Constandina Pospori **UCL**

PhD thesis

Tumour Immunology

WT1 TCR gene transfer into haematopoietic stem cells: In vivo functional analysis of WT1-specific T cells

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Declaration

I, Constandina Pospori confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the thesis.

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Abstract

The Wilms tumour antigen is a promising target for T cell-based tumour immunotherapies. Vaccines against WT1 peptides tested in cancer patients showed immunological and molecular responses. However, the clinical responses observed were partial and it is currently not known whether physiological levels of WT1 expression in some healthy tissues results in the deletion or tolerance induction of WT1-specific T cells. In this PhD project, TCR gene transfer into purified haematopoietic stem cells (HSCs) was used to study the thymic development of WT1-specific T cells and their fate in the periphery. Lentiviral constructs containing the genes for an HLA-A2 allorestricted, murinised WT1 TCR or the genes for a control, viral peptide-specific, LMP2 TCR, were generated. The conditions for lentivirally-transduced HSC transplants were optimised. The results obtained from WT1 TCR tranduced HSC transplants in HLA-A2Kb transgenic mice demonstrated that thymocytes expressing this high-avidity WT1 TCR were positively selected into CD8 T cells and emerged in the recipient's periphery. WT1-specific T cells exhibited a memory, CD44hi phenotype correlating with rapid antigen specific killing, proliferation and cytokine secretion of WT1-specific T cells in the absence of vaccination. LMP2-specific T cells exhibited a naive-like, CD44low phenotype without any antigen specific function. WT1-specific T cells persistent long-term in the periphery of transplanted mice, and no autoimmunity was noted. The results presented in this thesis show for the first time that T cell specificity for a tumour-associated, self-antigen did not result in tolerance induction,

but instead mediated the spontaneous generation of functionally competent, memory phenotype T cells.

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List of abbreviations

Ag – Antigen

AIRE – Autoimmune Regulator

APC - Antigen Presenting Cell

APECED - Autoimmune Polyendocrinopathy-Candidiasis-Ectodermal Dystrophy

AT - Adoptive Transfer

BM - Bone Marrow

BrdU - Bromodeoxyuridine

CECs – Cortical Epithelial Cells

CFSE - Carboxyfluorescein Diacetate Succinimidyl Ester

CLPs - Common Lymphoid Progenitors

CMPs - Common Myeloid Progenitors

cTECs - cortical Thymic Epithelial Cells

CRP – C-Reactive Protein

CTL - Cytotoxic T Lymphocyte

DC - Dentritic Cell

DL4 – Delta Ligand 4

DL – Delta Like

DN - Double Negative (thymocytes)

DP - Double Positive (thymocytes)

EBV - Epstein–Barr Virus

ELISA - Enzyme-Linked Immunosorbent Assay

ER - Endoplasmic Reticulum

ETP – Early Thymic Progenitors

FACS - Fluorescence-Activated Cell Sorting

FCS - Fetal Calf Serum

FTOC - Fetal Thymic Organ Cultures

GFP - Green Fluorescent Protein

HEV - High Endothelial Venules

HLA - Human Leukocyte Antigen

HSC - Hematopoietic stem cell

IFNγ - Interferon-gamma

IMDM - Iscove's Modified Dulbecco's Media

IRBP - Interphotoreceptor Retinoid Binding Protein

IRES - Internal Ribosomal Entry Sites

LB – Lysogeny Broth

Lin-ve - Lineage negative

LIP - Lymphopenia-Induced Proliferation

LM - Listeria Monocytogenes

LN – Lymph Node

LMP2 - Latent Membrane Proteins 2

LTR – Long Terminal Relief

MAbs - Monoclonal Antibodies

MECs - Medullary Epithelial Cells

MHC - Major Histocompatibility Complex

MOI – Multiplicity of Infection

mTECs - medullary Thymic Epithelial Cells

NK cells – Natural Killer cells

OVA – Ovalbumin

PBS - Phosphate-Buffered Saline

PCR - Polymerase Chain Reaction

PGE - Promiscuous Gene Expression

PI - Propidium iodide

pTα - pre-TCRα chain

RAG - Recombination Activating Gene

RIP – RAT Insulin Promoter

RNA - Ribonucleic Acid

RPMI - Roswell Park Memorial Institute medium

RT-PCR - Reverse Transcription Polymerase Chain Reaction

SP – Single Positive

SCID - Severe Combined Immunodeficiency

TAP – Transporter Associated with Antigen Processing

TAA - Tumour Associated Antigens

Tcm – Central Memory T Cells

TCR – T Cell Receptor

Tem – Effector memory T Cells

Tg- Transgenic

TIL - Tumour Infiltrating Lymphocytes

TRA – Tissue Restricted Antigen

Treg – Regulatory T cell

TSP – Thymus Settling Progenitors

UEA - Ulex Europaeus Agglutinin

VM cells - Virtual Memory cells

VSV-G - Vesicular Stomatitis Virus Glycoprotein

WT - Wild Type

WT1 – Wilms Tumour protein

1.1 Commitment to the T cell lineage and Early T cell development

1.1.1 From HSC to T cell precursor in the thymus

T cells, unlike all other haematopoietic cells require a specialised organ, the thymus, dedicated to their development. T cell progenitors derived from HSCs are imported from the blood into the thymus via the blood vessels near the corticomedullary junction, (Petrie and Zúñiga-Pflücker, 2007) in a periodic fashion(Donskoy et al., 2003, Foss et al., 2001). However, the nature of the extrathymic T cell progenitors that seed the thymus is not yet conclusively decided. Early thymic progenitors (ETPs) are the most immature thymocytes, but a number of progenitor cells in BM and blood feature as candidates for the actual T cell progenitors that enter the thymus and give rise to ETPs.

A model of T cell lineage development long-thought to be true was based on the identification of common lymphoid and common myeloid progenitors (CLPs and CMPs) in the BM on methylcellulose cultures (M. Kondon et al Cell 1997, A Galy et al Immunity 1995, K. Akash et al Nature 2000) and suggested that lymphoid progenitors separate from myeloerythroid progenitors early on in haematopoiesis. According to this, the cells that settle in the thymus and eventually differentiate into T cells would either be CLPs or their progeny and as such should only be able to develop into T, B and NK cells. However, in recent studies where single cell ETP cultures on OP9:OP9-Delta ligand 4 (DL4) stroma that can support both lymphoid and myeloid development, showed that ETPs could differentiate towards either of the two lineages given the right conditions (Wada et al., 2008, Bell and Bhandoola, 2008). These

studies therefore disputed the established dichotomy between lymphoid and myeloid lineage commitment and instead proposed a model were ETPs arise from multipotent progenitor cells with T, B and myeloid potential.

The newly established model instead suggests that Notch1 signalling in the thymus drives the commitment towards the T cell lineage by repressing gene expression programs characteristic of other lineages. In vitro studies in OP9 and OP9-DL1 stroma cell cultures have shown that Notch1 signals sequentially restrict the lineage potential of progenitor cells as these move from the DN1 to the DN3 stage (Schmitt et al., 2004). However, the involvement of Notch1 signalling in T cell lineage commitment was known even earlier. In 1999 Pui et al using BM chimera experiments where donor BM cells, were retrovirally transduced to express a constitutively active form of Notch1, demonstrated that this resulted in the development of immature αβ T cells in the BM while B cell lymphopoiesis was blocked (Pui et al., 1999). The reverse was observed in the absence of Notch1 signalling in developing thymocytes in a study by Radtke et al published the same year; in BM chimeras reconstituted with induced Notch1$^{-/-}$ BM cells, T cell development was blocked early before the DN2 stage. Instead thymocytes derived from these progenitors appeared to differentiate into phenotypically normal, immature B cells within the thymus (Radtke et al., 1999).

Recent evidence provided independently by two groups (Hozumi et al., 2008, Koch et al., 2008), identified DL4 ligand as the partner for the Notch1 receptor expressed in developing thymocytes which is necessary and sufficient to impose T cell lineage commitment. Both groups by located the site of DL4

expression to be in the thymic epithelial cells of the cortex, using DL4 antibody and cytokeratin staining in immunohistochemistry analysis. They also generated foxN1Cre:DL4 floxed mice to conditionally switch off DL4 expression in thymic epithelial cells. As this resulted in a block in T cell development at the DN2 stage and in the ectopic development of immature B cells in the thymus, it recapitulated the phenotype of mice bearing Notch1-/- BM progenitors described above and provided the functional evidence for the significance of DL4 expression by the thymic microenvironment, in T cell lineage commitment and development. In accordance with these results, the studies rejecting the long-established dichotomy between lymphoid and myelo-erythroid lineages, reported that the myeloid potential of ETPs was most clearly manifested in the absence of Notch1 signalling (Bell and Bhandoola, 2008, Wada et al., 2008).

1.1.2 The transition of DN thymocytes to CD4+CD8+ DP thymocytes

The transition between the DN2 to DN3 stages of T cell development primarily depends on V(D)J recombinations of the *Tcrγ*, *Tcrδ* and *Tcrβ* gene loci, directed by RAG1 and RAG2 (Mombaerts et al., 1992b, Shinkai et al., 1992) which make up the lymphoid-specific recombinase RAG. Following successful TCRβ VDJ rearrangement the pre-TCR complex, consisting of the rearranged TCRβ chain, an invariant pre-TCRα chain (pTα) and the CD3 molecule (Groettrup et al., 1993, Saint-Ruf et al., 1994) is assembled on the cell surface of developing thymocytes. Notch 1 signalling plays a decisive role at this stage of T cell development as well. In mice where the Notch1 gene was specifically inactivated at the DN2 stage, V-DJ rearrangement of the TCRβ genes was

impaired and resulted in a block at the pre-TCR checkpoint (Wolfer et al., 2002).

The analysis of T cell development in TCRβ-/- mice (Mombaerts et al., 1992a), as well as in pTα deficient mice (Fehling et al., 1995), showed that the expression of both of these components of the pre-TCR complex on DN3 thymocytes is necessary for the proliferation and survival of thymocytes committed to the TCRαβ lineage, as well as for their differentiation into CD4+CD8+ DP T cells. The pre-TCR signalling which mediates this process, called β-selection, has been shown to be ligand-independent in studies with transgenic mice expressing a truncated form of TCRβ and pTα lacking their extracellular domains (Irving et al., 1998). As β-selection is a developmental checkpoint that tests the productive outcome of the TCRβ locus rearrangement but not the specificity of this molecule, intuitively it is not surprising that an extracellular interaction is not required for the signalling that drives differentiation to the next stage. However, autonomous pre-TCR signalling alone does not seem to be sufficient in driving differentiation to the DP stage, as isolated DN3 cells fail to differentiate in vitro in the absence of a supporting thymic microenvironment (Petrie et al., 1990). More recent data from studies where Rag2-/- thymocytes - arresting in the DN3 stage - were transduced with the genes for a TCRβ chain and subsequently cultured in OP9 or OP9-DL1 stroma cultures, showed that only in the presence of Notch1 signalling, TCRβ gene transfer could mediate β-selection and the differentiation of Rag2-/- DN3 thymocytes into DP cells (Ciofani et al., 2004). It was therefore concluded that cooperative signals from Notch1 and pre-TCR are required during β-selection.

1.1.3 From pre-TCR to TCR expression on developing DP thymocytes

β-selection is followed by a transient downregulation of recombinase activity to block further TCRβ gene rearrangement and in this way ensure that a single TCRβ chain is expressed in each T cell. This process which is a classic example of allelic exclusion is followed by a proliferative burst and the differentiation of developing DN cells into CD4+ CD8+ Double Positive (DP) cells migrating to the thymic cortex. Once these cells become quiescent DP thymocytes, RAG1 and RAG2 activity resume and *Tcr α- chain* locus rearrangement is established (Wilson et al., 1994, Petrie et al., 1995). The aim of the recombination events at this site is not only to generate productive rearrangements of the TCR α chain locus, but also to provide a TCRα chain which when coupled to the already selected TCRβ chain gives rise to a self-MHC restricted TCR. Nevertheless, if a newly formed TCRαβ specificity fails to recognise self-p:MHC complexes on cortical Thymic Epithelial cells (cTECs), it is still possible for such a DP thymocyte to be rescued as gene rearrangement at this locus resumes giving rise to a different TCRα chain. A number of attempts of forming an appropriate specificity are possible in the 4-5 days that the cell spends in the thymic cortex.

The first evidence that allelic exclusion at the TCRα chain locus is not as stringent as in the case of the TCRβ locus came about following a study by Von Boehmer's group published in 1992. The authors looked into the expression of a transgene and endogenous TCRα chains in TCR Tg mice, at different stages of thymocyte development. T cells expressing the transgenic class I-restricted HY TCR were destined to commit to the CD8 lineage. The

introduced TCR, as in most TCR Tg mice, was expressed earlier than in the physiological T cell development. In this system the authors observed that while the introduced TCRαβ heterodimer was expressed in CD8 SP cells, the few CD4 SP thymocytes that were detected expressed the Tg TCRβ chain paired with an endogenous TCRα chain. This was the case even though the Tg TCRα chain was still produced and was present intracellularly in those cells. These results, in combination with the observation that RAG1 and RAG2 activity only ceased after a thymocyte is positively selected, allowed them to conclude that unlike the case of the *Tcrβ* locus, productive α chain rearrangement is not enough to signal the end of the recombination events at this site. Instead, it continues until the developing thymocyte assembles a positively selecting TCRαβ heterodimer (Borgulya et al., 1992).

The main events in T cell lineage commitment and early T cell development are schematically represented in figure 1.1.

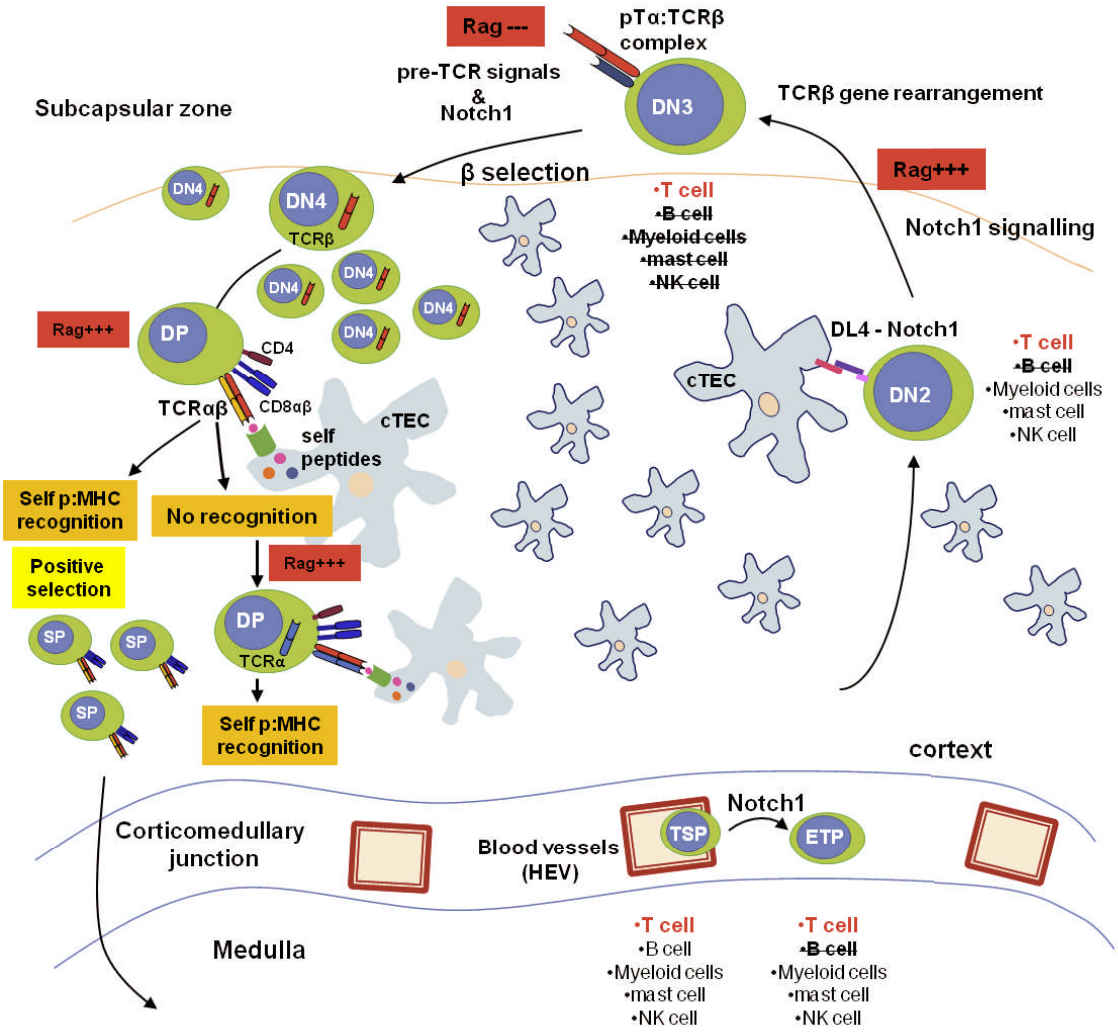


Figure 1.1 Schematic Representation of early T cell development and T cell lineage commitment.

T cell progenitors home to the thymus and enter this organ through the High Endothelial Venules (HEV) that are present in the corticomedullary junction. Notch 1 signalling in the thymus progressively represses other haematopoietic lineage fates, including the B cell lineage, driving in this way T cell lineage commitment.

TCR expression is a stepwise process in T cell development. RAG expression drives V(D)J recombinations at the TCRβ locus commensing at the DN2 stage. A rearranged TCRβ chain pairs with an invariant pre-TCRα chain (pTα) at the DN3 stage, forming the pre-TCR. Signalling through the pre-TCR confirms successful TCRβ chain rearrangement and together with Notch1 signalling mediates β-selection and the transition of DN3 thymocytes to the DP stage. At the DP stage, TCRα chain locus rearrangement takes place and pairing of a succesfully rearranged TCRα chain with the previously selected TCRβ chain results in the expression of a complete TCR molecule on the cell surface. Positive selection

Figure 1.1 (continued)

occurs at this point, assessing the expressed TCR for its ability to recognise peptides on the self-MHC complex. if a newly formed TCR$\alpha\beta$ molecule fails to be positively selected TCRα chain rearrangement restarts to produce a different TCR$\alpha\beta$ molecule. This process can be repeated a few times to allow the positive selection of DP T cells, followed by migration to the medulla where negative selection is generally thought to take place.

1.2 T cell development and Central Tolerance

The aim of positive selection is to identify immature DP T cells expressing TCRs with intermediate affinity for self-peptide:self MHC complexes (self-p:MHC) and induce them to differentiate into mature single positive (SP) CD4 or CD8 thymocytes. Once developing thymocytes reach the SP stage, specificity for self p:MHC has the opposite effect and leads to their deletion, a process called negative selection. Negative selection, one of the major mechanisms of central tolerance, ensures that autoreactive T cells are purged from the circulating T cell repertoire to avoid autoimmune damage on healthy tissues. In this simplified description of positive and negative selection, the paradox that has troubled scientists over the years quickly becomes apparent. Recognition of self is what seems to positively select DP T cells in the cortex, but also what appears to delete them as SP T cells in the medulla. A number of groups took up addressing this contradiction and 3 main theories have emerged from these studies, namely the altered peptide, the affinity and the avidity models of thymocyte selection.

1.2.1 The altered peptide model of thymic selection

The altered peptide model suggested that the peptides displayed by APCs mediating positive selection in the cortex differ both from those presented in the medulla where self-Ag recognition leads to cell death, as well as from those that mature T cells would encounter in the periphery. In that way the cells that successfully go through positive selection are not doomed to be deleted in the next step of thymic development, but at the same time they are also unlikely to cause autoimmunity as they are not positively selected on the

same self-peptides that they see in the periphery (Marrack and Kappler, 1987). A schematic illustration of the altered peptide model is shown in figure 1.2. The same group proposing this model went on to test it by isolating and comparing peptides from MHC class II proteins in the spleen and the thymic cortical epithelium. Their data showed that there was no fundamental difference in self-peptides presented by MHC class II by the two different cell types and consequently the altered peptide hypothesis was rejected (Marrack et al., 1993).

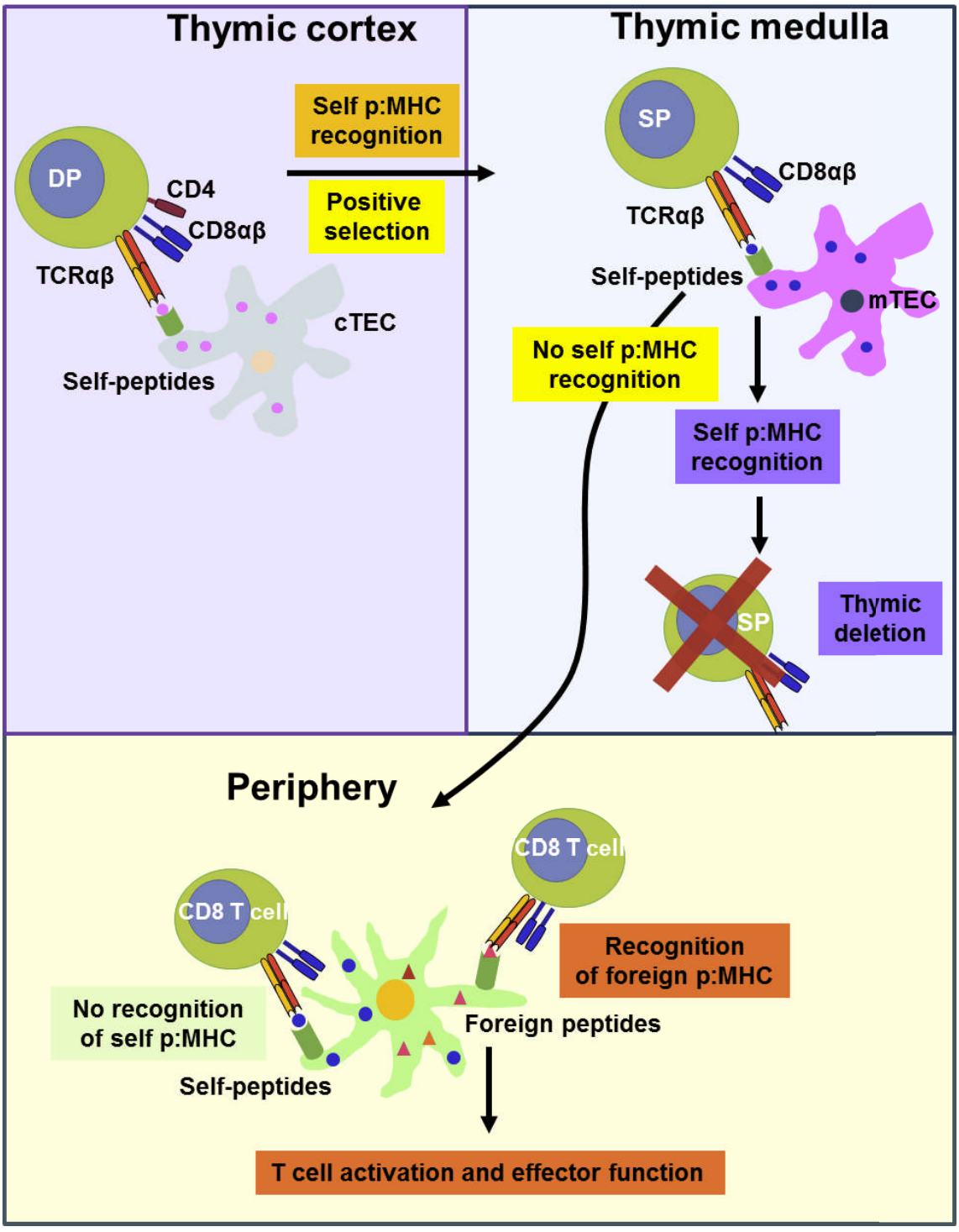


Figure 1.2 Schematic diagram of the altered peptide model of positive and negative thymic selection. The altered peptide model predicted that in the thymic medulla, generally accepted to be the site of negative selection, the peptides presented would consist of peripheral tissue antigens but would be different from the peptides presented to developing thymocytes in the thymic cortex, the site of positive selection. In this way, self p:MHC recognition in the cortex would allow positive selection, whereas self p:MHC recognition in the medulla (where the self p pool is different from the cortex) would drive thymic deletion.

1.2.2 The affinity model of thymic selection

The affinity model of positive and negative selection, first suggested by Sprent and co-workers (Lo et al., 1986) on the other hand, has been supported by a substantial amount of data by different groups and has been widely accepted. As illustrated in figure 1.3, this model suggests that thymocytes expressing TCR with no or very low affinity for self p:MHC die by neglect, whereas very high affinity contacts lead to death through negative selection. In 1996, Alam et al used a cloned OVA-specific TCR, in plasmon resonance experiments that allowed them to measure the kinetics of TCR-p:MHC interactions with different positive and negative selecting ligands in fetal thymic organ cultures (FTOCs). The purpose of this experiment was to correlate the affinity of each p:MHC ligand to the selection outcome. They were able to clearly demonstrate that positive selecting affinities were always up to 1-log lower than negative selecting affinities (Alam, 1996).

