This experiment faithfully recapitulated the phenotype of AIRE-HA × TCR-HA mice (**Fig. 5a**). In contrast, TCR-HA$^+$ CD4$^+$ SP cells in AIRE-HA × TCR-HA→WT bone marrow chimeras had a phenotype identical to that of control TCR-HA→WT chimeras, indicating that *Aire* locus–driven expression of HA in hematopoietic cells, if existent, was too low to measurably affect T cell development (**Fig. 5b**).

The TCR-HA system has been used in many settings to study T_{reg} cell development[11,12,14,15]. Therefore, we addressed whether T_{reg} cell differentiation after intrathymic antigen encounter might possibly reflect an inherent predisposition of TCR-HA$^+$ thymocytes. Targeting of HA to DCs with a transgenic construct driven by the promoter of the gene encoding CD11c[39] resulted in complete deletion of TCR-HA$^+$ thymocytes, indicating that TCR-HA$^+$ thymocytes can be deleted in the thymus when appropriate conditions are met (**Supplementary Fig. 3** online).

We next addressed whether our findings in the AIRE-HA × TCR-HA model could be reproduced with a second model antigen and another TCR transgene. We used a fusion protein of HA and ovalbumin (OVA) containing the OVA epitope (amino acids 323–339) recognized by the DO11.10 TCR and targeted the fusion protein to mTECs under control of the *Aire* locus with a bacterial artificial chromosome transgenic approach similar to that described above. The T cell phenotype of the resultant AIRE-OVA × DO11.10 mice strongly resembled that of AIRE-HA × TCR-HA mice; we detected Foxp3$^+$CD25$^+$ T_{reg} cells expressing the DO11.10 TCR in the thymus and periphery (**Supplementary Fig. 4** online). These data collectively indicated that self antigen expressed in mTECs can efficiently induce the differentiation of Foxp3$^+$CD25$^+$ T_{reg} cells.

DCs and mTECs present mTEC-derived antigen

Our experiments so far had established that expression of HA exclusively in mTECs was sufficient for the differentiation of TCR-HA$^+$ thymocytes into T_{reg} cells. The issue remained open, however, of whether this confined expression pattern coincided with a similarly restricted pattern of HA(107–119) presentation exclusively by mTECs or whether mTEC-derived HA was eventually taken up, processed and presented by thymic DCs. To directly measure presentation of HA(107–119) by various thymic stromal cell populations from AIRE-HA mice, we used the A5 T cell hybridoma, which expresses the clonotypic TCR-HA and carries a GFP reporter transgene driven by elements controlling the gene encoding interleukin 2 (ref. 40). Titration of HA(107–119) into cocultures of A5 cells and wild-type thymic DCs showed a dynamic range of GFP expression crossing three orders of magnitude and starting from a detection limit of approximately 10 ng/μl (**Fig. 6a**). The dose-response curve obtained with wild-type mTECs as APCs was essentially superimposable (**Fig. 6a**).

Antigen presentation assays with cTECs, mTECs and DCs isolated *ex vivo* from AIRE-HA mice showed strong stimulation of A5 cells by mTECs and weak yet significant stimulation by DCs, but no significant stimulation by cTECs (**Fig. 6b**). To confirm that presentation of HA(107–119) by DCs was indeed due to capture of mTEC-derived antigen rather than to processing of endogenously expressed antigen, we generated a series of bone marrow chimeras. Indeed, thymic DCs isolated from WT→AIRE-HA chimeras stimulated A5 cells as efficiently as did those from control AIRE-HA→AIRE-HA chimeras; in contrast, DCs obtained from AIRE-HA→WT chimeras did not stimulate A5 cells (**Supplementary Fig. 5** online).

Presentation of HA(107–119) by MHC class II–high B7.1^{hi} and MHC class II–low B7.1^{lo} mTECs isolated from AIRE-HA mice reflected in a more pronounced way the difference between these 'mature' and 'immature' mTECs in terms of mRNA quantities (**Fig. 3c**). Although MHC class II–high B7.1^{hi} mTECs strongly stimulated A5 cells, presentation by MHC class II–low B7.1^{lo} mTEC was barely measurable (**Fig. 6c**). These results collectively indicated that antigen exclusively expressed by mTECs can be presented concomitantly by mTECs themselves as well as by DCs.

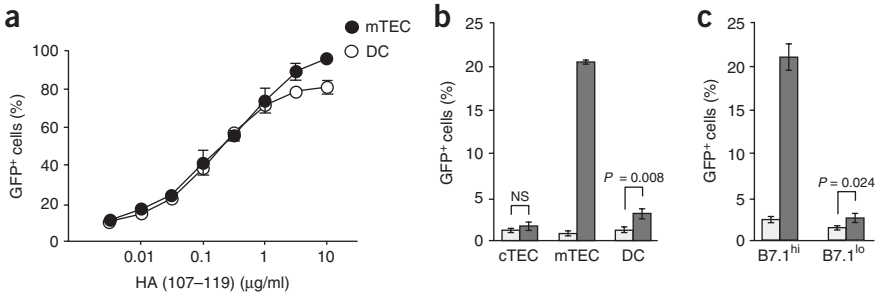

Figure 6 Display of HA(107–119) by isolated mTECs and DCs but not by cTECs from AIRE-HA thymi. (**a**) Dose-response curve of activity of the GFP reporter construct in A5 T hybridoma cells stimulated by wild-type mTECs or DCs (key) plus HA(107–119) (concentration, horizontal axis). The frequency of GFP$^+$ cells after 17 h of coculture was determined by flow cytometry. Data are representative of two experiments. (**b**) Stimulation of A5 cells after 17 h of culture together with cTECs, mTECs or DCs from AIRE-HA thymi (dark gray) or wild-type thymi (light gray). NS, not significant. Data are representative of more than five independent experiments. Stimulation of A5 cells after 17 h of culture together with MHC class II-high B7.1^{hi} and MHC class II–low B7.1^{lo} mTECs from AIRE-HA thymi (dark gray) or wild-type thymi (light gray). Data are representative of two independent experiments.

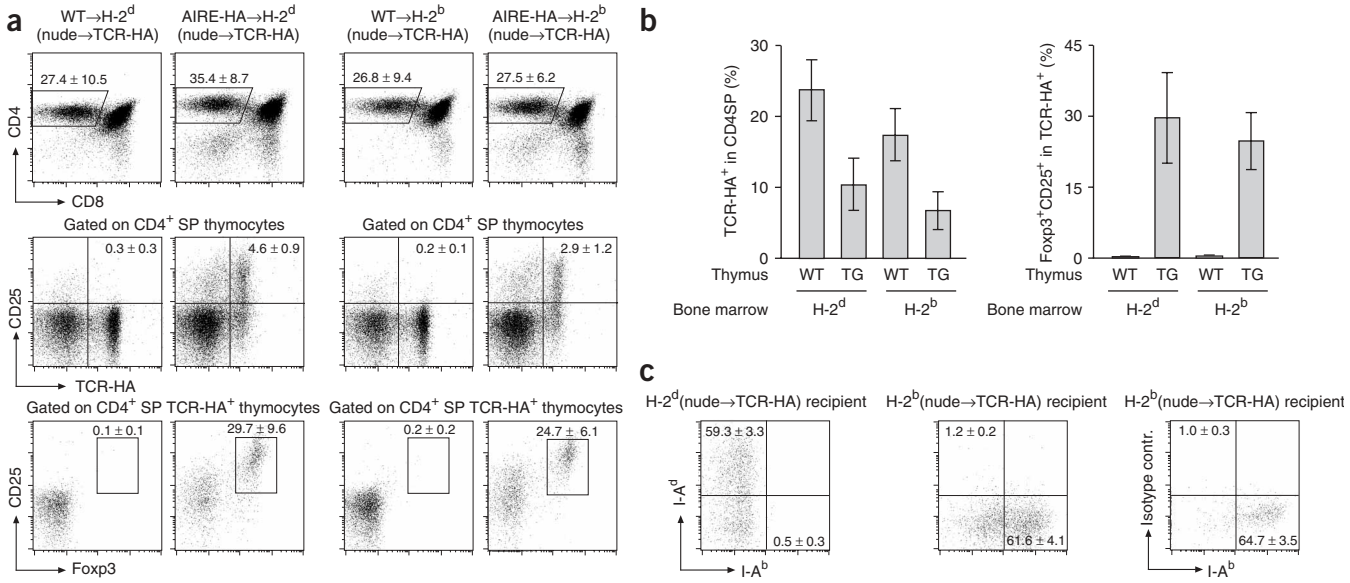

Figure 7 Efficient generation of Foxp3$^+$CD25$^+$ T_{reg} cells independently of cross-presentation by hematopoietic cells. (**a**) Cellular composition of deoxyguanosine-treated AIRE-HA or wild-type thymi (H-2^d) at 6–10 weeks after being grafted into H-2^d(nude→TCR-HA) or H-2^b(nude→TCR-HA) recipients. Numbers above outlined areas or in top right quadrants indicate the frequency (± s.d.) of CD4$^+$ SP cells (top row), of TCR-HA$^+$CD25$^+$ cells in the CD4$^+$ SP population (middle row) or of Foxp3$^+$CD25$^+$ cells among gated CD4$^+$ SP TCR-HA$^+$ cells (bottom row). All plots show data after gating on CD45.1$^+$ cells to identify TCR-HA thymocytes (nude mice and thymus grafts were CD45.2$^+$). Data were calculated from three independent experiments (WT→H-2^d(nude→TCR-HA), $n = 8$ chimeras; AIRE-HA→H-2^d(nude→TCR-HA), $n = 8$ chimeras; WT→H-2^b(nude→TCR-HA), $n = 7$ chimeras; AIRE-HA→H-2^b(nude→TCR-HA), $n = 19$ chimeras). The cellularity of grafts varied considerably within experimental groups, but was not substantially different between groups (data not shown). (**b**) Summary of the data in **a**. Left, percent TCR-HA$^+$ cells among CD4$^+$ SP cells; right, percent Foxp3$^+$CD25$^+$ cells among gated CD4$^+$ SP TCR-HA$^+$ cells. TG, AIRE-HA transgenic. (**c**) Carryover of donor-derived CD11c$^+$ DCs in deoxyguanosine-treated grafts. Expression of I-A^d and I-A^b on gated CD45$^+$CD11c$^+$ cells in H-2^d(nude→TCR-HA) or H-2^b(nude→TCR-HA) recipients was analyzed in thymic cell suspensions prepared by enzymatic digestion. Numbers in quadrants indicate percent cells (± s.d.) in each. Data are representative of at least four grafts each.

T_{reg} cell selection independent of hematopoietic cells

The detection of mTEC-derived antigen presented on thymic DCs raised the issue of whether the generation of T_{reg} cells in AIRE-HA × TCR-HA mice was orchestrated autonomously by mTECs or by DCs that captured and presented mTEC-derived antigen. If the latter were true, experimental interference with the capacity of DCs to present antigen would abolish T_{reg} cell differentiation. The fact that mice with targeted mutation of the gene encoding I-E^d, to which the TCR-HA is restricted, have not been generated rendered a straightforward approach with I-E^d-deficient bone marrow impossible. Therefore, as an alternative strategy, we made use of the fact that TCR-HA$^+$ T cells neither 'alloreact' to C57BL/6 (H-2^b) APCs nor recognize cognate HA peptide in the context of I-A^b (data not shown).

We generated bone marrow chimeras with athymic mice by reconstituting irradiated C57BL/6 nude mice (H-2^b) with H-2^b TCR-HA bone marrow (called 'H-2^b(nude→TCR-HA)' here) or BALB/c nude mice (H-2^d) with H-2^d TCR-HA bone marrow (called 'H-2^d(nude→TCR-HA)' here). Subsequently, we engrafted those chimeras with deoxyguanosine-treated AIRE-HA thymi (AIRE-HA→H-2^b(nude→TCR-HA) and AIRE-HA→H-2^d(nude→TCR-HA)) or wild-type thymi (WT→H-2^b(nude→TCR-HA) and WT→H-2^d(nude→TCR-HA)) obtained from BALB/c (H-2^d) E14 donors. Positive selection of CD4$^+$ TCR-HA$^+$ cells would in all situations be mediated by H-2^d thymic epithelium. The fate of TCR-HA$^+$ cells in AIRE-HA→H-2^d(nude→TCR-HA) grafts was expected to emulate that of AIRE-HA × TCR-HA mice. In AIRE-HA→H-2^b(nude→TCR-HA) grafts, however, T_{reg} cell development, depending on its requirement for the presentation of mTEC-derived antigen by hematopoietic APCs, would or would not occur.

We analyzed grafts 6–8 weeks after transplantation. The size of the CD4$^+$ SP compartment was not very different among the experimental groups (**Fig. 7a**). Somewhat unexpectedly, we consistently detected fewer TCR-HA$^+$ cells among CD4$^+$ SP cells for WT→H-2^b(nude→TCR-HA) thymi than for WT→H-2^d(nude→TCR-HA) thymi (in the absence of cognate antigen; **Fig. 7b**). We deem it likely that this was a passive, indirect effect due to less-stringent DC-imposed 'censorship' of thymocytes expressing endogenously rearranged TCRs (such as TCR-HA$^-$ CD4$^+$ SP cells) in thymi with a MHC-mismatched (H-2^b) hematopoietic compartment. Given that, the phenotypes of AIRE-HA→H-2^d(nude→TCR-HA) thymi and AIRE-HA→H-2^b(nude→TCR-HA) thymi were very similar. The frequency of TCR-HA$^+$ cells among CD4$^+$ SP cells was in both cases lower than that in control thymi (**Fig. 7b**). TCR-HA$^+$ CD4$^+$ SP populations in both experimental groups that received AIRE-HA thymi contained similarly high frequencies of Foxp3$^+$CD25$^+$ cells, whereas only minute populations of cells with that phenotype were detectable in the absence of cognate antigen (**Fig. 7a**,**b**).

To verify expression of the expected MHC haplotypes by hematopoietic APCs, we digested one third of each graft with collagenase and analyzed the resulting cell suspensions by flow cytometry. Most DCs from H-2^b(nude→TCR-HA) recipients expressed I-A^b, whereas cells expressing I-A^d were essentially undetectable (**Fig. 7c**). These data confirmed efficient seeding of thymic grafts with host-derived hematopoietic APCs. In summary, our findings indicate that T_{reg} differentiation of TCR-HA$^+$ thymocytes resulting from expression of HA exclusively in mTECs did not require cross-presentation of HA by thymic DCs but instead was autonomously mediated by mTECs.

DISCUSSION

Using two complementary approaches, we have provided evidence here for an autonomous function of mTECs, which act concomitantly as antigen-expressing and antigen-presenting cells in T_{reg} cell generation. Our initial experiments with RTOCs from material rigorously purged of hematopoietic cells provided unambiguous evidence in favor of the idea that TECs are sufficient to support polyclonal T_{reg} cell development. Our observations support a model whereby TEC-thymocyte interactions shape the T_{reg} cell compartment and dominant tolerance, whereas hematopoietic APCs (mainly thymic DCs) mediate deletional tolerance.

A study of the selection of 'superantigen-reactive' T_{reg} cells in the absence or presence of MHC class II on hematopoietic cells reached similar conclusions in terms of the general function of the thymic epithelium during T_{reg} cell development[41]. However, delineation of the respective contribution of medullary versus cortical TECs remained experimentally challenging. When MHC class II expression is restored in MHC class II–deficient mice with a purportedly cTEC-specific transgene, a functional $CD25^+CD4^+$ T cell pool of 'normal' size is selected[17]. Those results suggest that interactions of developing thymocytes with MHC class II on cTECs are sufficient to support T_{reg} cell differentiation. However, in that system it remains unclear whether mTECs truly lack MHC class II; this caveat and concern is particularly valid, given the notorious propensity of mTECs to 'ectopically' express transgenes[26].

Taking advantage of the clonal origin of medullary 'islands', we generated chimeric RTOCs in which individual medullae either expressed or lacked MHC class II (ref. 35). The significantly fewer $Foxp3^+$ T_{reg} cells in medullae lacking MHC class II supported the idea of involvement of mTEC-thymocyte interactions in generating the polyclonal T_{reg} cell pool. Several scenarios might account for the residual $Foxp3^+$ cells in MHC class II–deficient medullae. First, small clones of MHC class II–positive mTEC positioned adjacent to MHC class II-negative regions may have disappeared eventually, whereas their 'footprint' in the local T_{reg} cell compartment may have persisted for some time. This dynamic nature of medulla morphology over time has been reported before[42,43]. Second, the possibility of lateral movement of $Foxp3^+$ cells generated in more distant MHC class II–positive medullae cannot be formally excluded. Finally, initially stochastic (antigen-independent) specification of T_{reg} cell fate may initially occur in the cortex. After migration of thymocytes from the cortex to the medulla, interactions with cognate antigen on mTECs may then serve to selectively induce the survival and/or population expansion of otherwise short-lived precursors.

The phenotype of mice deficient in tumor necrosis factor receptor–associated factor 6 (TRAF6) supports the idea that mTEC-thymocyte interactions are critical for T_{reg} cell generation[44]. Absence of TRAF6 from the thymic epithelium is associated with general autoimmunity, most likely as a result of a disorganized medullary compartment lacking 'mature' $AIRE^+$ mTECs, the exact cell type to which HA was targeted in our model. The $CD4^+$ SP compartment of TRAF6-deficient mice was mostly devoid of $CD25^+$ T_{reg} cells. A complementary study of the involvement of TRAF6 in T cells has shown that this lack of T_{reg} cells is not due to a T cell–intrinsic requirement for TRAF6, as functional T_{reg} cells arise when TRAF6 is ablated specifically and only in T cells[45]. Our findings may explain why the autoimmunity caused by TRAF6 deficiency in thymic epithelium seems more severe than that in AIRE-deficient mice[20]. AIRE deficiency leads to lower expression of peripheral antigens in otherwise apparently normal 'mature' mTECs[23]. TRAF6 deficiency more profoundly results in a complete absence of such cells and thus also affects any tolerogenic function of mTECs beyond their function as a reservoir of tissue-specific antigens.

A chief limitation inherent to analysis of the polyclonal T_{reg} cell repertoire is that the function of specific antigen during T_{reg} cell selection remains mostly obscure. Therefore, we complemented our observations with studies in systems involving defined antigen specificity. Targeting of HA as well as OVA to mTECs efficiently directed TCR-transgenic $CD4^+$ T cells of the cognate TCR specificity into the T_{reg} cell lineage, whereas DC-specific expression of HA led to the deletion of $TCR\text{-}HA^+$ thymocytes.

Those data suggested that the APC type specifies the outcome of the recognition of self antigen, with the caveat that expression of self antigen in mTECs cannot necessarily be interpreted to mean that the ensuing mode of tolerance is in fact a result of antigen presentation by mTECs[29]. Indeed, direct assessment of antigen presentation in the AIRE-HA model demonstrated several salient features of antigen routing in this system. First, both mTECs and DCs, but not cTECs, presented substantial amounts of HA(107–119). Second, presentation of HA(107–119) by DCs was the result of acquisition and processing of mTEC-derived antigen. Third, presentation by mTECs was most likely due to endogenous MHC class II loading rather than antigen transfer in the mTEC compartment. Regardless of their limited ability to process exogenous antigens, mTECs presented HA(107–119) far more efficiently than did DCs, which are arguably better indicators of freely available antigen[46].

Using chimeras with MHC-mismatched thymi and bone marrow, we ruled out the possibility that transfer of mTEC-derived antigen to DCs was essential for the generation of $TCR\text{-}HA^+$ T_{reg} cells. This finding contrasts with the strict requirement for transfer of mTEC-derived antigen to hematopoietic cells during deletion of OT-II $CD4^+$ thymocyes in the 'RIP-mOVA' model[29]. These findings collectively support the idea that the modalities of routing of mTEC-derived self antigens are indeed a critical parameter in specifying recessive or dominant mechanisms of tolerance. We deem it likely that such considerations might likewise apply to different epitopes derived from a given self antigen.