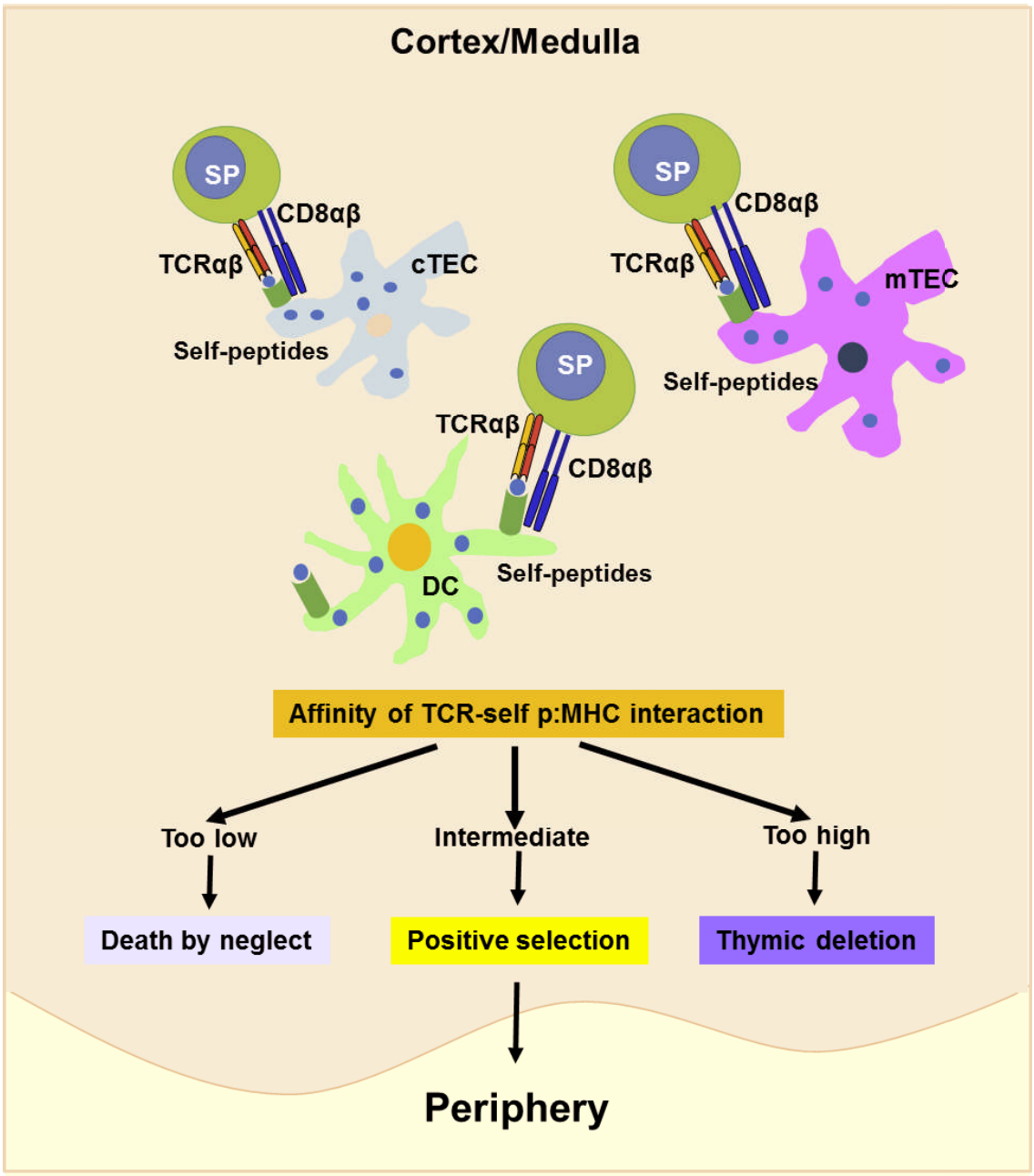


Figure 1.3 The affinity model of thymic selection. The simplest form of the affinity model of thymic selection suggests that positive and negative selection are both decided by the affinity of the TCR-self p:MHC interaction, irrespective of the location of this interaction and whether it takes place between T cell and cTEC, mTEC, or BM-derived APCs. An intermediate affinity interaction allows developing T cells to be positively selected. If the affinity of the interaction is too low, then developing T cells die by neglect whereas if the interaction is of too high affinity the cells are deleted to prevent autoreactive T cell specificities from emerging in the periphery.

1.2.3 The avidity model of thymic selection

The avidity model predicts that the quantity of interacting TCR-self p:MHC together with the quality of the individual interactions decides whether a thymocyte is positively or negatively selected. It came about following two studies demonstrating that the administration of negative selecting peptides at extremely low doses could result in positive selection of the relevant developing T cells (Sebzda, 1994, Ashton-Rickardt, 1994). The data provided by Alam et al, could be in agreement with both models.

However, an elegant study published in 2006 by Daniels et al has provided further evidence in favour of the affinity model. This group studied positive and negative selection of OT-I transgenic DP thymocytes in FTOCs in the presence of different OVA variant ligands exhibiting a hierarchy of affinities for this TCR. As the affinity of the ligands for the TCR was measured by Tetramer binding, this assay was performed on live cells and the data obtained took into account the participation of the CD8 co-receptor in the TCR-p:MHC complex interaction. Since among the ligands the least potent negative selector was only 1.5 times more potent than the strongest positive selector, the authors were able to show that very slight increases in the affinity of the TCR-p:MHC-CD8 interaction, but importantly not in the amount of added peptide, resulted in a shift from positive to negative selection. It was therefore appropriate to conclude that only a very narrow range of positively selecting autoreactive affinities could allow developing T cells to escape negative selection (Daniels, 2006).

1.2.4 The altered peptide model returns and complements the affinity model.

Despite the great amount of evidence in support of the affinity model, on its own this theory does not take into account the spatial and temporal compartmentalisation of thymic selection processes and provides an explanation for negative selection, but not for other central tolerance mechanisms such as the deviation of autoreactive T cells to the Treg lineage. Furthermore despite the sophistication of these studies, the peptides used were never shown to be physiologically expressed and presented in cTECs or indeed known to contribute to positive selection under natural conditions.

In this context, recent studies comparing the pathways through which MHC-bound peptides are generated and presented in cortical TECs (cTECs), medullary TECs (mTECs) and peripheral APCs put forward the idea that the affinity model and the previously rejected altered peptide hypothesis for positive and negative selection might actually complement each other (Klein et al., 2009). In all cells, proteolysis that gives rise to the antigenic peptides presented by MHC class I molecules is mediated by multicatalytic proteinase complexes called proteasomes (Brown et al., 1991, Rock et al., 1994). These cylindrical structures consist of 4 heteroheptameric rings, 28 subunits in total and of these the β1, β2 and β5 subunits mediate the catalytic function. IFNγ-induced versions of these particular subunits are present in immune cells. These proteasomes are more efficient in producing antigenic peptides and are called immunoproteasomes. Almost coincidentally, Murata et al came across the gene for a different version of the β5 proteosome subunit. They established

that this was exclusively expressed in the thymus and so they called it β5t (t for thymus). Using immunostaining of mouse thymus sections for β5t, the Ly51 and UEA-I and CD11c markers of cTECs, mTECs and DCs respectively, they were able to demonstrate that this newly discovered proteosomal subunit was exclusively found in the thymic cortex and in particular was expressed in cTECs (Murata, 2007). Immunoblot analysis of different thymic cell populations not only confirmed these results but also revealed that cTECs mainly contain thymoproteasomes defined as proteasomes containing β5t, β1i and β2i subunits. Thymoproteasomes, unlike immunoproteasomes and proteasomes were found to have weaker chymotrypsin-like activity and consequently are expected to cleave proteins in a different fashion. This suggests that an altered pool of peptides might be available and be presented by cTECs during positive selection, (than during negative selection). In the absence of the thymoproteasome in β5t-/- knockout mice, thymic CD8 T cell development did not progress beyond the DP stage and there was an associated dramatic decrease in the size of the CD8+ T cell compartment. In view of these data and the fact that cTECs are known to be instrumental in mediating positive selection, the same group went on to further investigate the role of the thymoproteasome at this stage of T cell development by crossing HY-TCR Tg mice with β5t deficient mice.

Normally in female HY-TCR Tg mice, HY-specific CD8 T cells are positively selected, while in male mice developing HY-specific T cells are deleted by negative selection as they recognise the HY-self antigen. In the absence of β5t in these mice, positive selection was highly impaired in female mice, whereas negative selection in male mice remained unaffected. This proved the

essential role of β5t in positive selection. Beyond these results, the authors were able to show that the reduced numbers of CD8 T cells generated in β5t deficient mice remained functional in response to TCR engagement, but instead displayed an altered and diminished TCR repertoire compared to wild-type mice and hence were unable to mount an effective immune response to allogeneic and viral antigens (Nitta et al., 2010).

The importance of the thymoproteasome in positive selection, strongly proposes a come-back of the altered peptide theory of positive and negative selection, even if at this point the available technologies only allowed Nitta, Murata and co-workers to speculate in the nature and uniqueness of the peptide repertoire presented by cTECs facilitating positive selection.

The comeback of the altered peptide model and the well established affinity model together, as illustrated in figure 1.4, provide an explanation as to how recognition of self-antigen can result in the divergent developing T cell fates of positive or negative selection. However, they do not give us an insight into the nature and mechanisms by which peripheral self-antigens appear in the thymus.

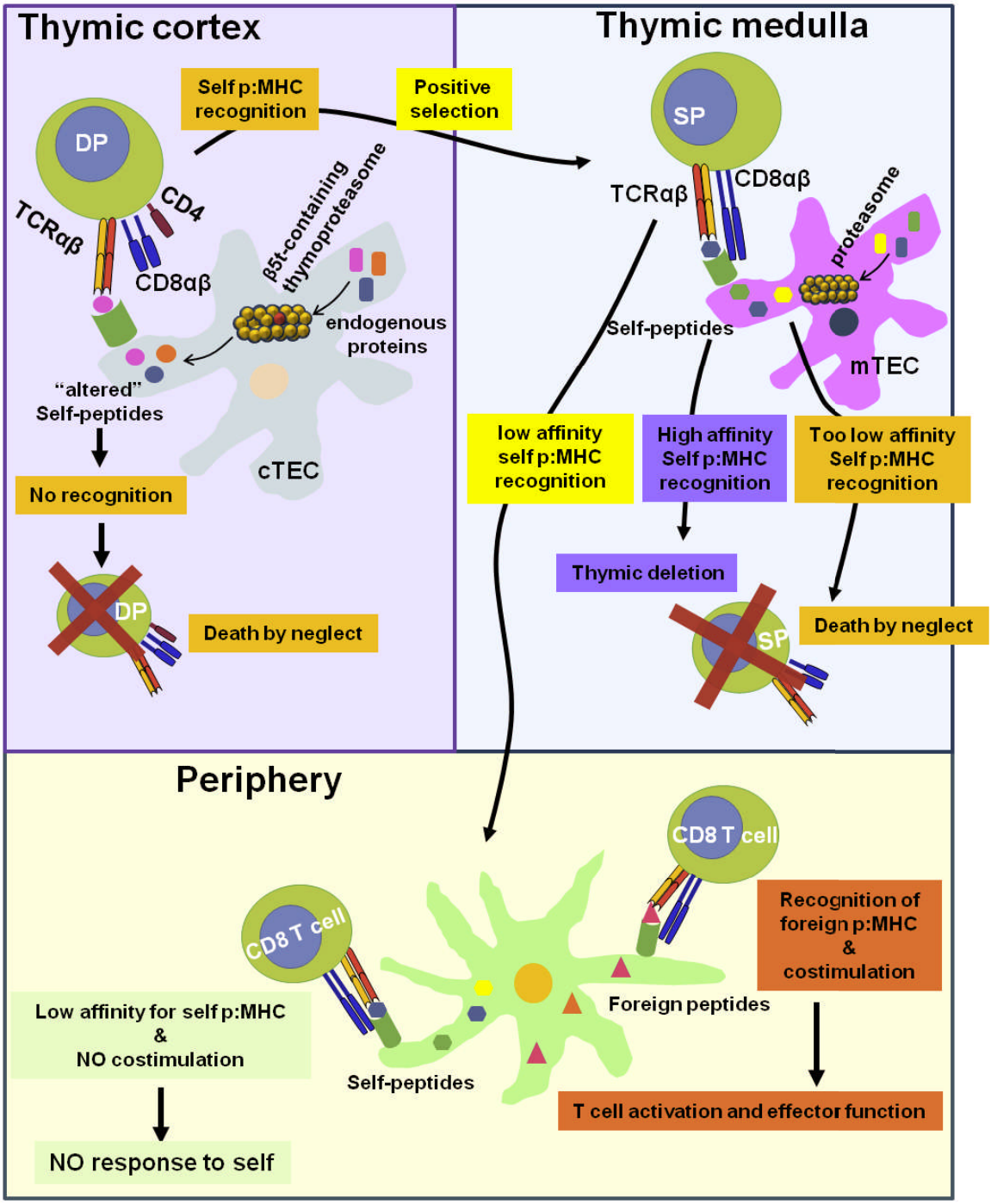


Figure 1.4 Schematic diagram of the current model of positive and negative selection combining the altered peptide and affinity models. A combination of the altered peptide and affinity models of positive and negative selection provides a model that takes into account the spatial and temporal compartmentalisation of these processes. In cTECs a distinct proteasome is present (the β5t-containing thymoproteasome) and this cleaves proteins in a different fashion than proteasomes present in mTECs and BM-derived APCs. Therefore, a different pool of self-peptides is available in the cortex compared to the medulla. Self-Ag expression in the medulla

Figure 1.4 (continued)

on the other hand, mirrors that of peripheral tissue self-Ags. In this way it becomes possible that a TCR-self p:self MHC interaction in the cortex will allow the positive selection of a developing T cell, but once this cell migrates to the thymic medulla the affinity of the TCR-self p:self MHC interaction (where the self p in the medulla is different from that in the cortex) decides whether the cell survives negative selection or not.

1.2.5 Clonal deletion – a focal point for central tolerance

Despite early seminal work in the 50s suggesting the presence of mechanisms preventing autoimmunity, for years it was presumed that tolerance was established merely by the inactivation of mature, autoreactive lymphocytes. This hypothesis was based on in vitro experiments demonstrating the presence of autoreactive clones in mature lymphocyte populations (Bruce Smith and Pasternak, 1978, Glimcher and Shevach, 1982). In particular, Glimcher and Shevach in their attempt to produce antigen specific T cell hybridomas, came across a large population of cells exhibiting a response to H-2-matched splenocytes, in the absence of the priming antigen. The authors were able to identify the stimulatory self-antigen to be the I-A^d subregion of the MHC complex. The very presence of self-MHC specific T cells with autoreactive potential was later on used by Kappler et al to provide the first strong evidence in favour of clonal deletion and central tolerance. In two accompanying papers the authors first described a monoclonal antibody that was reactive and could hence be used to identify TCRs containing the Vβ17α chain (Kappler et al., 1987b). Having shown that a great proportion of Vβ17α T cells were reactive to an IE class II MHC molecule, they then demonstrated that such T cells were present in a mouse strain that lacks expression of the IE molecule, but not in mice where this molecule was expressed. Further experiments showing that Vβ17α+ T cells were absent in the periphery of mice expressing the IE molecule, but present at normal levels up to a certain stage in thymic T cell development allowed them to confidently conclude that the clonal deletion of autoreactive immature thymocytes is an important tolerance mechanism (Kappler et al., 1987a).

The concept that immunological tolerance to self is largely obtained during thymic T cell development was soon established. Whilst the consensus initially was that the scope of central tolerance was restricted to self-antigen coming from ubiquitously expressed proteins and from abundant blood-born self-antigens, the 1990s saw a rising number of papers reporting the expression of tissue-specific genes at very low levels in the thymus.

1.2.5.1 Peripheral Antigens are ectopically expressed in the thymus

The first publication to broach the idea that ectopic expression of tissue-restricted antigens could be a physiological property of thymic stromal cells came from Jolicoeur et al in 1994. This group utilised the rat insulin promoter (RIP) to express a viral antigen (Tag) in pancreatic β islet cells and characterise the antigen-specific T cells response against these cells in the presence of different amounts of antigen. They were surprised to find that irrespective of the amount of antigen expressed, Tag-specific T cells were profoundly tolerant to the β cell antigen and subsequently examined transgene and endogenous insulin gene expression in the thymus, to find that these and other pancreatic genes were indeed expressed at low levels intrathymically (Jolicoeur et al., 1994). This report was followed by numerous others showing the “ectopic” expression of tissue-specific parenchymal peptides conferring a protective advantage against corresponding autoimmune conditions, both in mouse and human (Egwuagu et al., 1997, Vafiadis et al., 1997, Pugliese et al., 1997, Heath et al., 1998, Sospedra et al., 1998). Klein et al went further to show that the expression of CRP, an acute inflammatory phase inducible protein, is expressed in the thymus even when it is not expressed

peripherally(Klein et al., 1998), providing in this way evidence that thymic expression of peripheral antigens is not only independent of the location of each antigen, but also of the time at which antigens are expressed.

Each of these and other studies reporting promiscuous gene expression in the thymus identified different thymic cell subsets as the ones where this phenomenon was taking place. In 2001 Derbinski et al shed light on this controversy with the publication of a comprehensive analysis of promiscuous gene expression (PGE) in highly purified thymic stromal cell subsets, combining density fractionation and cell sorting techniques. Their findings showed that all of the genes assessed were expressed in mTECs, while some of those were also present in either of the other 3 cell subsets, cTECs, DCs and macrophages. However, the thymic expression of 50% of the genes analysed was exclusive to mTECs. Interestingly they also showed that the promiscuous expression of any particular antigen was limited to 100-1000 mTECs per thymus and on analysing mTECs at the single cell level only a minor fraction of those exhibited PGE (Derbinski et al., 2001).

A year later Anderson M.S. et al, went on to answer the question of what drives this phenomenon. They followed up on developments of the late 1990s relating to the identification of Autoimmune Regulator (AIRE) as the single gene defect causing the rare autoimmune condition Autoimmune Polyendocrinopathy-Candidiasis-Ectodermal Dystrophy (APECED) (Nagamine et al., 1997, Rosatelli et al., 1998) and the localised and exclusive high level expression of this gene in the thymus and in particular in mTECs (Heino et al.,

1999). Their working hypothesis was that loss of AIRE expression in mTECs would result in the prevention or modification of PGE in these cells.

Firstly they produced BM chimeric mice to control the expression of aire in haematopoietic (radiosensitive) and TECs (radioresistant) cells and were able to show that autoimmunity only occurred when the radioresistant TECs were Aire-/-. Then to directly assess whether this autoimmunity was due to a related change in PGE in TECs, they probed 12000 mouse genes in mTEC RNA preparations from Aire-/- and WT mice using labelled complementary RNA. A link was indeed strongly suggested as the expression of a substantial number of genes, including previously identified promiscuously expressed genes was silenced or significantly repressed in these cells in the absence of Aire. As 2 known autoantibody-targets in patients with APECED syndrome were shown to be abolished in Aire-/- MECS, this was the first data directly implying that absence of Aire impedes the thymic expression of a particular self-Ag and subsequently results in the escape of the corresponding autoreactive T cells in the periphery which cause autoimmunity(Anderson, 2002). However, the first formal evidence to substantiate this link was presented by DeVoss et al a few years later.

DeVoss et al pursued the cause of spontaneous autoimmune uveitis in Aire-/- mice, the eye being one of the organs targeted by autoimmunity in this setting. Sera from Aire deficient mice, previously shown to contain autoantibodies specific for the photoreceptor layer of the retina (Anderson et al., 2002), were used to immunoblot whole-eye extract identifying the Interphotoreceptor retinoid binding protein (IRBP) as the main target Ag. Quantitative RT PCR on

cDNA from WT and Aire-/- purified thymic stroma cells showed that IRBP was expressed in the thymus in an aire-dependent fashion. Finally, to prove that autoimmune uveitis in aire deficient mice was linked to the absence of IRBP expression in the thymus, DeVoss et al crossed IRBP-/- with Aire-/- mice. Double knockout animals did not show any evidence of autoimmunity against the eye, while other organs usually targeted by autoreactive cells in aire-/- mice where still affected. These results illustrated that, in the absence of Aire, autoimmune uveitis is an IRBP-specific response (DeVoss et al., 2006).

1.2.5.2 Models of Promiscuous Gene Expression (PGE) in the thymus

Interestingly despite the clear link between Aire and PGE, regulation of the latter at the cellular and molecular level is not based on a straightforward relationship between the two. Derbinski et al performed a detailed gene expression analysis in each of the progressively more differentiated subsets of mTECs, defined and FACS sorted on the basis of varying expression levels of lectin UEA binding sites, CD80 and MHC class II molecules. Comparison of global gene expression performed by microarray analysis and RT-PCR in the different thymic cell populations confirmed that mTECs are the cells specialising in PGE and the authors were able to show that strikingly almost every single tissue was represented by the expression of at least one tissue restricted Ag (TRA) in these cells. On examining the expression of casein β and testis lipid binding protein, the former a gene expressed in the mammary gland of female mice in late pregnancy and the latter a gene expressed in male mice in the testes, the levels of each of these two proteins in mTECs did not differ among male and female animals. With this experiment the authors

demonstrated that PGE is not subject to the normal tissue-, sex- and development gene regulations.

They then went on to show that PGE of 21 out of 22 genes studied and Aire expression correlated with increasing CD80 expression levels on mTECs. As the authors worked on the presumption that progressively differentiated mTECs are marked by increasing levels of CD80, they concluded that their results were clearly in favour of a terminal differentiation model of PGE. This model proposed that as mTECs matured and differentiated they expressed increasing levels and numbers of peripheral Ags in a cumulative fashion. A contrasting model to interpret the same results was then proposed by Farr et al. This group had a different view on the differentiation profile of mTECs and suggested a progressive restriction model of PGE where the earliest thymic epithelial progenitors are not yet committed to the thymic lineage and are multipotent. Therefore, multiple conserved transcriptional programmes are still able to be active and consequently a broad spectrum of peripheral Ags is expressed in immature mTECs. As these progenitors then mature their developmental potential is progressively restricted and most of the transcriptional programs sequentially become silenced. Subsequently each mTEC commits to one of numerous terminally differentiated cell gene expression profiles, expressing a discrete set of TRA according to the peripheral lineage fate it has taken. Consequently all mature mTECs put together compose a mosaic of peripheral tissue antigen expression in the thymus (Gillard and Farr, 2005).

This disagreement was addressed with an elegant study by Gray et al, published in 2007. As one model was predicting that PGE is prominent in the terminally differentiated subset of mTECs and the opposing model was suggesting that this was a property of immature, uncommitted mTECs the authors decided to investigate the differentiation state and the dynamic relationship between different mTEC subsets distinguished by Aire and MHC class II expression. To do this they first identified 3 subsets of TECs, the cortical epithelial cells (CECs), the medullary epithelial cells expressing high levels of CD80, and MHC class II (MEChi) and the medullary epithelial cells expressing low levels of these two molecules (MEClo) and they confirmed by intracellular staining that only a very small proportion of MEClo cells express Aire while this molecule is highly expressed in 50-60% of MEChi cells. BrdU incorporation and decay kinetics determined in in vivo experiments suggested that Aire+ve MEChi cells are not cycling and are derived from proliferating Aire-ve MEChi cells. The post-mitotic, most differentiated Aire+ve MEChi subset appeared to also have a rapid turnover. Based on these findings the authors wished to investigate the possibility that Aire promotes the apoptosis of the MEChi cells that express it. They therefore transfected a MEC cell line with an Aire-GFP fusion protein or the GFP protein alone, and assessed proliferation and apoptosis of these cells by BrdU incorporation and Annexin V FACS analysis respectively. Their results showed that Aire did not affect proliferation but promoted apoptosis.

Overall, the authors felt that their results strongly supported the terminal differentiation model and proposed an intriguing refinement to it. Aire-ve MEClo cells differentiate to Aire+ve MEChi cells possibly through an Aire-ve

MEChi intermediate stage and the Aire molecule induces apoptosis either directly by turning on an apoptosis program or indirectly by upregulating peripheral tissue antigen expression to the extent where protein synthesis overwhelms the cells and causes death through ER stress (figure 1.5). Whatever the mechanism, cell death of Aire+ve MEChi cells that express high levels of peripheral tissue Ags, makes these Ags available for cross-presentation by the DCs that are present in the medulla (Gray et al., 2007).

Clonal deletion is the hallmark of central tolerance. Shifting into the Regulatory T cell (Treg) lineage has also been associated with self-antigen specificity of developing CD4 T cells in the thymus. However, the mechanism behind commitment to the Treg fate in the thymus remains controversial and as it is not linked CD8 T cell central tolerance, it will not be discussed further.