How likely is it that the nature of the stromal cell presenting mTEC-derived antigen (mTECs themselves versus 'cross-presenting' DCs) is the sole determinant of whether recessive or dominant tolerance is induced? Differentiation of $TCR\text{-}HA^+$ cells into the T_{reg} cell lineage in AIRE-HA × TCR-HA mice coincided with much lower absolute numbers of these cells than in TCR-HA control mice. According to a strictly qualitative model, this may have occurred through deletion of a certain fraction of $TCR\text{-}HA^+$ cells after antigen recognition on DCs concomitant with T_{reg} cell selection by mTECs. However, elimination of the capacity of DCs to present mTEC-derived antigen did not restore the frequency of $TCR\text{-}HA^+$ cells. Thus, it seems that even in conditions in which HA is presented exclusively by mTECs, deletion and T_{reg} cell selection occur 'side by side'. Several mutually nonexclusive explanations exist. First, it is possible that the strength of agonistic signals delivered by mTECs in our model is close to a threshold that may distinguish deletion from T_{reg} cell selection; hence, small fluctuations in antigen expression in mTECs may 'tip the balance' in favor of one or the other mode of tolerance. Second, functional heterogeneity in the mTEC compartment (for example, varying amounts of costimulatory molecules) may influence the outcome of deletion versus T_{reg} cell fate specification. Evidence suggests that CD28-B7 interactions may be involved[47–50]. Finally, the possibility of stochastic heterogeneity among thymocytes, even those expressing an identical TCR, regarding the predisposition to

differentially respond to antigenic stimuli delivered by mTECs cannot be ruled out.

Our conclusion that interactions with AIRE$^+$ mTECs shape the T_{reg} cell repertoire is not incompatible with the fact that the autoimmunity in AIRE-deficient mice has been ascribed mainly to a defect in deletional rather than dominant tolerance[23]. Mutations in *Aire* may change the profile of peptide–MHC class II complexes displayed on mTECs in a very subtle way. At the same time, *Aire*-deficient mTECs can still be expected to present a plethora of self antigens and thus may contribute to the generation of a sufficiently diverse T_{reg} cell repertoire. Consistent with that idea, even mice expressing a single MHC class II–bound peptide have a T_{reg} cell repertoire of considerable diversity[9]. It remains unclear in how far antigen-specific 'holes' in the T_{reg} cell repertoire may eventually predispose to autoimmunity.

We propose that mTECs provide a critical niche that fosters T_{reg} cell development. Based on considerations pertaining to T_{reg} cell ontogeny and positioning in the thymic microenvironment, the existence of such a niche has been postulated before, although its exact nature (hematopoietic or epithelial) has remained unclear[18]. What is the function of this niche? At first, the enormous increase in the number of antigen-specific T_{reg} cells triggered by the presentation of endogenously expressed antigen by mTECs may suggest *de novo* induction of a T_{reg} cell phenotype. However, our observations are also compatible with a stochastic component of T_{reg} cell development[51]. Thus, selection and/or population expansion of stochastically 'primed' T_{reg} cell precursors may require, in addition to agonistic antigen, external cues specifically provided by mTECs. Deciphering the molecular 'signature' of this mTEC niche in terms of critical surface molecules and/or soluble factors should provide additional insights into T_{reg} cell development. Signaling via CD28 (ref. 49) as well as through the thymic stromal lymphopoietin receptor[52] has been shown to directly induce Foxp3 expression in thymocytes *in vitro*. Consistent with our proposition, their ligands (B7 and thymic stromal lymphopoietin, respectively) are abundantly provided by mTECs.

METHODS

Mice. Animals were bred in the animal facility of the Research Institute of Molecular Pathology in individually ventilated cages. C57BL/6 nude and BALB/c nude mice were from Taconic; other strains were from 'in-house' breeding colonies. All animal studies were approved by local authorities (MA58) and were done according to Austrian regulations. The generation of transgenic mice is described in the **Supplementary Methods** online.

Antigen presentation assay. Thymic stromal cells (2×10^4) from wild type or AIRE-HA mice were cultured together with A5 T hybridoma cells (2×10^4) in 200 μl Iscove's modified Dulbecco's medium (IMDM) supplemented with 1% (vol/vol) FCS in 96-well round-bottomed plates. Then, 17 h later, stimulation was measured by flow cytometry of GFP reporter expression on gated TCR-HA$^+$CD4$^+$ cells. Equal input numbers of APCs (except in experiments with B7.1^{hi} and B7.1^{lo} mTEC subsets) were ensured by verification that similar stimulation was achieved with saturating amounts (10 μg/ml) of exogenous HA(107–119).

Suppression assays. Sorted CD25$^+$TCR-HA$^+$ CD4$^+$ SP T cells (2×10^4) from AIRE-HA thymi and/or naive TCR-HA$^+$ CD4$^+$ T cells (2×10^4) enriched by magnetic-activated cell sorting from spleen and lymph nodes of TCR-HA mice deficient in recombination-activating gene were cultured together with irradiated (3,000 rads) BALB/c splenocytes (2×10^5) in the presence of 10 μg/ml of HA(107–119). Proliferation was measured by scintillation counting after cells were pulsed with 1 μCi [^{3}H]thymidine per well for the last 20 h of a 96-hour incubation period.

Preparation of thymic stroma. Thymi from 2-week-old mice were cut into small pieces and were digested at 37 °C in IMDM containing 0.2 mg/ml of collagenase (Roche), 0.2 mg/ml of dispase I (Roche), 2% (vol/vol) FCS, 25 mM HEPES, pH 7.2, and 25 μg/ml of DNase I, followed by incubation for 5 min in 5 mM EDTA. Cells were washed and were resuspended in Percoll (ρ, 1.115; GE Healthcare). A discontinuous gradient was then generated by the addition of a layer of Percoll (ρ, 1.050) followed by a layer of PBS on top of this cell suspension. Gradients were spun for 30 min at 1,350*g* at 4 °C, and low-density cells were collected from the upper interface, washed and stained for sorting by flow cytometry.

RTOC. Single-cell suspensions of E14 fetal lobes were prepared by enzymatic digestion (described above). Samples were depleted of CD45$^+$ cells with biotinylated CD45-specific monoclonal antibody and streptavidin MACS beads (Miltenyi Biotech) according to standard procedures. Cells were 'spun down' and were resuspended at a density of approximately 1×10^6 cells/μl. Drops of 0.2–0.4 μl were deposited onto 0.45-μm nylon membranes (Millipore) supported by Gelfoam sponges (Pharmacia & Upjohn) in six-well plates containing 3 ml IMDM and 10% (vol/vol) FCS. RTOCs were incubated for 48 h before transplantation.

Deoxyguanosine treatment. E14 thymic lobes were placed on 0.45-μm membrane filters (Millipore) supported by Gelfoam (Pharmacia&Upjohn) and were incubated for 5 d with 10% (vol/vol) IMDM supplemented with 1.35 mM 2-deoxyguanosine before transplantation.

Immunofluorescence. Frozen sections 11 μm in thickness were fixed in cold acetone, were washed and were blocked for 20 min with 10% (vol/vol) FCS in PBS, followed by incubation overnight with the appropriate primary antibodies: Foxp3-specific polyclonal rabbit serum, MTS10 supernatant (rat immunoglobulin M) and/or biotinylated antibody to MHC class II (anti–MHC class II; P7/7). Sections were incubated with secondary antibodies for 2 h at 21 °C after being washed three times for 15 min each in 0.1% (vol/vol) Tween 20 in PBS. Secondary reagents were Alexa Fluor 488–conjugated anti-rat immunoblobulin M (Molecular Probes), indocarbocyanine-conjugated anti-rabbit (Jackson ImunoLaboratories) and/or indocarbocyanine-conjugated streptavidin (Jackson ImmunoLaboratories). MHC class II–positive or MHC class II–negative medullae were identified in consecutive sections through entire grafts for verification of the absence of MHC class II–positive cells in three dimensions. The volume of the respective medullary segments was determined as follows: area of the MTS10$^+$ medullary region (in μm^2) × thickness of the section (in μm). The number of Foxp3$^+$ cells per unit volume in MHC class II–positive and MHC class II–negative medullary regions was determined and the mean value was calculated.

Statistical analysis. Statistical significance was assessed by the two-tailed Student's *t*-test.

Note: Supplementary information is available on the Nature Immunology website.

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AUTHOR CONTRIBUTIONS

K.A. generated and analyzed AIRE-HA × TCR-HA mice; L.M.D. did the RTOC experiments; E.H.V. generated and analyzed AIRE-OVA × DO11.10 mice together with J.E.; M.H. contributed to the generation and analysis of nude chimeras; L.K.S. and A.R. generated and analyzed CD11c-HA × TCR-HA mice (**Supplementary Fig. 3**); and L.K. prepared the manuscript.

COMPETING INTERESTS STATEMENT

The authors declare no competing financial interests.

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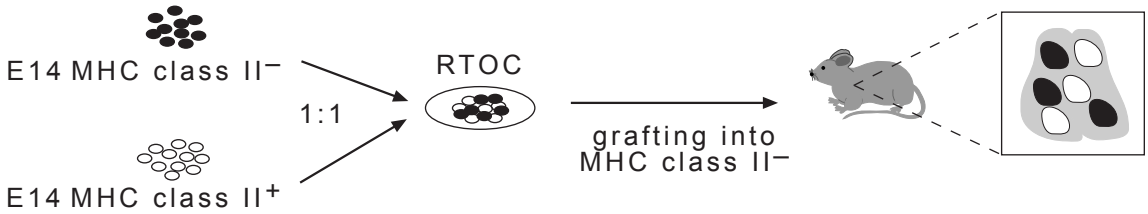
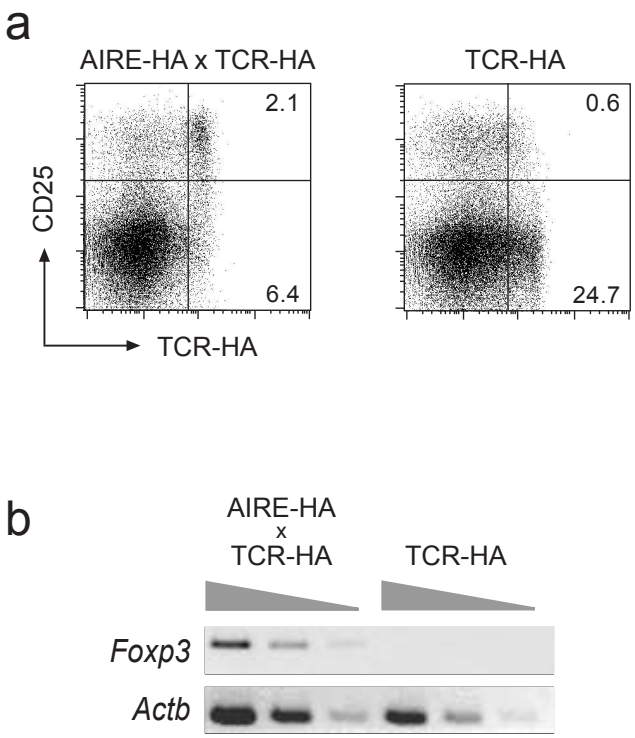
a



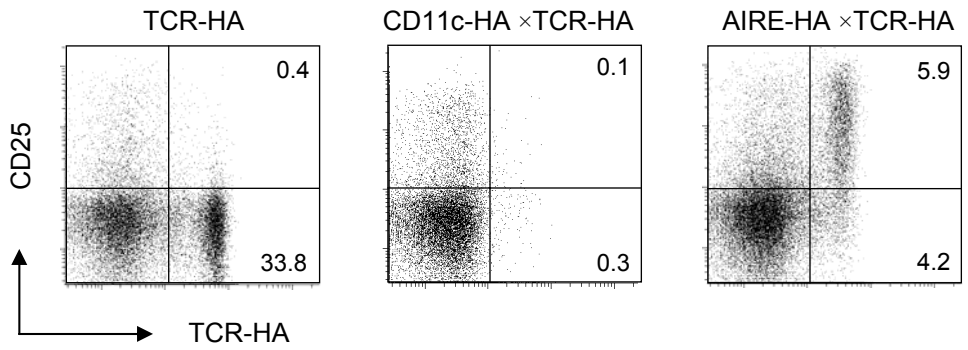
Supplementary Figure 1 Generation of mixed RTOC (outline of the experimental procedure). Equal numbers of CD45-depleted fetal thymic stromal cells were combined to generate mixed RTOCs. Six to eight weeks after grafting into MHC^{-} recipients, $MHCII^{+}$ and $MHCII^{-}$ medullary “islands” of clonal origin are found.

Suppl. Fig. 1



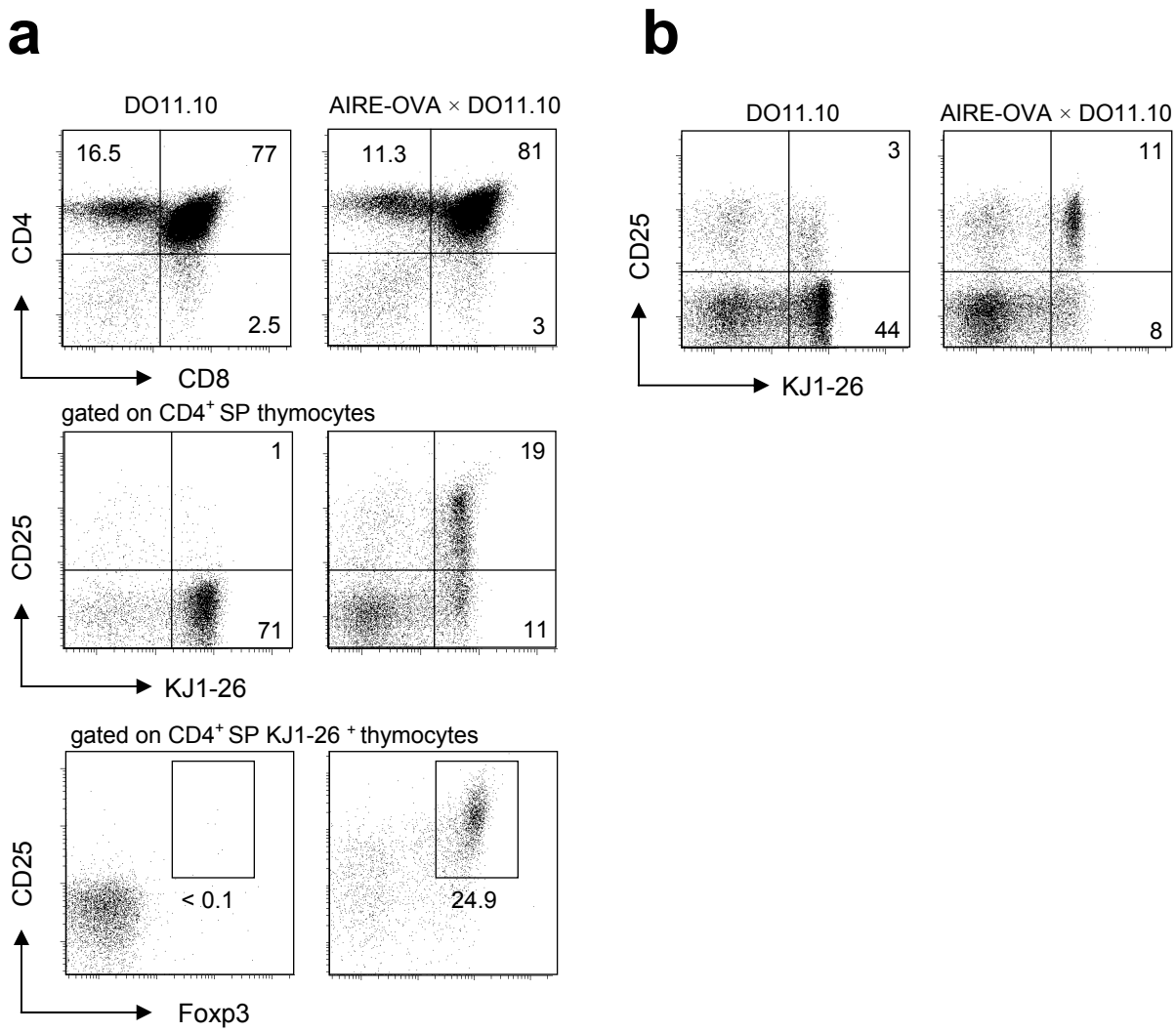
Supplementary Figure 2 A distinct population of TCR-HA$^+$CD25$^+$Foxp3$^+$ cells among peripheral CD4$^+$ T cells in AIRE-HA • TCR-HA mice. (**a**) Lymph node cells of AIRE-HA • TCR-HA mice and TCR-HA controls were stained with antibodies specific for CD4, CD8, TCR-HA and CD25. Expression of TCR-HA versus CD25 is shown on gated CD4$^+$ T cells. Frequencies of cells representative of more than 9 mice analyzed from each group are depicted in the respective quadrants. (**b**) Expression of *Foxp3* mRNA in sorted TCR-HA$^+$ CD4$^+$ T cells from AIRE-HA • TCR-HA mice and TCR-HA controls was measured by RT-PCR using serial dilutions (5-fold) of RNA. Data are representative of two independent experiments.

Suppl. Fig. 2



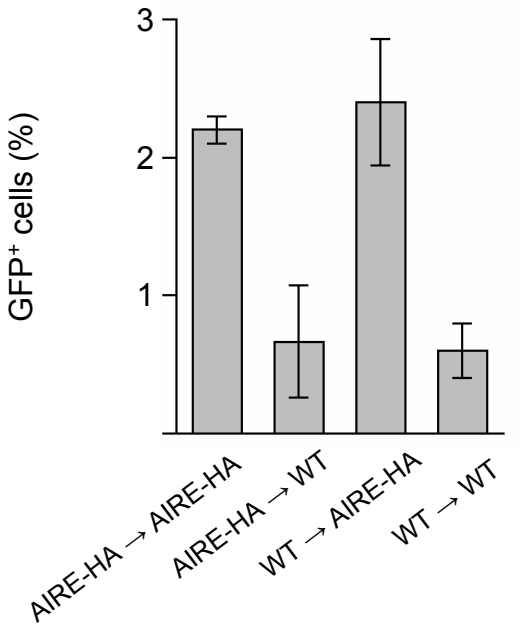
Supplementary Figure 3 Targeting of HA to DCs in CD11c-HA × TCR-HA mice results in efficient intra-thymic deletion of TCR-HA+ cells. To test whether TCR-HA cells are deleted when HA is abundantly expressed by thymic DCs, we targeted HA expression to DCs under control of the promoter driving expression of the gene encoding CD11c. Thymocytes from CD11c-HA × TCR-HA mice and TCR-HA controls were stained with the indicated antibodies. Expression of TCR-HA versus CD25 is shown on gated CD4+ SP thymocytes. The phenotype of AIRE-HA × TCR-HA mice is shown on the right for comparison.

Suppl. Fig. 3



Supplementary Figure 4 Targeting of OVA to mTECs leads to generation of OVA-specific CD25+Foxp3+ Treg cells. A fusion protein of HA and OVA containing the OVA323-339 epitope recognized by the DO11.10 TCR was targeted to mTECs using a BAC transgenic approach. (**a**) Thymocytes of AIRE-OVA × DO11.10 or DO11.10 control mice were stained with the indicated antibodies and analyzed by flow cytometry. Numbers in quadrants indicate the percentage of cells of the respective phenotype. (**b**) Thymic selection of KJ1-26+ Treg cells in the periphery of AIRE-OVA × DO11.10 mice. Plots depict CD4+ lymph node T cells from indicated mice. Data in (**a**) and (**b**) are representative of at least 5 animals of the respective genotype.

Suppl. Fig. 4



Supplementary Figure 5 Presentation of HA107-119 by DCs resulting from capture of mTEC-derived antigen. Thymic DCs were isolated from the indicated groups of bone marrow chimeras and their ability to stimulate *Il2* GFP-reporter expression in A5 T hybridoma cells was assessed. Data are representative of two independent experiments.

Suppl. Fig. 5

Supplementary Methods

Transgenic mice

To generate AIRE-HA mice, a cDNA encoding a fusion protein of HA and GFP followed by a poly-A signal was inserted by homologous recombination in bacteria[1] into the mouse BAC RP23-1146F6 (BACPAC). In brief, the BAC was modified using a targeting vector containing homology boxes spanning nucleotides -379 to -2 (5') and 17 to 399 (3') surrounding the HA-GFP cDNA and a poly-A signal followed by a neomycin resistance (*neo*^{r}) gene flanked by FRT-sites. After selection of successfully recombined clones, the *neo*^{r} cassette was removed. Transgenic mice were generated by injection of supercoiled BAC DNA into pronuclei of C57BL/6 × CBA F1 zygotes. Mice were backcrossed to the BALB/c background for at least three generations before analysis. For the generation of AIRE-OVA mice, a cDNA encoding a fusion protein of HA and OVA was inserted into BAC RP23-77O11 (BACPAC) replacing the start codon of the *Aire* gene using the strategy depicted above. Briefly, a portion of cDNA encoding the 71 C-terminal amino acids (314-385) of OVA was cloned 3′ of the HA cDNA. The resulting membrane-bound fusion protein carries the $OVA_{323-339}$ epitope in its cytoplasmic tail. Transgenic mice were generated by injection of supercoiled BAC DNA into pronuclei of C57BL/6 × CBA F1 zygotes. Mice were backcrossed to the BALB/c background for at least three generations before analysis. For the generation of CD11c-HA mice, the HA cDNA was inserted into the *Eco*RI site of a vector containing the promoter of the gene encoding CD11c (kindly provided by T. Brocker, Munich, Germany) as previously described[2]. The resulting plasmid DNA was linearized by *Xho*I and *Not*I digestion and was injected into fertilized BALB/c oocytes.

Antibodies and flow cytometry

Biotin-conjugated monoclonal antibodies (mAbs) specific for CD4 (GK 1.5), CD8 (53-6.7), I-A^d, CD80 (B7.1, 16-10A1), phycoerythrin-conjugated mAbs specific for CD25 (PC61), CD45.1 (A20), Ly51 (6C3), the DO11.10 TCR (KJ1-26), mAbs specific for CD8 (53-6.7), CD45 (Ly-5), CD4 (GK1.5), phycoerythrin-Cy7-conjugated mAb specific for CD25 (PC61), allophycocyanin-Cy7-conjugated mAb specific for CD4 (GK1.5), allophycocyanin-conjugated streptavidin and cychrome-conjugated streptavidin were purchased from Becton Dickinson. mAbs specific for TCR-HA (6.5), EpCAM (G8.8), I-A^b (Y3P) and pan-MHC class II (P7.7) were purified from hybridoma supernatants and conjugated to FITC or Alexa 647 in our lab. Foxp3 intracellular staining was performed according to the manufacturer's recommendations using PE- or APC-conjugated anti-mouse Foxp3 (eBiosciences). Flow cytometric analysis was performed on a FACSCanto flow cytometer (Becton Dickinson) using FACS DIVA software (Becton Dickinson).

Semi quantitative RT-PCR

mRNA was extracted using the High Pure RNA Isolation Kit (Roche). RNA was subjected to oligo(dT)-primed reverse transcription with a cDNA synthesis kit (Invitrogen). The PCR primers were: *Aire*, 5′-ACCATGGCAGCTTCTGTCCAG-3′ and 5′-GCAGCAGGAGCATCTGCAGAG-3′; HA-GFP, 5′-CTAATGGATCTTTGCAGTGC-3′ and 5′-GGCTGTTGTAGTTGTACTCC-3′; *Actb*, 5′-TGGAATCCTGTGGCATCCATGAAAC-3′ and 5′-TAAAACGCAGCTCAGTAACAGTCCG-3′; *Foxp3*: 5′-CAG CTG CCT ACA GTG CCC CTA G-3′ and 5′-CAT TTG CCA GCA GTG GGT AG-3′. PCR conditions for *Aire* were

94°C for 3 min., 30 cycles of 94°C for 45 s, 61°C for 45 s, 72°C for 1.5 min. PCR conditions for GFP were 94°C for 3 min, 37 cycles of 94°C for 45 s, 60°C for 45 s, 72°C for 1.5 min. PCR conditions for *Actb* were 94°C for 3 min., 29 cycles of 94°C for 45 s, 58°C for 45 s, 72°C for 1 min. PCR conditions for *Foxp3* were 94°C for 3 min, 36 cycles of 94°C for 30 s, 61.8°C for 30 s, 72°C for 30 s.

Bone marrow chimeras

Mice were lethally irradiated (2 x 450 Rad for BALB/c and 2 x 550 Rad for C57BL/6) and received a total of 8 x 10^6 T cell-depleted bone marrow cells. Chimeras were analyzed 6-8 weeks after reconstitution. For combined bone marrow and thymus chimeras, BALB/c nude (CD45.2$^+$) or C57BL/6 nude (CD45.2$^+$) mice were lethally irradiated as above and reconstituted with TCR-HA$^+$CD45.1$^+$H-2^d or TCR-HA$^+$CD45.1$^+$H-2^b bone marrow. 3-4 weeks after bone marrow transfer mice received grafts of dGUO-treated WT or AIRE-HA H-2^d CD45.2$^+$ E14 thymi under the kidney capsule. Grafts were analyzed 6-8 weeks after transplantation. Some grafts in C57BL/6 nude recipients were left in place for up to 4 months without evidence for tissue-rejection.

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6.3

Title: Expansion of peripheral NTreg by FLT3L treatment

Lee Kim Swee[1], Nabil Bosco[1], Bernard Malissen[2], Rhodri Ceredig[1,3] and Antonius Rolink[1]

[1]Departement Biomedizin Basel, Division of Molecular Immunology, University of Basel, Mattenstrasse 28, 4058 Basel, Switzerland.

[2]Centre d'Immunologie de Marseille–Luminy, Université de la Méditerrannée, INSERM U631, CNRS UMR6102, 13288 Marseille Cedex 9, France.

[3]INSERM U645/UPRES EA2284, IFR 133 Université de Franche-Comté, Etablissement Français du Sang Bourgogne Franche-Comté, BP1937, F-25020 Besançon, France.

Correspondence: Professor Antonius Rolink, Departement Biomedizin Basel, Division of Molecular Immunology, University of Basel, Mattenstrasse 28, 4058 Basel, Switzerland. E-mail: antonius.rolink@unibas.ch

Phone: +41 61 695 3072

Fax: +41 61 695 3070

Abstract

Fms-like tyrosine kinase 3 ligand (FLT3L) plays a major role in dendritic cell (DC) biology. Deficiency of FLT3L causes a dramatic decrease in DC numbers whereas increasing its availability (by repetitive injections for 7-10 days) leads to a 10-fold increase in DC numbers. Here, we show that FLT3L treatment indirectly leads to an expansion of peripheral naturally occurring T regulatory (NTreg) cells. The FLT3L-induced increase in NTreg was still observed in thymectomized mice, ruling out the role of the thymus in this mechanism. Instead, the increased number of NTreg was due to proliferation of pre-existing NTreg, likely due to favoured interactions with increased number of DC. In vitro, we show that DC induce Treg proliferation by direct cell contact and in an IL-2 dependent, TCR independent manner. FLT3L treatment had no effect on normal immune response but could prevent death induced by acute Graft vs Host Disease (GvHD). This study demonstrates unique aspects in the regulation of Treg homeostasis by DC, which were unappreciated until now. It also re-enforces the relevancy of FLT3L treatment in transplantation or autoimmune settings by its ability to increase both the number of immature tolerizing DC and NTreg.

Introduction

Fetal-liver kinase 2/FMS-like tyrosine kinase 3 (FLK2/FLT3) is a receptor tyrosine kinase, which was initially cloned from fetal liver population enriched with hematopoietic stem cell activity [1]. FLT3 has sequence and structural homology with c-kit and is expressed mostly by hematopoietic cell progenitors and dendritic cells (DC) [2-4]. FLT3L is the only known ligand for FLT3.

Both FLT3 and FLT3L-deficient mice are viable, fertile and hematopoiesis is maintained in the absence of FLT3 signalling [5,6]. The most striking phenotypes of these mice are the decrease in lymphoid and myeloid bone marrow progenitors as well as a decrease in DC and NK cell numbers. As expected from data obtained in FLT3L-deficient mice, administration of FLT3L by repetitive injections leads to an increase in the number of bone marrow progenitors as well as DC and NK cells [7-10]. Moreover, our previous work showed that increased FLT3L availability had an inhibitory effect on B cell lymphopoiesis suggesting that, when present in supra-physiological levels, FLT3L could enhance myeloid over lymphoid differentiation [10].

DCs play a dual role in the immune system. They are responsible for inducing an immune response or tolerance to self or non-innocuous antigens depending on their maturation/activation state [11,12]. In an immature state, DC express low levels of MHC class II antigens and costimulatory molecules and are thought to induce T cell tolerance by several distinct mechanisms[12]. Maturation of DC is induced by pathogens or endogenous “danger signals” sensed through a variety of receptors, which upon ligand binding induce MHC class II and costimulatory molecule up-regulation as well as inflammatory

cytokines secretion. Once in a mature state, DC prime T cells and induce immune response[13].

There is a considerable literature suggesting that DC influence regulatory T cell biology. Regulatory T cells (Treg) encompass T cell population, which prevent harmful immune responses against self [14,15]. Among Treg cells, the so-called naturally occurring CD4 regulatory T cells (NTreg) are the best characterized. This lineage is defined by the expression of the transcription factor Foxp3 [16-18], a natural mutation or conditional deletion of Foxp3 leads to lethal autoimmune disease, due to absence of functional NTreg [19-22]. NTreg develop in the thymus [15] but conversion from effector to regulatory cells can take place in the periphery and in some experimental settings, DC were demonstrated to be instrumental in this process [23-26].

Little is known about the rules governing NTreg homeostasis in the periphery in normal conditions but it is clear that IL-2 is indispensable for NTreg survival whereas it is dispensable for their development in the thymus [27,28]. In IL-2 deficient mice, NTreg development still takes place in the thymus but cells die rapidly after thymic egress because of lack of IL-2-mediated survival signals. Consequently, IL-2 deficient mice suffer from autoimmune disease because of the lack of peripheral NTreg [29,30]. In addition, CD28 signalling has been shown to be implicated in thymic NTreg development as well as peripheral survival [31,32]. In a diabetes model, CD28 blockade or deficiency exacerbates autoimmune symptoms [32]. Finally, NTreg are known to proliferate upon antigen encounter in vivo [33-36].

In the present study, we investigated the influence of FLT3L-mediated increase of DC numbers on regulatory T cell homeostasis. We demonstrate

that repetitive injections of FLT3L lead to expansion of NTreg cells in the periphery. NTreg increase was due to proliferation of pre-existing Treg, likely due to favoured interactions with DC. Experiments conducted in vitro showed that DC-induced Treg proliferation was IL-2 dependant, TCR independent and required an additional contact-dependent DC-mediated signal whose nature currently remains unknown.

FLT3L treatment prevented death induced by acute GvHD but did not impair normal immune response. This represents a new aspect of Treg homeostasis and might provide alternative ways of controlling Treg biology in vivo.

Materials and methods

Mice

C57Bl/6, C57Bl/6.Ly5.1, DBA/2 and (C57Bl/6 x DBA/2)$_{F1}$ FLK3L k.o.[6] mice were bred in our animal facility. MHC class II deficient mice [37] were obtained from Taconic farms and C57Bl/6 Foxp3EGFP reporter mice [38] were a kind gift from Prof. Malissen. All mice were used between 7 to 16 weeks of age and were maintained in specific pathogen–free conditions. All animal experiments were carried out within institutional guidelines. Mice were humanely killed by CO_2 inhalation and organs removed by standard procedures.

Thymectomies

At 4 to 6 weeks of age, mice were anesthetized and thymus was removed by suction through a small upper sternal incision. That thymectomy had been complete was verified in each animal by anatomical inspection at the time of sacrifice.

FLT3L treatment

Recombinant human FLT3L (rFLT3L) was a kind gift of Amgen (Thousand Oaks, CA). A stock solution containing 1mg rFLT3L/mL was prepared in PBS and aliquots stored at –20°C until use. For FLT3L treatment, mice generally received 10-20 µg rFLT3L (0.2 mL) by intraperitoneal injection daily or every second day for 10 days, a treatment schedule previously used to increase DC number. In titration experiments ("Results"), mice were treated with graded doses of 2 or 10 µg/0.2-mL injections.

Bone marrow chimeras

Bone marrow cell suspensions from donor mice were prepared by flushing femurs and tibias with PBS using a 23-g needle. After red blood cell lysis, T cells were depleted by re-suspending cells in a mixture of rat IgM anti-CD90 (AT83) anti-CD4 (RL172) and anti-CD8 (31 M) mAb hybridoma supernatants and incubated for 20 min at 4°C. Following a washing step, antibody-coated cells were lysed by adding rabbit complement dissolved in serum-free Dulbecco's modified Eagle's medium (DMEM). After incubation for 45 min at 37°C, cells were washed and resuspended in DMEM prior to injection. Hosts were lethally γ-irradiated with a single dose of 950 cGy using a Cobalt source (Gammacell 40, Atomic energy of Canada, Ltd) prior to receiving $5x10^6$ cell intravenously.

Cell preparation

Single-cell suspensions from thymus or spleen were prepared by pressing through a 100-µm nylon mesh into 2% FCS IMDM. For flow cytometry, cells were washed and resuspended in PBS containing 2% FCS and 0.1% sodium azide (FACSwash). Viable cells were stained with trypan blue and counted in a hemocytometer. The total number various lymphocytes subpopulations were calculated from the frequency estimated by FACS analysis and the total number of living cells recovered per organ.

Flow cytometry

The following mAbs were used: anti-B220 (RA3-6B2), anti-CD3 (145.2c11),

anti-CD4 (RM4-5), anti-CD11c (HL3), anti-DX5 (DX5) and anti-Ki67 (B56) were purchased from BDbioscience; anti-NK1.1 (PK136) and anti-FoxP3 (mAb FJK-16S) were purchased from eBioscience; anti-CD4 (GK1.5), anti-CD8α (53-6-72) anti-CD19 (1D3), anti-CD45.2 (1D4-2.1) and anti-pan MHC class II (M5) antibodies were purified from the hybridoma supernatant and labeled by standard methods. Cell surface staining was performed as previously described, and analyses were performed on a FACSCalibur interfaced to a Macintosh computer with BD Biosciences/CellQuest or Flowjo software. Dead cells were excluded from analysis by a combination of light scatter and/or absence of propidium iodide (PI) staining. Intracellular staining for FoxP3 and Ki67 were performed according to the manufacturer's instructions (eBioscience) and (BDbioscience). Cell sorting was performed on a FACSAria (BD Biosciences).

In vitro assay

Bone marrow derived dendritic (BMDC) cells were differentiated in vitro using standard protocol[39]. Differentiated DC were harvested for stimulation after 6-10 days. Bulk lymph node cells or sorted cells from C57BL/6 Foxp3EGFP reporter mice were labelled with PKH26 (sigma, PKH26GL) and incubated alone or together with sorted CD19+ B cells or BMDC in 2-5% FCS IMDM. IL-2 supernatant or anti-IL-2 (S4B6) 10 μg/ml was added when indicated. Proliferation was monitored by measurement of PKH26 fluorescence in FL2 channel using a FACS calibur.

Immunizations

To induce a T-dependent antibody response, mice were injected intraperitonealy with 50µg NIP-ovalbumin in 200µl Alum. Sera were obtained from tail bleeding prior to and 14 days after immunization and stored at –20°C. Total and hapten-specific IgG levels were determined by enzyme-linked immunosorbent assay (ELISA)[10].

GvHD exeriment

GvHD was induced by intravenous injection of pooled cells suspension form lymnode and spleen cells from C57Bl/6 into $(C57Bl/6 \times DBA/2)_{F1}$. Animals were followed on a daily basis and euthanized when necessary.

Results

Changes in T lymphocytes subsets following FLT3L treatment.

The effect of increased FLT3L availability on dendritic cells (DC) development in mice is well documented[4-9,40], however, little is known about the direct or indirect impact of prolonged FLT3L treatment on T cell populations. In order to study the influence of FLT3L levels on T cell populations, WT mice were injected daily with 10μg/day of recombinant cytokine for 10 days and compared with WT control and FLT3L knock-out (k.o.) mice[6]. Figures 1A and B show the percentages and absolute numbers of the indicated splenic subpopulations in FLT3L k.o., WT control or WT treated mice. Proportionally, changes in FLT3L level had the most striking effect on conventional DC (FLT3L k.o.: $0.1x10^6$; WT: $1.7x10^6$; WT Treated: $31x10^6$) and plasmacytoid DC (FLT3L k.o.: $0.02x10^6$; WT: $0.7x10^6$; WT Treated: $10.6x10^6$) as well as NK cells number (FLT3L k.o.: $1x10^6$; WT: $3.3x10^6$; WT Treated: $13.4x10^6$) as we already reported previously[10]. Expansion of the DC compartment was also observed in the bone marrow, thymus and lymphnodes of treated mice (data not shown). Despite the differences of NK and DC numbers, the absolute numbers of CD4 or CD8 T cells were not drastically altered by changes in FLT3L levels (Figure 1B) and entire thymopoiesis was similar between control and treated mice (data not shown). We observed a decrease in B cell number in FLT3L k.o. compared to WT mice, which is due to decreased number of B cell precursors in the bone marrow (data not shown).

The role for DC in extrathymic development of Tregs has been reported by many groups [23-26,41]. Therefore, we wondered whether the influence of FLT3L level on DC numbers would have any influence on NTreg population.

Variations of FLT3L concentration had no influence on the percentage or the absolute number of NTreg cells in the thymus (Figure 1C). However there was a significant, dose-dependent increase in percentage and absolute number of Foxp3+ NTregs in the spleen (Figure 1D). Amongst CD4 T cells, FLT3L k.o. had 2 fold less Treg than WT mice (6.7% vs 12.8%) and in the best case FLT3L treatment increased Treg cells by 2 fold (12.8% vs 24%). Both DC and NTreg numbers went back to normal values after stopping FLT3L treatment (data not shown).

Because the total CD4 T cells number in FLT3L k.o., WT or treated mice was similar (Figure 1B), we conclude that FLT3L level correlates with NTreg population size in the periphery but not in the thymus.

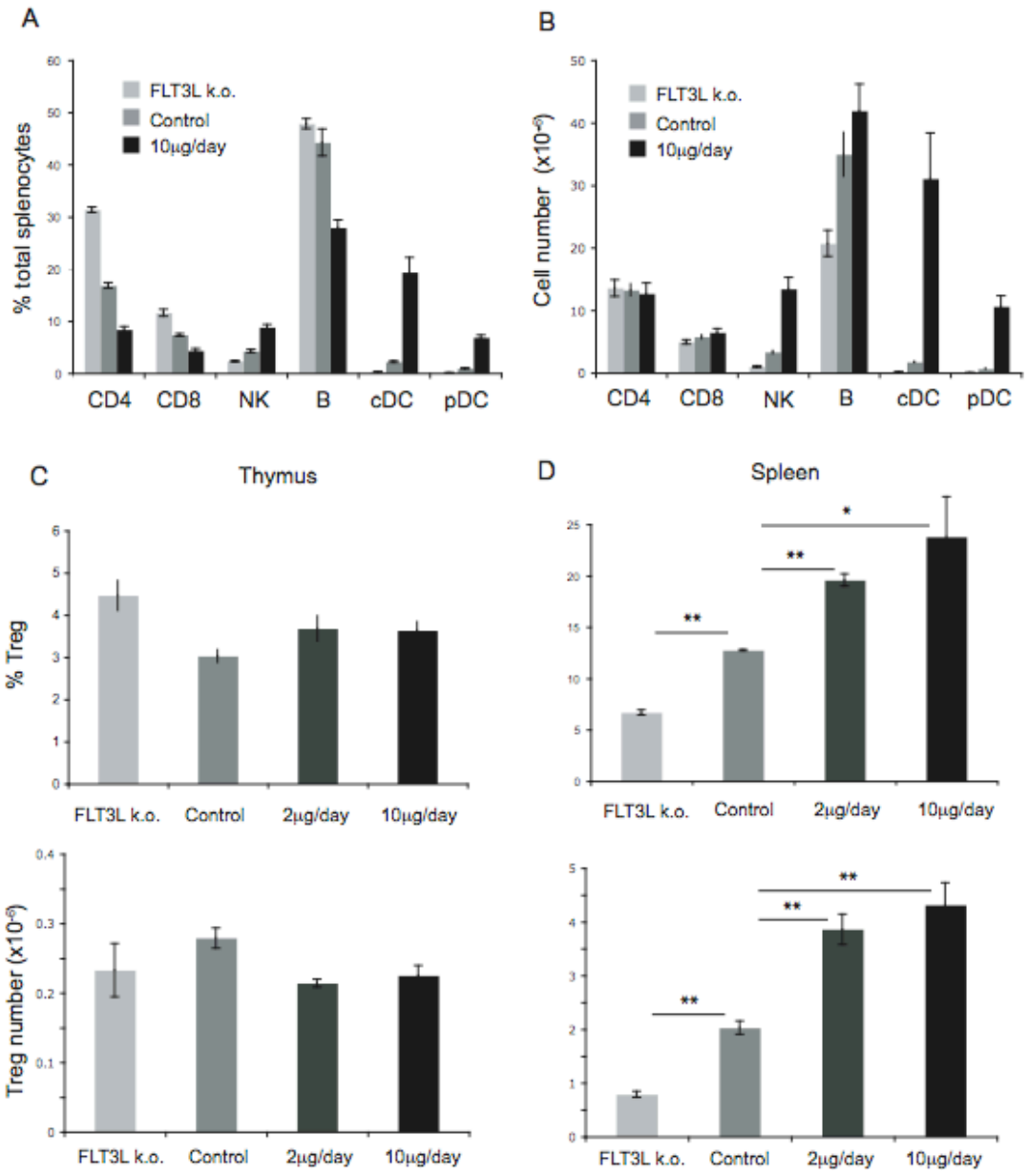

Figure 1. Influence of FLT3L treatment on lymphocyte subpopulations.