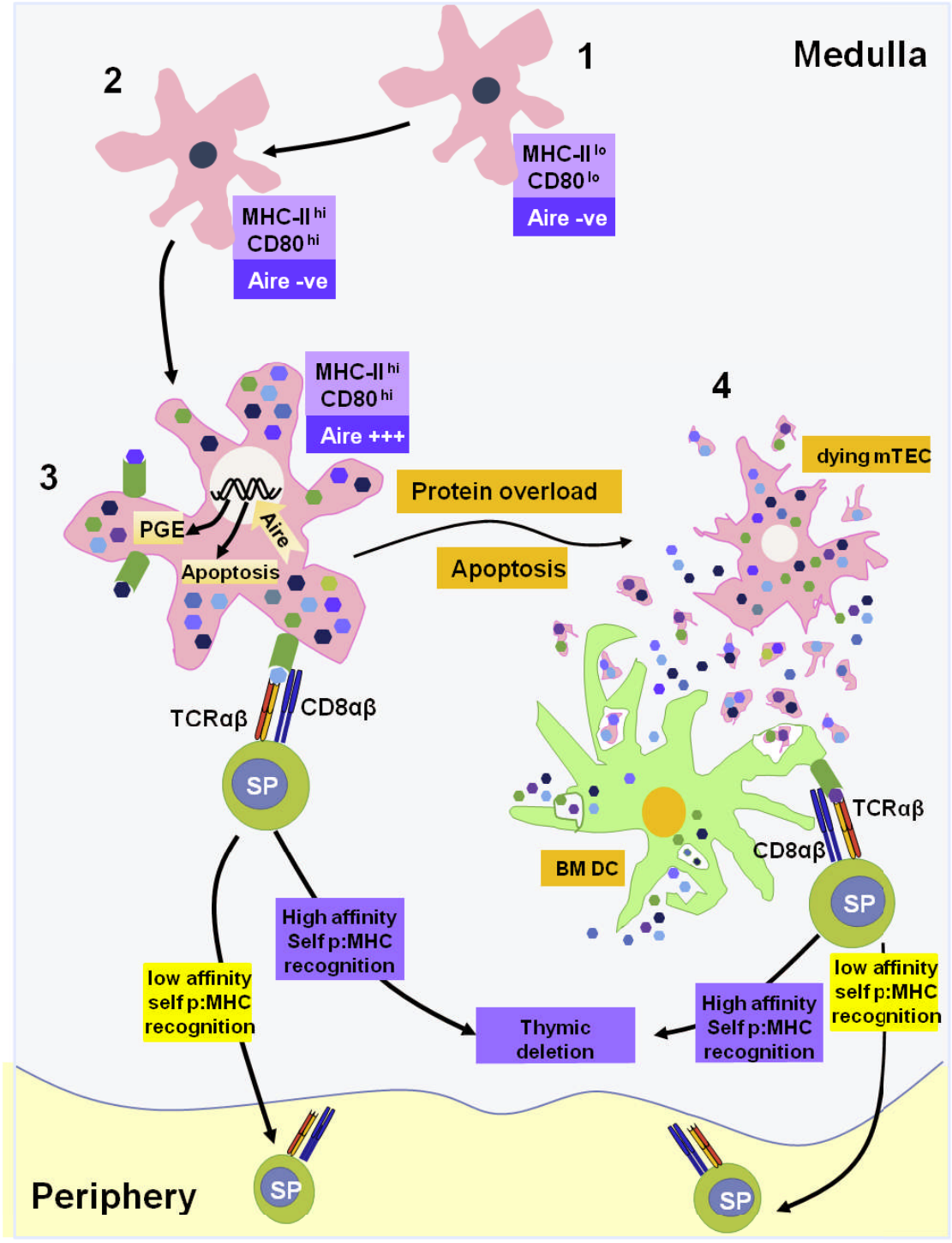


Figure 1.5 Progressive differentiation model of Aire driven PGE. Aire-ve medullary epithelial cells (MEC) differentiate to Aire+ve cells upregulating the expression of MHC-II and CD8 at the same time. Aire expression induces apoptosis of the terminally differentiated MECs. In the terminally differentiated stage, MECs express great amounts of peripheral tissue Ags at this terminally differentiated stage and a link has been suggested between protein overload and apoptosis induction.

Figure 1.5 (continued)

The promiscuously expressed self-Ags are disseminated in the medulla following the MECs death and obtained by BM-derived APCs for cross-presentation. This is thought to facilitate negative selection.

1.3 CD8 T cells in the periphery and Peripheral Tolerance

Despite the elaborate processes of central tolerance, it is well established that some autoreactive T cells escape to the periphery. More often than not, such T cells are comprised of cells with low-avidity self-specificities (Liu et al., 1995). Rarely, high avidity, self-reactive specificities are found, if the Ag was not presented in the thymus. It is therefore no surprise, that peripheral tolerance mechanisms are employed to compromise the function of these T cells and prevent autoimmunity. Clonal ignorance, deletion, anergy and suppression are the 4 main mechanisms of peripheral tolerance. The latter spans central and peripheral tolerance as Tregs that mediate suppression, can be generated both in the thymus and in the periphery, but act in the periphery. Prior to discussing peripheral tolerance, the parameters contributing to CD8 T cell activation will be discussed.

1.3.1 Antigen processing and presentation to CD8 T cells

CD8 T cells recognise antigen presented to them on MHC class I molecules. Intracellular proteins produced in the cytosol - viral, mutated as in a tumour cell, and normal proteins - are degraded by proteasomes as part of a continuous turnover process. Peptides longer than 7 amino acids are transferred to the ER through two ATP-dependent peptide transporters, called transporters associated with antigen processing (TAP). In the ER the amino acids' length is adjusted to 8-mers and 9-mers, the size of class I restricted antigenic epitopes. In this organelle they also meet and bind to the MHC class I molecules, forming a stable p:MHC class I complex which is subsequently transferred to the cell surface. Naive, as well as memory CD8 T cells scan

these complexes that are in fact a sample of the intracellular compartment of each cell. While effector and memory CTLs can respond to p:MHC complexes on any cell, naive CD8 T cells are more resilient to activation and require Ag presentation by activated professional APCs.

1.3.2 Cross presentation and its role in T cell activation and peripheral tolerance.

In the mid-70s Bevan et al immunised animals with allogeneic cells bearing different minor and major histocompatibility antigen genes from the host and observed that a number of CTLs specific for the donor minor antigens presented on host MHC class I molecules were generated (Bevan, 1976). These results revealed for the first time that class I antigens could somehow be transferred from one cell to an APC which can then prime a CTL response. The phenomenon of cross-presentation has been extensively studied since. In the 1990s DCs and macrophages were identified as the specialised cells capable of cross-presentation (Kovacsovics-Bankowski et al., 1993, Rock et al., 1990, Rock et al., 1993), acquiring exogenous antigen through phagocytosis (Shen et al., 1997) and macropinocytosis (Norbury et al., 1995). Nevertheless, how extracellular proteins taken up in endosomes and phagosomes, access the MHC I processing machinery which is typically found in the cytosol, and how peptides derived from extracellular proteins meet the MHC class I molecules to form the cross-presenting p:self MHC I molecules, remain matters under investigation. Almost 10 years ago it was shown that the necessary elements for peptide loading onto the MHC class I molecule, classically thought to be exclusively found in the ER, are also found in

phagosomes (Ackerman et al., 2003, Houde et al., 2003). The same year Guermonprez et al showed that in the case of DCs - the APCs that are most effective in cross-presentation - fusion of the ER to the phagosomes is what provides the peptide loading machinery to the latter (Guermonprez et al., 2003). The processes of direct class I antigen presentation and cross presentation are described in the schematic diagram in figure 1.6.

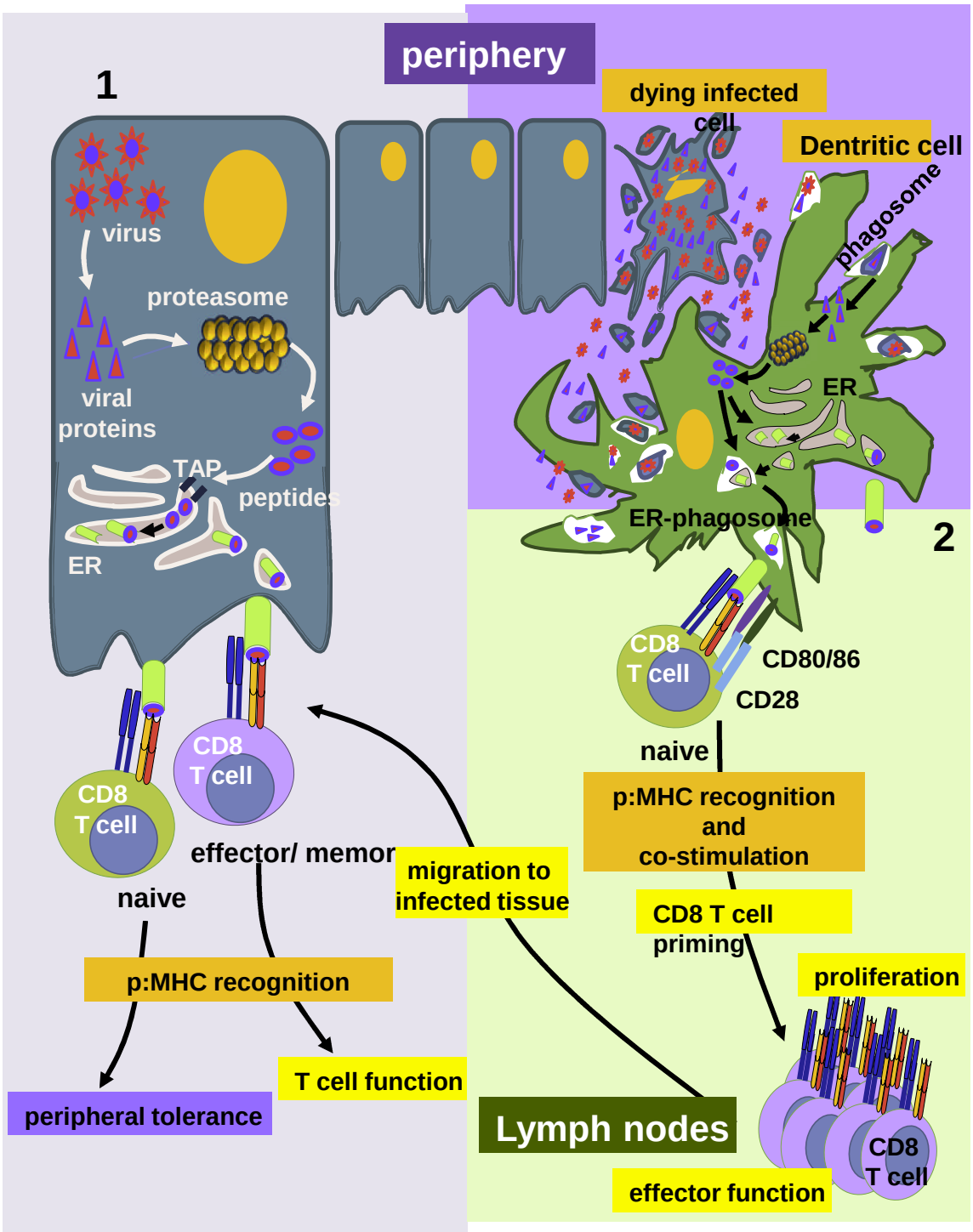


Figure 1.6 Class I Antigen processing and presentation. Class-1 antigen processing and presentation takes place in all cells expressing MHC class I molecules and is the means to present the intracellular environment to CD8 T cells. In the example shown on the left in the above diagram (1) a virally infected cell produces viral peptide epitopes through the proteolytic degradation of viral proteins by the proteasome. These viral peptides are transferred in the endoplasmic rediculum (ER) through two ATP-dependent peptide transporters (TAP). In the ER

Figure 1.6 (continued)

the class I epitopes bind to MHC class I molecules, forming a stable p:MHC class I complex which is then transferred to the cell surface. Naive and memory CD8 T cells can both recognise Ag presented in this way, however in the absence of co-stimulation only previously primed, memory T cells are expected to produce a functional, Ag-specific response. Cross-presentation, shown in (2) refers to the processing and presentation of extracellular antigenic material by professional APCs on class I, rather than class II molecules. It involves the uptake of extracellular proteins by macropinocytosis and phagocytosis of dying, infected (or transformed) cells. The mechanism by which the phagocytosed extracellular proteins then enter the class I antigen processing and presentation pathway is unclear. Some studies suggest that fusion of the ER to phagosomes facilitates Ag loading on class I molecules. Cross-presentation of class-I peptides on professional APCs can stimulate memory T cells, but also prime naive CD8 T cells which in this setting will recognise Ag in the presence of co-stimulation. Primed CD8 T cells can then migrate to the periphery as effector/memory T cells and respond to direct Ag presentation by infected or transformed cells.

Cross-presentation can be used to prime a CTL response, whilst it can also orchestrate the containment of autoreactive T cell specificities that circulate in the periphery. Kurts et al first reported that self-Ags, from constitutively expressed proteins, can be cross-presented by professional BM-derived APCs in the draining LNs of the organs expressing them and that this can lead to the activation of autoreactive T cells (Kurts et al., 1996). They then went on to study the fate of these autoreactive T cells that see their cognate Ag in the periphery through cross-presentation. As their model-self Ag, OVA, was expressed in kidneys, pancreas as well as in the thymus, they first thymectomised and grafted a B6 thymus in their RIP-OVA Tg animals to avoid OVA thymic expression. Then they replaced the BM of these mice with BM cells from OT-I TCR Tg mice. The Tg self-Ag specific OT-I T cells that were generated in the recipient animals, escaped thymic negative selection and appeared to be activated in the periphery. However, despite their initial Ag-specific proliferation they were subsequently gradually deleted and the mice did not develop diabetes. Thus, cross-presentation was shown to also mediate peripheral tolerance to self-Ags and this phenomenon was named cross-tolerance (Kurts et al., 1997).

1.3.3 Requirements for CD8 T cell activation vs tolerance

When a CD8 T cell encounters its cognate Ag, with the exception of very low affinity specificities that render such a meeting an insignificant event, a decision is made as to whether the cell will be activated or tolerised. Therefore studies aiming to elucidate the prerequisites of T cell priming have also

contributed important information regarding the requirements for tolerance following TCR - p:MHC interaction.

1.3.3.1 Co-stimulation is required for naive CD8 T cell activation

The 2 signal hypothesis for effective CD8 T cell priming is well established. The first signal involves Ag recognition and engagement of the TCR with its cognate p:MHC complex presented on a professional APC. Binding of co-stimulatory receptors on the responding T cell, of which CD28 is the prototype, with a choice of ligands such as CD80 and CD86 on the activated APC presenting the Ag, leads to IL-2 production by the T cell. In that way, CD28 signalling conducts signal 2 which promotes the proliferation and survival of primed T cells (Thompson et al., 1989, Harding et al., 1992). Co-stimulatory receptor ligands are upregulated on the APCs with the provision of CD4 T cell help when these cells simultaneously recognise a related class II Ag presented on MHC class II on the same cell (Bennett et al., 1997). Alternatively, binding of Toll-like Receptor and other Pattern Recognition Receptors to their appropriate ligands found on the invading microbes can also result in the maturation of APC.

1.3.3.2 The three-signal model of CD8 T cell activation and peripheral tolerance

Incomplete activation in the absence of signal 2 is considered to be the major cause of T cell anergy and deletion, and this was first shown for CD4 T cells. In the case of CD8 T cells, signal 1 and 2 will only lead to a dampened Ag-specific response if a third signal, in the form of IL-12 and IFNα, is not provided. This concept was first put forward by Curtsinger JM et al in 1999.

This group found that in vitro, IL-12 was essential for highly purified naive CD8 T cells to progress from being merely clonally expanded following signal 1 and 2 induction by artificial APCs, to becoming differentiated cytolytic effectors. In vivo peptide immunisation experiments, demonstrated that administration of IL-12 could replace adjuvant in stimulating an antigen specific CD8 T cell proliferative response in vivo (Curtsinger et al., 1999). The role of IL-12 in mounting a full blown CD8 T cell response that leads to the formation of a memory population, was then confirmed with adoptive transfer of TCR Tg T cells and peptide immunisation experiments in the presence of adjuvant or IL-12 (Schmidt and Mescher, 1999). The contribution of signal 3 in the decision between CD8 T cell activation versus tolerance was subsequently highlighted in a later study by Curtsinger et al. The authors showed that IL12 not only was necessary for antigen specific T cell differentiation into functional effector cells, but also that CD8 T cells expanding in response to antigen and costimulation alone remained unable to show in vivo functional activity upon restimulation in the presence of all 3 signals and were therefore tolerised (Curtsinger et al., 2003).

1.3.4 Modes of peripheral T cell tolerance

1.3.4.1 Clonal ignorance

In the early 90s, Pircher et al used an LCMV TCR Tg model where the T cells exhibited a range of reactivities to different LCMV virus variants to compare the avidity of TCR-p:MHC interaction required to cause negative selection in the thymus with that required to cause activation in the periphery. Their experiments revealed that the avidity threshold for thymic deletion was lower than for T cell activation and suggested that this “safety margin” between the different strengths of TCR-p:MHC interactions required to induce each of these two phenomena protects the periphery from low avidity self-Ag specific T cells that evade central tolerance (Pircher et al., 1991).

The higher activation threshold of T cells in the periphery than in the thymus can, to some extent, prevent the activation of autoreactive low affinity T cells when these meet their cognate self-Ag in the periphery. Nevertheless, high-affinity autoreactive T cells are also infrequently present in the periphery. The physical separation of naive T cells from parenchymal cells that might express their cognate Ag of such high affinity self-specificities is another protective mechanism preventing autoimmunity. When in the blood naive T cells express CCR7 which binds to the CCL21 and CCL19 chemokines, expressed by lymphoid tissue, and leads them to migrate to the lymph node cortex. Naive T cells also express a receptor for a lipid molecule called sphingosine 1-phosphate (S1P). The expression of this receptor is downregulated upon T cell activation in the LN. However, if TCR ligation does not take place this receptor remains expressed and binds to S1P which is expressed in an increasing

concentration gradient between lymphoid tissue and blood. Therefore, this interaction mediates the return of the naive T cells to blood. Using a TCR Tg mouse model, where all T cells were specific for the LCMV epitope GP - expressed in the β islets of the pancreas - Ohashi et al demonstrated that GP-specific T cells escaping central tolerance remained ignorant of their cognate Ag in the periphery and caused no autoimmunity. This was the case even though they were fully functional upon stimulation in in vitro and in vivo assays. The authors therefore proved the hypothesis of clonal ignorance. Nonetheless, once Tg mice were infected with the CMV virus, the previously ignorant GP-specific T cells encountered their cognate Ag in the context of infection, became fully activated and infiltrated the pancreas in an Ag-specific manner consequently causing autoimmunity in the form of diabetes. Therefore, the authors also proved that this clonal ignorance is an inherently unreliable mode of peripheral tolerance. (Ohashi et al., 1991).

The ease with which clonal ignorance can be overcome is not surprising given that even though naive T cells navigate only between blood and lymph nodes, upon their passage through secondary lymphoid organs they scan APCs that previously sampled the antigenic environment of parenchymal tissues and maybe able to cross-present it. As mentioned above cross-presentation by immature APCs, in the absence of co-stimulation and cytokines such as IL-12, typically fail to prime naive T cells. However, it is often the case that certain infections can precipitate autoimmune disease and one of the mechanisms behind this phenomenon is thought to be cross-presentation of self-Ags by APCs that have been activated by the microbial challenge (Mills, 2011).

1.3.4.2 Deletion and Anergy

Peripheral tolerance induced by the cross-presentation of self-Ags mainly derived from apoptotic cells, in the absence of inflammation, occurs through either clonal deletion or anergy. Each of these two mechanisms are considered to be more important in safeguarding the periphery from autoreactive T cells than clonal ignorance. While numerous studies have attempted to distinguish the different requirements driving one or the other, both deletion and anergy are processes that can take place together in the same experimental system or physiological situation.

1.3.4.2.1 Peripheral Deletion

Clonal deletion of autoreactive T cells in response to the cross-presentation of their cognate Ag by professional APCs was described earlier in section 2.2. The peripheral deletion of autoreactive T cells would obviously be the most permanent solution in the body's attempt to avoid autoimmune T cell damage. However, it seems that this decision is not taken at the time that an autoreactive CTL first encounters its cognate Ag(Redmond et al., 2003), but rather depends on further antigenic exposure. A number of studies suggested that deletion occurs when low-levels of the tolerising Ag are present systemically and long term (Kurts et al., 1997, Mamalaki et al., 1993, Barron et al., 2008, Redmond et al., 2005, Rocha et al., 1995).

Barron et al demonstrated that peripheral deletion of T cells encountering their model self-Ag systemically in vivo depends on Bim-mediated apoptosis. By subsequently knocking down Bim to prevent apoptosis they learned that tolerance in these conditions was maintained because Bim-/- autoreactive T

cells only survived to become anergic (Barron et al., 2008). This interesting study suggested that clonal deletion in the periphery can sometimes be disposable and replaced by anergy. However, a different study suggested that while deletion prevents highly pathogenic autoreactive T cells -which could not be otherwise contained by Treg-induced suppression- from causing autoimmune damage, anergy is responsible for containing the remaining autoreactive T cell specificities(Gavanescu et al., 2007)

1.3.4.2.2 CD8 T cell Anergy

Anergy is defined as a state of functional unresponsiveness, were proliferation and IL2 production are defective on subsequent antigenic challenges even in the presence of a full assembly of costimulatory signals (Schwartz, 2003). CD8 T cell anergy was first described in 1991 by Otten and Germain. In this in vitro study the authors showed that TCR-p:MHC interactions under conditions preventing co-stimulation by the APCs, abolished IL-2 production and proliferation but had no effect on the cytotoxic function of CD8 T cells (Otten and Germain, 1991).

Dubois et al in an in vivo model studying CD8 T cell peripheral tolerance showed that initially responsive F5 Tg CD8 T cells, were rendered anergic by repeated peptide injections as they did not proliferate and were also unable to kill. Both functions were rescued with the addition of IL-2 in the cell culture (Dubois et al., 1998). This was also one of a number of studies which demonstrated that anergy is a transcriptionally active event requiring the translocation of transcriptional activators to “turn-on” anergy-inducing genes.

Activation of such genes has been shown to downregulate TCR signalling pathways (Redmond and Sherman, 2005).

1.3.4.2.3 Inhibitory signals inducing anergy

Apart from the lack of co-stimulation, co-inhibitory signals are now known to contribute to T cell hyporesponsiveness. Two structurally similar molecules, CTLA-4 and PD-1 are present on T cells and play an important role in suppressing the CTL response. CTLA-4, the expression of which is dramatically increased following TCR engagement (Lindsten et al., 1993), is an inhibitory homologue of CD28 and shares the same ligands with the latter. The inhibitory role of CTLA-4 was clearly suggested in 1995, when two groups showed that CTLA-4 -/- mice suffered a fatal lymphoproliferative disease (Waterhouse et al., 1995, Tivol et al., 1995). The autoimmune nature of this disease also hinted at a role for CTL4 in tolerance. The contribution of CTLA-4 to anergy induction has since been extensively studied. Greenwald et al, utilised naive T cells from TCR Tg, CTLA4 -/- or CTLA4 competent mice in adoptive transfer experiments to prove that T cells lacking CTLA4 remained functional despite a tolerogenic stimulus. CTLA4 appeared to exert its tolerigenic effects by mediating a cell cycle arrest (Greenwald et al., 2001).

Similarly to CTLA-4 the first evidence for the importance of PD-1 in peripheral tolerance came from PD1-/- developing a lupus-like proliferative arthritis and glomerulonephritis, as well as autoimmune dilated cardiomyopathy (Clay et al., 1999a, Latchman et al., 2001). This molecule belongs to the immunoglobulin superfamily, is expressed in T, B and myeloid cells (Agata et al., 1996) and exerts its effects upon ligation to the PD Ligand 1 and 2 (PDL-1, PDL-2)

expressed on APCs, lymphocytes and non-lymphoid tissues (Freeman et al., 2000, Latchman et al., 2001). Probst et al investigated the effects of both these molecules in peripheral tolerance using mixed BM chimeras of PD1-deficient and Rag-DIETER mice (DC-specific inducible expression of T cell epitopes by recombination' mice), and by blocking CTLA-4 signalling with a monoclonal blocking antibody. In DIETER mice the expression of 3 Tg CTL epitopes can be induced with the administration of Tamoxifen. Depending on the activation state of the DCs the outcome of Ag presentation is either T cell activation or tolerance(Probst et al., 2003). In the absence of either PD-1 or CTLA-4 signalling, not only peripheral tolerance was impaired, but the antigen-specific CD8 T cells were genuinely primed under what would normally be tolerogenic conditions; they proliferated and secreted IFNγ in an antigen specific manner and were even able to clear an LCMV infection in vivo. PD-1 and CTLA-4 pathways seemed to have an additive effect in tolerance induction. These results suggested that the balance between co-stimulatory and co-inhibitory receptor-ligand interactions between T cells and APCs at the time of TCR engagement, contributes to the decision between T cell activation and tolerance (Probst et al., 2005).

1.3.4.2.4 CTL anergy linked to TCR and CD8 downregulation

In some cases, anergy appears to be linked with the downregulation of TCR and CD8 molecules. Schonrich et al, came across this mode of CTL anergy when studying peripheral tolerance induction in a monoclonal T cell population. They created a class I restricted TCR Tg mouse and a Tg mouse expressing the Tg TCR's cognate Ag in very few peripheral tissues. Double transgenic

mice derived from crosses between the TCR and Ag transgenic lines, accepted skin grafts and allowed lymphoid tumour cells (EL4) expressing the Ag to grow. Therefore, in this system tolerance was induced not only in the possible absence of costimulation, but also in its presence. Phenotyping the Tg T cells in these mice pointed at a different mechanism mediating T cell hyporesponsiveness. Both CD8 and Tg-TCR expression were downregulated among T cells of the double Tg, but not in the TCR Tg mice. Following ex-vivo antigenic stimulation of FACS sorted Tg-TCR positive and negative T cells from double Tg animals, TCR and CD8 expression was upregulated among Tg-TCR negative T cells and Ag-specific cytotoxicity was recovered. These data confirmed that Ag specific T cells were in fact present in the double Tg mice but their TCR and CD8 phenotypes were altered under tolerogenic conditions. Subsequently the authors also confirmed a correlation between anergy and the expression levels of these two molecules (Schönrich et al., 1991).

In a more recent study, where Gutermuth et al used a TCR Tg model of CD8 T cell-mediated autoimmunity to explore self-peptide vaccination as the means to diminish autoreactive responses, they were able to study CD8 and TCR downregulation in the setting of peripheral tolerance further. In their model OT-I TCR Tg mice crossed with mice expressing OVA in a number of epithelial tissues and in the thymus, developed a lethal CD8 T cell driven autoimmunity. Only 14% of these double transgenics escaped this fate spontaneously, but upon prophylactic or even therapeutic injection with soluble OVAp the majority were protected from autoimmunity and their survival was prolonged. Even though thymic deletion was shown to play a part, great numbers of OT-I

specific CD8 T cells were present in the LNs of spontaneously healthy or treated healthy animals suggesting that peripheral tolerance mechanisms were also involved in holding these cells at bay. FACS staining showed that the majority of OTI-specific T cells in mice that remained healthy had downregulated either the CD8 molecule or the Vα chain of the OTI TCR. Not surprisingly, they were also shown to be anergic in terms of antigen-specific proliferation in ex-vivo studies. Impaired OVA-pentamer staining only evident in healthy double Tg mice, allowed the authors to attribute the observed T cell hyporesponsiveness to the inability of these cells to bind to their cognate Ag (Gutermuth et al., 2009).