C57BL/6 mice were injected intraperitoneally daily for 10 days with different dose of recombinant FLT3L. (A) Percentages of indicated splenic subpopulations in FLT3L k.o., WT control or WT treated mice. (B) Absolute cell numbers of indicated splenic subpopulations in FLT3L k.o., WT control or WT treated mice. (C) Percentages of Foxp3+ cells among CD4sp (upper) or absolute numbers of Foxp3+ cells (lower) in thymus of FLT3L k.o., WT control or WT treated mice. (D) Percentages of Foxp3+ cells among CD4 T cell or absolute numbers of Foxp3+ cells in spleen of FLT3L k.o., WT control or WT treated mice.

Each Bars shows mean +/- standard error of the mean. *, $p<0.05$; **, $p<0.01$.
FLT3L k.o., light grey; WT control, grey; WT treated dark grey and black.

Origin of Treg increase

Several non-exclusive mechanisms could explain the increase in NTreg after repetitive FLT3L injections. Firstly, we addressed the question whether the extra number of peripheral Tregs was due to enhanced thymic NTreg production. Although we could not observe an increase in CD4SP Foxp3+ thymocytes (Figure 1C), FLT3L could potentially increase the rate of Treg development and output from the thymus. Therefore mice were thymectomized prior to FLT3L treatment. We observed that even in the absence of thymic output, there was still a FLT3L-dependent increase in the percentage and absolute numbers of splenic Treg cells (Figure 2A). This increase was comparable to that observed in non-thymectomized mice (Figure 1A), implying that the thymus does not contribute significantly to the increase in peripheral Ntreg.

Thus, if peripheral NTreg expansion was thymus independent, it could be due either to i) the conversion of a CD4 T helper, non-Treg cell to a Treg phenotype and/or ii) the proliferation of pre-existing Treg upon increased FLT3L availability. To address the first hypothesis, we investigated whether CD4+ Foxp3- cells could become Foxp3+ upon FLT3L treatment. For this purpose, we injected $2x10^6$ sorted CD4+Foxp3- CD45.2 cells from Foxp3EGFP reporter mice [38] into lightly (100rad) irradiated CD45.1 hosts and two weeks later, treated mice with FLT3L for 10 days or left them as control. As shown in figure 2B, treatment induced a significant increase in total Treg cells but did not induce Foxp3 expression in transferred cells (Figure 2C). We therefore conclude that FLT3L-mediated Treg increase was not due to conversion of effector to regulatory cells.

To challenge the second hypothesis, we determinated NTreg proliferation upon FLT3L treatment by the expression of the proliferation-associated marker Ki67. As shown in figure 2D, FLT3L treatment significantly increased the percentage of Ki67+ CD4+Foxp3+ and, to a lesser extent CD4+Foxp3-, in a FLT3L dose-dependent manner. This strongly suggests that FLT3L treatment induced a preferential increase proliferation of regulatory versus effector cells.

Taken together, these results show that expansion of the peripheral NTreg compartment is due to proliferation of NTreg cells upon FLT3L treatment.

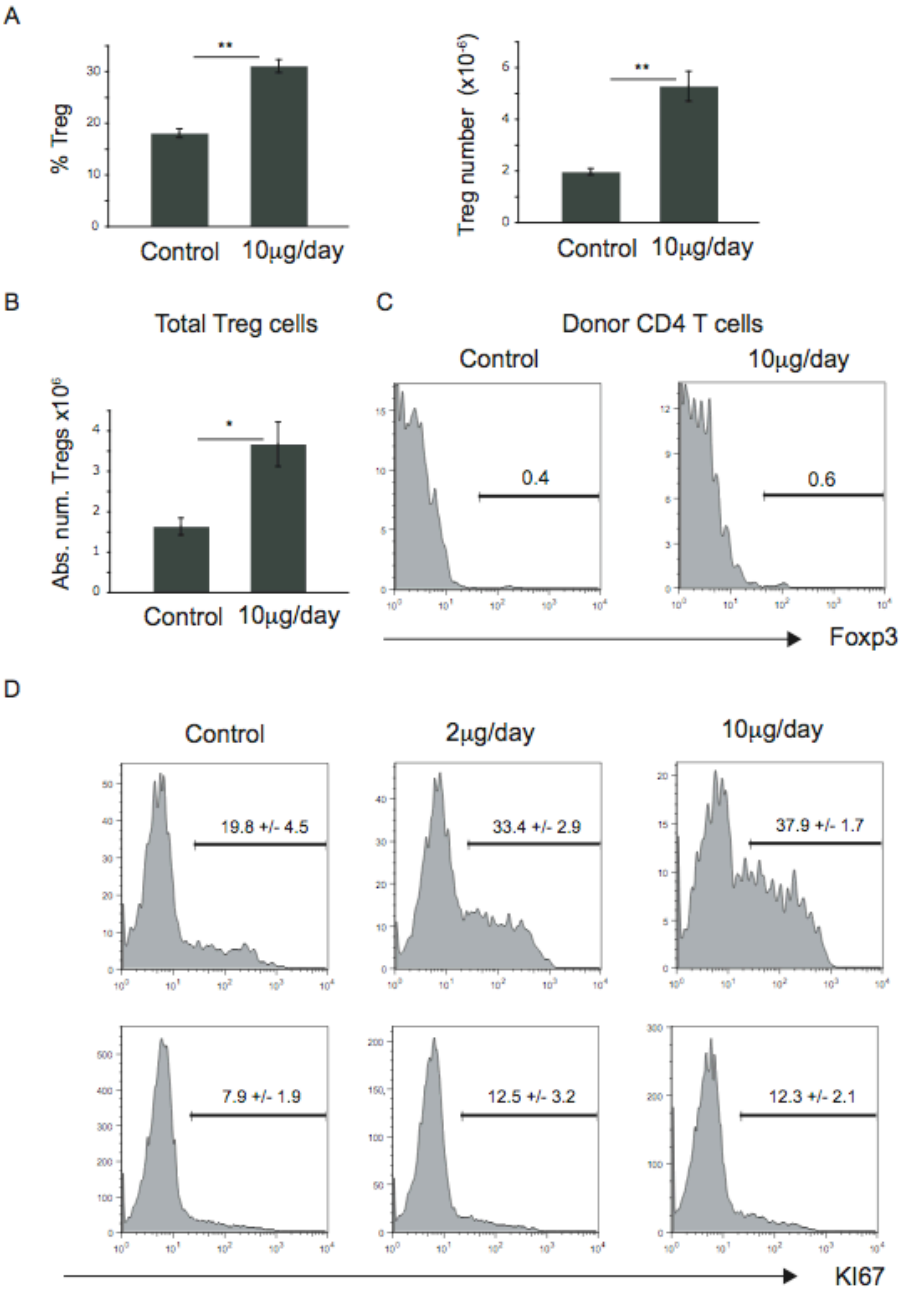

Figure 2. Expansion of peripheral Treg compartment due to increased proliferation of Foxp3+ CD4 T cells.

(A) Mice were thymectomized prior to FLT3L treatment (10µg intraperitoneal injection daily for 10 days). Histograms show percentages of Foxp3+ among CD4 T cells (left) and absolute numbers (right) in the spleen of control or treated mice. Each bars shows mean +/- standard error of the mean. **, p<0.01. (B, C) CD45.1 WT mice were irradiated (100rad) prior to intravenous injection of $2x10^6$ sorted CD4+Foxp3- cells from CD45.2 Foxp3EGFP reporter mice. After two weeks, mice were treated with FLT3L (10µg intraperitoneal injection daily for 10 days) or left as control. (B) Absolute numbers of CD4+Foxp3+ cells in the spleen of control or treated mice. Histogram shows mean +/- standard error of the mean. *, p<0.05. (C) Foxp3 expression in CD45.2+CD4+ donor cell population. (D) Histograms show percentages of Ki67 positive cells among Foxp3+ (upper panels) or Foxp3- (lower panels) CD4 T cells in control or treated animals.

Dendritic cells support Treg proliferation

FLT3L-induced Treg proliferation could be due either to a direct signalling of the cytokine on the Treg population or to an indirect effect. Since neither CD4 helper nor Treg cells express detectable FLT3 at protein level (data not shown), we favoured the hypothesis of an indirect effect.

FLT3L treatment increased both NK and DC numbers in the spleen (Figure 1A-B). FLT3L-induced expansion of NTreg took place even when mice were depleted of NK cells by injection of anti NK1.1 mAb (data not shown), suggesting that NK cell were not instrumental in the expansion of NTreg cells.

Given their ability to stimulate T cells, DC were the most plausible candidates responsible for the expansion of NTreg, We therefore addressed the capacity of DC to induce NTregs proliferation. As there is no simple, reliable, in vivo model available for long-term DC depletion, we addressed this hypothesis in vitro.

Figure 3A shows the proliferation of NTreg and CD4 helper cells as measured by PKH26 staining. Neither CD4 helper nor NTreg cells proliferated alone (not shown) or in the presence of purified syngeneic B cells (Figure 2Aii). In contrast, co-culture with syngeneic in vitro FLT3L-induced bone marrow-derived dendritic cells (BMDC) [39] led to a preferential proliferation of Foxp3+ cells (Fig 2Aiii). Cell division in these cultures was IL-2-dependent since it could be inhibited by addition of blocking anti-IL-2 antibody (Figure 2Aiv). Proliferation took place in the absence of any extra FLT3L and could not be blocked by addition of blocking anti-FLT3 antibody, again suggesting that there is no direct link between FLT3L and NTreg proliferation (data not

shown). Addition of blocking anti-TGFβ mAb in co-culture had no effect and sorted CD4+Foxp3- cells did not convert to a regulatory phenotype when incubated together with BMDC (data not shown), ruling out any conversion mechanisms as demonstrated in vivo (Figure 2B).

Sorted NTreg proliferated only when they were cultured in presence of extra IL-2 and BMDC (Figure 3Biv), whereas IL-2 (Figure 3Biii) or BMDC (Figure 3Bii) alone only induced a weak proliferation. These results argue firstly that neither BMDC nor NTreg produce IL-2 and secondly that NTreg need a DC-mediated additional signal in order to proliferate in the presence of IL-2. This additional signal required direct cell-contact, since Treg did not divide significantly when they were incubated separately from BMDC in a transwell cultures (Figure 3Di) compared to cells incubated in direct contact with BMDC (Figure 3Dii).

We next investigated whether CD4 helper and/or CD8 T cells were providing IL-2 to Treg cells. As shown in Figure 3C, sorted Tregs proliferated in the presence of BMDC plus CD4 helper or CD8 (Figure 3Ci-ii) and this proliferation could be inhibited by addition of blocking anti-IL-2 antibody (Figure 3Ciii-iv). This demonstrates that, unlike BMDC (Figure 3B), both CD4 helper and CD8 T cells can provide IL-2 in these settings.

We show here that unlike B cells, BMDC induce NTreg proliferation. Proliferation was IL-2 dependent and required an additional cell-contact dependent signal supplied by DC.

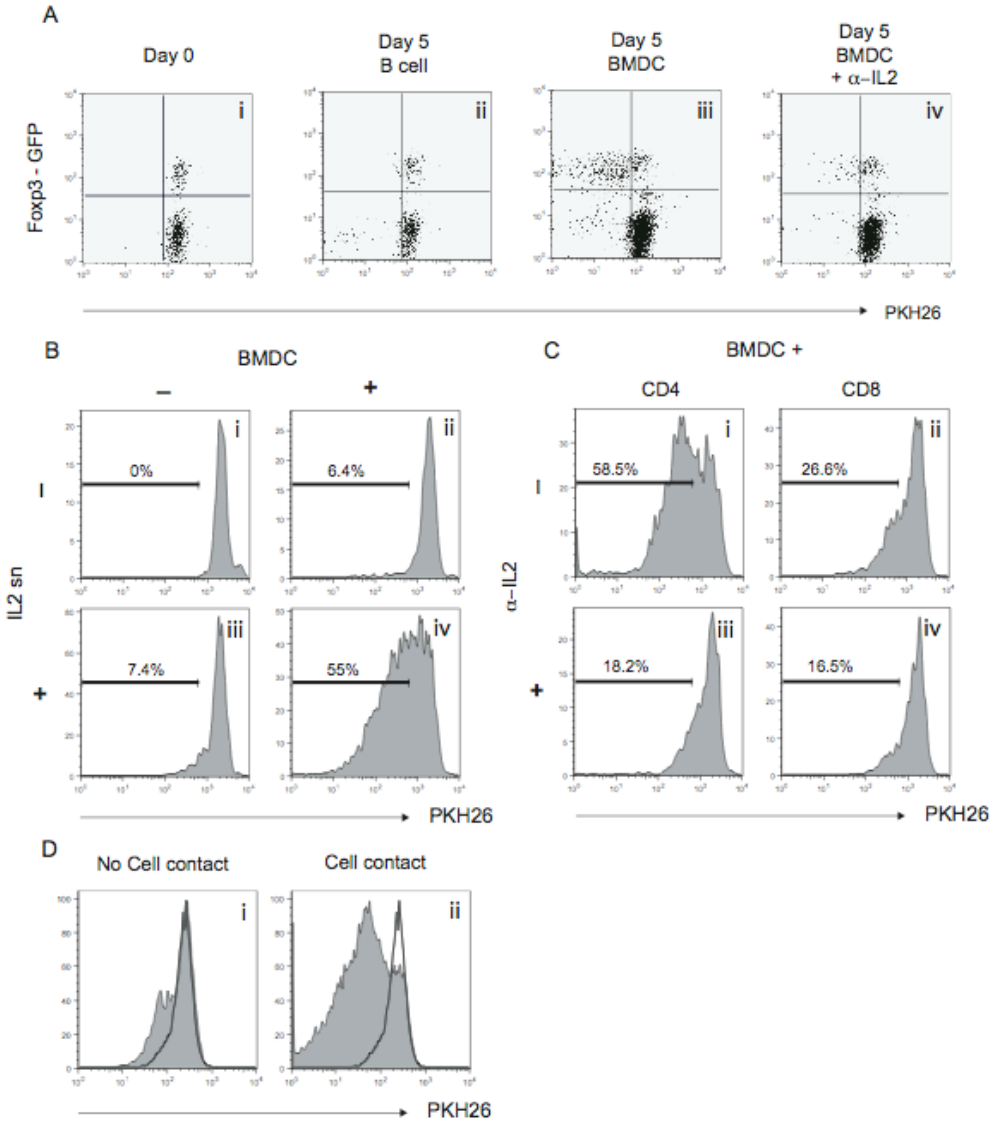

Figure 3. FLT3L-differentiated bone marrow-derived dendritic cell induce proliferation of Treg.

(A) PKH26-labeled lymph node cell suspension from Foxp3EGFP reporter mice were incubated with sorted syngeneic B cell or syngeneic in vitro FLT3L-differentiated BMDC for 5 days with or without antagonistic anti-IL-2. Dot plots show PKH level on CD4 T cells. (B) PKH26-labeled CD4+Foxp3+ cell sorted from Foxp3EGFP reporter mice were incubated with or without extra IL-2 and with or without BMDC. Histograms show PHK26 level on CD4+Foxp3+ cells after 5 days of incubation. (C) PKH26-labeled CD4+Foxp3+ cell sorted from Foxp3EGFP reporter mice were incubated with BMDC and sorted CD4+Foxp3- (CD4:Treg, ratio 4:1) or CD8+ (CD8:Treg, ratio 2:1) T cells. Co-culture were done in presence or absence of antagonistic anti-IL-2. Histograms show PHK26 level on CD4+Foxp3+ cells after 5 days of incubation. (D) PKH26-labeled CD4+Foxp3+ cell sorted from Foxp3EGFP reporter mice were incubated with extra IL-2 only (empty histogram) or with BMDC without (left panel) or with (right panel) contact. Histograms show PHK26 level on CD4+Foxp3+ cells after 5 days of incubation.

DC mediated Treg expansion is TCR-independent.

We concluded from figure 3B that IL-2 was necessary but not sufficient to induce sustained NTreg proliferation in vitro and we postulated that BMDC were providing a second signal, which was enhancing Treg proliferation. A strong TCR signal together with CD28 co-stimulation is known to induce NTreg proliferation in vitro in the presence of extra IL-2, hence we looked to see whether DC-induced Tregs proliferation was TCR dependent. As shown in figure 4A, MHC class II k.o. BMDC [37] were as potent as WT BMDC in inducing the proliferation of sorted NTreg when extra IL-2 was provided. This argues that DC induced NTreg proliferation is independent of TCR-MHC class II interactions. Since sorted NTreg cells did not proliferate when stimulated with plate bound anti-CD28 plus IL-2 (data not shown), we conclude that BMDC were inducing NTreg proliferation by a TCR-independent CD28-independent mechanism.

Next we wondered whether FLT3L treatment would induce Treg expansion in the absence of TCR-MHC class II interactions in vivo. To test this hypothesis, we lethally irradiated WT mice and reconstituted them with bone marrow from MHC class II k.o.. In such chimeras, MHC class II molecules are still expressed on radio-resistant cells of the thymus, allowing CD4 T cells to develop, but are completely absent on radiosensitive cells. As expected, in these chimeras, CD4SP and mature CD4 T cells could be found in the thymus (Figure 4B) and spleen (Figure 4C). In the spleen, MHC class II expression could not be detected either by FACS (Figure 4D) or by immuno-histology (data not shown). As expected, FLT3L treatment led to an increase in the number of dendritic cells and had no influence on the expression of MHC

class II in the spleen (Figure 4E). FLT3L induced an increase both in the percentage and absolute number of NTreg (Figure 4F-G).

Taken together, we conclude that MHC class II-TCR interactions are dispensable for DC induced Treg proliferation both in vitro and in vivo. The nature of the second signal delivered by DC, which is necessary for enhanced Treg proliferation is still under investigation.

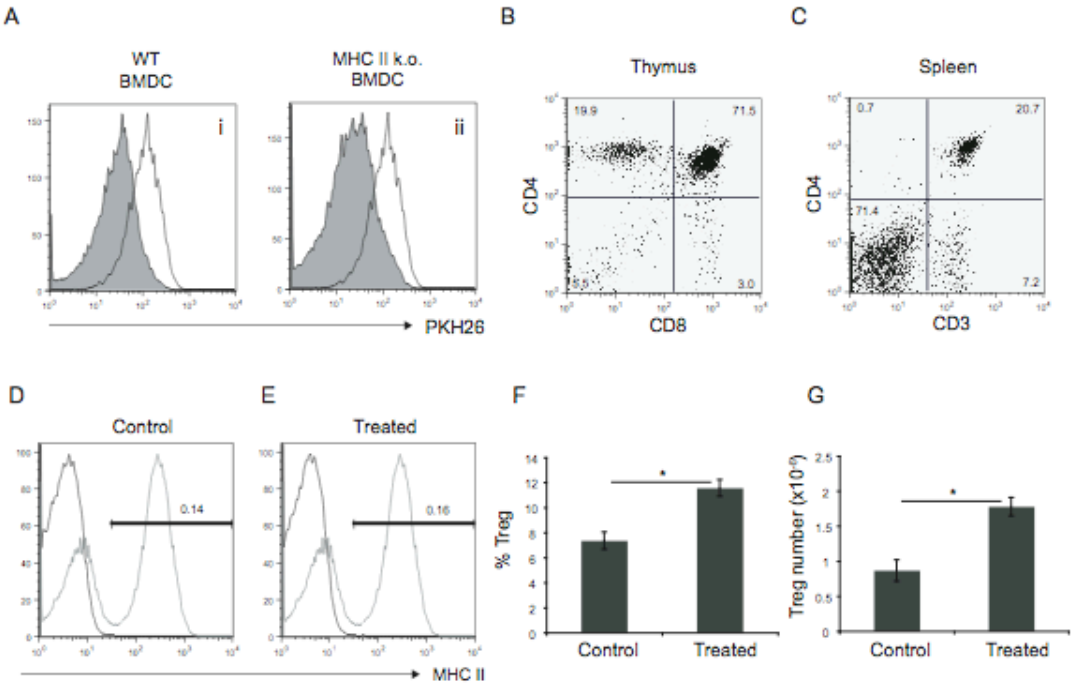

Figure 4. Mechanism of FLT3L-induced Treg proliferation is TCR-independant.

(A) PKH26-labeled CD4+Foxp3+ cell sorted from Foxp3EGFP reporter mice were incubated with WT BMDC (filled histogram, left panel), MHC class II k.o. BMDC (filled histogram, right panel) or alone (empty histogram) in presence of extra IL-2. Histograms show PHK26 level on CD4+Foxp3+ cells after 5 days of incubation. (B-G) WT mice were lethally irradiated and reconstituted with MHC class II k.o. bone marrow. (B) CD4 versus CD8 expression in thymus of MHC class II k.o. -> WT chimera. (C) CD4 versus CD3 expression in spleen of MHC class II k.o. -> WT chimera. (D, E) MHC II expression in the spleen of control (C) or FLT3L-treated (D) MHC class II k.o. -> WT chimera (black line) versus WT control (grey line). (F, G) Percentages of Foxp3+ cells among CD4 T cell or absolute number of Foxp3+ cells in spleen of treated vs control MHC class II k.o. -> WT chimera. Each histogram shows mean +/- standard error of the mean. *, $p<0.05$.

FLT3L treatment does not impair normal immune response but can prevent mice dying from by acute Graft versus Host disease.

We wondered what would be the influence of increasing the number of DC and NTreg by FLT3L treatment on a T-dependent antibody response as well as in a GvH model. As showed in figure 5A, FLT3L treatment had no influence on an anti-NIP antibody response as measured by ELISA for specific Ig. Both control and treated animals had similar titres of anti-NIP antibody following immunisation. However, as it was reported previously [40], FLT3L treatment completely prevented death due to acute GVHD caused by injection of C57Bl/6 lymphocytes into $(C57Bl/6 \times DBA/2)_{F1}$ animals (Figure 5B). FLT3L treatment could partially prevent death even when treatment was initiated 1 week after disease induction (Figure 5B). In addition, FLT3L treatment protected mice from GvHD-induced death even when mice were depleted of NK cells by injection of anti-NK1.1 mAb at time of GvHD induction (data not shown), suggesting that, in accordance with previously published data[40], NK cells had no role in FLT3L-mediated protection of GvHD.

In conclusion, we show here that FLT3L treatment had no influence on an antibody response whereas it had a clear, beneficial effect in preventing death caused by acute GvHD.

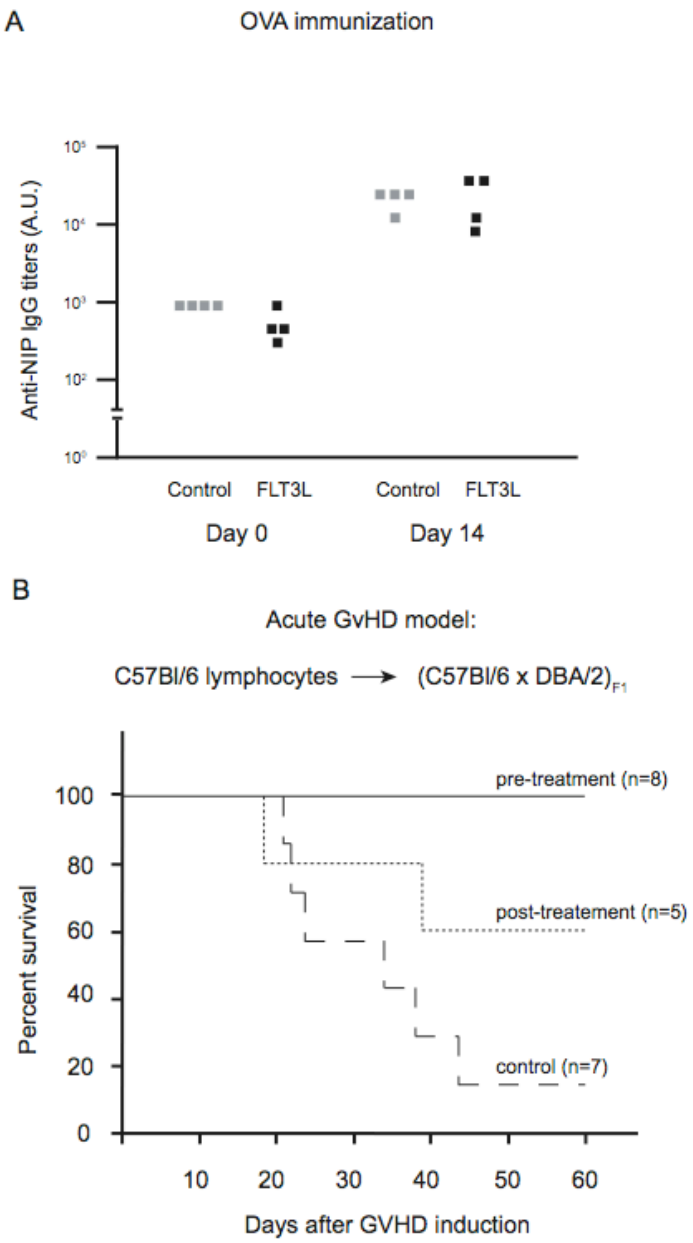

Figure 5. FLT3L treatment does not impair normal immune response but can prevent mice dying from by acute Graft versus Host disease.

(A) C57Bl/6 mice were treated with FLT3L (10μg intraperitonal injection daily for 10 days) or untreated. Mice were then immunized with 50μg NIP-OVA in Alum intraperitonealy. Anti-NIP IgG titers were determined at day 0 and 14 by ELISA. (B) $(C57Bl/6 \times DBA/2)_{F1}$ were injected with 50×10^6 pooled Lymphnode/spleen from C57Bl/6 mice. Recipient mice were either kept as control, treated with FLT3L for 10 days prior to GvHD initiation (”pre-treatment”) or 1 week after (”post-treatment”). Curves show percentage survival of each group.

Discussion

Factors controlling NTreg homeostasis are not yet completely understood, however it would seem that there is a niche of pre-determined size for the NTreg population. Thus the proportion of NTreg amongst total CD4 T cells is set early on during ontogeny and kept fairly constant until advanced adulthood [42]. This is also true regarding the proportion of NTreg in different mouse strains, the ratio between regulatory and effectors cells is varying significantly between strains but is very constant within each strain [43]. Altogether, it strongly suggests that a combination of factors keep the NTreg niche to a given size in homeostatic conditions. Moreover, it seems that even in lymphopenic situation like in pTα or IL-7 deficient mice, the size of the NTreg compartment is preserved [44].

The unique role of IL-2 in NTreg biology is well described and there is clearly a direct correlation between cytokine availability and NTreg numbers. In mice, increasing IL-2 availability by injection of highly potent IL-2/anti-IL-2 complex expand NTreg number in the periphery [45,46] whereas neutralizing IL-2 by injection of mAb induce a decrease in NTreg numbers and eventually leads to autoimmune disease due to absence of NTreg-mediated tolerance [47]. Since IL-2 production by NTreg is minimal [48-50], they rely for their survival on IL-2 produced by conventional CD4 helper cells [51].

The role of CD28 signalling in thymic NTreg development has been dissected [31], however its precise role in NTreg homeostasis in periphery is less clear. Although inhibition of CD28 by injection of blocking antibody leads to reduced numbers of peripheral NTreg [32], it is not completely understood to what

extent, this effect is NTreg intrinsic or due to a decrease in IL-2 production by CD4 effector cells caused by a lack of costimulatory signals. If injection of anti-CD28 “superagonist” antibody were reported to induce NTreg proliferation in vitro and in vivo [52], it does not mimic the physiological situation where CD80 and CD86, the natural ligands of CD28, also bind to the inhibitory molecules CTLA-4. NTreg are also known to proliferate upon antigen encounter in vivo [33-36] and in vitro; NTreg can be stimulated with antigen loaded APC [35,53]. However, in homoestatic conditions, one can wonder what proportion of Treg receive such a strong TCR signal.

So far, therefore the only reported ways of inducing NTreg proliferation have been either by TCR stimulation, increased IL-2 availability or CD28 co-stimulation. However, in this report, we show that FLT3L treatment likely by increasing DC number also results in an increase in NTreg number.

Thus repetitive FLT3L injections expanded peripheral Treg probably by favouring interactions between DC and NTreg. This increase in NTreg was not due to phenotypic conversion from conventional to NTreg cells (Figure 2B). Moreover, FLT3L treatment had no influence on the $CD103^+$ DC population (data not shown), which was recently reported to be particularly efficient at promoting extrathymic NTreg differentiation [26].

We could show in vitro that DC control NTreg proliferation by cell-contact mechanisms in a TCR independent manner, allowing cells to divide in the presence of IL-2. Addition of blocking anti-TGFβ mAb had no effect on the outcome of the co-culture, suggesting again the absence of differentiation mechanisms. We can postulate that the signals delivered by DC is either

positively influencing the IL-2 signalling pathway and/or giving an independent additional signal. Since a) neither FLT3L treatment nor NTreg stimulation by BMDC had an effect on CD25 or IL-2Rβ expression by NTreg and that b) IL-2 was not a limiting factor in our in vitro experiments, we rather think that DC provide an additional signal (or signals) to NTreg. This signal seems to determinate very clearly whether or not cells will enter cell cycle in the presence of IL-2 and explain the absence of sustained proliferation of sorted NTreg cells in the presence of saturating IL-2. These conclusions imply that the expansion we observe in vivo and in vitro is very likely to be polyclonal. It might guarantee polyclonality of the NTreg repertoire in homeostatic conditions and reciprocally to avoid oligoclonality in a situation where a considerable subpopulation of the NTreg is in constant presence of their cognate antigen [54].

We suggest that DC play a privileged role in the control of the NTreg niche in the periphery. This is strengthened by several facts. Firstly, increasing DC numbers by, for example GM-CSF treatment, induces similar increases in NTreg numbers [55]. Secondly, both DC and NTreg numbers go back to normal values shortly after DC numbers decrease after stopping FLT3L treatment (data not shown). Thirdly, in FLT3L k.o. mice, in which the most striking phenotype is the decrease in DC number (Figure 1B), there is a significant decrease in Treg number and percentage of CD4 T cell in the periphery only. Finally, the fact that J_h k.o. mice [56], which have no B cells, have comparable percentages of splenic NTreg compared to WT mice suggests that B cells, which represent the vast majority of splenic APC in normal mice, do not play any role in controlling the size of the peripheral NTreg niche. This last point

underlines the unique role of DC in setting the size of peripheral NTreg niche. Overall this might be a mechanism for the immune system to ensure equilibrium between the number of professional APC and the number of NTreg. Given their key role in T cell priming, it is probably necessary to keep a homeostatic link between DC and Treg, as it is the case for CD4 helper cells and NTreg.

FLT3L treatment did not interfere with an antibody immune response, despite the increase of immature DC and Treg numbers (Figure 5A). We hypothesize that in the presence of a strong adjuvant, most of the DC induced by FLT3L treatment will mature and will therefore have a positive impact rather than tolerizing effects on immune response. In such a situation, it would not be surprising that increasing the number of NTreg had no effect on the final immune response.

In contrast, the effect of FLT3L treatment on GvHD outcome was very dramatic. When FLT3L injections were performed prior to GvHD initiation, treatment could rescue 100% of mice. Strikingly, up to 60% of mice could be rescued when treatment was started 1 week after GvHD induction. We conclude from the latter results that FLT3L treatment is able to stop or decrease GVHD even after allo-T cell priming, suggesting that the mechanism of tolerance involved are likely to be dominant. In some situation of GvHD, recipient NK cells can reject donor cells; a mechanism called “hybrid resistance”. We therefore wondered if FLT3L treatment was enhancing rejection of allo-T by increasing the number of NK cells. However, we could exclude a role for NK cells. It was reported that FLT3L-mediated protection

was due to increased numbers of immature DC and that in vitro, DC from FLT3L-treated animals were far less potent in allo-T cell priming compared to DC from control animals [40]. Although we agree with these conclusions, we think in addition that it is not the only way by which FLT3L treatment protects from GvHD. We have previously shown that Treg transfer can prevent death from syngeneic GvHD after bone marrow transplantation [57] and others have demonstrated the importance of Treg in allograft acceptance [58]. Therefore we think that FLT3L might prevent death due to GVHD partly by increasing the number of NTreg as well as immature DC.

We conclude that, by increasing both immature DC and Treg numbers, FLT3L treatment might be a very valuable way of improving GvHD or autoimmune disease outcome without impairing immune response.

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Authorship

Contribution: L.K.S. and N.B. performed experiments and analyzed data; L.K.S. and R.C. wrote the paper; L.K.S., N.B., R.C. and A.R. designed research; B.M. contributed vital reagent.

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Correspondence: Professor Antonius Rolink, Departement Biomedizin Basel, Division of Molecular Immunology, University of Basel, Mattenstrasse 28, 4058 Basel, Switzerland. E-mail: antonius.rolink@unibas.ch

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6.4

Auto-reconstitution of the T cell compartment by radio-resistant hematopoietic cells following lethal irradiation and bone marrow transplantation

Short title: T cell auto-reconstitution following BMT

Nabil Bosco[1], Lee Kim Swee[1], Angèle Bénard[1], Rhodri Ceredig[1,2] and Antonius Rolink[1]

[1] Department of Biomedicine, Division of Molecular Immunology, University of Basel, Mattenstrasse 28, 4058 Basel, Switzerland.

[2] INSERM U645/UPRES EA2284, IFR 133 Université de Franche-Comté, Etablissement Français du Sang Bourgogne Franche-Comté, BP1937, F-25020 Besançon, France.

Correspondence to:

Professor Antonius Rolink, Department of Biomedicine, Division of Molecular Immunology, University of Basel, Mattenstrasse 28, 4058 Basel, Switzerland. E-mail: antonius.rolink@unibas.ch

Phone: +41 61 695 3072

Fax: +41 61 695 3070

ABSTRACT

It has been known for more than thirty years that in lethally irradiated bone marrow chimeras, part of the reconstituted T cell compartment is derived from the irradiated host. However, the detailed origin and functional activity of these host-derived T cells has not been thoroughly analysed. Here, we generated bone marrow chimeras by reconstituting lethally irradiated C57BL/6 mice with either syngeneic Rag2-deficient or CD3-epsilon-deficient BM neither of which is capable of generating T cells and therefore, all surviving T cells were exclusively host-derived. We show that in the absence of donor-derived cells, host-derived T cells can reconstitute 35% of the normal T cell pool. By comparing thymectomized versus non-thymectomized host, we show that host-derived T cells, comprised a major (70%) subpopulation of *de novo* generated, thymus-derived, polyclonal, naïve cells and a minor subpopulation of surviving, peripheral, oligoclonal, memory-like cells. Host-derived thymocytes regenerated from conventional DN1-2 prothymocytes and their differentiation recapitulated normal thymic ontogeny. Thus, host-derived T cells might provide a first line of defence against infections during recovery from lymphopenia after BMT. This conclusion is supported by the fact that host-derived T cells were fully functional.

INTRODUCTION

In man, syngeneic or allogeneic bone marrow (BM) transplantation (BMT) with BM donors is a frequently used treatment modality which has curative results for a variety of haematological disorders such as malignant or genetic-based immune deficiencies. Nevertheless, the restoration of an adaptive immune system (with both B and T cells) in transplanted patients is a very slow process and during the initial reconstitution phase, patients are lymphopenic. T cell recovery plays a key role in the clinical recuperation of patients post-BMT as they lack adequate T cell-mediated immunity: they often succumb to one of several life-threatening infections, especially cytomegalovirus (CMV) infections (reviewed in (Gress et al., 2007; Hakim and Gress, 2005)). Roosnek and colleagues recently showed that the presence of residual host- (surviving the conditioning regimen) or donor-derived (persisting and expanding from the BM inoculum) CMV-specific $CD8^+$ memory T cells could provide efficient anti-viral immunity and help patients combat CMV infections in the lymphopenic period following BMT (Chalandon et al., 2006). Therefore, in man, it seems that donor and recipient immune infection history should be taken into account in designing the best BMT protocol strategy including whether the donor BM should be T cell depleted and how recipients should be conditioned. The ability of patients, especially adults, to regenerate T lymphocytes after BMT or conditioning therapy-related depletion of the host mature T lymphocyte compartment has emerged as a critical problem in clinical medicine. However, studies in man are difficult to control experimentally and animal models are required to dissect and improve BMT outcomes.

We and others have shown that host-derived T cells are present in lethally irradiated mouse BM chimeras (Anderson et al., 2004; Benard et al., 2006; Komatsu and Hori, 2007). However, the origin and function of these cells were not fully-characterized. This prompted us to examine more carefully the host-derived T cell pool in chimeric mice. We recently showed that host-derived T cells were enriched in extra-thymically located, radio-resistant, functional $CD4^{+}CD25^{+}$ regulatory T cells which could prevent a syngeneic graft-versus-host (GvH) disease following BMT (Benard et al., 2006).

The object of the current study was to characterize further host-derived T cell development and function in chimeric mice. To this end, we generated chimeras by reconstituting lethally-irradiated C57BL/6 mice with either syngeneic $Rag2^{-/-}$ or $CD3\varepsilon^{-/-}$ BM. In such chimeras, donor-derived BM progenitors are not able to generate T cells and surviving T cells will be exclusively host-derived. We showed that host T cells contain a mixture of *de novo*-generated naïve T cells and surviving peripheral memory-like cells. We found that host-derived thymopoiesis was initiated by DN1-2 prothymocytes having a conventional ($CD44^{high}$, $CD117^{high}$, $CD25^{-/+}$) phenotype and we further demonstrate that T lymphopoiesis recapitulates normal thymic ontogeny after BMT. Additionally, by comparing host-derived T cell numbers in non-thymectomized (NTX) versus thymectomized (TX) hosts, we observed that the differentiation of host-derived thymocytes provided an important cohort of naïve, functional, mature T cells having a large TCR repertoire and accounting for up to 35% of the total T cell number found in a control chimeric or unmanipulated mice. Moreover, by using TX hosts, we could show that there was a second population of extra-thymically located, $CD44^{high}CD62L^{-}$ memory-phenotype, functional T cells

having an oligoclonal TCR repertoire. These host-derived T cells might provide a first line of defence against infections during recovery from lymphopenia after BMT.

RESULTS

1. Host-derived T cells following BMT in lethally-irradiated mice.

More than 30 years ago, it was already recognised that in lethally irradiated BM chimeras, part of the T cell compartment was of host origin (Ceredig and MacDonald, 1982; Hirokawa et al., 1985; Kadish and Basch, 1975; Lesley et al., 1990; Sharrow et al., 1983). However, the precise origin and functional activity of these host-derived T cells has not been thoroughly analysed. In order to study host-derived lymphocytes, we reconstituted lethally-irradiated C57BL/6 CD45.2 mice with T-cell depleted (TCD) BM from C57BL/6 CD45.1 donors. After six months, to characterise the host- and donor-derived lymphoid compartments of chimeric mice, we stained splenocyte suspensions with combinations of CD45 allotype-specific antibodies, CD3 for T cells, and CD19 for B cells and analysed them by FACS. As expected, in these syngeneic bone marrow transplantation (BMT) experiments, all recipient mice survived (10/10) indicating a good BM engraftment and there was no evidence of Graft-vs-Host disease (GvHD). However, a sizeable host-derived $CD45.2^+$ lymphocyte population survived even as long as 6 months after BMT. Host-derived lymphocytes in chimeric mice were exclusively $CD3^+$ and represented about 5% total splenic T cells (Figure 1A). Importantly, the absence of host-derived $CD19^+$ B cells ruled out the possibility that host-derived multi-potent cells had survived in our chimeras; this was further confirmed by analysis of the BM where all progenitor

compartment were donor-derived (data not shown). Additional phenotypic analysis of host-derived T cells showed that both $CD4^+$ and $CD8^+$ cells were present and that they were enriched in $CD44^{high}$ $CD62L^{+/-}$ cells compared with their donor-derived partners (Figure 1B). Thus, host-derived T cells are still present in chimeric mice and are mostly composed of $CD44^{high}$ memory-like cells (Tough and Sprent, 1994). To evaluate whether host-derived T cells could be found in other locations, we studied lymphocytes isolated from the blood, gut, liver, lung, lymph nodes and skin of chimeric mice 6 months after reconstitution. Again, none of these organs contained host-derived B cells (data not shown) but did contain host-derived T cells in different proportions. As shown in figure 1C, about 3-5% of blood, LN and spleen, and 10-20% of gut, liver, lung and skin $CD3^+$ T cells were host-derived. Together, these data revealed the presence of a substantial pool of host-derived mature T cells in chimeric mice despite lethal irradiation preceding BMT.