1.3.5 Memory T cells

1.3.5.1 Classical T cell memory development

The establishment of peripheral tolerance is expected following the encounter of a T cell with its cognate antigen under the tolerogenic conditions described above. However, when T cells specific for foreign Ags are presented with the corresponding epitopes on activated professional APCs, more frequently than not the outcome of this interaction will be an effective immune response that will clear the infection. Following antigenic clearance, the expanded population of effector T cells contracts, leaving behind 5-10% of these cells which have in the meantime differentiated to memory T cells (Jacob and Baltimore, 1999). Memory T cells in general respond faster and more effectively, than naive T cells, in a subsequent antigenic challenge even in the absence of co-stimulation and cytokines.

The event triggering memory T cell development and its timing within a primary immune response have been studied extensively by numerous groups. In 2001, Kaech and Ahmed stimulated CFSE-labelled, naive, TCR Tg CD8 T cells in vitro with a limited number of peptide-pulsed APCs and observed that T cells continued to proliferate even after antigen clearance in the cell culture. In another experiment they stimulated CFSE-labelled T cells in vitro for 24 hrs only and then purified CD8 T cells from the cell culture prior to transferring them into recipient Ag-free mice. In this in vivo system they confirmed that the T cells continued to proliferate for a few days in the absence of Ag and 43 days after Adoptive Transfer (AT) the surviving cells exhibited memory T cell function upon a secondary challenge. Therefore the authors concluded that initial antigenic stimulation is enough to instruct the differentiation of naive T cells into memory (Kaech and Ahmed, 2001).

A central issue however, remained unanswered from the above study regarding the exact identity of the memory T cell progenitors. Opferman et al reported that in AT experiments, memory CD8 T cells could only be generated from effector T cells that had already divided more than five times in vitro following their first antigenic encounter in vitro. They considered this to be evidence that all antigen-reactive T cells follow a linear differentiation pathway from naive to effector T cells during a primary response and subsequently a low proportion of the effector T cells are selected to differentiate into memory T cells (Opferman et al., 1999).

Sallusto et al first described the existence of 2 subsets of memory T cells in the human system and the definitions they have provided for Central Memory

(Tcm) and Effector Memory (Tem) T cells are still being used. Tem are CCR7 negative, CD62L low and migrate to sites of inflammation readily exhibiting effector functions. In mice they correspond to CD62L negative CD44 low CD8 T cells. Tcm are CCR7 positive CD62L positive and therefore home to the LNs. In mice this subset corresponds to CD44 positive CD62L positive T cells. These were thought to be less functional, but able to rapidly differentiate to effectors in a secondary antigenic challenge. In this same study the authors sorted Tcm and stimulated them in vitro showing that 10 days later almost all cells converted to an effector phenotype and function. In contrast repeating this experiment with sorted Tem cells did not result in the conversion of these cells to the Tcm phenotype. They therefore suggested that naive T cells differentiated stepwise to Tcm and subsequently to Tem/effector cells (Sallusto et al., 1999). However, as Sallusto et al pointed out this hypothesis was only derived from in vitro experiments leaving open the possibility that memory T cell differentiation is a more complicated issue.

A few years later Wherry et al further investigated the differentiation pathway of CD8 memory T cells in an in vivo model infecting TCR Tg mice containing T cells, specific for particular epitopes of LCMV or Listeria Monocytogenes (LM). Unlike the initial description of Tcm and Tem by Sallusto et al, they showed that both subsets showed similar functional responses in ex-vivo peptide re-stimulation assays. Despite this, sorted, adoptively transferred Tcm were markedly better than Tem at controlling both viral and bacterial infections in vivo. In order to address the lineage relationship between these two memory T cell subsets the authors again adoptively transferred sorted Tcm and Tem into separate naive recipients to observe that 25 days later the former maintain

their phenotype, whereas half of the transferred Tem converted to a Tcm phenotype. The Tcm were additionally shown to have better antigen-driven and homeostatic proliferation in vivo. Overall their results allowed Wherry et al to propose a progressive differentiation model according to which Tem is an intermediate cell type, rather than a different memory lineage, as antigen specific T cells differentiate from effector to Tcm following an antigenic stimulation (Wherry et al., 2003).

In 2007 Chang et al CFSE-labelled, naive P14 TCR Tg CD8 T cells and adoptively transferred them into WT recipients that were infected with LM 24 hrs prior to the AT. 32 hrs after the transfer they FACS sorted undivided donor T cells to examine them during their first division using live cell confocal microscopy for the expression of various T cell signalling molecules. They were in this way able to demonstrate an asymmetric distribution of these molecules in naive T cells during their first antigen-driven cell division. With subsequent AT experiments they showed that the daughter cells from this first division, differed in function in a way that correlated to the effector or Tcm phenotype they each exhibited. Therefore this group suggested that at the beginning of an immune T cell response effector and memory T cells are generated from the same naive cell rather than sequentially (Chang et al., 2007). The same year in a technically impressive study Stemberger et al attempted to provide a definitive answer to the question of memory T cell subset origin by adoptively transferring a single naive OT-I T cell into recipient mice. With their experiments, this group unequivocally demonstrated that the full spectrum of effector and memory T cell subsets can be generated from a single naive precursor. More recently, Gerlach et al also using a

technologically advanced in vivo model, where individual naive T cells carried unique genetic tags, confirmed the findings of Stemberger et al. One naive T cell, was shown to give rise to both effector and memory CD8 T cell progeny under different infection conditions and in different anatomical sites (Gerlach et al., 2010). Even though all three studies – Chang et al, Stemberger et al and Gerlach et al - discussed here clearly provide strong evidence in favour of effector and memory T cells being derived from a single naive T cell, they do not exclude a scenario where after the first division of a naive T cell the effector daughter cell can give rise to memory T cells in subsequent cell divisions.

1.3.5.2 Homeostatic proliferation and memory T cell development.

Antigen-driven memory T cell development following a primary immune response is the classical paradigm of memory T cell development. However, it is well established that homeostatic proliferation also leads to memory T cell differentiation in a physiological as well experimental conditions and this phenomenon is now called Lymphopenia-Induced Proliferation (LIP) – driven memory development (Surh and Sprent, 2008). In 2000, two back to back papers in Journal of Experimental Medicine described the cognate Ag-independent generation of memory T cells in lymphopenia. Both groups showed that these cells exhibited a Tcm-like phenotype and function, rapidly secreting IFNγ and becoming cytotoxic in response to their cognate Ag both in vitro and in vivo (Goldrath et al., 2000, Cho et al., 2000).

Cho et al studied the kinetics of LIP-driven memory development by transferring CFSE labelled, naive TCR Tg T cells into Rag1-/- recipients and

monitoring the phenotype of the dividing cells over a time course. In these experiments they observed that naive T cells differentiated to memory without passing through an intermediate effector stage. They also showed that whilst cognate Ag was not required for memory development, expression of the appropriate MHC by the host was needed, as the transfer of naive T cells into allogeneic, NK-depleted Rag1-/- recipients did not allow the cells to proliferate, let alone differentiate into memory T cells. Importantly, the authors also showed that CD28 signaling and IL-2 were not required for LIP-driven memory T cell development by using naive T cells from CD28-/- and IL-2-/- donor mice (Cho et al., 2000). Goldrath et al performed similar experiments, but used sublethally irradiated B6 mice instead of Rag1 -/- animals as recipients and their results were mostly similar to Cho et al. However, they demonstrated that in their system, but not in Rag1-/- hosts, the majority of memory cells eventually reverted to a naive phenotype once the mice were no longer lymphopenic (Goldrath et al., 2000).

A few years later, Min et al published a study highlighting the fact that the physiologic lymphopenia of the neonatal period also supports LIP-driven memory T cell development. The authors showed that AT of naive polyclonal CD4 T cells in newborn mice supported LIP, promoting memory phenotype development in 20% of the donor-derived T cells. In contrast to memory T cells generated in response to an adult lymphopenic environment, memory T cell development in the neonate was dependent on CD28 co-stimulation along with the interaction with MHC class II molecules (Min et al., 2003).

More recently, Haluszczak et al reported on a very exciting finding that suggested a physiological contribution of LIP-driven T cell memory development to the unprimed T cell repertoire. Using MHC class I tetramers loaded with 3 different foreign peptide antigens, magnetic column separation and FACS sorting they were able to isolate small numbers of nominal Ag-specific CD8 T cells from unimmunised normal B6 mice. After transferring them into syngeneic recipients that were neither immunised nor irradiated, they were surprised to notice that both CD44hi and CD44low donor cells were present. The authors then showed that these Ag-specific, but Ag-inexperienced memory phenotype T cell were also present in unprimed gnotobiotic mice strengthening the hypothesis that these cells were generated through homeostatic mechanisms rather than due to cross-reactivity with any commensal flora-derived Ags. They named these cells Virtual Memory (VM) T cells. Phenotypic analysis of the VM T cells was consistent with an LIP-derived memory CD8 T cell phenotype and their functional activity was superior to that of their naive counterparts. In view of these results the authors concluded that VM T cells contribute to the unprimed CD8 T cell repertoire and speculated that they might have a role in primary immune responses especially in situations of physiological lymphopenia such as the neonatal period (Haluszczak et al., 2009).

1.4 Tumour immunology

1.4.1 Tumour immunosurveilance and immunoediting

1.4.1.1Tumour Immunosurveilance

More than 50 years ago Burnet discussed the possibility that despite being of the “body’s own pattern”, cancer cells might exhibit enough antigenic differences from healthy tissues to trigger an immune response against them which could clear the tumour before it even becomes clinically apparent. In this publication the author even suggested that what might be required for the effective treatment of cancer is to enhance the immune system in order for it to be able to recognise these small differences, possibly by means of genetic manipulation (Burnet, 1957).

This formed the tumour immunosurveillance hypothesis which was further substantiated in the 1990s when Dighe et al demonstrated that IFNγ had a central role in mediating the rejection of Meth A- carcinogen induced tumours. As inoculating IFNγ sensitive or insensitive tumour cells in SCID mice lacking lymphocytes, unlike in BalbC animals, showed no difference in tumour growth it was clear that the IFNγ effect related to the tumour being recognised and attacked by the adaptive branch of immunity (Dighe et al., 1994).

1.4.1.2 Tumour Immunoediting

In 2001, Shankaran et al presented a set of striking data where an absent lymphocyte compartment either coupled or not with defective IFNγ signalling resulted in increased susceptibility not only to carcinogen-induced malignancy, but more importantly to spontaneous epithelial tumour formation. Even though

these results clearly supported the tumour immunosurveilance hypothesis, the authors where also interested whether the immune response to a tumour can also influence its immunogenicity. To investigate this, they transplanted carcinogen-induced tumours growing in WT and Rag2 deficient mice into syngeneic immunocompetent mice and noticed that while tumours derived from mice lacking a lymphocyte compartment only grew in 40% of the transplanted animals, 100% of the transplanted tumours obtained from WT animals grew progressively in their recipients (Shankaran et al., 2001). Therefore, having documented that tumours arising in immunocompetent mice become less immunogenic as they establish amidst an immune response against them, the authors proposed an update to the tumour immunosurveillance hypothesis introducing the concept of cancer immunoediting. According to this, the immune system paradoxically can both fight off cancer but in doing so sculpts it into a version evolved to escape its defences.

Schreiber et al have recently suggested that cancer immunoediting can be seen as a process characterised by three consecutive phases. The elimination step, being the first phase in any cancer's natural history, incorporates the tumour immunosurveilance hypothesis. In this phase, relatively sub-clinical numbers of cancer cells are detected and destroyed by the immune system. A cancer enters the next phase only if at least one transformed cell survives the elimination phase. At that point, the immune system might still be able to keep those few malignant cells at bay as the still clinically undetectable cancer enters the equilibrium phase. The fact that tumour cells are genetically unstable allows them to evolve through the immune selection pressure that they are found under during this phase. They evolve into less immunogenic cells, and in this way they escape the body's defences, proliferate and establish themselves into clinically detectable disease, this being the third stage of cancer immunoediting (Schreiber et al., 2011).

Despite the more negative picture painted by the concept of tumour immunoediting in comparison to the older more hopeful immunosurveilance hypothesis, the advances in our knowledge of the interaction between cancer and immunity, allow the development of a variety of tumour immunotherapies aimed at modulating the patient's immune system so that it can recognise already immunoedited transformed cells and effectively clear them.

1.4.2 Tumour Immunotherapy

1.4.2.1 Tumour Antigens

Tumour antigens can be broadly divided into tumour-specific (TSA) and tumour-associated Ags (TAAs). TSAs are epitopes arising from mutated proteins and are exclusively expressed and presented in tumour cells. They represent good targets for T cell based cancer therapies as they can mark the tumour cells as non-self. On the other hand TAA are expressed both in healthy and transformed tissues, typically overexpressed in the latter. Many more TAA than TSA have been identified over the years. Their obvious and important disadvantage is that they are self-Ags and as such any T cell specificities present against them are very likely to be tolerised either in the thymus or in the periphery. Nevertheless, the very fact that they are more common and often shared among different malignancies has the practical advantage that one possible successful T cell based immunotherapy may be applicable for more than one tumour type.

1.4.2.2 WT1 antigen and its role as a tumour immunotherapy target

The Wilms tumour antigen 1 (WT1) is a TAA overexpressed in nearly all haematological malignancies (Menssen et al., 1995, Yong et al., 2008, Tamaki et al., 1999) and in solid tumours such as breast, colon and ovarian cancer (Oji et al., 1999, Oji et al., 2002). WT1 expression at low level is only observed in a limited number of healthy tissues; in a subset of HSCs, renal podocytes, ovarian granulosa cells and sertoli cells (Sugiyama, 2002, Oka et al., 2006). Therefore, the prospect of only destroying transformed cells with a T cell based immunotherapy directed against this TAA makes it a very appealing target.

Apart from its pattern of expression in malignant and healthy tissues, WT1 has further advantages as a TAA. Even though it was originally identified as a tumour suppressor gene in the context of the paediatric Wilms tumour (Haber et al., 1993, Haber et al., 1990, Santos et al., 1993), evidence for its role as an oncogene are also present. Yamagami et al suggested that overexpression of WT1 in most haematological malignancies has a role in the leukaemogenesis process. To investigate this they synthesised WT1 antisense oligonucleotides and in support of their hypothesis they were able to show that these molecules specifically inhibited tumour cell growth both in leukaemic cell lines and fresh leukaemic cells isolated from patient's at the time of diagnosis (Yamagami et al., 1996). Algar et al also cultured leukaemic cell lines with antisense oligonucleotides obtaining similar results. In addition, they were able to demonstrate that when WT1 protein expression was inhibited, the reduction in cell growth was associated with an increased frequency of cells with apoptotic features. As the WT1 gene was found not to be mutated in those cell lines, they concluded that overexpression of the normal WT1 protein was important to leukaemic cell viability and proliferation (Algar et al., 1996). Therefore, targeting and eliminating tumour cells expressing WT1 could have a bold effect on both the tumour load but perhaps more importantly on tumour growth. A more recent study suggested an additional role for WT1 in solid tumour metastasis, as siRNA knockdown of WT1 expression in serous ovarian carcinoma cell lines interfered with cellular migration and hindered their invasion into 3D collagen cultures (Barbolina et al., 2008). As this molecule appears to be vital for the malignancies that overexpress it, it is unlikely that its

expression will be downregulated as a tumour immune evasion mechanism and this is an important advantage in the context of cancer immunoediting.

1.4.2.3 T cell based tumour immunotherapies

1.4.2.3.1 Vaccination

The identification of naturally occurring CTL clones recognising TSAs and TAAs in a variety of malignancies encouraged research in the development of vaccination strategies to enhance such populations in cancer patients. WT1-specific T cells in particular were isolated for the first time from the blood of an HLA-A2-positive healthy donor following numerous cycles of in-vitro stimulations with wild type, class I-presented WT1 peptides (Freeman et al., 2000). Based on this discovery, the same group performed WT1-DNA vaccinations in mice to demonstrate that vaccination could generate detectable numbers of WT1-specific CD8 T cells able to reject WT1-expressing tumour cells in subsequent challenges (Oka et al., 2000a). Clinical trials with different WT1-vaccination protocols including peptide, DNA and dentritic cell-based, followed (Rezvani et al., 2008, Oka et al., 2004, Van Tendeloo et al., 2010, EC Morris, 2010). Even though these have only been phase I/II clinical trials, primarily assessing the safety of each proposed treatment, they have all shown variable degrees of success in containing WT1-expressing malignancies correlating to an immunological response in terms of an increase in WT1-specific CTLs in the patients' blood.

Despite the somewhat encouraging results from WT1-vaccination trials, their effectiveness as a tumour immunotherapy is limited when compared to the triumphant clinical results of vaccines in the prevention of a multitude of

infections. In part, this is because most trials are limited by recruiting end-stage cancer patients where the antigen-specific response elicited by the vaccination is simply not adequate to overcome a substantial tumour load. Another key issue is that vaccination strategies rely on naturally occurring TAA-specificities to be present in the immunised patients T cell repertoire. As TAAs are self-Ags, the TAA-specific T cell repertoire is tolerised and therefore compromised both in terms of numbers and responsiveness. Previous work done by the Stauss/Morris group identified naturally occurring, WT1 specific CTLs from draining LNs of breast cancer patients, using WT1 tetramers. While these cells exhibited antigen-specific cytokine secretion and cytotoxicity, killing HLA-A2 positive breast cancer cell lines was only possible following treatment of the tumour cells with IFNγ which increased the HLA A2 expression levels on these targets (Gillmore et al., 2006). Therefore this study suggested that perhaps the biggest limitation of WT1 vaccination-strategies for the induction of antitumour immunity is not the fact that WT1-specific T cells are limited in numbers, but the fact that higher avidity WT1-specific T cells are required to be effective in the context of the tumour microenvironment. The schematic diagram in figure 1.7 describes the principle behind WT1 vaccination and the potential limitations of such a tumour immunotherapy strategy.

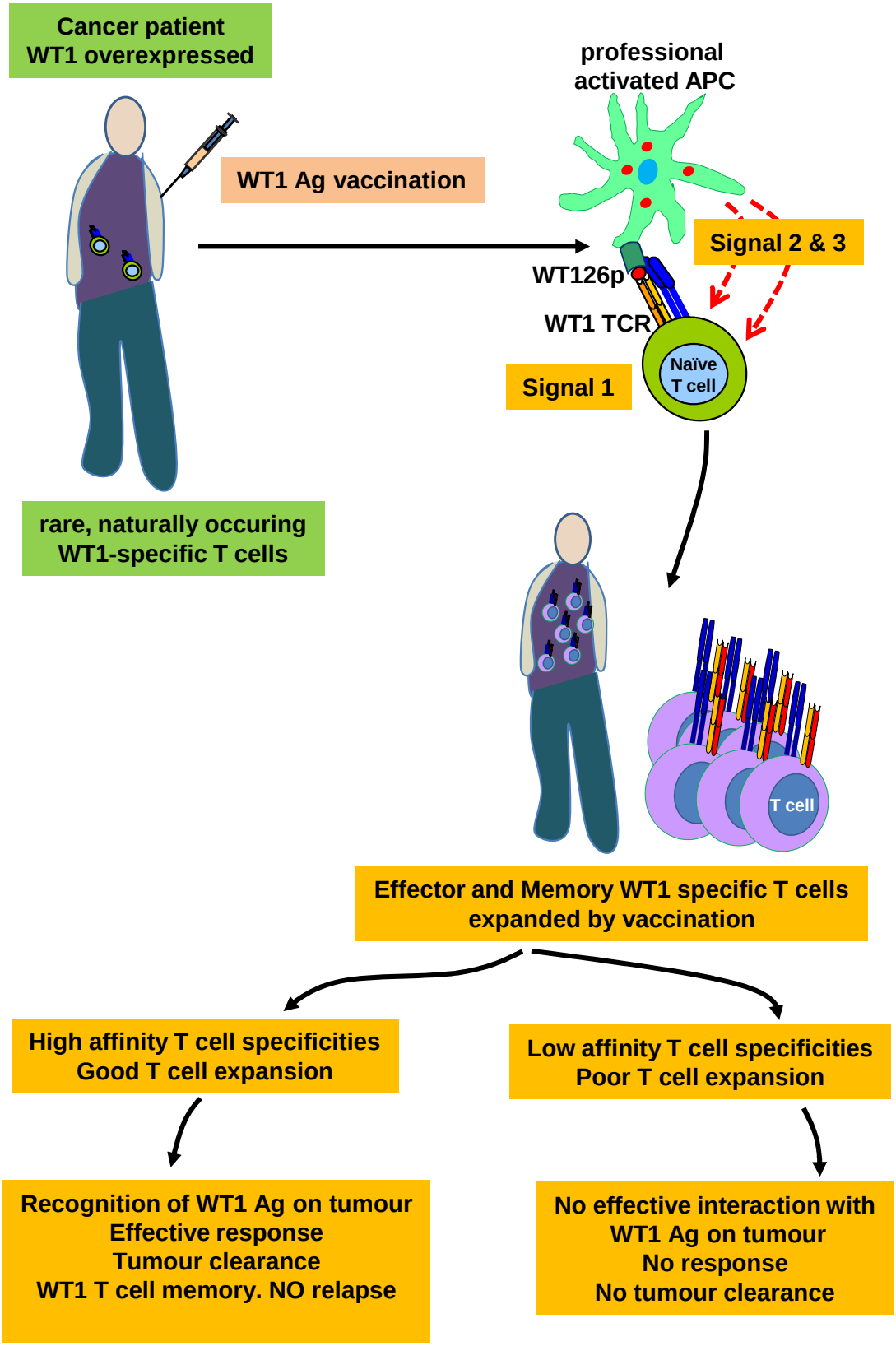


Figure 1.7 WT1 Ag vaccination as a tumour immunotherapy strategy. Patients with malignancies that overexpress the WT1 TAA can be vaccinated with WT1 Ag to expand a naturally occuring WT1 specific T cell population already present in the patient and drive its differentiation into memory T cells. Upon recognising tumour

Figure 1.7 (continued)

cells the WT1 specific T cells can then mount an effective immune response leading to tumour clearance. However, the success of vaccination strategies relies on naturally-occurring high affinity WT1 T cell specificities being present in the patient prior to vaccination. As WT1 is a self-Ag it is likely that high-affinity WT1 specific T cells will be deleted in the thymus during T cell development. The thymic fate of high affinity WT1 specific T cells is examined in this project.

1.4.2.3.2 Adoptive T cell transfer

Adoptive transfer of a particular number of tumour-specific T cells aiming to passively provide the patient with tumour-reactive, functional T cells, is an alternative more interventional approach to vaccination. Clinical trials where tumour infiltrating lymphocytes (TILs) are adoptively transferred to cancer patients, have so far provided encouraging results. In 2002 Dudley et al reported that the adoptive transfer of TILs found in a melanoma metastasis could result in primary and metastatic partial tumour regression in a considerable percentage of the patients recruited in the trial. In vitro IL-2 treatment was required to reverse the anergic character of these autologous T cells that were expanded prior to their return to the patients they were derived from. Receiving non-myeloablative lymphodepleting chemotherapy prior to adoptive T cell transfer, proved to contribute to the treatment's success as the proliferation of the transferred T cells was in part homeostatic and in these conditions regulatory T cells were absent to impede their action (Dudley et al., 2002). Despite its promising outcome, such an approach has a number of practical limitations. Firstly, tumour-responsive TIL cannot be successfully harvested from all patients. Even in cases where this proves not to be a problem, a lengthy in vitro culture period is necessary for the cells' expansion to the appropriate numbers and as this procedure is labour intensive and therefore expensive, making it widely available would be difficult. The schematic representation in figure 1.8 described the basic principles of Adoptive T cell Transfer as a Tumour Immunotherapy strategy.

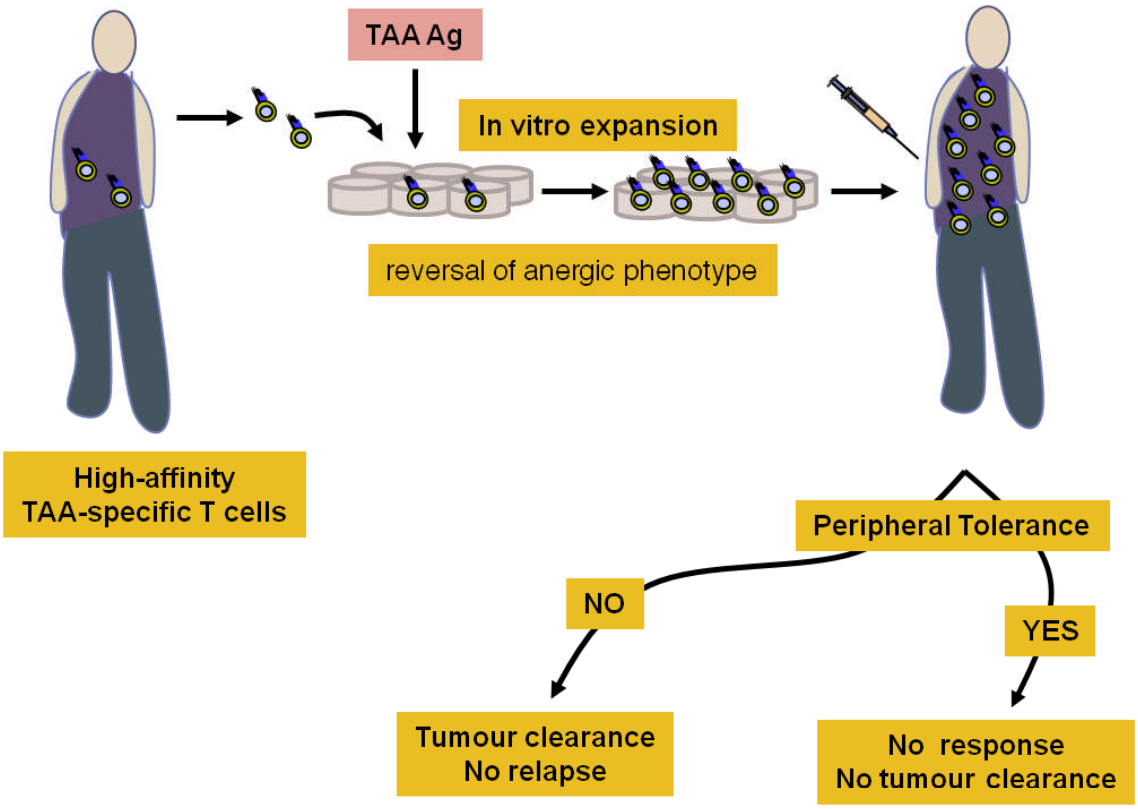


Figure 1.8 Adoptive Transfer of WT1-specific T cells as a tumour immunotherapy strategy. The autologous transfer of TAA-specific T cells into cancer patients, involves the isolation of such cells from the patient and the subsequent in vitro expansion in order to obtain cell numbers that could clear the tumour once returned to the patient. Therefore this strategy relies on naturally occuring TAA-specific T cell being present in the patient in the first place. Even though the isolated TAA-specific T cells can be manipulated in vitro to overcome any anergic character they might still be susceptible to peripheral tolerance when they are adoptively transferred into the patient.

1.4.2.3.3 TCR gene therapy

An alternative approach to obtaining TAA-specific T cells is by employing TCR-gene transfer into the patients' primary T cells. Once the genes for the TCR of a T cell clone that demonstrated TAA-specific function are isolated and cloned into a viral vector, any given number of a patient's T cells - of initially varied specificities - can be easily and rapidly redirected to express that particular TCR. TCR gene therapy can therefore serve as an almost generic treatment for many cancer patients. In the case of WT1-TCR gene therapy this could be applied to patients with most leukaemias, as well as to patients with breast or colon cancer. Nevertheless, as the specificity of any TCR is defined not only by its cognate peptide, but also by the HLA molecule on which the antigen is presented, selecting the patients for which a certain TCR-gene therapy would be suitable not only depends on their malignancies overexpressing the targeted TAA, but also on their HLA phenotype.

Clay et al isolated the genes for a MART-1 specific, HLA-A2 restricted TCR from the TIL culture of a melanoma patient, and were the first to report successfully transducing primary human T cells with TCR$\alpha\beta$ genes and in that way redirecting their specificity. The genetically modified CD4 and CD8 T cells exhibited antigen-specific cytokine secretion and about half the transduced CD8 T cell clones lysed HLA-A2+ melanoma cell lines(Clay et al., 1999a). Soon after, Kessels et al strengthened the case of TCR gene transfer-based tumour immunotherapies, by developing an in vivo model where the adoptive transfer of TCR-gene modified murine splenocytes into syngeneic recipients,

provided antigen specific protection against both infection and a tumour expressing the relevant Ag (Kessels et al., 2001).

Although TAA-specific T cells can be easily obtained with TCR gene transfer, identifying a T cell specificity that can efficiently recognise a TAA and result in the killing of the tumour cells that express it, remains an essential prerequisite. In fact, the cell surface expression of the TCR introduced into a primary T cell is often lower than desired. Given that one of the most well recognised tumour evasion mechanisms is the downregulation of tumour antigens from the transformed cell's surface, lower expression levels of the TCR recognising such an Ag is problematic in the context of tumour immunotherapy.

A number of techniques are currently being developed to improve the transduced-TCR lower expression levels, but employing high-affinity TCRs for TCR gene therapies can also compensate for this considerably as the T cell avidity for a target cell is a function of both the number of TCRs on the T cell surface and the individual TCR's affinity. However, identifying tumour reactive T cell clones bearing a high affinity TCR for the targeted TAA is a challenging matter. TAA-specific T cells essentially recognise self-Ags and are therefore subject to central and peripheral tolerance mechanisms. High-affinity TAA-specific T cells are unlikely to pass through the thymus unnoticed, so they are scarce in the periphery. The few that would emerge from the thymus, like any other autoreactive T cell, will be compromised by the multiple tolerance mechanisms the periphery has to offer.

1.4.2.3.3.1 Allo-restricted specificities for TCR gene therapy

The same year that the Nobel prize was awarded to Doherty and Zinkernagel for their discovery that virus-specific CTL responses are self-MHC restricted (Zinkernagel and Doherty, 1974), Stauss et al suggested that peptide-specific recognition in the context of allogeneic, rather than self, MHC molecules could provide a unique opportunity to bypass this problem and obtain high-avidity TAA-specific CTLs. In a proof-of-principle murine study, they used a TAP-deficient cell line to present a TAA-peptide on the Kb MHC class I molecule to lymphocytes from Balb/c mice. Such co-cultures yielded Balb/c CTL clones that could lyse a Kb-lymphoma cell line endogenously presenting the particular peptide (MDM2-derived peptide) in an antigen-specific manner, while they spared healthy Kb cells expressing MDM2 at lower levels (Sadovnikova and Stauss, 1996).

Stauss' group then went on to identify TAA allorestricted specificities in the human setting. Using a synthetic WT1 peptide - pWT126 - they generated HLA-A2 allorestricted WT1-specific CTLs. In vitro analysis determined that these cells were both pWT126-specific and HLA-A2 specific and could efficiently clear CD34+ HLA-A2 positive leukaemia cell lines and fresh leukaemia cells from patients whilst sparing healthy CD34+ BM cells that expressed the antigen at lower levels (Gao et al., 2000). Further experiments, in an in vivo xenotransplantation model, showed that the allorestricted WT1 T cells could selectively inhibit the engraftment of human leukaemic, but not healthy stem cells in the NOD/SCID mouse (Gao et al., 2003). Therefore, early in the past decade, the idea of generating and adoptively transferring such

allorestricted CTLs in cancer patients as an immunotherapeutic strategy was discussed. Nevertheless, the already mentioned limitations of T cell adoptive transfer coupled with the recognised complication that T cells derived from a mismatched donor are to be rejected by the recipient, advocated in favour of utilising such high-avidity TAA-specificities by cloning their TCR genes and developing TCR gene transfer-based tumour immunotherapies.

1.4.2.3.3.2 WT1-TCR gene therapy: Challenges and Limitations

Indeed Stauss' group confirmed in an in vivo model that human WT1-TCR gene modified T cells obtained the specificity and function of the allorestricted WT1-specific CTL from which the TCRα and TCRβ genes were cloned. Lymphocytes from a patient with leukaemia were redirected to selectively kill autologous leukaemic CD34+ cells expressing this Ag, while transduced T cells from a healthy donor did not attack healthy CD34+ stem cells that also express WT1 at low levels. For the first time, this study demonstrated that TCR td human T cells can be functionally active in vivo, since WT1-TCR td but not mock td T cells showed therapeutic efficacy against a human leukaemia cell line in the NOD/SCID murine model (Xue et al., 2005). In preparation for a WT1-TCR gene therapy trial in acute myeloid leukaemia patients, these results were taken a step further; in a recent paper where Xue et al showed that a patient's cells can be genetically engineered in this fashion to eliminate autologous leukaemia progenitor cells in vivo. In this same study a number of modifications to the TCR vector construct used were examined for the purpose of optimising the expression of the WT1-TCR in primary human T cells and improving the safety of TCR gene therapy (Xue et al., 2010).

These promising pre-clinical results are in support of the phase I/II clinical trial of the high avidity HLA A2 allorestricted WT1-TCR gene therapy for the treatment of leukaemia that is currently underway by the Stauss/Morris group. The effect that the physiological low-level WT1 expression in some healthy tissues has on the phenotype and function of genetically redirected WT1-specific T cells when these are adoptively transferred in patients is not known. Will such cells be tolerised by the periphery and rendered ineffective in fighting cancer, or will their high avidity for the WT1-self Ag instruct them to cause autoimmune damage? Furthermore, given the evidence showing that naturally occurring WT1-specific T cells can be identified both in healthy individuals and cancer patients, a number of WT1 vaccination trials are in development. Nevertheless, eliciting low-avidity WT1-specific T cells by any means of immunisation is not adequate for tumour immunotherapy purposes. Hence, even though there is evidence that central tolerance against WT1-specific T cells is incomplete, the question of how thymic education shapes the high affinity WT1-specific T cell repertoire, is more pertinent. This is because it is such a population that could be effectively enhanced following WT1-immunisation, both in terms of numbers and functional activity to target WT1 expressing malignancies. The schematic diagram in figure 1.9 describes the identification of the high avidity, HLA-A2 allorestricted WT1 TCR and its use in a WT1-TCR gene therapy for the treatment of WT1-overexpressing malignancies.

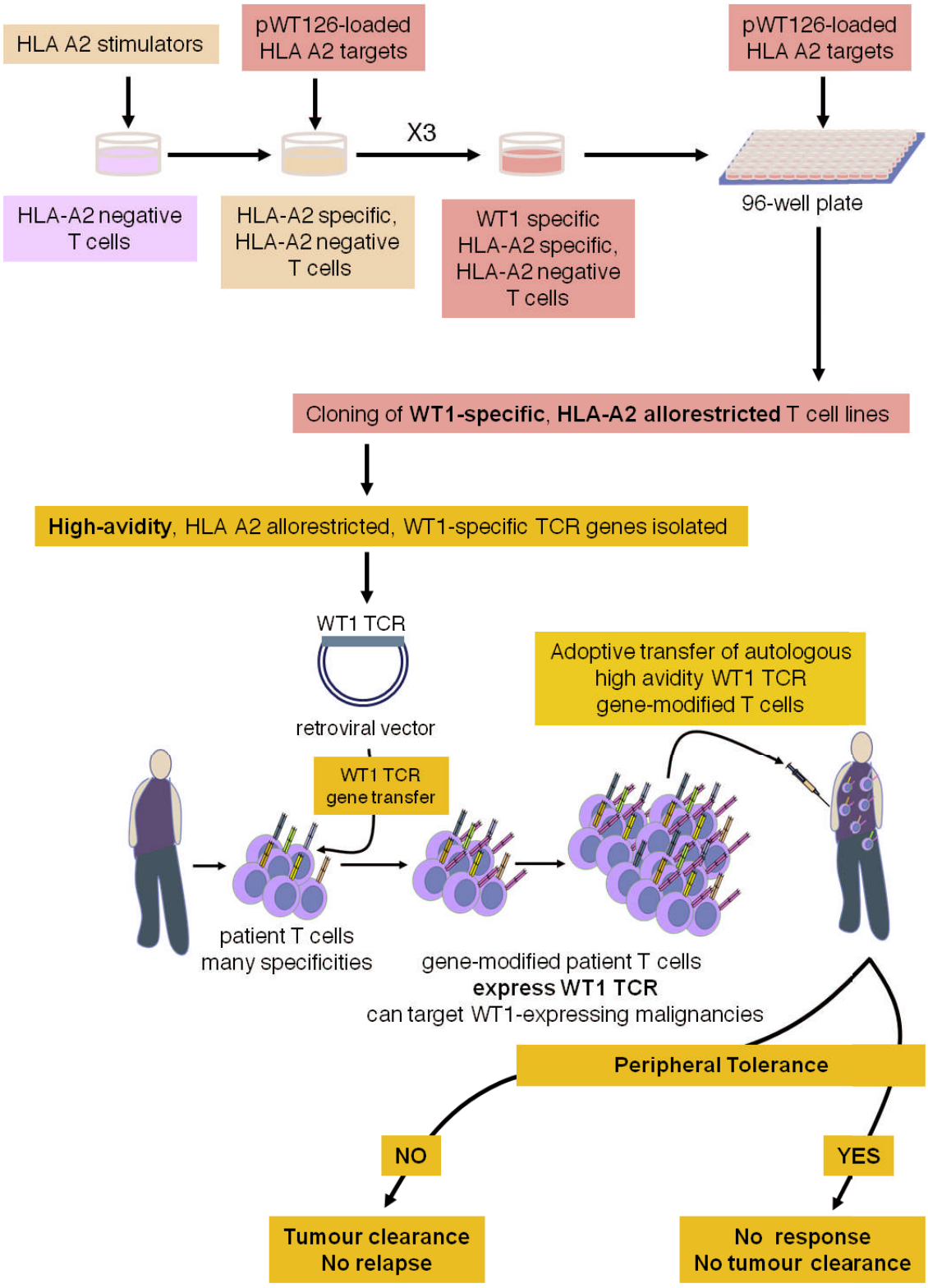


Figure 1.9 Identification of a high-avidity, HLA-A2 allorestricted WT1-specific T cell line and using the genes for this WT1 TCR in TCR gene therapy for the treatment of WT1-TAA overexpressing malignancies. T cells from HLA A2 negative individuals were stimulated with HLA-A2 positive cells to identify HLA A2 specific T cells. HLA-A2 specific, HLA-A2 negative T cells were subsequently

Figure 1.9 (continued)

stimulated with pWT126 loaded HLA-A2 target cells, to identify WT1 specific, HLA-A2 allorestricted T cells. Following 3 rounds of stimulation these cells were transferred into 96-well plates in limiting dilution cultures in order to isolate WT1-specific T cell lines. A high-avidity WT1-specific, HLA-A2 allorestricted T cell line was identified and the genes for this TCR were cloned. These genes have now been placed in a single retroviral vector to be used in a WT1 TCR gene therapy trial for the treatment of leukaemia. WT1 TCR gene therapy involves harvesting the patient T cells and transducing them with the genes for the WT1 TCR. Expression of the WT1 TCR will redirect the patient T cells to recognise and clear leukaemic cells overexpressing the WT1 Ag, upon their transfer back into the patient. However, as WT1 is a self-Ag, ithe in vivo function of these high-avidity, WT1 specific T cells might be compromised by peripheral tolerance mechanisms.

1.5 Project Aims

This PhD project aimed to examine the thymic development of high-avidity, HLA-A0201-restricted WT1-specific T cells as well as their phenotype and function in the periphery, in a clinically relevant murine model. In particular we were interested in answering the following questions regarding T cells expressing the WT1-TCR:

1. Are these cells subject to central tolerance during their development?

2. If following thymic development, high avidity WT1-specific T cells emerge into the periphery, do they then become tolerised in the periphery? Do they become deleted or anergic due to their self-specificity?

3. How does their specificity for the WT1-TAA define their phenotype and function?

4. Do these cells cause autoimmune damage in healthy tissues expressing the WT1-antigen at low levels, in particular in the BM and kidney?

1.6 The experimental model

In order to address the above questions the WT1-TCR genes were transferred into purified lineage negative (Lin-ve) murine HSCs and subsequently transplanted into HLA-A0201 Tg, syngeneic recipients. As described in the schematic representation of the experimental model, in figure 1.10, the transduced murine T cell progenitors home to the thymus where they express the HLA-A2 restricted WT1-TCR in a timely fashion with the CD3 molecule, at the DN stage. Thymic development of the WT1-specific thymocytes in this

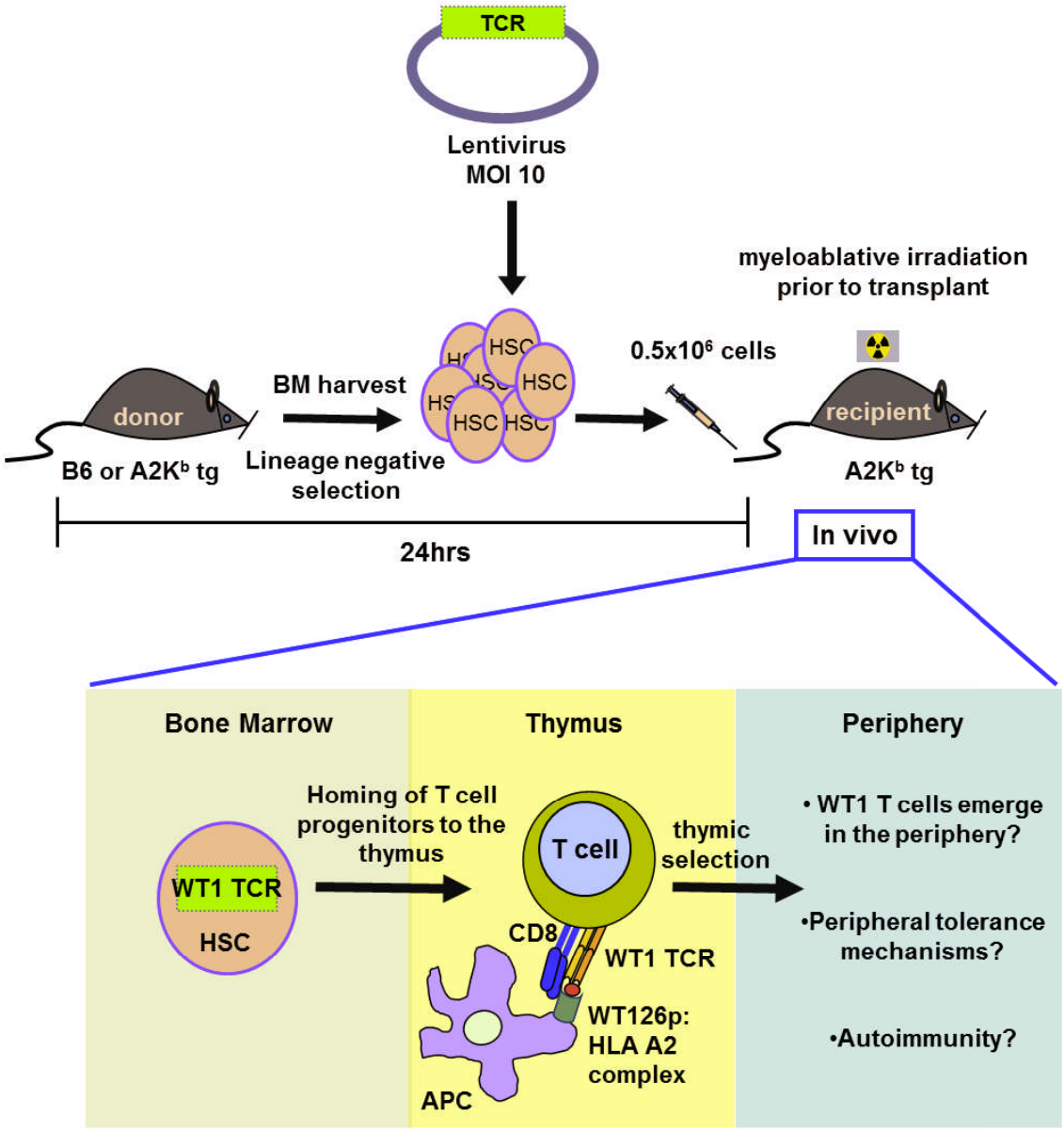


Figure 1.10 Schematic diagram of the experimental model. BM was harvested from donor mice and the cells were taken through a magnetic Lineage negative selection, facilitating the enrichment of the BM cells in HSCs. Following negative selection the Lin-ve BM cells were transduced with a lentiviral construct containing the genes for the WT1 TCR. The next day $0.5x10^6$ transduced HSCs were transferred in each A2Kb tg, lethally irradiated recipient by intravenous injection into the tail vein. A2Kb tg mice express a hybrid HLA A2 molecule. Once in vivo, the transduced T cell progenitors homed from the BM to the thymus, where they expressed the WT1 TCR. In the thymus WT1-specific developing thymocytes went through positive and negative selection . It was likely that during thymic development they would be presented with the WT1 Ag in the context of HLA A2. Therefore, in

Figure 1.10 (continued)

this system it was possible to ask whether high affinity, WT1 specific T cells can escape thymic deletion. Subsequently, if they would escape central tolerance, emerging in the periphery, it was possible to ask whether they were susceptible to peripheral tolerance mechanisms and if not whether they could cause any autoimmune damage because of their self-specificity.

system takes place in the context of HLA-A2 positive thymic epithelial cells. In both human and mouse, WT1 is expressed at low levels in the periphery - in a fraction of CD34+ HSCs and kidney podocytes – but is expressed widely and at high levels during prenatal development (Hohenstein and Hastie, 2006). Therefore it is expected that WT1 will be one of the many self-antigens promiscuously expressed in the thymus facilitating the negative selection of potentially autoreactive T cells. Stauss' group has previously observed WT1 expression in the mesothelial lining of the thymus, but not in the epithelial stroma were positive and negative selection take place (unpublished data). However, at present time no report has been made to ascertain that the WT1 Ag is not promiscuously expressed by mTECs in particular. Such promiscuous expression in only a small number of mTECs could be enough to shape the thymic development of WT1-specific T cells.

Therefore, in the absence of this information, the experimental model presented here addresses the question of whether high-affinity WT1-specific T cells are subject to central tolerance mechanisms. The phenotype and function of WT1-specific T cells emerging in the periphery in this system, was studied extensively to explore the possibility of these cells being either tolerised or causing autoimmune damage. To confirm that the results obtained related to the WT1 specificity, rather than being an artefact of the HSC isolation and lentiviral transduction protocol, a second construct containing the genes for the high avidity, HLA A2 restricted, LMP2-specific TCR was used in control experiments. T cells bearing this TCR, are specific for an Ebstein-Barr Virus epitope derived from the LMP2 protein, a non-self epitope. Finally, the results produced by our model can also to some extent be extrapolated to inform us

about the fate of high affinity self-specific T cell in the thymus and the periphery, especially in view of recent data suggesting that self-antigen can mediate the differentiation of naive T cells into memory in the context of homeostatic proliferation (Haluszczak et al., 2009).

Below the important parameters of the experimental model used in this project are discussed.

1.6.1 WT1 expression in human and mouse.

WT1 expression is highly conserved between human and mouse and the tissue distribution of this protein is also very similar among these two and other species (Kent et al., 1995). In 2000, Oka et al, being one of the groups in support of an oncogenic role for wildtype WT1, especially in the context of leukaemia pathogenesis, went on to explore whether it could also serve as a target Ag for antitumour immune responses and potentially in the context of tumour immunotherapy. Using a peptide library-based method for predicting MHC class I-binding peptides (Udaka et al., 1995, Stryhn et al., 1996) from the WT1 protein sequence, they identified and synthesised 4 H-2Db-restricted WT1 peptides. One of these three peptides, the Db126 peptide, induced antigen-specific CTLs in response to in vivo immunisation that subsequently lysed and rejected WT1-expressing tumour cells introduced in the immunised animals. The authors then confirmed that this is a naturally occurring antigenic epitope, by testing peptide fractions from WT1-expressing tumour cell lysates for their sensitising activity to Db126-specific CTLs (Oka et al., 2000b). In the same year, this group showed that this same epitope could also bind to the human class I molecule, HLA-A2.1. As with the case of murine leukemic cells,

HLA-A2.1 positive leukaemic cells endogenously expressing the WT1 protein were lysed in an HLA-A2 restricted, WT1 antigen-specific fashion by Db126-specific human CTLs (Oka et al., 2000a). The HLA A2 allorestricted high avidity WT1-TCR, currently evaluated in a TCR gene therapy clinical trial conducted by our lab, is specific for the pWT126 that has been shown to be identical in human and mouse. This and the similar tissue distribution of WT1 in the two species, allowed the generation of the in vivo murine model presented above to study the effects of physiological WT1 expression in healthy tissues on the development and functional activity of T cells expressing this particular WT1-TCR.