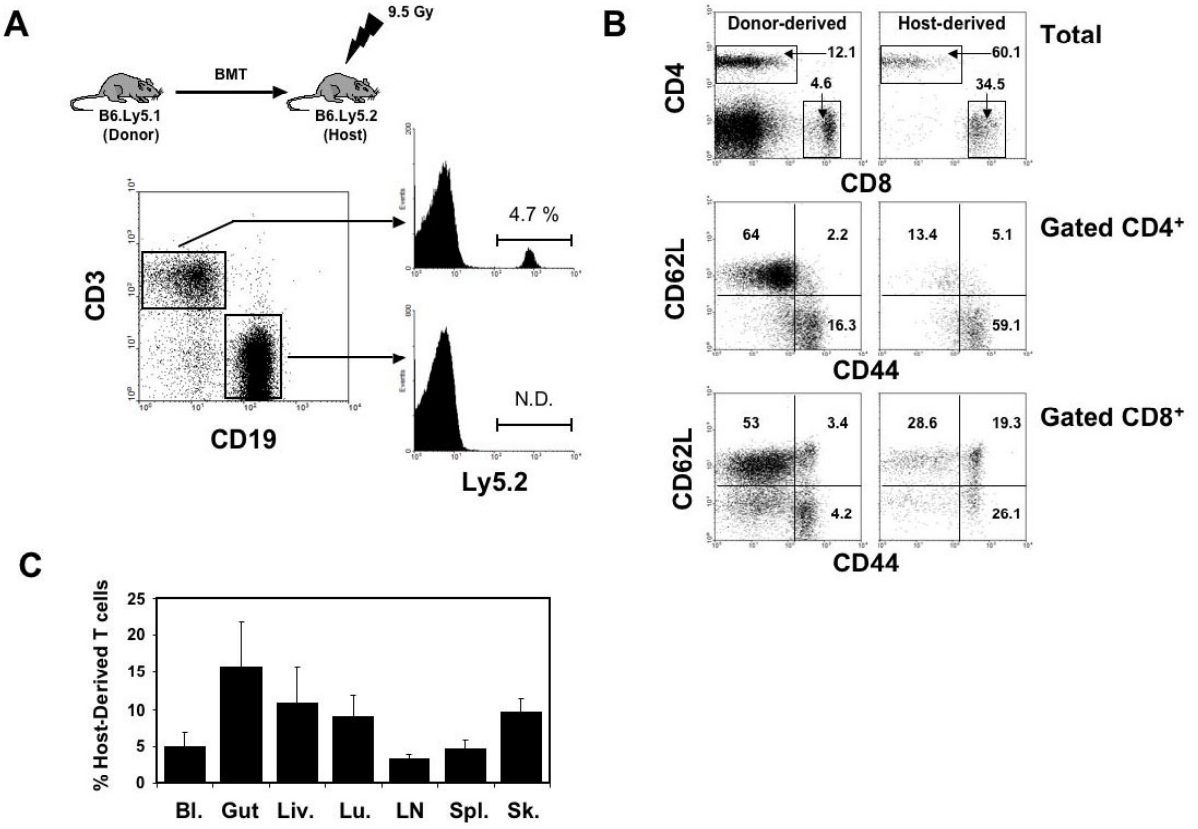


Figure 1. Host-derived T cells are present in lymphoid and peripheral organs of mouse BM chimeras. (A) Lethally-irradiated C57BL/6 CD45.2 mice were reconstituted with T-cell depleted (TCD) BM from C57BL/6 CD45.1 donors. After six months, splenocyte suspensions were stained for CD3, CD19 and CD45 allotype. A typical result of FACS analysis is shown. $CD3^+$ T cells and $CD19^+$ B cells were gated as shown in left dot-plot and the proportion of $CD45.2^+$ (host-derived cells) was quantified as shown in the right histograms (N.D., not detected). **(B)** In the upper dot-plots, shown are CD4 and CD8 distribution in splenocytes. In the middle and lower dot-plots data shown are CD44 and CD62L expression on gated $CD4^+$ (middle dot-plots) or $CD8^+$ (lower dot-plots). Cells were either $CD45.1^+$ donor-derived (left dot-plots) or $CD45.2^+$ host-derived (right dot-plots). Shown in A-B are representative results of 5-10 mice analyzed. Numbers in the quadrants indicate percentages of cells. **(C)** T cell chimerism in blood (Bl.), gut, liver (Liv.), lung (Lu.), lymph nodes (LN), spleen (Spl.) and skin (Sk.) was analysed. Bar histogram represents the mean ± SD of host-derived T cells ($CD3^+CD45.2^+$) from 3-5 mice.

2. Following BMT most host-derived T cells are derived from a single wave of thymic T cell differentiation.

Multiple origins could explain the persistence of host-derived mature T cells in chimeric mice. Firstly, host-derived T cells progenitors could survive and generate T cells. These progenitors could reside either within the thymus or elsewhere and generate either thymus-derived T cells or extrathymic-derived T cells respectively. Secondly, some host-derived mature T cells could represent radio-resistant, resident T cells. These hypothesis are not mutually-exclusive and to discriminate between them, we generated several chimeric mouse combinations as described below.

To demonstrate that the host thymus is still able to produce T cells following lethal irradiation and BMT, chimeras were generated by reconstituting lethally irradiated C57BL/6 mice with C57BL/6.Rag2$^{-/-}$ BM. In such chimeras, donor-derived BM progenitors are not able to generate T cells and surviving T cells will be exclusively host-derived. We tracked host-derived T cells in the thymus and spleen from 1 to 6 weeks following BMT. The total number of thymocytes rapidly decreased and reached about 2 x 10^6 cells one week post-BMT. Reduction of cell number occurred in each CD4 and CD8-defined thymocyte subset, but the degree of reduction varied among the subsets, possibly reflecting their relative radio-sensitivity ($CD4^+CD8^+$ double positive (DP) > $CD4^+CD8^-$ or $CD4^-CD8^+$ single positive (SP)). Figure 2A shows the changes in proportion of each T cell subsets in thymus and spleen. As reported by others (Penit and Ezine, 1989), DP thymocytes are highly susceptible to irradiation and, in the first week following BMT, decreased rapidly from 89.6% in control un-irradiated mice to 3.3%. In contrast, at one week post-BMT, we observed a relative enrichment of $CD4^+$ SP cells, representing 73.2% of total

thymocytes whereas they represented only about 10% of thymocytes in control mice. At two weeks post-BMT, thymocyte number had increased to reach a maximum of 45 x 10^6 cells, representing about 50% of control thymus cellularity. At that time, the CD4 and CD8 distribution was very similar to controls. Additionally, phenotypic analysis revealed that SP thymocytes in chimeric mice were indistinguishable from controls, expressing high level of CD3 and TCRβ, and about 5% of $CD4^+$ SP cells were FoxP-3^+ (data not shown). At six weeks, thymus cellularity had decreased to less than 1 x 10^6 cells. At this time, the thymic rudiment contained exclusively host-derived SP cells.

In the spleen of these chimeras, results reflected what we observed in the thymus. Thus, as early as one week post-BMT, there was a dramatic loss of cellularity and spleens contained a mean of only 10.8 x 10^6 cells. $CD4^+$ T cells represented almost all radio-resistant T cells. At 2 weeks post-BMT, splenocyte number peaked at 17.1 x 10^6 cells, finally decreasing to ~8-10 x 10^6 cells at 6 weeks. Throughout this time, the numbers of $CD4^+$ T cells exceeded that of $CD8^+$ T cells. The percent $CD4^+$ T cells at 1, 2 and 6 weeks post-BMT were 8.5, 2.9 and 39.8 whereas those of $CD8^+$ cells were 1.1, 1.4 and 6.7 respectively. Interestingly and as already documented by others, host splenocytes contained about 0.4 % DP cells; this could reflect some degree of so-called extra-thymic T cell differentiation (Allman et al., 2001; Antica and Scollay, 1999; Garcia-Ojeda et al., 1998; Terra et al., 2005). Thus, as we (Ceredig and MacDonald, 1982) and others (Kadish and Basch, 1975; Penit and Ezine, 1989; Sharrow et al., 1983) have previously shown, following lethal irradiation and BMT, a single wave of host-derived reconstitution occurred (herein referred as *auto-reconstitution*), but the nature of the radio-resistant T cell precursors

responsible for this remained undefined. Additionally, we observed a significant number of mature T cells in the periphery as long as 6 weeks post-BMT. It should be noted that this pool of host T cells could comprise a mixture of (i) radio-resistant mature peripheral T cells that had survived and expanded (ii) T cells derived *de novo* from the host thymus and (iii) some not well-characterized T cells generated via extra-thymic differentiation.

Next, to clarify the relative contribution of these processes to the host-derived T cell pool, we generated chimeras where C57BL/6.CD3$\varepsilon^{-/-}$.CD45.1 BM was transferred into lethally irradiated C57BL/6.CD45.2 hosts which were either thymectomized (referred herein as TX) or not thymectomized (referred herein as NTX). In these chimeras, all mature T cells would be host-derived with those in TX recipients being of only extra-thymic origin and those in NTX recipients a mixture of extra-thymically and thymus-derived cells. Thus, the difference in T cell numbers between these two groups of chimeras would reveal the contribution of thymic-derived T cells to the overall pool. As shown in figure 2B, the donor-derived B cells in the spleen of NTX and TX were very similar to controls and represented 50-60 x10^6 cells or 70-87% splenocytes respectively 2-3 months post-BMT. In contrast, the mean number of host-derived T cells was about 20 x10^6 in control chimeras, decreasing to ~7 x10^6 cells in NTX and to only about 2 x10^6 cells in TX chimeras. Thus, in the spleen, 5 x10^6 host-derived T cells were thymus-derived whereas only 2 x10^6 were from an extra-thymic origin. In TX chimeras, histological analysis of spleen sections revealed abnormal follicular architecture with small, scattered, T cell areas reflecting their severe lymphopenia. In contrast, spleen sections from NTX animals displayed normal features (figure 2C). Our results show that (i) in chimeras where all T cells are host-derived, about 35% [(7/20) x100] of the normal T cell pool could be

formed and maintained and (ii) more importantly, at least 71% [100 – (2/7 x100)] of host-derived T cells are derived from the thymus. This value may be an underestimate because it assumes that the extra-thymically derived T cell pool remained constant in TX versus NTX recipients.

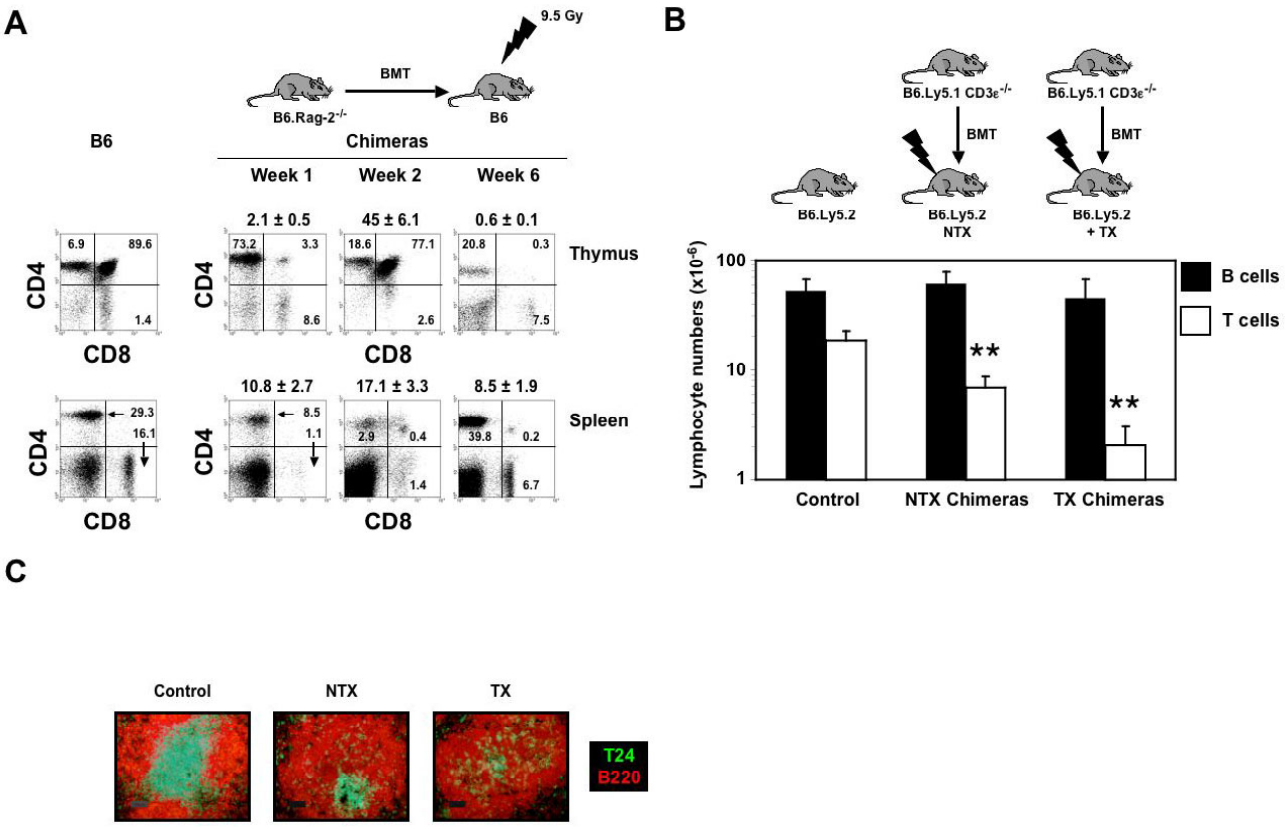


Figure 2. Host-derived T cells in lethally irradiated chimeras are mainly coming from a transient wave of T cell differentiation within the thymus. **(A)** Lethally-irradiated C57BL/6 mice were reconstituted with total BM from C57BL/6.Rag2$^{-/-}$ donors. Thymus and spleen were analyzed at week 1, 2 and 6 post-BMT. Shown are CD4 versus CD8 staining on viable lymphocyte gate. Numbers on the top of the quadrants and in the quadrants indicate mean organ cell numbers ± SD and percentages of cells respectively. **(B)** Lethally-irradiated C57BL/6.CD45.2 mice were thymectomized (TX) or not (NTX) and reconstituted with BM from C57BL/6.CD3ε$^{-/-}$.CD45.1 donors. Chimeras were analyzed 8-12 weeks post-BMT. Bar histograms represent total B (black bars) and T (white bars) lymphocyte numbers ± SD (n=10) in controls and chimeras. ✶✶, *P* <0.01. **(C)** Frozen spleen sections from chimeras described in figure 2B were prepared and stained as described in materials and methods. B cells appear in red and T cells in green. Scale bar, 100 µm.

3. Thymus-derived host T cells originate from radio-resistant DN1-2 like thymocytes.

The above results demonstrated that most (>70%) host-derived mature T cells were derived from a single wave of thymocyte differentiation. The presence of a host-derived thymocyte precursor has been proposed (Ceredig and MacDonald, 1982; Kadish and Basch, 1975; Penit and Ezine, 1989; Sharrow et al., 1983), however, whether it is an intra-thymic "classical" progenitor or an "atypical" cell was not resolved. Indeed, it had been proposed recently (Maillard et al., 2006) that reconstitution of the thymus in the BM chimeras was derived directly from an "atypical" $CD25^{+}CD44^{-}CD117^{-}$ DN3-like precursor. Therefore, complementary approaches were undertaken to further characterise host-derived thymocyte precursors.

As host-derived mature lymphocytes were almost exclusively T cells, we concluded that this radio-resistant precursor should reside within the thymus where T cell precursor commitment is thought to take place. In order to demonstrate that a pool of intra-thymic T cell precursors survive lethal irradiation and can differentiate into mature thymocytes, we carried out foetal thymus organ culture (FTOC) experiments with foetal thymus lobes from lethally irradiated pregnant mice. As shown in figure 3A (and data not shown), one week after FTOC, irradiated thymi contained $TCR\beta^{+}$ DP and SP cells in a proportion similar to that of controls. The same experiments were carried out with pieces of adult thymus in organ culture (ATOC). Again, we observed typical signs of thymocyte differentiation and despite lethal irradiation, organ-cultured adult thymi contained $TCR\beta^{+}$ DP and SP cells one week after ATOC (data not shown).

Next, C57BL/6 mice were reconstituted with $Rag2^{-/-}$ BM and treated with anti-IL-7Rα mAb immediately after BMT (figure 3B, right graph). At two weeks post-BMT, following anti-IL-7R α treatment, the number of host-derived T cells was greatly diminished with thymocyte cellularity reduced from ~50 to 1 x 10^6 cells. This reduction in cellularity was associated with a block of DN to DP stage transition (figure 3B, left panels). We conclude that some T cell precursors survive lethal irradiation, reside within the thymus and that their differentiation is IL-7 dependent.

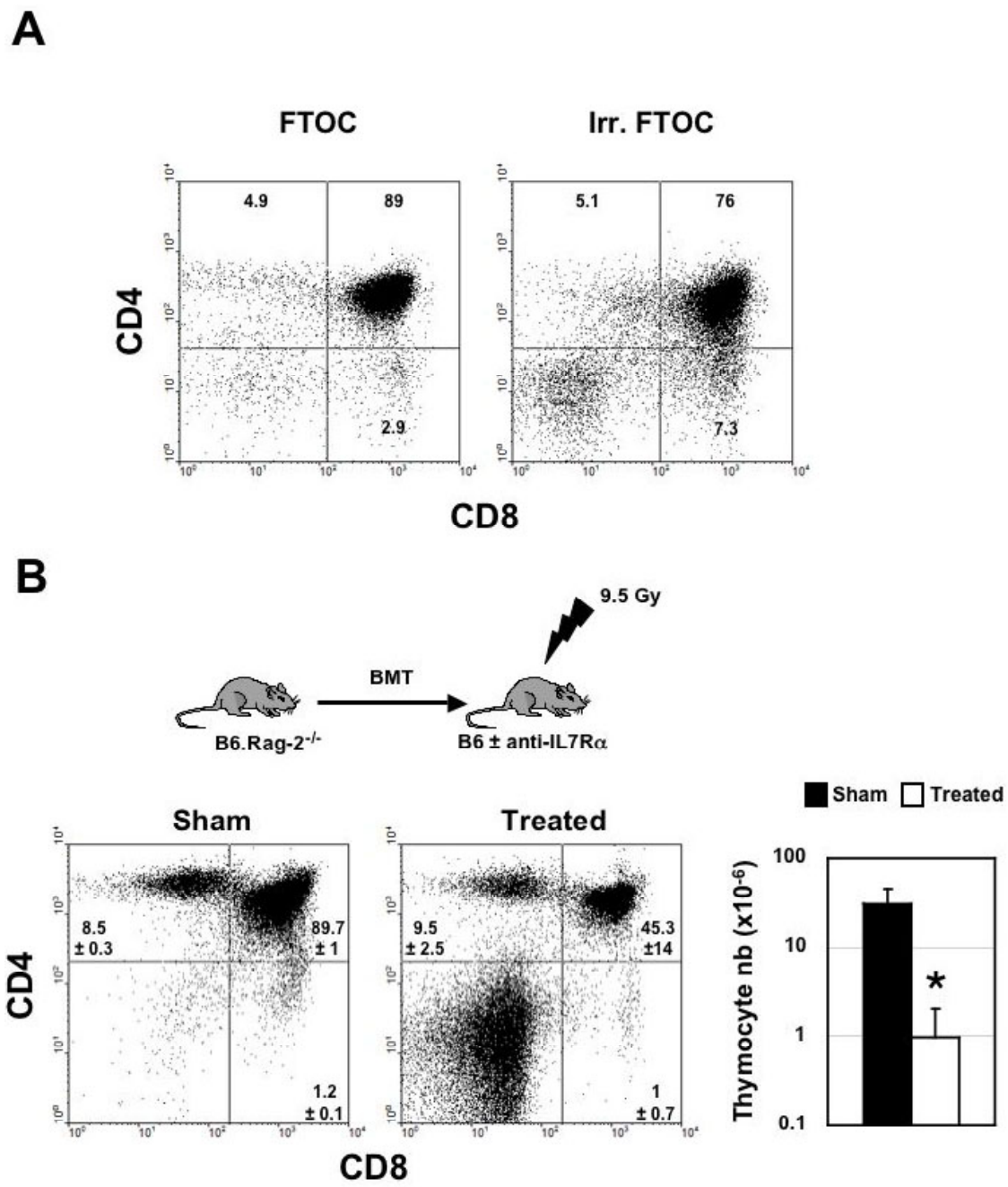


Figure 3. Radio-resistant DN1-2 thymocyte precursors reside in the thymus following lethal irradiation. **(A)** Shown are the CD4 versus CD8 profiles of day-7 FTOC with (right) or without (left) lethal irradiation (950 cGy) at day 0. Numbers in the quadrants indicate percentages. Shown is one representative experiment out of 5 conducted independently. **(B)** The same chimeras as in figure 2A were done and following BMT either injected i.p. with 300 µg of anti-IL-7R blocking antibody (A7R34) in 200 µl of PBS once a week (treated) or with PBS only (sham). Two weeks post-BMT, mice were killed and their thymus analysed by FACS. Shown are CD4 versus CD8 profiles (left dot-plots) and mean thymocyte numbers ± SD (n=3) in a logarithmic scale (right histogram bars). ★, P <0.05.