1.6.2 The WT1-TCR

The interaction between the WT1-specific TCR with the hybrid HLA A2 molecule used in this study is described in figure 1.11. The variable TCRα and TCRβ chains are derived from a high-avidity HLA-A2 allorestricted pWT126-specific CTL clone. The identification and characterisation of this specificity has been described in detail in section 3.2.3.3.1. The constant regions of the human WT1-TCR have been murinised for the purposes of the TCR gene therapy clinical trial, as it has been shown that this modification improves the dimerization between the introduced TCRα and TCRβ chains (Cohen et al., 2006). In this way the risk of mispairing between endogenous and exogenous α and β chains, which has been shown to give rise to autoreactive specificities in the context of TCR gene transfer in primary T cells (Bendle et al., 2010), is minimised. Hybrid TCRs appear to enhance the function of transduced T cells when compared to fully human molecules of the same specificity and affinity

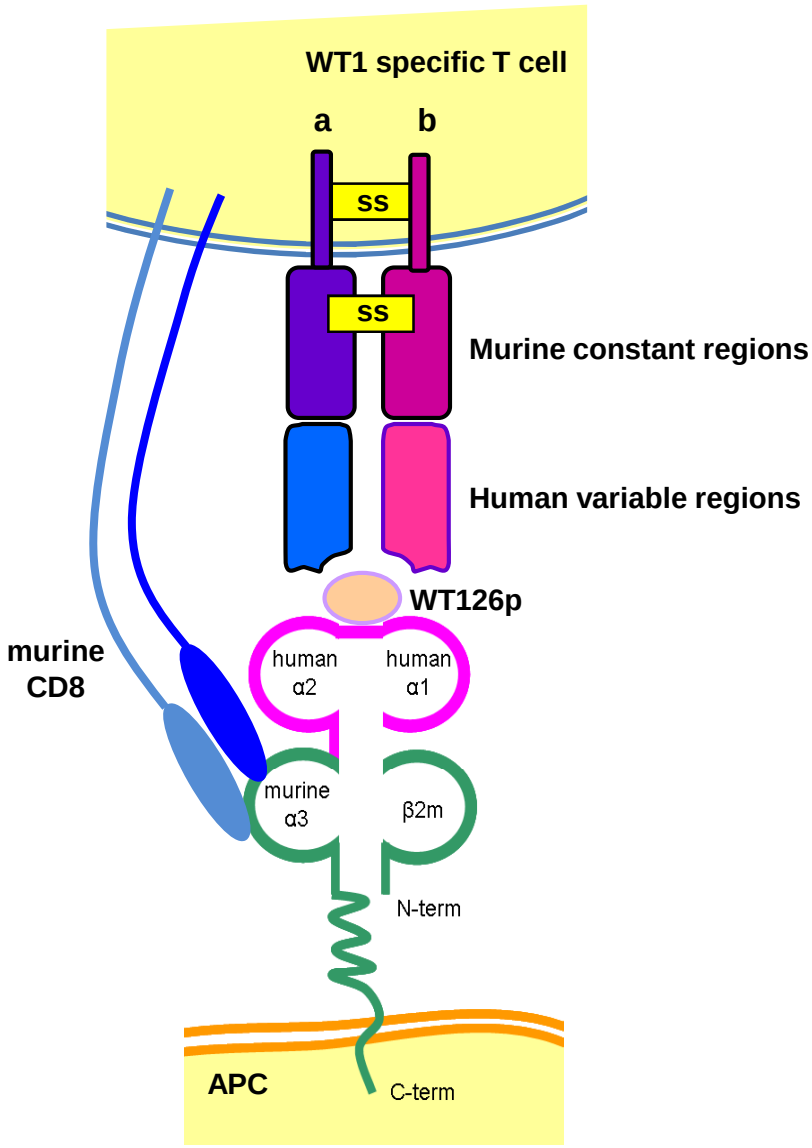


Figure 1.11 Schematic representation of the interaction between the transgenic HLA A2/Kb molecule presenting WT126p and the murine CD8 T cell expressing the murinised, Cys 1- modified, human WT1 TCR. The hybrid HLA-A2Kb molecule expressed in HLA-A2Kb (A2Kb) tg mice contains the human alpha1 and alpha2 domains that interact with the human HLA-A2-restricted TCR and are fused to a murine alpha 3 domain, which facilitates the interaction of this molecule with the murine CD8 coreceptor.

and this is thought to be due to a stronger and more stable interaction between murine constant regions and the human CD3 molecule.

Another modification also made to this TCR molecule to enhance specific pairing between the introduced TCR chains is the addition of a second disulphide bond between the two constant chains (Cohen et al., 2007, Kuball et al., 2007, Thomas et al., 2007). Finally, the genetic sequence for the hybrid WT1-TCR was codon optimised, which has been shown to improve TCR expression levels and therefore enhances their in vivo functional activity (Scholten et al., 2006, Jorritsma et al., 2007).

This set of modifications were performed for the purposes of the recently commenced WT1-TCR gene therapy clinical trial, as they are documented to cumulatively improve the surface expression of the introduced TCR and the antigen-specific function of human transduced T cells in vivo. Nevertheless, these modifications are likely to have similar effects when the TCR is expressed in murine T cells and therefore are likely to increase further the avidity of the WT1 T cells, thus facilitating the study of the thymic selection and peripheral function of high avidity WT1 TAA-specific T cells. Furthermore, using the same TCR as in the clinical trial offers clinically relevant information about the in vivo behaviour of T cells bearing this particular TCR.

1.6.3 The HLA A2 Tg mouse

The HLA A2Kb transgenic mice used in this project were a kind gift from Mathias Theobald. These animals expressed hybrid HLA-A0201 molecules consisting of human α1 and α2 domains, while the α3 domain is of murine origin. An antigenic class I epitope binds in the groove formed by the α1 and α2

domains, and from this position it is presented to CTLs. Upon TCR-pMHC complex binding, the interaction between T cell and its target is strengthened by the CD8 co-receptor present on cytotoxic T cells, binding to the α3 domain of the MHC class I molecule. In fact this interaction partly defines T cell avidity. In our model, the human-murinised TCR is expressed on murine CD8 T cells. It is the use of HLA A2Kb transgenic mice that not only allows the human variable regions of the WT1-TCR to bind to the pWT126:HLA A2 complex in the same way they would in human, but also enables optimal binding of the murine CD8 to the murine α3 domain of the hybrid HLA A2Kb molecule. A schematic representation of the interaction between the modified pWT126-specific TCR and the pWT126:HLA A2Kb complex is shown in figure1.11.

1.6.4 TCR gene transfer in HSCs for the in vivo study of T cell selection and function

TCR transduction of HSC was first reported by Clay, who interestingly back in 1999 suggested that this could be an adoptive immunotherapy strategy and performed in vivo experiments to prove that it was technically possible to generate antigen specific cells (Clay et al., 1999b). Later, Baltimore and Yang revived and further explored this idea by investigating the phenotype and functional activity of OTI and OTII-specific T cells generated in vivo following transfer of OTI and OTII TCR-transduced HSC-enriched BM cells into irradiated recipients. These cells emerged from the thymus exhibiting a naive phenotype, and following antigenic stimulation they rapidly differentiated into fully functional effector and memory T cells that were also able to confer

significant tumour protection against an OVA-expressing tumour (Yang and Baltimore, 2005).

Essentially mice reconstituted with TCR-td HSC, were comparable to TCR Tg mice. The main differences between the two are technical; given that the TCR genes are available, the former only takes a few weeks to generate. However, unlike TCR Tg mice it is not possible to propagate a mouse line containing the particular antigen-specific T cells by breeding. Holst et al named these mice retrogenic mice, in reference to the retroviral vectors used to transduce the HSCs and to distinguish them from TCR Tg mice. In their 2006 paper these authors demonstrated that retrogenic mice can serve as models for the in vivo study of T cell selection and function. In particular they transfered male antigen-specific TCR-td HSCs into male and female mice and, as predicted, observed the thymic deletion of such developing thymocytes in male, but not female mice. In the case of the HY TCR, negative selection was incomplete with the surviving HY-specific T cells being tolerised in the periphery of male recipients (Holst et al., 2006), as was the case of HY-TCR Tg equivalents described many years before (Kisielow et al., 1988). In this work, as in some of their previous publications (Szymczak et al., 2004, Arnold et al., 2004) the Vignali group constructed and used 2A peptide-linked TCR vectors. Linking the TCRα and TCRβ chain genes with the 2A peptide, rather than the commonly used IRES, allows for the stoichiometric expression of the two genes. When placed in the same open reading frame the 2A-linked genes are transcribed as one. Through a ribosomal ‘skip’ mechanism the 2A consensus motif, results in the co-translational separation of the proteins encoded upstream and downstream of this typically 18-20aa long peptide. The only remnant of this

cleavage process is that the C-terminal of the 2A peptide remains attached to the upstream protein. Despite the theoretical possibility of these virus-derived, few amino acids to be immunogenic, this has not yet been described.

1.6.5 Lentiviral vectors for TCR gene transfer in HSC

In this project, unlike the first studies performing TCR gene transfer into HSCs, a lentiviral construct was preferred over a retroviral vector. This made the choice of a porcine tchechovirus 2A sequence (P2A) even more pertinent, as there is evidence that the non-stoichiometric expression observed with the use of an IRES element is consistently a problem with bicistronic lentiviral vectors with the second transgene always being underexpressed. The main advantage of lentiviral over retroviral vectors is that they can transduce non-dividing cells (Sakuma et al., 2012). One of the technical objectives of this project is the efficient transduction of HSCs rather than more differentiated progenitors, to achieve the long-term and unlimited in vivo provision of T cell progenitors destined to express the introduced TCR when in the thymus. Abolishing the requirement for activation and proliferation of the cells prior to their infection with the viral vector carrying the genes of interest, secures to a certain extend the stem cell potential of the transduced HSCs.

Chapter 2 – Materials and Methods

2.1 Mice

C57Bl/6 mice were purchased from Charles River Laboratories or the Comparative Biology Unit of University College London, Royal Free Campus. HLA-A2Kb transgenic (A2Kb Tg) mice of C57/Bl6 background were a kind gift from Theobald M (University Medical Centre, Utrech, The Netherlands). C57Bl/6, CD45.1 C57Bl/6, A2Kb mice as well as A2Kb mice crossed to CD45.1 mice (A2Kbx CD45.1) were bred and maintained in the Comparative Biology Unit of University College London. All animals were housed in pathogen-free conditions in individually ventilated cages and were kept in accordance with the University and United Kingdom Home Office regulations. All procedures were performed aseptically, and irradiated animals received 2.5% enrofloxacin (baytril, Bayer) in their water for 1 week before irradiation and for 2 weeks after irradiation, unless otherwise specified in individual experiments. Donor mice were 7 to 10 weeks old at the time of bone marrow (BM) harvest. Recipient mice were 12 weeks old at transplantation and received 9.4Gy irradiation in 2 divided doses at day -2 and day 0. Secondary adoptive transfer recipients were sublethally irradiated (5Gy) 4 hrs before T cell transfer.

2.2 Lentiviral vector constructs

2.2.1 PCR & primers

For the purposes of cloning the hybrid WT1-TCR sequence in the pSin second-generation lentiviral vector containing a spleen focus forming virus LTR

promoter and the HIV-1 central polypurine tract cis-active element, the human WT1 Vα and murine Cβ (mCβ) gene segments were PCR amplified from the pGA4 cloning plasmid, as described in Chapter 3, section 3.2.1.

The primers used are listed in table 2.1. The mixture used for each PCR amplification reaction was prepared using the following amounts of reagents: 10ng DNA template, 2.5mM of each of dATP, dTTP, dCTP, dGTP (dNTP mixture), 50μM of each 5' and 3' primers, 1μl Pfu turbo polymerase, 10μl 10x Buffer. Final volume of the reaction mixture was 100μl made up with distilled H_2O (ddH_2O).

	Primer sequence	Highlighted sequence
5'primer to amplify Vα WT1 TCR chain	GGATCCAGCCACCATGCTGCTG CTGCTGGTGCCC	BamH1 restriction site
3' primer to amplify Vα WT1 TCR chain	CCGCGGGCTCGGGGTTCTGGA	Sac2 restriction site
5'primer to amplify mCβ chain	CTCGAGGACCTGCGGAACGTG ACCCCCCCC	Xho1 restriction site
3' primer to amplify mCβ chain	TGTACAGCGGCCGCGTCGACT CATCAGCTGTTCTTCTTCTT	Not1 restriction site

Table 2.1 List of primers used for PCR amplification reactions to clone the hybrid WT1 TCR sequence from the pGA4 cloning plasmid into the pSin second-generation lentiviral vector.

2.2.2 Restriction digests

Restriction digests were performed both to confirm that the correct modifications in a particular construct/DNA sequence have been achieved (screening digests) and to prepare DNA for ligation/cloning (preparative digests). Mixtures used for each were the following:

Screening digests: 10μl mini prep DNA, 0.5μl of enzyme(s) required, 2μl 10xBSA, 2μl appropriate 10x NEB buffer. Final volume of the reaction mixture was 20μl made up with ddH_2O and this was incubated at 37^oC for 2hrs. 5μl of Ethidium Bromide was then added to the mixture and the digested DNA bands were visualised under UV light.

Preparative digests: 6μg DNA, 5μl appropriate NEB buffer, 5μl 10xBSA, 1.5μl of each enzyme. Final volume of reaction mixture was 50μl made up with ddH_2O. The mixture was incubated at 37^oC for 4hrs. This was then mixed with 10μl of Ethidium Bromide and run on 2% agarose gel. The digested DNA bands were visualised under UV light and the band of interest was removed from the gel with the use of a scapel. DNA was then purified using Qiagen DNA purification kit following the manufacturer's instructions.

2.2.3 Ligation Reactions

PCR amplified gene segments replaced sequences previously present in the cloning pGA4 and lentiviral backbone vectors. Two different ligation mixtures were prepared for each reaction; one containing insert and vector at an 1:1 molar ratio and a second one containing insert and vector at a 3:1 molar ratio. The ligation mixtures were prepared in a final volume of 10μl made up with

ddH2O. Each ligation and contained 1μl 10x Ligation Buffer, 0.5μl DNA ligase, 100ng of (digested) vector, and the appropriate amount of insert calculated with the following formula:

$$\frac{\text{ng of vector} \times \text{size of insert in Kb} \times \text{insert:vector molar ratio}}{\text{size of vector in Kb}} = \text{ng of insert to use}$$

Ligation mixtures were incubated at 4^{o}C overnight and the ligated DNA was then used to transform DH5 maximum efficiency competent E.coli (Invitrogen).

2.2.4 Transformations

Transformations of DH5 maximum efficiency E.coli were performed as per manufacturer's protocol. Following transformations with ligated DNA, up to 10 colonies from each transformation LB-agar plate were harvested and cultured overnight in LB-medium, at 37^{o}C in a shaker. Both LB-agar plates and LB-medium contained 100μg ampicillin ng/ml. DNA was then prepared using the Qiagen DNA Miniprep Kit as per manufacturer's protocol. The prepared DNA was screened with a restriction digest using the appropriate restriction digestion enzymes to confirm the presence of the insert used in the ligation, as described in section 2.2.2.

2.2.5 Maxipreps

To produce the necessary quantities of DNA to be used for the production of lentiviral particles the Qiagen DNA Maxiprep kit was used as per manufacturer's protocol.

2.3 Preparation of lentiviral particles

2.3.1 Preparation of lentiviral particles

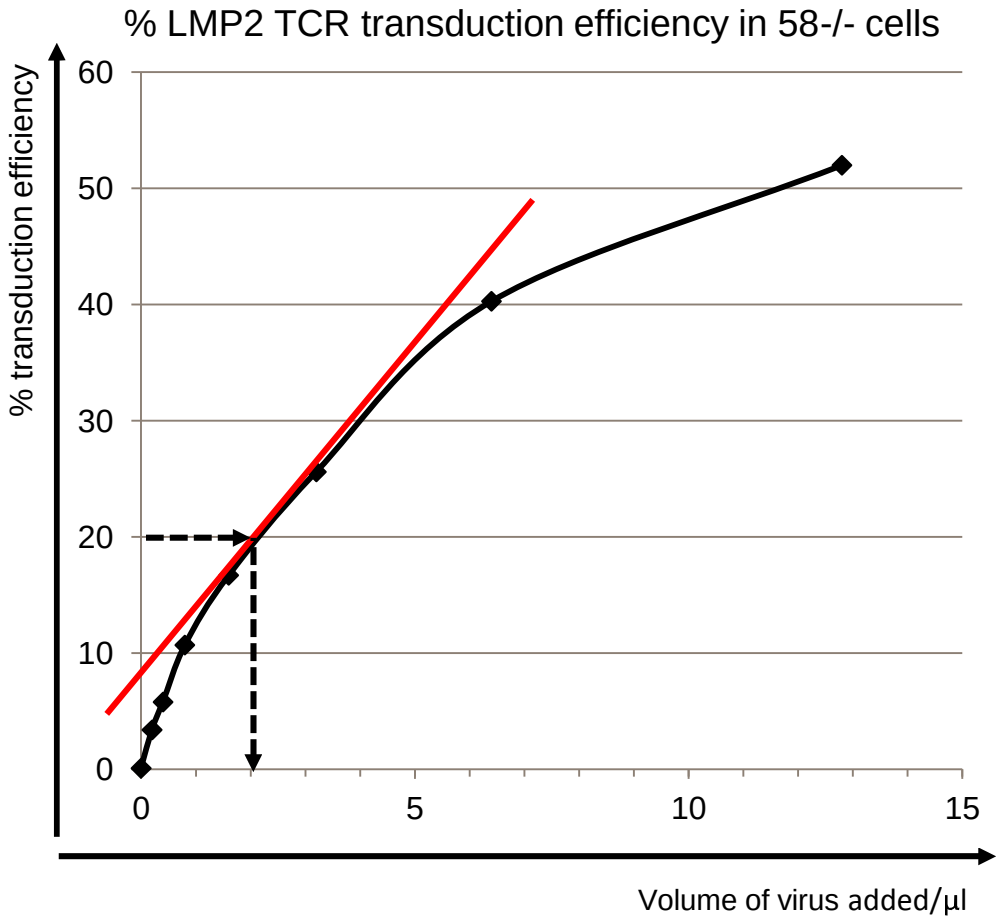
For the production of lentiviral particles, $2x10^6$ 293T packaging cells were cultured overnight in IMDM medium containing 10% FCS, 1% penicillin/streptomycin and 1% L-glutamine, in 175 cm^2 flasks for 24hrs at 37^oC, 5% CO_2 in a humidified atmosphere. 12-18 hours later the flasks were 70% confluent and packaging cells were co-transfected with pMD.G, a plasmid encoding the vesicular stomatitis virus glycoprotein (VSV-G) envelope and pTcmVΔ8.91, a plasmid encoding the genes required for the generation of functional viral particles, together with the pSIN lentiviral vector encoding the GFP or TCR genes. Medium was replaced with fresh IMDM supplemented as above 24hrs after transfection, and the viral supernatant was harvested 48hrs later and concentrated 100x using ultracentrifugation as described below.

2.3.2 Concentrating lentivirus

The lentiviral supernatant was harvested and centrifuged at 1500rpm for 5min to remove any cellular debris. It was then pipetted slowly onto 5ml of 20% sucrose solution (20% in water) in Ultra-clear Centrifuge tubes 25x89mm from Beckman, to prevent any further cellular debris from pelleting during ultracentrifugation. The lentiviral supernatant was ultracentrifuged at 25000rpm for 2hrs at 4^oC and was then discarded as the lentiviral particles were pelleted in an invisible pellet. The virus was then resuspended in StemSpan medium (100x concentrated), aliquoted in 200μl batches and stored at -80^oC for future use. Each aliquot could only be thawed once.

2.3.3 Quantification of viral particles

To estimate the number of viral particles per μl of the concentrated virus $5x10^5$ 58-/- cells in 1ml of RPMI medium supplemented with 10% FCS 1%Pen/Strep and 1% L-glutamine were transduced with a serial dilution of concentrated lentivirus (0.1μl to 12.5μl). This was repeated for each new batch of lentivirus. Transduction was performed by adding the specified amount of virus into each well and replacing with fresh medium 24hrs later. The transduced cells were kept in culture and FACS analysed 48hrs later for transgene expression. The percentages of GFP, WT1-TCR or LMP2-TCR were each time plotted in a graph as shown in figure 2.1 and a "standard curve" was drawn. As it is widely accepted that at low transduction efficiencies each transduced cell receives one functional virus, the total number of functional viral particles per μl of concentrated virus was calculated based on the volume of virus required to reach a transduction efficiency of 20%. For the purposes of our experiments an MOI (Multiplicity of Infection) 10 was used to transduce the Lin-ve BM cells.



20% transduction efficiency→2µl virus

X% transduction efficiency←1µl virus

Figure 2.1 "Standard Curve" to calculate the number of viral particles per µl of concentrated lentivirus. 5x10^5 58-/- cells in 1 ml of cell culture medium were transduced with a serial dilution of concentrated lentivirus (0.1-12.5µl). 24hrs later fresh medium was added and 48 hours later the cells were analysed by Flow Cytometry for transgene expression. The percentages of transduced 58-/- cells in relation to the amount of virus added were plotted in a graph to produce a "standard curve". Here the standard curve" for the LMP2 TCR lentivirus is shown. The volume of virus corresponding to 20% transduction was used to calculate the total number of functional viral particles per µl.

2.4 HSC isolation, infection and transfer

2.4.1 BM harvesting

Whole BM was harvested from 7- to 10- week old donor mice (C57Bl/6 or A2Kb Tg as specified in each experiment). Donor animals were sacrificed using Schedule 1 procedures and sprayed thoroughly with 70% ethanol. Femurs, tibias and pelvic bones were removed from the donor animals, using a sterile technique. Bones were kept in ice cold RPMI and the BM was harvested by cutting each end of every bone and flushing it with ice cold RPMI medium containing 1% Pen/Strep using a syringe attached to a 25-gauge needle. A single cell suspension of BM cells was then prepared by mixing the cells using a syringe attached to a 16-gauge needle.

2.4.2 Lineage negative selection

Uncommitted BM progenitors, highly enriched in HSCs, were negatively selected using either the lineage cell depletion kit for mouse (Miltenyi Biotec) or the Haematopoietic Progenitor Enrichment kit (StemCell Technologies) following the manufacturer's protocols. Lineage negative BM (Lin-ve BM) cells- highly enriched in HSCs- were placed in culture at $1x10^6$cells/ml in StemSpan medium (StemCell Technologies) containing 50U/ml penicillin/streptomycin, 100ng/ml murine Stem Cell Factor, 100ng/ml human Fms-like tyrosine kinase 3, 100ng/ml human IL-11, and 20ng/ml murine IL-3 (all cytokines purchased from Peprotech). In experiments where the transduction of Lin-ve cells was required this was performed by adding the necessary volume of the appropriate lentiviral particles to provide an MOI of 10. 18-24 hrs after transduction, the transduced Lin-ve BM cells were harvested, washed and

resuspended in RPMI containing 1% Pen/Strep. Cells were then counted and resuspended at a concentration that would allow the transfer of the required number of cells into recipient animals. In preliminary cell number titration experiments, mock transduced cells were used that had undergone the same procedures, apart from the addition of virus in the cell culture.

2.4.3 HSC transplant

HSC transplants with mock, GFP td, WT1-TCR or LMP2-TCR td Lin-ve BM cells were performed in 11-12 wk old A2Kb Tg mouse recipients that were lethally irradiated with 9.4Gy in two fractions, unless otherwise specified in individual experiments. In all experiments except the cell number titration experiments (section 3.3.2), each recipient received $5x10^5$ Lin-ve BM cells by tail vein injection. Tail bleeds were performed at weeks 5, 7, and 9 after transplantation. Recipient animals were sacrificed 11 weeks after transplantation and thymus, spleen, LNs and BM were harvested for phenotypic analysis and functional characterisation of T cells.

2.4.4 Standardised clinical monitoring

All transplated mice were monitored on a daily basis and scored according to the standardised clinical monitoring system presented in table 2.2 Mice scoring 4 or higher were sacrificed.

score	0	0.5	1	2
Fur	Normal	0 <Fur at angle ≤45	45 ≤ fur angle < 90	fur angle 90^{o}
Hunch	None	slight hunch, lost when mouse moves	Significant hunching, reduced on movement	severe hunching persistent on movement
Eyes	Normal	< 50% closed	<50% closed < 100%	Eyes closed
Activity	Normal	mild to moderate reduction in spontaneous movement, resist handling	Severe reduction in spontaneous movement , do not resist handling	No spontaneous movement

Table 2.2 Standardised clinical monitoring system for HSC transplant recipients. Mice were assessed on the appearance of their fur and eyes, the presence of a hunch, and their activity.

2.4.5 Histology

Samples of bone marrow and kidney from mice receiving WT1-TCR and LMP2-TCR td HSCs were fixed in 10% neutral buffered formalin, processed routinely and stained with haematoxylin and eosin (H&E). The blinded histological analysis of tissue samples from mice looking for evidence of T cell infiltration and autoimmune damage in these tissues that are known to physiologically express low levels of the WT1 Ag, was carried out by Dr. Emma Morris, Dept of Immunology, Royal Free and University College Hospital Medical School, UCL.

2.4.6 Adoptive T cell transfer into secondary recipients

Lentiviral TCR-transduced C57Bl/6 Lin-ve BM cells were transferred into lethally irradiated A2KbxCD45.1 Tg recipients as described above (section 2.4.3). Seventeen weeks after transplantation the mice were sacrificed, and their spleens were harvested. Splenocytes from 5 primary recipients were pooled and untouched CD3 T cell selection was performed using a mouse pan-T cell isolation kit (Miltenyi Biotech) as per manufacturer's protocol. A total of $3x10^6$ T cells were transferred to secondary A2KbxCD45.1 recipients that were sublethally irradiated (5Gy) 1 day prior to the transfer. Tail bleeds were performed at day 9 and 28 after adoptive transfer. On days 69 and 70, in vivo cytotoxicity assays (see section 2.5.1) were performed and splenocytes from these secondary recipients were also used in ex-vivo proliferation assays as described in section 2.5.2, materials and methods.

2.4.7 Serial HSC transplants

Serial HSC transplants were performed using BM cells from primary WT1-TCR HSC transplant recipients (A2KbxCD45.1 to A2Kb transplant). BM cells were pooled together and $5x10^6$ cells were transferred into each lethally irradiated (9.4Gy administered in two portions) C57Bl/6 secondary recipients. The mice were monitored on a daily basis and tail bleeds were performed at 3, 5, 8 and 12 weeks after transplantation, to monitor the reconstitution kinetics of all haematopoietic lineage at these time points by FACS analysis. BM cells from untreated A2KbxCD45.1 Tg mice were used to reconstitute lethally irradiated C57Bl/6 mice as a control for donor-derived haematopoietic reconstitution.

2.5 T cell phenotypic analysis and functional assays

2.5.1 In vivo cytotoxicity assays

Splenocytes from female A2Kb Tg mice were peptide loaded with 100μM of either relevant peptide (WT1-TCR pWT126, LMP2-TCR pCLG) or an irrelevant HLA-A0201 presented epitope before labelling them with 1.5μM carboxyfluorescein diacetate succinimidyl ester (CFSE; CFSEhi) or 0.15μM CFSE (CFSElo) respectively, for 5min at 37^oC. The cells were then washed once in ice-cold RPMI with 8% FCS and twice with ice-cold PBS. Labelled cells were mixed at a 1:1 ratio, relevant:irrelevant targets and a total of $10x10^6$ total cells were injected per mouse by tail vein injections. 18hrs later, injected animals were sacrificed and splenocytes were analysed by flow cytometry to identify CFSE high or CFSE low labelled targets. Control untreated A2Kb Tg mice were injected with the same mix of labelled target cells. Percentage antigen-specific in vivo cytotoxicity was determined using the following formula:

[1-((A:B)/(C:D))], where A= mean number of relevant peptide-loaded splenocytes at 18hrs in experimental mice; B = mean number of irrelevant peptide-loaded spelnocytes at 18 hrs in experimental mice; C = mean number of relevant peptide-loaded splenocytes at 18hrs in control mice and D = mean number of irrelevant peptide-loaded splenocytes at 18hrs in control mice.

2.5.2 Ex-vivo proliferation assays

Splenocytes from mice that had been transplanted with WT1 or LMP2-TCR td HSCs, or mice that had received adoptively transferred of T cells from primary transplant recipients, were labelled with 1.5μM CFSE as described in section 2.5.1 and placed in culture at $1x10^6$ cells/ml. The CFSE labelled cells were then stimulated with 100μM of relevant (pWT126 or pCLG accordingly) or irrelevant peptide for 5 days before the CFSE dilution of WT1 or LMP2-specific T cells were analysed by flow cytometry. WT1 and LMP2-specific T cells were identified by staining with human Vβ2 and human Vβ13 antibodies respectively. In the case of A2Kb Tg mice receiving C57Bl/6 TCR td HSCs, professional antigen presenting cells (APCs) were almost completely replaced with donor C57Bl/6 BM-derived APCs. Therefore, to ensure adequate antigen presentation, the assayed labelled splenocytes were stimulated with an equal number of A2Kb Tg splenocytes loaded with relevant/irrelevant peptides (100μM), with the final cell concentration being $1x10^6$ cells/ml.

2.5.3 Antibodies and FACS analysis

2.5.3.1 Antibodies

Optimum concentration of the monoclonal antibodies (MAbs) used was determined in a titration assay where a mixture of cells consisting of both cells staining positive and negative for each MAb was stained with increasing concentrations of the MAb. The staining profile was analysed by flow cytometry to identify the lowest concentration of the MAb at which a clear separation between the negative and positive population of cells was obtained. This was then used in subsequent experiments. The following fluorescently labelled anti-murine antibodies (BD Bioscience) were used in this project: CD45.1 APC Cy7, CD3 APC, CD3 FITC, CD4 FITC, CD8 PE Cy5, CD44 APC, CD62L FITC, B220 PE Cy5, CD11b APC, Lineage cocktail antibody APC, Sca1 PECy7, and streptavidin APC. Anti-human Vβ2.1 PE, Vβ2.1 biotin and Vβ13 were purchased from Immunotech. An LSR II cytometer (BD Bioscience) was used for flow cytometric analysis. Data were analysed using FACS DIVA (BD) and FlowJo Version 7 (TreeStar) software.

2.5.3.2 Preparing blood samples from tail bleeds for staining

Red blood cells were lysed prior to antibody staining blood samples for FACS analysis by transferring the sample in 9ml of H_2O. 10 seconds later 1ml of 10xPBS was added to restore isotonic conditions. The solution was then centrifuged at 1500rpm for 5min and stained as described below.

2.5.3.3 Cell Counting and viability

All cells were counted using a haemocytometer (Immune Systems) under a light microscope. Cell viability was assessed using 0.1% trypan blue (Sigma, UK) in PBS as live cells exclude the trypan blue dye.

2.5.3.4 Staining for cell surface molecules

Antibody staining for cell surface molecules for flow cytometric analysis, was performed in the following way. Cells were harvested, counted and washed in ice-cold staining buffer (PBS with 1% FCS) and pelleted. The cells were resuspended in 50μl of staining buffer containing the appropriate dilution of the relevant monoclonal antibodies, and the samples were incubated for 25 minutes at 4^{o}C in the dark. After the incubation, samples were washed twice in ice-cold staining buffer and resuspended in 500μl of staining buffer. Samples were acquired on an LSR II Flow cytometer and analysed using FACS DIVA (BD) and FlowJo Version 7 (Treestar) software.

2.5.4 ELISA

Supernatants from the ex vivo proliferation assays were harvested on day 5 and stored at -20^{o}C. IL-2 and IFN-γ production after peptide stimulation, described in section 2.5.2, was determined by performing ELISA on these supernatants using the BD OptEIA mouse IL-2 and IFNγ ELISA sets, as per manufacturer's protocol.

2.5.5 Peptides

The WT1 and LMP2, HLA A0201 restricted epitopes used in functional assays to determine the antigen specific T cell activity of WT1 and LMP2-specific T cells respectively were the following:

WT1 derived epitope, pWT126: RMFPNAPYL

EBV derived epitope, pCLG: CLGGLLTMV

The HLA A0201 restricted epitopes used as irrelevant controls, as specified in individual experiments were the following:

pWT235: CMTTWNQMNL

pEBV GLC: CLCTLVAML

pCMV NLV: NLVPMVATV

Chapter 3 – Results 1 - Preparation of the lentiviral constructs used for TCR gene transfer in HSCs and validation of the experimental in vivo model.

3.1 Introduction

The fate of high avidity, A2-restricted WT1-specific T cells in the thymus and in the periphery was studied in an HLA A2 transgenic mouse model. As discussed in the Introduction (Chapter 1) the cognate epitope for the WT1-TCR (pWT126), is expressed at low levels and presented in both species. The experimental model used was also described in the schematic representation of figure 1.10. Murine HSCs were transduced with the genes for the WT1-TCR and were subsequently transferred in HLA A2 Tg mice. In vivo, developing thymocytes expressing the WT1-TCR go through thymic development while in the thymus peripheral self-Ags are promiscuously presented on HLA A2 molecules. Depending on whether they can escape negative selection, WT1-specific thymocytes then emerge in the periphery where they are susceptible to the effects of WT1 Ag expression by rare BM progenitors and renal podocytes.

In this chapter the molecular work performed to prepare the lentiviral constructs used for TCR gene transfer in HSCs is described. Even though TCR gene transfer in HSCs has previously been validated and used to study the development and function of other T cell specificities (Yang and Baltimore, 2005, Arnold et al., 2004) these studies used retroviral vectors and enriched for HSCs by treating donor mice with 5-Fluorouracil prior to BM harvest. In this project, lentiviral vectors were preferred for gene transfer. In addition, BM

harvest from untreated donor animals was followed by the magnetic separation of Lin-ve BM cells which are highly enriched in HSCs. Therefore, it was necessary to optimise the conditions for and validate the experimental model before proceeding with TCR td HSC transplant experiments to study the fate of WT1-specific T cells in the context of the low level, physiological expression of WT1 Ag. The preliminary experiments performed to establish the in vivo model are presented in this chapter.

3.2 WT1-TCR and LMP2-TCR lentiviral constructs

3.2.1 Generation of pSin-WT1-TCRα-p2A-WT1-TCRβ vector.

Figure 3.1 shows a schematic representation of the cloning strategy used to generate the pSin-WT1-TCRα-p2A-WT1-TCRβ lentiviral vector. The genes for the WT1-TCRα-p2A- WT1-TCRβ were present in the pGA4 cloning plasmid and had to be transferred into the lentiviral vector backbone. To do this the restriction sites in the WT1-TCR pGA4 construct were modified. The existing WT1 Vα1.5 chain sequence was amplified by PCR with a 5' primer containing the BamH1 restriction site without a Not1 restriction site, thus removing the Not1 restriction site from the PCR product. Similarly, the murine Cβ chain was amplified with a 3'primer containing an additional Not1 restriction site after the stop codon and before the BsrG1 site. The original Vα and Cβ chains were removed from the WT1-TCR-pGA4 plasmid by BamH1/Sac2 and Xho1/BsrG1 double digestions respectively, to be replaced with the corresponding, mutated PCR-amplified sequences. Figure 3.2A shows the gel electrophoresis following screening restriction digestions of the modified hybrid WT1-TCR – pGA4 plasmid with XhO1/Not1. BamH1/Not1 restriction digests were then used to

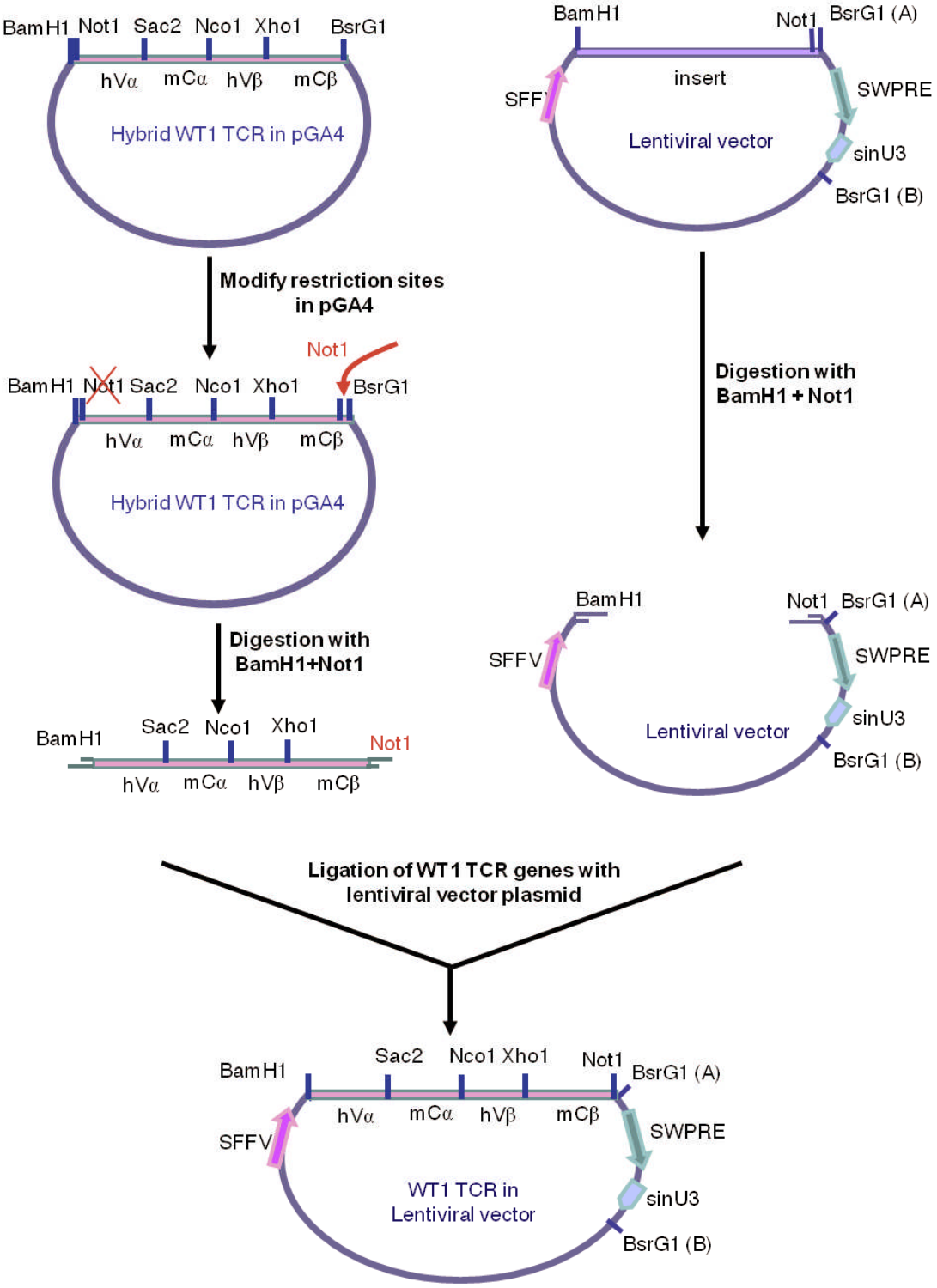


Figure 3.1 Schematic representation of cloning strategy to obtain WT1 TCR in lentiviral vector.

release the complete WT1-TCR sequence from the pGA4 cloning vector, and to remove the insert from the lentiviral vector backbone before the two were ligated together. The list of primer sequences used is found in chapter 2, Materials and Methods. A screening digestion with BamH1/Not1 was performed on the final hybrid WT1-TCR lentiviral construct to confirm that the required plasmid was produced. The relevant gel electrophoresis of the digested DNA is shown in figure 3.2B.

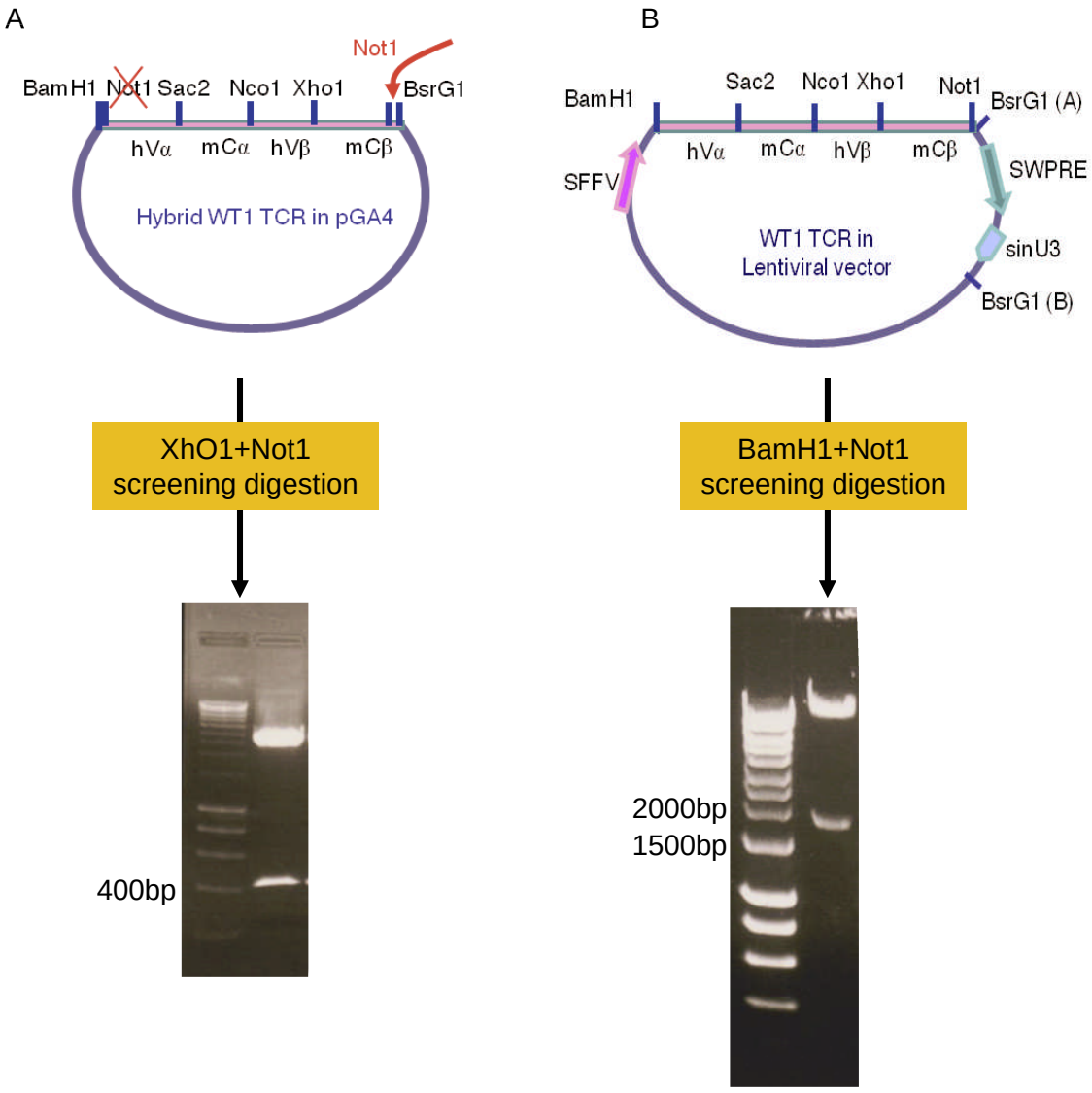


Figure 3.2 Screening restriction digests to confirm modifications performed in DNA constructs. A. screening restriction digestion of modified WT1 TCR in pGA4 with XhO1/Not1. The smaller band (around 400 bp) confirms that the original Not1 restriction site has been removed and a new Not1 restriction site has been inserted as indicated. B BamH1/Not1 restriction digestion on final construct (WT1 TCR in lentiviral vector) to confirm that the hybrid WT1 TCR has been successfully transferred into the lentiviral vector.

3.2.2 Cloning of the LMP2-TCR genes into the lentiviral vector

The hybrid LMP2-TCR was cloned from the pMP71 retroviral vector, into an empty lentiviral backbone as described in figure 3.3. The LMP2-TCRα-p2A-LMP2-TCRβ genes in the retroviral construct were preceded by a Not1 restriction site and followed by a BsrG1 restriction site. These sites were used to release the LMP2-TCR sequence fragment from the pMP71 backbone. Figure 3.3 describes the cloning strategy and shows the gel electrophoresis of the digested empty .lentiviral vector and hybrid LMP2-TCR in pMP71 vector digested with Not1/BsrG1. However, the lentiviral construct in which this sequence was to be cloned, originally contained two BsrG1 restriction sites. Site directed mutagenesis was used to remove the second BsrG1 site (BsrG1(B)) as described in the schematic diagram in figure 3.4. Primers which introduced a point mutation within the BsrG1(B) site were used to PCR amplify the entire lentiviral vector. The PCR product was then digested with Dpn1 to discard the original plasmid. XL10-Gold competent cells were then transformed with the PCR product containing the mutated lentiviral construct and colonies were screened by BsrG1 digestions. The appearance of a linearised DNA sequence, as shown in figure 3.4, confirmed that a single BsrG1 restriction site was present following site directed mutagenesis.

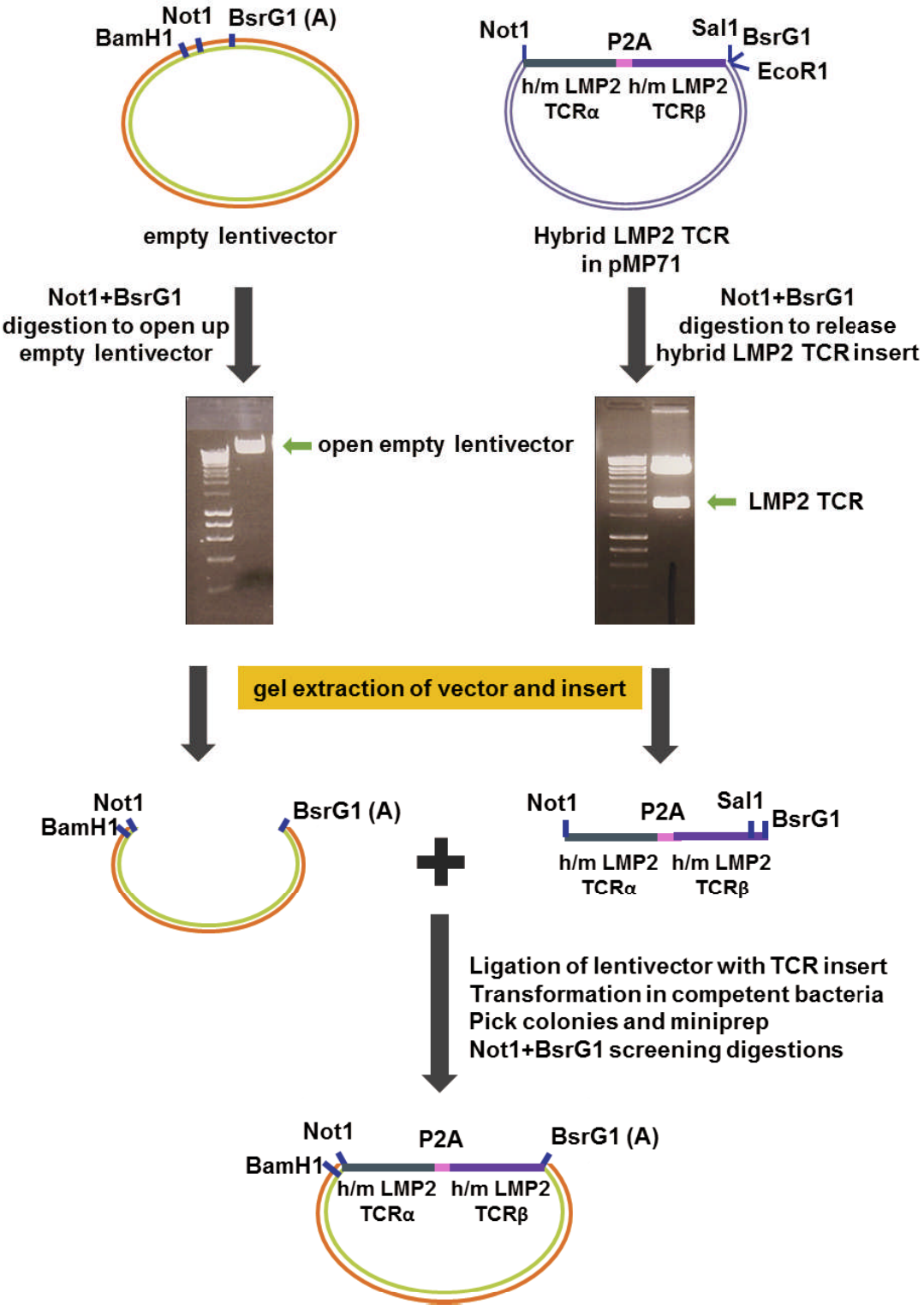


Figure 3.3 Schematic representation of cloning strategy for the transfer of the hybrid LMP2 TCR sequence from the pMP71 retroviral vector, into an empty lentiviral vector. Agarose gel electrophoresis of preparative digestions as indicated.

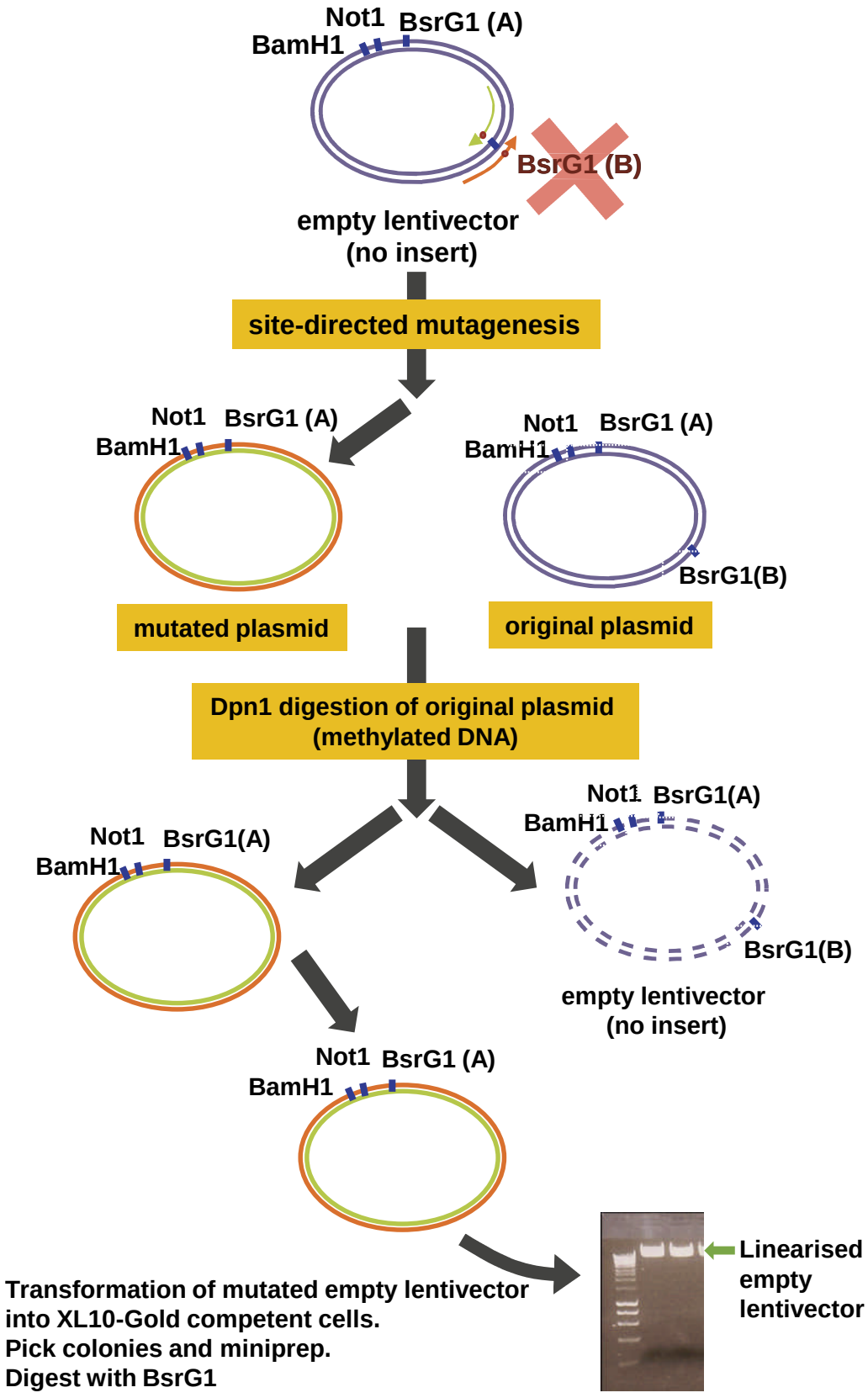


Figure 3.4 Schematic representation of site-directed mutagenesis.