The properties of these thymocyte progenitors were reminiscent of those of typical DN1-2 thymocyte precursors (Balciunaite et al., 2005; Balciunaite et al., 2005; Ceredig and Rolink, 2002). We therefore enriched DN cells from C57BL/6.CD45.1 → C57BL/6. CD45.2 chimeric mice from 1 to 3 weeks post-BMT and by CD44 and CD117 staining, we were able to reveal the presence of $CD44^{+}CD117^{high}$ cells corresponding to ~0.3 to 0.6% of DN preparations (Figure 4A, left dot plots). As shown in figure 4A, this population contained $CD117^{high}$ DN1 ($CD44^{+}CD25^{-}$) and DN2 ($CD44^{+}CD25^{+}$) T cell precursors. We then quantified the host- and donor-origins of DN1 and DN2 cells from chimeras 1 to 3 weeks post-BMT (Figure 4B, bar graphs). At 1 week post-BMT, DN1-2 cells were detected which were ~99% host-derived and this proportion decreased to ~90% at 2 weeks and finally to less than 1-5% at 3 weeks post-BMT. Thus, early "classical" T cell progenitors exist in the thymus of chimeric mice even though they are rare cells. Initially, the $CD117^{high}$ progenitor compartment is almost exclusively host-derived but later gets diluted by donor-derived cells.

We next addressed the T-cell development potential *in vitro* of host-derived thymic $CD44^{+}CD117^{high}$ cells found in chimeric mice at week 1 post-BMT. About $2x10^{3}$ cells were sorted and differentiation potential was assessed by plating them on OP9-DL1 stromal cells (supporting T-lineage development) in the presence of IL-7. Cells expanded rapidly, reaching about 10^{5} cells one week after culture when they were harvested and analyzed by FACS. They underwent typical T-lineage development features by up-regulation of CD25 and down regulation of CD44 expression (figure 4C left dot-plot). One week post-BMT, 95% of the cells were $CD45.2^{+}$ host-derived (figure 4C right histogram). Taken together, our results

characterize for the first time the nature of genuine radio-resistant T cell precursors. These cells are (i) intra-thymic in location, (ii) grow in an IL-7-dependent fashion, (iii) display a normal CD117^{high} phenotype and (iv) are functional *bona-fide* DN1-DN2 cells with canonical T cell developmental potential.

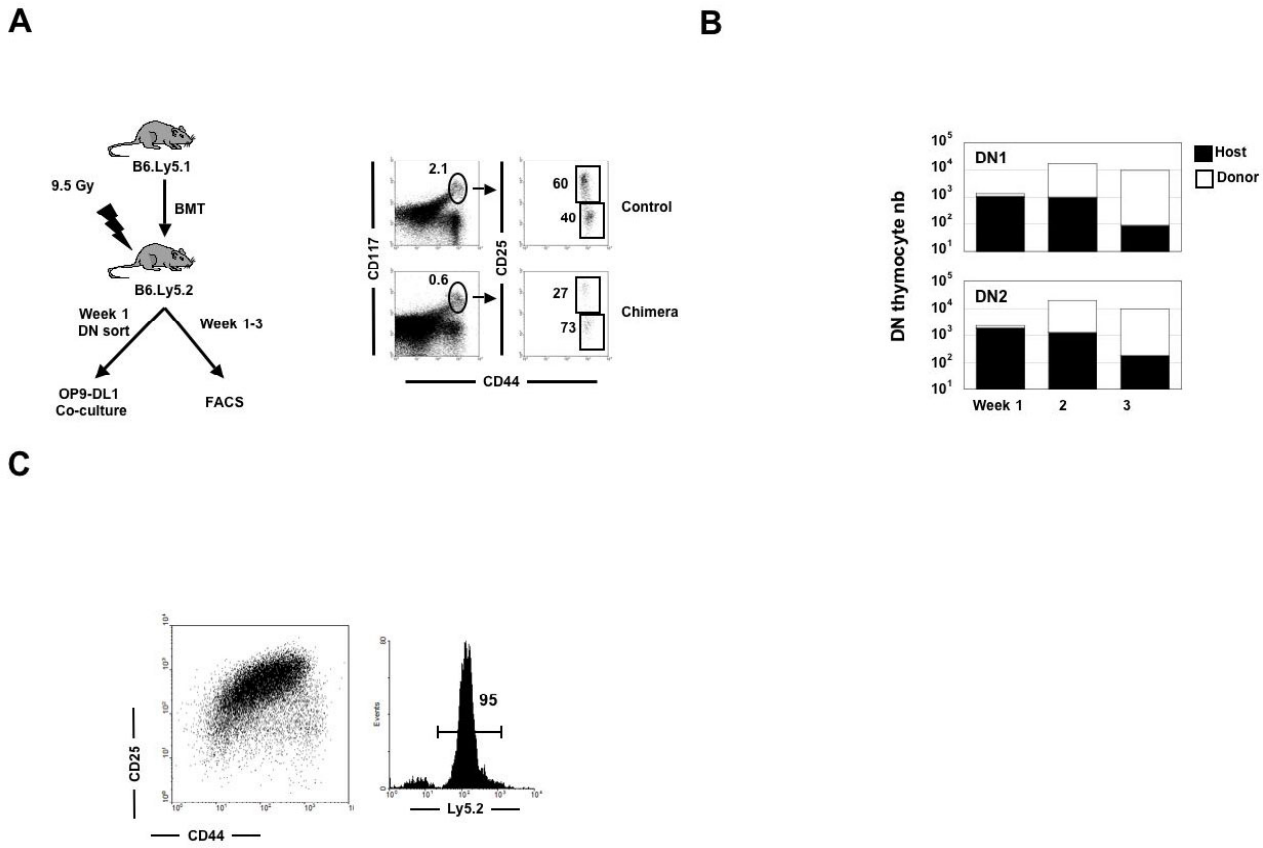


Figure 4. Radio-resistant DN1-2 thymocytes recapitulate normal thymic ontogeny in vitro. **(A)** Lethally-irradiated C57BL/6.CD45.2 mice were reconstituted with TCD BM from C57BL/6.CD45.1 donors. Thymi were analyzed at week 1, 2 and 3 post-BMT. Shown in the right are CD44 versus CD117 on DN preparation and CD44 versus CD25 staining on gated CD44^{+}CD117^{High} cells from an unmanipulated control or a chimeric mouse. CD44^{+}CD25^{-} and CD44^{+}CD25^{+} represent DN1 and DN2 respectively. Numbers in the quadrants indicate representative percentages. **(B)** Bar histograms display the kinetics and mean numbers (n= 3-5 mice for each time point) of host- and donor-derived DN1 and DN2 cells in black and white respectively from 1 to 3 weeks post-BMT. **(C)** The same chimeras as described in figure 4A were done, 5 mice were killed pooled and used to sort CD44^{+}CD117^{High} DN1 and DN2 cells one week after BMT. About 2000 cells were plated on OP9-DL1 stroma with IL-7 and analysed by FACS 7-10 days later to assess their in vitro T-cell development potential. Shown are CD44 versus CD25 profiles and CD45.1 (donor-derived) or CD45.2 (host-derived) proportion of recovered thymocytes.

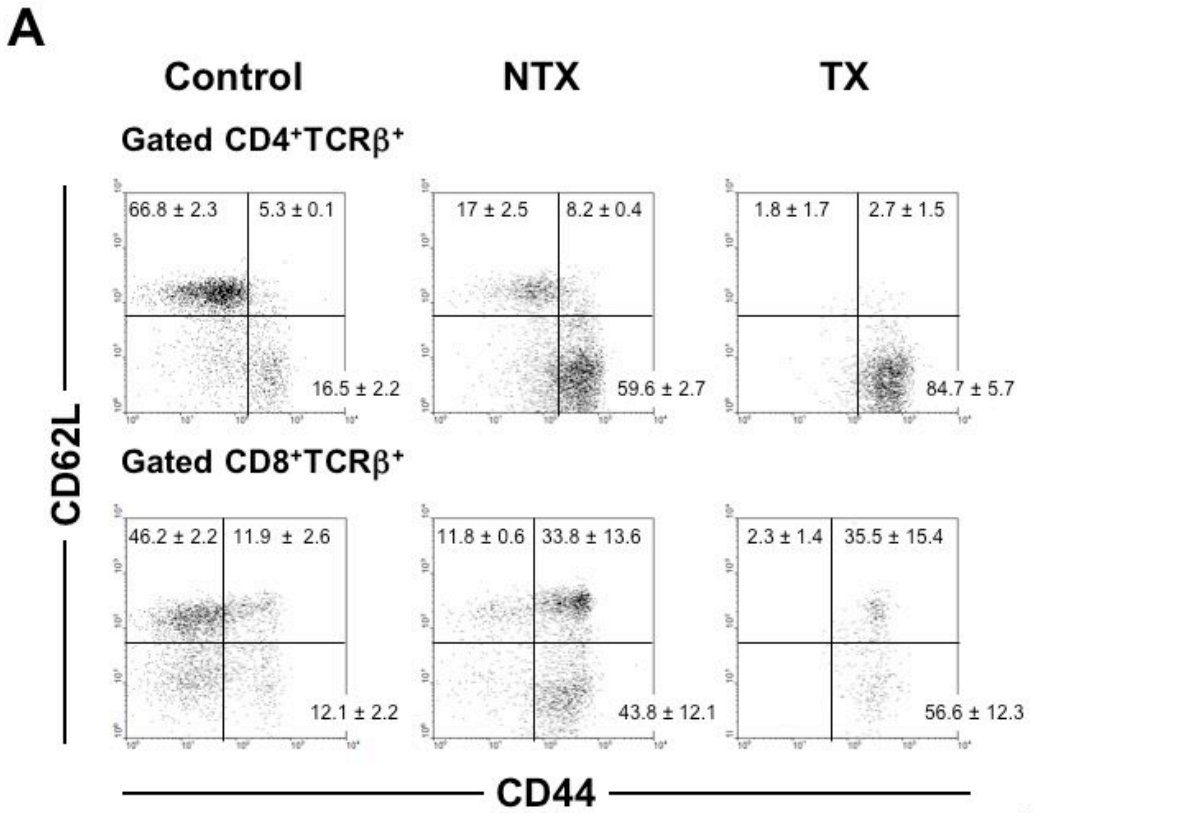
4. Host-derived T cells are functional, but presence of the thymus is required to ensure an unbiased TCR repertoire

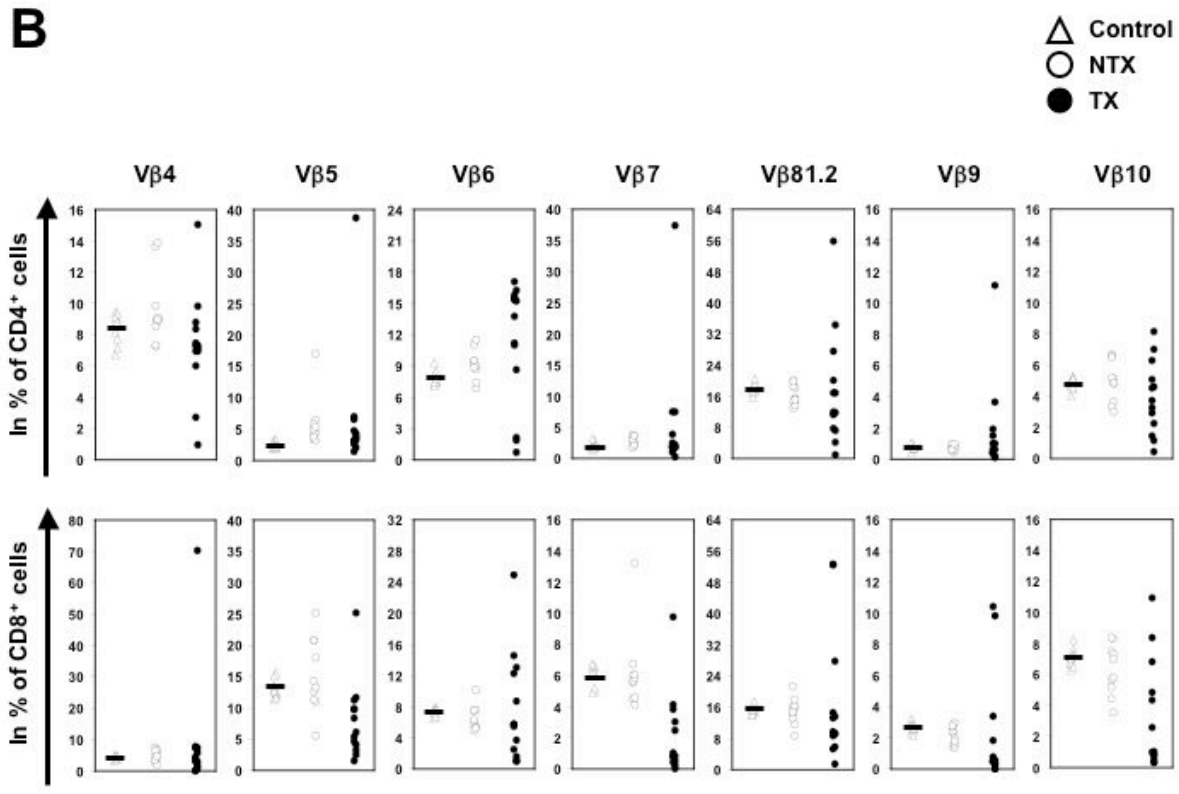
A series of chimeras comparing NTX and TX hosts were generated and analysed further to evaluate the functional importance of the thymus in auto-reconstitution. Phenotypically, the most notable difference between the two subpopulations was the increased proportion of naïve ($CD44^{low}CD62L^{+}$) T cells in NTX hosts (17% and 11.8% for $CD4^{+}$ and $CD8^{+}$ T cells respectively) compared with TX hosts (1.8% and 2.3% for $CD4^{+}$ and $CD8^{+}$ T cells respectively) (Figure 5A). In controls (un-manipulated or B6→B6 chimeras), most T cells displayed a naïve phenotype with 66.8% and 46.2% of $CD4^{+}$ and $CD8^{+}$ T cells respectively (Figure 5A left panels) having the $CD44^{low}CD62L^{+}$ phenotype. Thus, NTX chimeras contained a higher proportion of memory phenotype ($CD44^{High}CD62L^{-}$) T cells whereas in TX chimeras almost all T cells were of this phenotype.

The reduced T cell number in TX hosts, where T cells are only extra-thymically derived combined with their severe T cell lymphopenia might result in a repertoire bias and a consequent functional defect. To investigate the repertoire question further, we stained T cells from 10 mice from each group of chimeras with a panel of anti-TCR Vβ-specific mAbs. As shown in Figure 5B, the TCR Vβ repertoire of $CD4^{+}$ and $CD8^{+}$ T cells in NTX chimeras consistently resembled that found in controls. This was in clear contrast to that of TX chimeras where in many mice the TCR Vβ repertoire was frequently biased.

Next, to determine if TX hosts had functional defects, we measured their ability to mount a T-dependent (TD) antibody response. As shown in figure 5C, 14 days following NIP-OVA immunisation, NTX chimeras were able to mount a TD antibody response as efficiently as controls. This was in striking contrast to TX chimeras,

where only 4 out of 10 mice were able to mount a TD immune response. Taken together, our results show that host-derived T cells in chimeras are functional but in TX hosts, their oligoclonal nature frequently resulted in an inability to mount an efficient antigen-specific TD antibody response.


A
Control
NTX
TX
Gated CD4+TCRβ+
66.8 ± 2.3
5.3 ± 0.1
16.5 ± 2.2
17 ± 2.5
8.2 ± 0.4
59.6 ± 2.7
1.8 ± 1.7
2.7 ± 1.5
84.7 ± 5.7
Gated CD8+TCRβ+
46.2 ± 2.2
11.9 ± 2.6
12.1 ± 2.2
11.8 ± 0.6
33.8 ± 13.6
43.8 ± 12.1
2.3 ± 1.4
35.5 ± 15.4
56.6 ± 12.3
CD62L
CD44


B
Control
NTX
TX
Vβ4
Vβ5
Vβ6
Vβ7
Vβ81.2
Vβ9
Vβ10
ln % of CD4+ cells
ln % of CD8+ cells

C

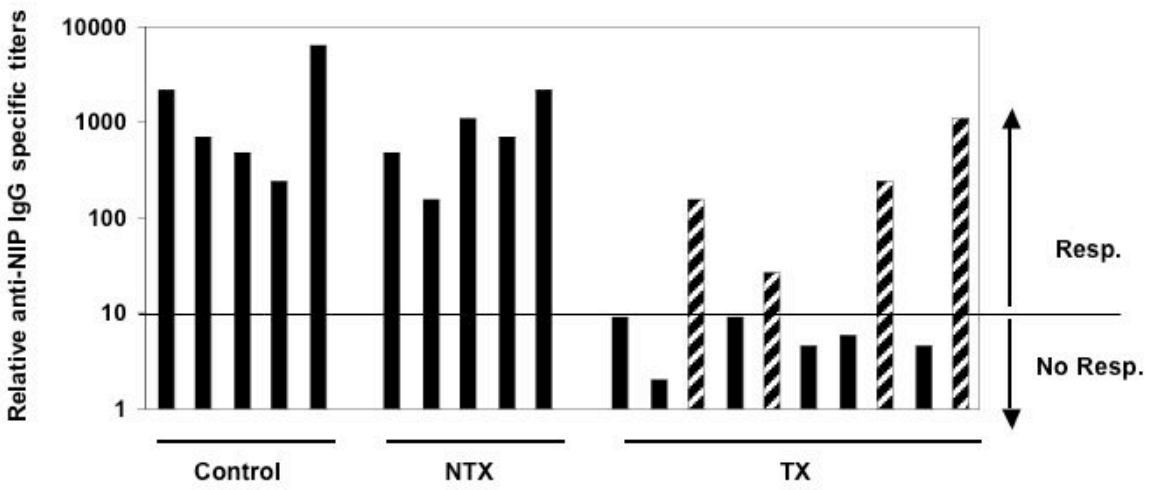


Figure 5. Phenotype, repertoire and functionality of host-derived T cells in lethally-irradiated BM chimeras. **(A)** Shown are CD44 and CD62L expression on gated $CD4^+$ $TCR\beta^+$ (upper dot-plots) or $CD8^+TCR\beta^+$ (lower dot-plots). Numbers in the quadrants indicate mean percentages ± SD from at least 5 mice analysed per group. **(B)** Vb repertoire analysis among $CD4^+$ and $CD8^+$ T cells by FACS in chimeras. Each symbol represents a single mouse from control (▲), NTX (O) or TX (●) group. Black bars represent mean value in control group. Ten mice per group were analysed. **(C)** Depicted is T cell-dependent antibody responses in different group of chimeras day 14 after NIP-OVA immunization. Shown are relative titers of IgG anti-NIP specific antibodies. Each bar represents a single mouse and 5-10 mice were analyzed. When the ratio [anti-NIP IgG titers after immunization / anti-NIP IgG titers before immunization] was below 10 (horizontal line) we considered that the mouse did not mount a T-dependent response. In control and NTX group, 5/5 mice made a specific response with ratio >100. Among TX group, only 4/10 mice made a specific response (hatched bars). Control, NTX or TX chimeras were the same as described in figure 2B.

DISCUSSION

By carefully studying the kinetics of development, distribution, phenotype and function of the host-derived T cells in BM chimeras, we have clarified their previously unappreciated, but important, contribution to the restoration of the immune system. We called this process “auto-reconstitution”. We revealed that following lethal irradiation, host-derived haematopoietic cells in secondary lymphoid organs were almost exclusively composed of T cells and totally devoid of B cells. In addition, host-derived T cells represented a considerable proportion of total T cells (~5% of all T cells six months post-BMT). Kinetic analysis showed that the proportion of host-derived T cells decayed as donor-derived T cell development slowly took place in B6→B6 chimeras. In these competitive settings, where the donor BM generated T cells, it was not possible to study host-derived T cells exclusively. Thus, we generated non-competitive BM chimeras in which BM donor cells (either $Rag2^{-/-}$ or $CD3\varepsilon^{-/-}$) were incapable of generating T cells. Then, we were able for the first time to quantify precisely host-derived T cells in these non-competitive chimeric settings. Surprisingly, auto-reconstitution could generate a pool of T cells equivalent to 35% that in normal mice. We could also show that T cell auto-reconstitution had two origins: (i) a major, thymic-dependent pathway (Thy-D) and (ii) a minor, thymic-independent pathway (Thy-I). Indeed, in $CD3\varepsilon^{-/-}$→B6 chimeras, by comparing host-derived T cell numbers in euthymic (NTX) versus athymic (TX) hosts, we could quantify Thy-D and Thy-I pathways. Thus, the Thy-D pathway accounted for 70% of host-derived T cells, thereby representing the main source of host T cells in an auto-reconstituted T cell compartment. This estimate is based on the assumption that the

size of the Thy-I compartment is not drastically altered by the Thy-D cohort of T cells and *vice versa*.

The Thy-D T cells were composed of the progeny of a single wave of T-cell differentiation which reached a maximum 2 weeks following BMT. As host-derived thymocyte differentiation is only transient and ceases around 3-4 weeks, the thymus does not contain any DP cells later on and its cellularity decreases dramatically. This has been previously characterized by others (Ceredig and MacDonald, 1982). The Thy-D cohort of T cells provides a large number of naïve T cells with a diverse TCR Vβ repertoire. Logically, this observation led us to investigate the nature of radio-resistant thymocyte progenitors.

Recently, when addressing the cellular origin of the host-derived cohort of thymocytes regenerating in BM chimeras, Maillard *et al.* (Maillard et al., 2006) suggested that they arose from an abnormal sequence of differentiation starting from a donor-derived $CD25^{+}$ $CD44^{-}$ $CD117^{-}$ "DN3-like" stage and therefore might involve an atypical thymocyte progenitor. In contrast, we could detect the presence of genuine, or "conventional" $CD25^{-/+}$ $CD44^{+}$ $CD117^{high}$ DN1-2 cells. Such conventional DN1-2 cells were few in number, representing around 2000 cells per mouse one week post-BMT. Further analysis indicated that these T cell progenitors were mainly (~95-99%) host-derived initially and were then replaced by donor derived progenitors around 3 weeks post-BMT.

The toxicity of ionizing radiation to cells is associated with massive apoptosis which is particularly acute in hematopoietic organs, well-known to be highly

radiosensitive. Lethal γ-irradiation triggers DNA breaks which in turn activate DNA repair mechanisms and/or p53-dependent apoptosis, unless DNA repair has been carried out correctly (Jeggo and Lobrich, 2006; O'Driscoll and Jeggo, 2006). There is a body of literature addressing the radio-sensitivity of lymphoid cells, but so far, there are no clear mechanisms demonstrating why only some subpopulations of cells can survive lethal irradiation damage. From in vitro experiments with cell lines, it has been proposed that the cell cycle status at the time of irradiation might determine their radio-sensitivity (Jeggo and Lobrich, 2006; Lobrich and Jeggo, 2007; Pawlik and Keyomarsi, 2004). Thus, cycling cells harbour much more DNA alterations than their quiescent counterparts and are more prone to die. This dogma by itself would hardly explain what is going on *in vivo* in lethally irradiated mice. Indeed, it would seem that memory T cells, which are known to be more rapidly cycling than their naïve counterparts, survive whereas B cells, some of which are known to be more cycling than others, are totally deleted. Thus, we believe that certain niches throughout the body might provide lymphocytes with a particular environment with specific radio-protective signals. In our study, the gut contained a higher proportion of radio-resistant T cells and could be an interesting candidate for such a radio-protective niche even though we cannot exclude a preferential relocalisation of surviving T cells following total body irradiation (TBI). Interestingly, a role for the gut in radioprotection was recently proposed because a polypeptide-drug derived from commensal salmonella flagellin, which binds to Toll-like receptor 5 and which activates nuclear factor-kB signalling, was able to prevent mice from gastrointestinal and hematopoietic disorders following lethal TBI(Burdelya et al., 2008). Furthermore, in recipient mice where the anti-apoptotic gene *Bcl-2* was constitutively over-expressed ($H2^K$-BCL-2 transgenic mouse), even host-derived B cells survived following TBI and BMT (data

not shown and (Domen et al., 1998)). Thus, the clear contrast in survival properties of B versus T cells might (at least partly) be due to intrinsic differences in their expression of pro- versus anti-apoptotic genes. By intracellular staining, we have already observed a higher baseline level of Bcl-2 expression in T cells versus B cells. Clearly, the issue of the differential survival of T versus B cells following lethal irradiation is warranted and might shed light on important pathways for radioprotection at the cellular level.

As shown in TX recipients, the minor, Thy-I-derived, T cell component of auto-reconstitution was composed of the progeny of mature, radio-resistant peripheral $CD4^+$ and $CD8^+$ T cells. Following their survival and expansion post-irradiation, they expressed a biased TCR Vβ repertoire. Phenotypically, these Thy-I cells had a $CD44^{High}$ $CD62L^{+/-}$ memory-like phenotype which could correspond to their initial phenotype or to a phenotype acquired upon lymphopenia-induced expansion (Bosco et al., 2005; Bosco et al., 2006). Additionally, as we and others have previously shown, these cells were enriched in FoxP-3^+ regulatory T cells (Anderson et al., 2004; Benard et al., 2006; Komatsu and Hori, 2007; Roord et al., 2008). Following BMT in mice, these Thy-I Treg cells protected the host from GvHD complications in syngeneic conditions (Benard et al., 2006). Others studies have shown that Treg, as well as memory T cells, are more resistant to γ-irradiation, chemotherapy or antibody-mediated depletion *in vitro* and *in vivo* (Anderson et al., 2004; Benard et al., 2006; Bourgeois and Stockinger, 2006; Gladstone et al., 2007; Walzer et al., 2002). The molecular mechanisms behind these interesting observations are still unknown and require further experiments.

Functionally, in NTX CD3$\varepsilon^{-/-}$→B6 thymus-bearing chimeras, host-derived $CD4^+$ T cells alone were sufficient to mount a TD immune response whereas in TX recipients, the immune response was frequently impaired. Clinically, it is recommended that patients be re-vaccinated following BMT in order to restore a high level of protection against many vaccine-preventable diseases such as measles, tetanus, hepatitis or polio (Avigan et al., 2001; Gangappa et al., 2008; Ljungman et al., 2005; Parkman, 2008; Patel et al., 2007). In this context, mouse BM chimeras might provide a relevant experimental system to study immune response or diseases associated with lymphopenia subsequent to conditioning regimens and BMT in humans. Published observations suggested that host-derived cytomegalovirus-specific (CMV) $CD8^+$ memory T cells could provide efficient anti-viral immunity and help patients survive the severe lymphopenic period following BMT (Chalandon et al., 2006). However, studies in man are difficult to control experimentally and animal models are required to dissect and improve the outcome of BMT.

In conclusion, our results demonstrate a so-far unappreciated, but important, residual host-derived thymic activity following conditioning and BMT. These thymus-derived T cells constitute a significant population of T cells with a polyclonal TCR Vβ repertoire and normal functional properties. By analysing thymectomised recipients, we could demonstrate that in their absence, lymphopenia was more severe, the T cell repertoire was frequently oligoclonal and immune responses were compromised. Therefore, strategies should be developed to improve and expand the Thy-D and/or the Thy-I cohort of T cells which together could serve as a first line of defence in lymphopenic hosts until donor-derived T cell reconstitution takes place. In this regards, improving thymic reconstitution and peripheral T cell survival by, for

example, infusing IL-7/anti-IL-7 complexes (Boyman et al., 2006; Boyman et al., 2008) during thymus regeneration would appear to be a promising approach.

MATERIALS & METHODS

Mice.

Female C57BL/6, C57BL/6.Rag2$^{-/-}$, and C57BL/6.CD3$\varepsilon^{-/-}$ mice of either CD45.1 or CD45.2 genotype were maintained in our SPF animal facility. As BM donors, mice were used at 4-8 weeks of age and recipients were killed by CO_2 inhalation prior to analysis. These studies were approved by the institute animal care and user committee.

Thymectomies.

At 4 to 6 weeks of age, mice were anesthetized and thymus was removed by suction through a small upper sternal incision. That thymectomy had been complete was verified in each animal by anatomical inspection at the time of sacrifice.

Cell preparation, immunofluorescence staining and FACS analysis.

Lymphoid organs (thymus, spleen or lymph nodes) were removed from adult unmanipulated or chimeric mice at the indicated times. Single-cell suspensions were generated by disruption of organs through a 40 µm nylon mesh via a syringe plunger and were washed once with 2% FCS-supplemented DMEM. Spleen cell suspensions were depleted of red cells by NH_4Cl treatment prior to staining. Lymphocyte suspensions from liver, lung and skin were prepared by standard procedures and Percoll density gradient centrifugation performed as previously described(Schleussner and Ceredig, 1993) prior to cell recovery. Biotin-, FITC-, PE-, PE.Cy7-, or APC-conjugated mAbs were either home made or purchased from BD Biosciences or eBioscience (San Diego, CA). Cell staining was performed as

previously described (Bosco et al., 2005; Bosco et al., 2006) prior to FACS analysis with a FACScalibur and CELLQuest Pro software (BD Bioscience). The data presented are of live-gated cells based on a combination of forward- and side-scatter signals and when possible propidium iodide exclusion.

Bone marrow transplantation.

Bone marrow cell suspensions from CD45 allogenic donor mice were prepared by flushing femurs and tibias with PBS using a 23g needle. After a red blood cell lysis step, T cells were depleted when necessary by resuspending cells in a mixture of rat IgM anti-CD4 (RL172) and anti-CD8a (31M) mAb hybridoma supernatants and incubated for 20 min at 4°C. Following a washing step, antibody-coated cells were lysed by adding a 1:10 dilution of screened rabbit serum as source of complement in serum free Dulbecco's modified Eagle's medium (DMEM) for 45 min at 37°C. Then, cells were washed, resuspended in serum-free DMEM and counted prior to injection. Host mice were lethally g-irradiated with a single dose of 950 cGy at a dose of 80 to 90 cGy/min using a Cobalt-source (Gammacell 40, Atomic energy of Canada, Ltd) 4h prior to receiving $5x10^6$ bone marrow cells intravenously. Chimeric mice were analysed at the indicated time points and the host and donor origin of lymphoid cells determined by means of labelled CD45.1- or CD45.2-specific mAbs.

Fetal thymic organ cultures

Fifteen day pregnant C57BL/6 mice were lethally irradiated (950 cGy) prior to embryo removal. Thymus lobes from irradiated or control un-irradiated embryos were then used for FTOC. For staining, lobes were dissociated by passing through needles of

decreasing size as previously described (Balciunaite et al., 2005), washed in DMEM medium and then stained and analyzed by flow cytometry.

Thymus DN preparation, cell sorting and culture on OP9-DL1 stroma.

From total thymocytes, $CD4^-CD8^-$ double negative (DN) cells from control or chimeric mice were prepared by complement-mediated lysis of CD4 and CD8 expressing thymocytes as described above. DN thymocytes were then stained with combinations of fluorescently-labelled CD25, CD44 and CD117 (c-kit) mAbs and pooled $CD25^-$ DN1 and $CD25^+$ DN2 cells sorted as $CD117^{high}CD44^+$ cells using a FACSAria sorter (BD Biosciences). Reanalysis of sorted cells indicated that their purity was routinely ≥ 98%.

OP9 stromal cells expressing the Notch ligand delta-like-1 (OP9-DL1) were kindly provided by Professor Juan-Carlos Zúñiga-Pflücker (University of Toronto, ON, Canada) and maintained in IMDM supplemented with 5 x 10^{-5} M β-mercaptoethanol, 1 mM glutamine, 0.03% (wt/vol) Primatone (Quest, Naarden, The Netherlands), 100 U/mL penicillin, 100 µg/mL streptomycin, and 2% heat-inactivated fetal bovine serum (FBS). Two days prior to coculture, 10^4 stromal cells were seeded per well of a 24-well plate. At day 0, stromal cells had grown to semi-confluency and were then γ-irradiated with 3000 cGy, culture medium removed and replaced by supplemented DMEM plus IL-7 (~10ng/mL). To these cultures were added ~5x10^3 sorted DN1 and DN2 thymocytes. At day 10-14 of culture, cells were either analysed by FCM or kept in culture after transfer to fresh OP9-DL1 stromal cells.

T cell-dependent antibody responses.

To induce a T cell-dependent antibody response, reconstituted chimeric mice were injected subcutanously with 200µl complete Freund's adjuvant emulsion containing 50 µg total NIP-ovalbumin. Sera were obtained after bleeding prior to and 14 days after immunization and stored at –20°C. Hapten-specific IgG antibody titres were determined by enzyme-linked immunosorbent assay (ELISA) as previously described (Ceredig et al., 2006).

Immunofluoresecent staining of spleen sections.

Spleens were snap frozen with dry ice in Tissue-Tek OCT compound and then 5 to 7-µm sections cut on a cryostat. Sections were then fixed for 10 min in acetone and stored at -80°C. For staining, sections were covered with an antibody solution at saturating concentration diluted in PBS-1% FBS for 1h at room temperature. Anti-$B220^{Biotin}$ and anti-$CD90^{FITC}$ were used to discriminate B and T cell areas respectively, and PNA^{Biotin} (Peanut Agglutinin, Vector Laboratories) and anti-IgM^{FITC} (M41) used to reveal germinal centres (GC). For the second step, sections were washed in PBS (three times 10 min) and incubated 30 min at room temperature with a Neutralite Avidin-Texas-Red reagent (Southern Biotech) diluted in PBS. Then, sections were washed, coverslipped and analyzed under a fluorescence microscope (Zeiss axioskope) with a 10 to 20x objective.

Statistical analyses.

Data are presented as the mean and SEM. Comparisons between groups were done using a Student's two-tailed *t* test for independent events. *P* values of less than 0.05 were considered significant (★, *p* value <0.05 and ★★, *p* value <0.01).

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CONFLICTS OF INTEREST

The authors have no financial interest to disclose.

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7. General discussion

In this work we have investigated several aspects T cell central and peripheral tolerance, I would like now to compare and contrast two different experiments related to the treatment of autoimmune and GvH diseases.

In the first study, we demonstrated the CD11c-HA x TCR-HA double transgenic mice could be partially rescued from premature death by transfer of HA-specific Treg whereas treatment failed to prevent overall signs of autoimmunity.

In the second study, we could show that the treatment of BDF1 mice with FLT3L prior to GvHD induction was able to rescue as much as 100% of the mice and that no other major signs of disease could be observed (summarized in table 1).

Model	Disease	Treatment	Survival upon treatment
CD11c-HA x TCR-HA	Systemic autoimmunity	Injection of sorted HA-specific Treg	Partial rescue
B6 -> BDF1	Acute GvH disease	Semi-therapeutic FLT3L injections	Complete rescue

Table 1. Treatment of autoimmune and GvH diseases.

Even though, these two systems are completely different in both case CD4 T cells play an instrumental role in the development of the disease and the establishment of T cell tolerance seems to be a key point in the success of the therapy. We can hypothesize on why the first treatment failed to prevent disease whereas the second seems to be very efficient in this regard.

Chronic vs acute disease

A major difference between the two models is that in CD11c-HA x TCR-HA mice, auto-reactive T cells are constantly produced by the thymus whereas GvHD is induced by injection of a fixed number of alloreactive T cells. In the latter situation the resulting disease has a more acute form.

In other words, the success of the treatment of CD11c-HA x TCR HA depends on long-term establishment of tolerance because self-reactive T cells are always exported. In contrast, the control and/or deletion of fixed number of alloreactive T cells might be sufficient to prevent GvHD. As already discussed, chronic defects in central tolerance might be difficult to cure, this could be an explanation why autoimmunity in CD11c-HA x TCR-HA is difficult to treat.

Cellular vs cytokine therapy

An obvious limitation of cellular therapy, e.g. transfer of Treg cells, is the restricted survival and migration of injected cells. Unfortunately we did not monitor the fate of donor cells and we have no data regarding these issues however we can speculate that a majority of injected cells might die because of lack of survival signals and/or inability to migrate in lymphoid organs. We do not know either whether Treg can persist in the constant presence of their cognate antigen or whether cells get exhausted similarly to effector cells. Altogether, the limited number of cells and their long-term survival is a clear problem in the treatment of chronic disease.

In contrast, FLT3L treatment induces expansion of DC and Treg compartment in situ. The treatment is therefore much more efficient because cells expand in their physiological environment where survival signals are provided and where they can deliver they tolerogenic function.

Therefore considering “Treg therapy”, expansion of pre-existing cells (with relevant specificity) is probably a better approach than transfer. FLT3L is a highly potent cytokine to increase the number of DC and we have shown here that it expands peripheral Treg compartment. However it is doing so in a rather inefficient manner regarding the amount of cytokine needed compared to the expansion. Other ways of expanding Treg in vivo have been published among which injection of IL-2/anti-IL-2 complexes seems to be particularly efficient [70]. Authors could actually demonstrate that such treatment could prevent spontaneous diabetes in NOD mice. It is important to notice here that treatment by complexes is expanding Treg to a level that is probably impossible to achieve by transfer of cells.

Treg vs DC therapy

In the first study, the treatment is based on transfer of HA-specific Treg cells whereas in the second, the strategy was to expand DC and Treg compartment by repetitive injection of FLT3L. We are currently trying to dissect the role of DC and Treg in therapeutic effect of FLT3L however, given the respective expansion of both compartment we hypothesized that most of the therapeutic effect is DC-mediated.

In other words, we can speak here about Treg- versus DC-mediated therapy. As already mentioned, it is not fair to compare both therapies because models

are different and unfortunately we could not apply these two therapies on the same model. However conceptually, we can make a contrast between a “repressor” versus an “educational” strategy. The goal of Treg therapy is to control self-reactive T cells, to “repress” whereas the objective of DC therapy is to differentiate allo/self-reactive T cell toward an anergic or regulatory phenotype, to “educate” T cells and/or eventually to delete them. This makes DC-mediated therapy more interesting at least in theory.

Clinical applications

One last point of comparison is the potential application of such therapies to human. Cellular therapy like Treg transfer seems to be highly difficult to achieve, because it needs to manipulate cells from the patient ex-vivo e.g. convert Ag-specific T cells toward regulatory phenotype in order to re-inject them in the patient. Such protocols have tremendous costs in time and money and require extreme cautions due to the ex-vivo manipulation of the cells. In contrast, treatment of patients with recombinant cytokines is much more simple.

For these reasons, cytokine-mediated expansion of Treg or DC are probably much more interesting in clinical perspective.

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Curriculum Vitae

General

First name: Lee Kim

Last name: SWEE

Date of birth: 03.02.1981

Nationality: Swiss (Isorno/TI)

Education

2004-2008:

PhD in immunology. Developmental and Molecular Immunology

Departement Biomedizin, university of Basel

1999-2004:

Diploma in biology

University of Lausanne

1996-1999:

Baccalauréat / Maturité fédérale

High school Yverdon