3.2.3 Cell surface expression of WT1 and LMP2-TCRs following lentiviral vector transduction of 58-/- cells

To confirm that the two new lentiviral constructs generated could mediate the surface expression of the WT1 and LMP2-TCRs, the 293T cell line was transfected with each construct to produce the corresponding viral particles and the 58-/- murine thymoma cell line was transduced with the produced recombinant lentiviral particles. The same protocol was later used throughout the study for the lentiviral transduction of HSCs and is described in detail in Materials and Methods, chapter 2. The FACS analysis plots in figure 3.5 demonstrate that 3 days post-transduction levels of WT1 and LMP2-TCR surface expression were as expected, dependent on the MOI of the concentrated lentivirus added to the cell culture. TCR expression was determined by FACS analysis on gated viable 58-/- cells, using anti-Vβ2 and Vβ13 antibodies respectively.

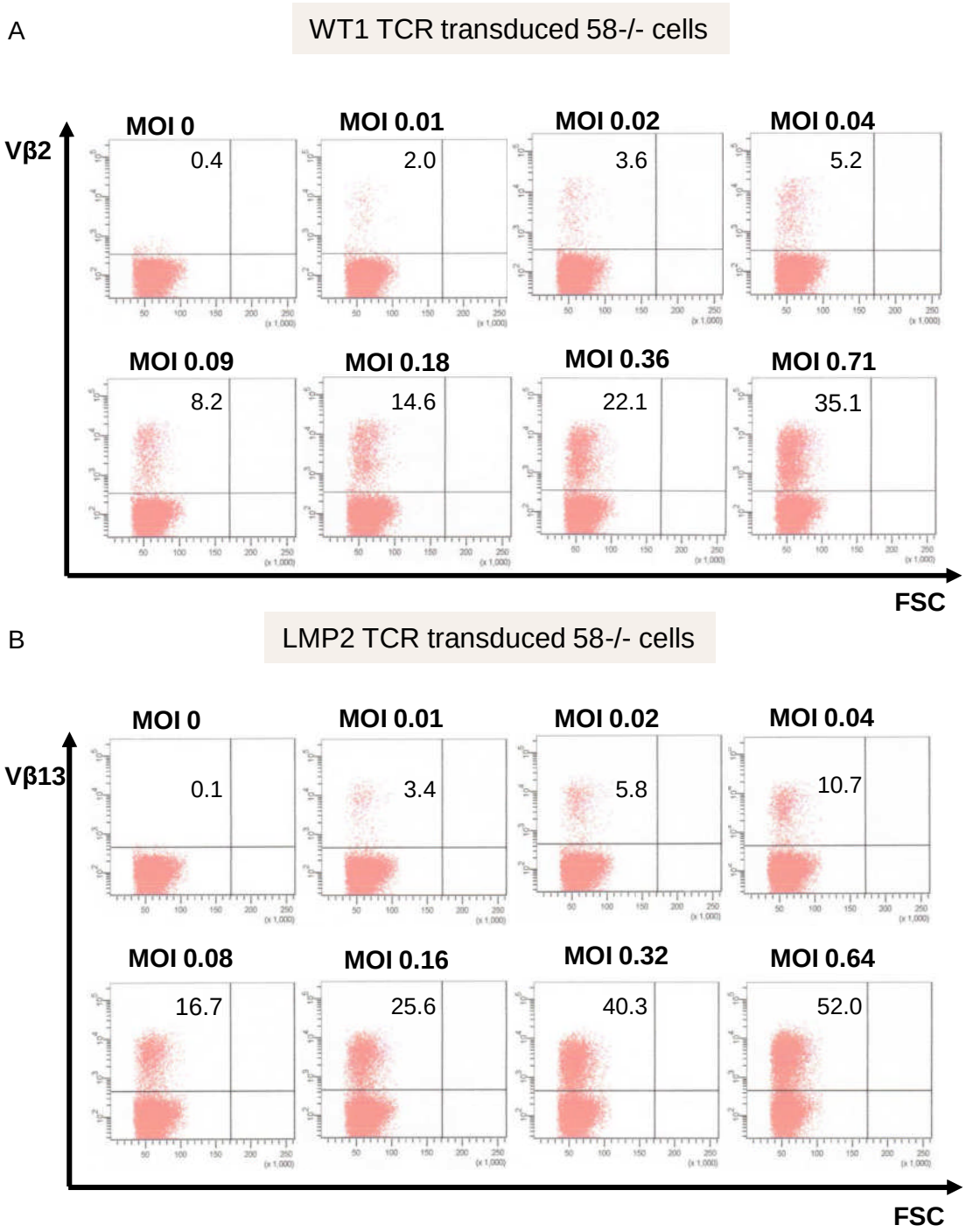


Figure 3.5 Lentiviral constructs containing hybrid TCR sequences effectively transduce the 58-/- cell line resulting in the expression of the introduced TCRs. A. $0.5x10^6$ 58-/- cells are transduced with increasing amounts of concentrated lentiviral particles (increasing MOI) containing the sequence for the hybrid WT1 TCR. B.$0.5x10^6$ 58-/- cells are transduced with increasing amounts of concentrated lentiviral particles containing the sequence for the hybrid LMP2 TCR. A&B 72hrs post-transduction the cells were stained with human Vβ2 and Vβ13 antibodies respectively and TCR expression was measured by FACS.

3.3 Establishment of the in vivo murine model to study the development, phenotype and function of WT1-specific T cells

3.3.1 Enrichment and lentiviral transduction of Lineage Negative BM cells.

Uncommitted BM progenitors, highly enriched in HSCs were negatively selected using either the Miltenyi Biotech Lineage cell depletion kit for mouse, or the Haematopoietic Progenitor Enrichment kit from StemCell Technologies following the manufacturers' protocols. A negative selection kit was preferred to ensure that the HSCs were unaffected from the magnetic separation process. Purity and cell surface phenotype of Lin-ve BM cells before and after Lin-ve selection were assessed by FACS analysis following staining with Lineage cocktail and Sca-1 antibodies. The yield after the Lin –ve BM stem cell separation was around 1% and the purity ranged between 87-94% (representative FACS plots before and after Lin-ve selection, shown in figure 3.6). The Lin-ve Sca-1+ve compartment of BM cells has been shown to contain pluripotent HSCs (Uchida and Weissman, 1992, Spangrude et al., 1988).

Prior to in vivo experiments it was important to confirm that lentiviral transduction of Lin-ve BM cells efficiently mediates the expression of the introduced transgene in this cell population. This was not possible to test in vitro using the TCR lentiviral constructs, as TCR surface expression requires the similteneous expression of the CD3 molecule, an event taking place after progenitor BM cells have entered the thymus and committed to the T cell lineage. For this reason, a GFP lentiviral construct was used. Lin-ve BM cells were transduced with lentiviral GFP particles (MOI 20) and kept in culture.

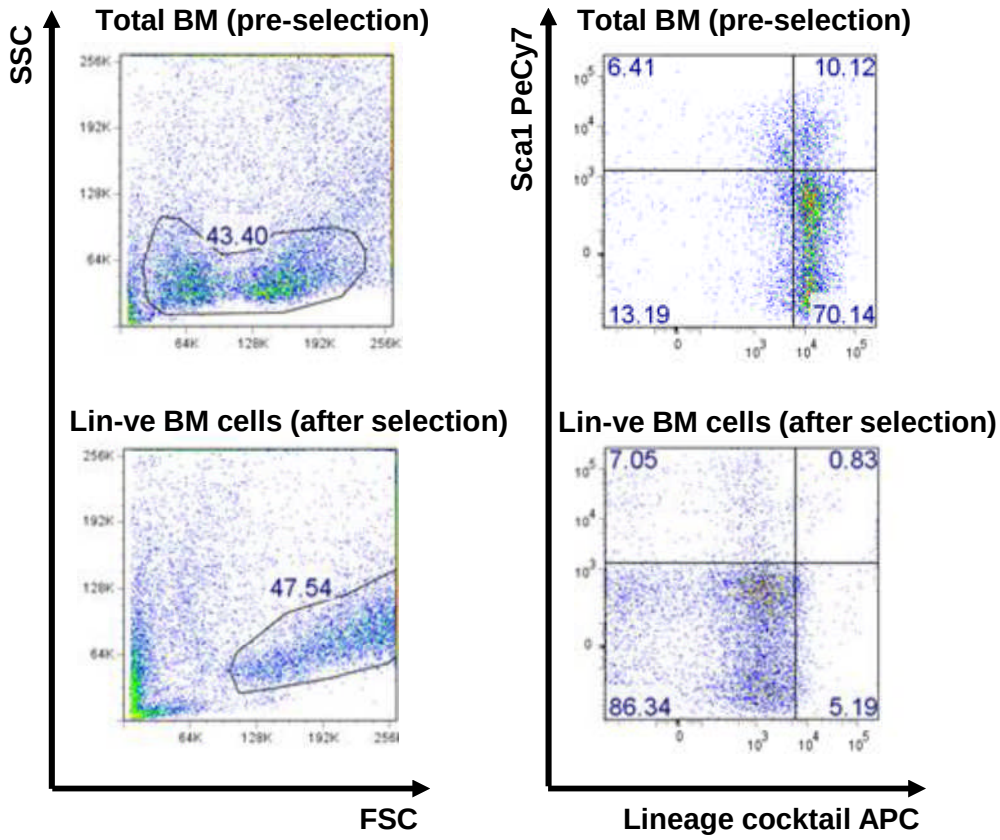


Figure 3.6 Enrichment of Lineage negative HSCs and lentiviral transduction. BM cells were harvested from 7-10 wk old donor mice and enriched in HSCs by means of Lineage Negative Selection prior to being transduced with a lentiviral vector. Biotin-streptavidin APC conjugated Murine haematopoietic Lineage cocktail (CD5, CD11b, CD45R, anti-Ly6g, 7-4, Ter-119) and PECy7 Sca1 antibodies were used to stain BM cells prior and after magnetic separation.

After 3 days 95% of the Lin-ve BM cells expressed GFP, as determined by FACS analysis. The presence of two GFP positive cell populations suggested that integration of more than one GFP gene occurred in a proportion of transduced cells (figure 3.7) when an MOI of 20 was used. Therefore, an MOI of 10 was used for all subsequent experiments. Achieving lower transduction efficiencies with this MOI was in fact desirable to allow WT1-specific T cells to develop in a polyclonal environment of endogenous T cell specificities.

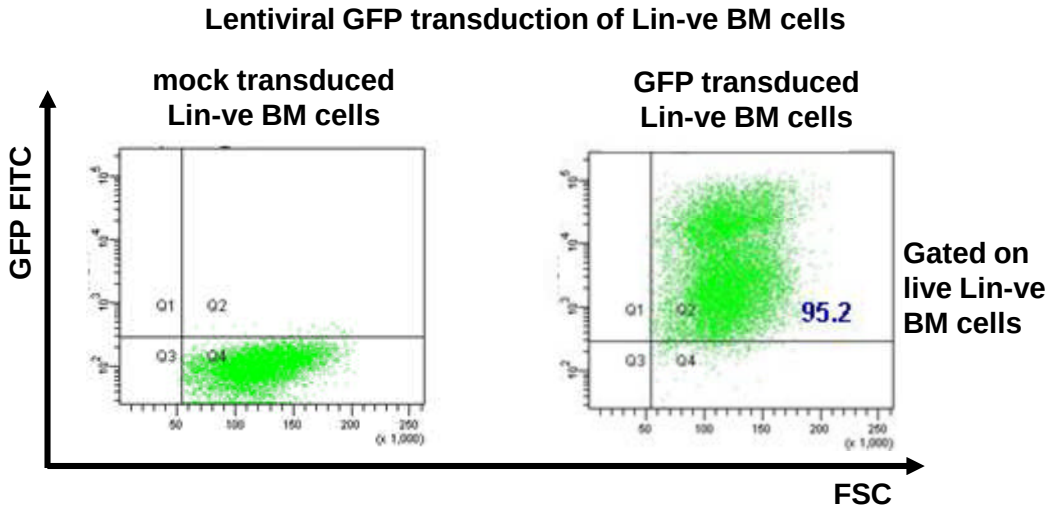


Fig.3.7 Lin-ve BM cells are efficiently transduced with a lentiviral GFP vector. GFP-lentiviral supernatant was added to $0.5x10^6$ Lin-ve BM cells, at an MOI of 20. In mock transductions no lentivirus was added. 24 hrs later the cell culture medium and virus were replaced with fresh StemSpan medium. 72 hrs later the cells were analysed by Flow Cytometry for the expression of the GFP transgene. The presence of two distinct GFP positive populations suggests that integration of more than one GFP genes occured in a proportion of the transduced cells.

3.3.2 Optimisation of HSC transplant experiments

All experiments utilised $5x10^5$ cells and an irradiation dose of 9.4Gy administered in 2 fractions. Transplant recipients were also kept on antibiotics (Baytril) for 1 week prior to the transplant and 2 weeks after. With these experimental conditions no transplant-related mortality was observed.

3.3.3 Donor-derived haematopoietic reconstitution in recipients of lentivirally td HSCs.

To test the engraftment potential of genetically modified BM stem cells, congenic transplants were performed in C57Bl/6 mice. Donor CD45.1 positive C57Bl/6 Lin-ve BM cells were transduced with lentiviral GFP control vector (MOI 10) prior to transfer into lethally irradiated (9.4Gy) wild-type (WT) C57Bl/6 animals. Tail bleeds were performed at 5, 6 and 9 weeks and at 6.5 months post-transplant. Peripheral blood samples were stained with anti- CD45.1, anti-CD3, anti-CD8 and anti-B220 antibodies to determine donor chimerism in the T and B cell lineages. Figure 3.8A shows representative FACS plots of peripheral blood samples stained for CD8 T cells (CD3, CD8 positive lymphocytes) and B cells (B220 positive lymphocytes) and the congenic marker CD45.1 used to identify donor-derived cells. The graph in figure 3.8B showing the percentage donor chimerism in the T and B cell lineage at the various time points, illustrates that donor B cell engraftment occurred more rapidly than T cell engraftment. In recipients receiving $5x10^5$ cells, 30.8% (median) of T cells were donor-derived 5 weeks post-transplant, increasing to 80% (median) 9 weeks post-transplant. At 6.5 months post-transplant the levels of donor T cell chimerism were > 90%. Therefore, in subsequent

experiments, recipients of TCR td HSC transplants were allowed to reconstitute for longer than 9 weeks (11 wks) post-transplant, prior to performing the phenotypic analysis and functional assays of the in vivo generated WT1-specific T cells.

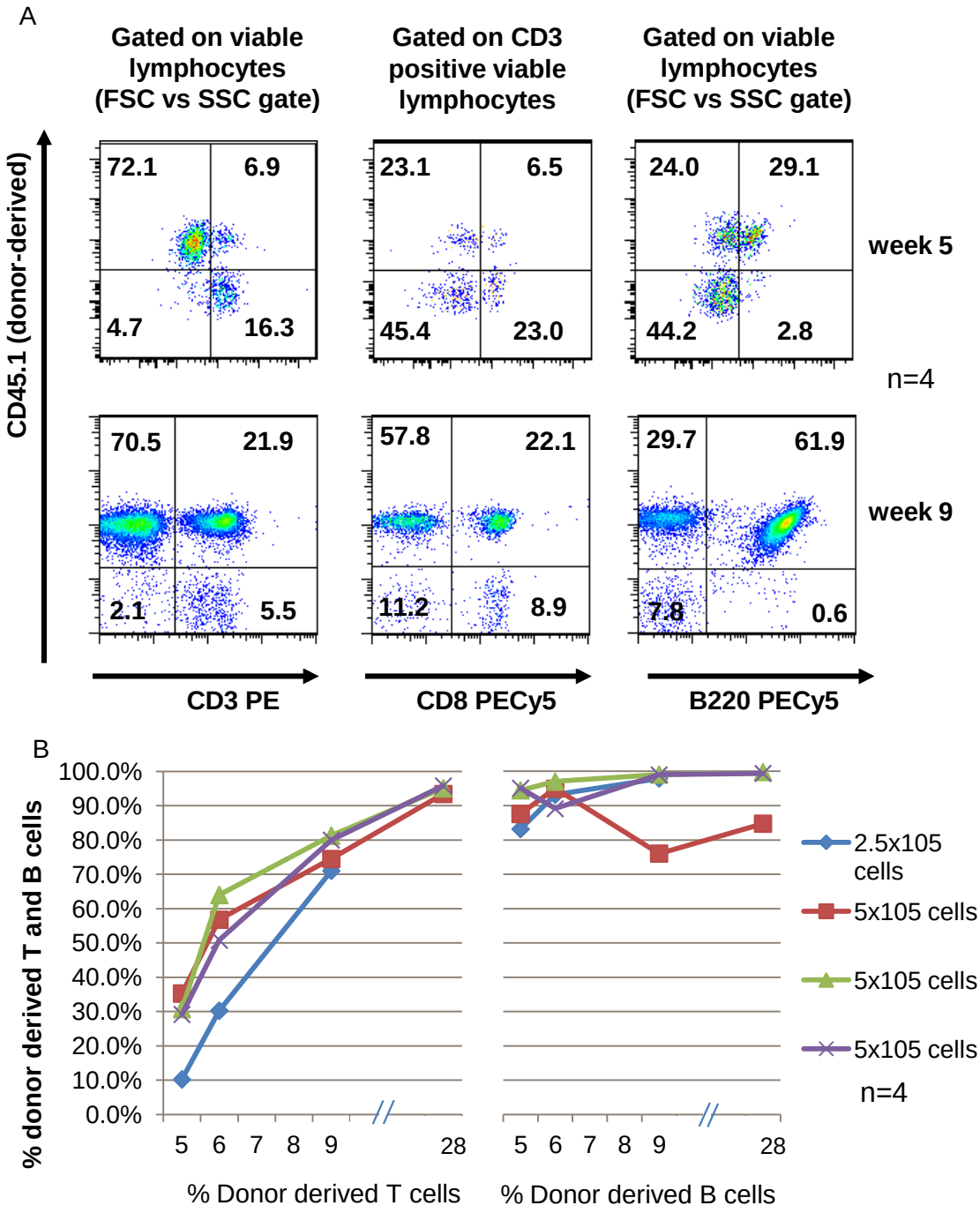


Figure 3.8 Donor-derived haematopoietic reconstitution at different time points in recipients of lentivirally transduced HSCs.

C57Bl/6 Lin-ve BM cells were transduced with a lentiviral construct containing a GFP transgene, prior to their transfer into lethally irradiated congeneic recipients. tail bleeds were performed at 5, 6, 9 and 28 weeks post-transplant to monitor donor T and B cell reconstitution by FACS analysis. Peripheral blood samples were stained with murine anti- CD45.1, CD3, CD8 and B220 antibodies. (A) Representative FACS plots of peripheral blood samples, 5 and 9 weeks post-transplant as indicated. The

Figure 3.8 (continued)

CD45.1 congeneic marker identified donor-derived, T cells, CD8 T cells and B cells. Percentages shown are on gated viable lymphocytes and gated viable CD3 positive lymphocytes as indicated. (B) Graphs show percentage donor (CD45.1 positive) T or B cells in total T or B cells at the indicated time points, in individual transplant recipients. n=4 (3 mice received $5x10^5$ td Lin-ve BM cells and 1 mouse received $2.5x10^5$ cells).

3.3.4 Long term gene expression following transplantation of lentivirally transduced HSCs.

The C57Bl/6 mice receiving GFP-td HSC transplant were used to assess not only whether lentivirally transduced HSC transplant can result in the haematopoietic reconstitution of myeloablated recipients but also to confirm that they can give rise to a differentiated progeny expressing the introduced gene. As illustrated in figure 3.9A and B, GFP was expressed in both T and B cells at all recorded time points. Therefore, donor HSCs were successfully transduced with the lentiviral GFP construct and the transduced uncommitted progenitor cells were able to differentiate into T and B cells expressing the transgene.

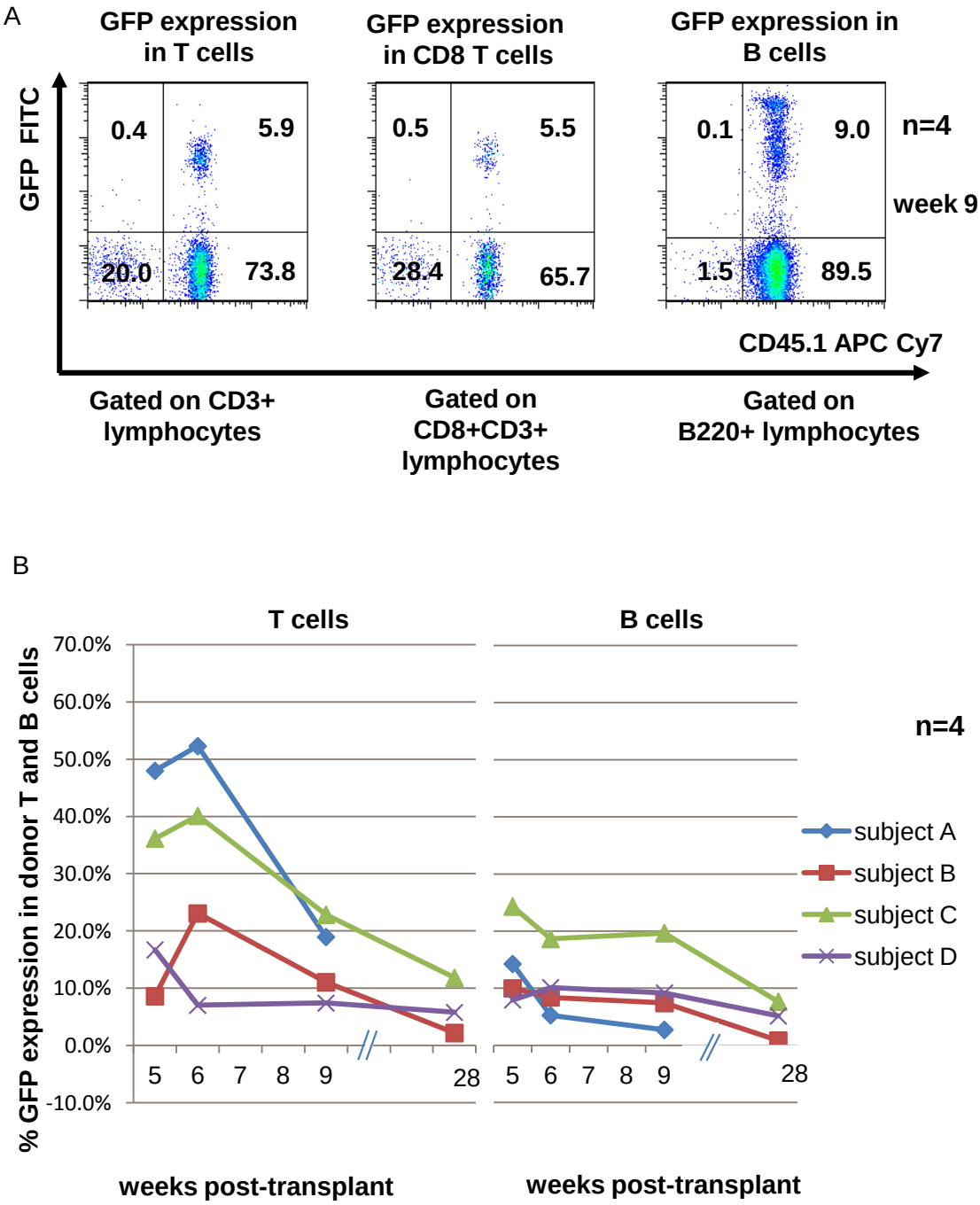


Figure 3.9 Lentiviral transduction of HSCs results in the long term expression of the introduced gene in differentiated donor-derived lymphocytes. C57Bl/6 Lin-ve BM cells were transduced with a lentiviral construct containing a GFP transgene, prior to their transfer into lethally irradiated congeneic recipients. Tail bleeds were performed at 5, 6, 9 and 28 weeks post-transplant to monitor GFP expression in the T and B cell lineage. A .Representative FACS plots of peripheral blood samples taken 9 weeks post transplant and stained with anti-CD45.1, anti-

Figure 3.9 (continued)

anti-CD8 and anti-B220 antibodies. Top right quadrant in each FACS plot shows the percentage of GFP transduced cells in gated viable T cells, CD8 T cells and B cells respectively. B.% GFP expression in peripheral blood donor-derived T and B cells, at 5, 6, 9 weeks and at 6.5 months.

3.3.5 Donor derived haematopoietic reconstitution in recipients of TCR td HSC transplants

The in vivo experimental model to study the development and function of WT1-specific T cells in the context of physiological WT1 Ag expression, involved TCR td HSC transplants. In these experiments the introduced TCR would only be expressed on T cells, because TCR surface expression is linked to CD3 expression. It was therefore important to establish that TCR transgene expression solely on T cells did not affect donor T cell reconstitution. C57Bl/6 Lin-ve BM cells were transduced with the WT1-TCR lentiviral construct, prior to their transfer into 9.4Gy irradiated A2Kb recipients. Tail bleeds were performed at 6 and 8 weeks post transplant and the peripheral blood samples were stained with anti-CD45.1, anti-CD3, anti-CD8 antibodies to monitor donor-derived T cell reconstitution. Recipient mice were sacrificed at 11 weeks post-transplant and single cell suspensions were prepared from the spleen, Lymph Nodes (LNs) and BM of each animal and stained with the same antibodies prior to FACS analysis. Figure 3.10 demonstrates that 6 weeks post-transplant the mean percentage of donor derived, CD45.1 positive T cells in 9 recipients was 49.5% and 43% among donor CD8 T cells (range 41.4%-58% and 29.5%-60.9% respectively) increasing to >90% (range 88.3-95.2% and 92-96.3% for total and CD8 T cells respectively) at 11 weeks in the spleen and LNs and 84% and 87.8% in the BM. Therefore, donor T cell chimerism was similar to that observed in GFP-td HSC transplant recipients.

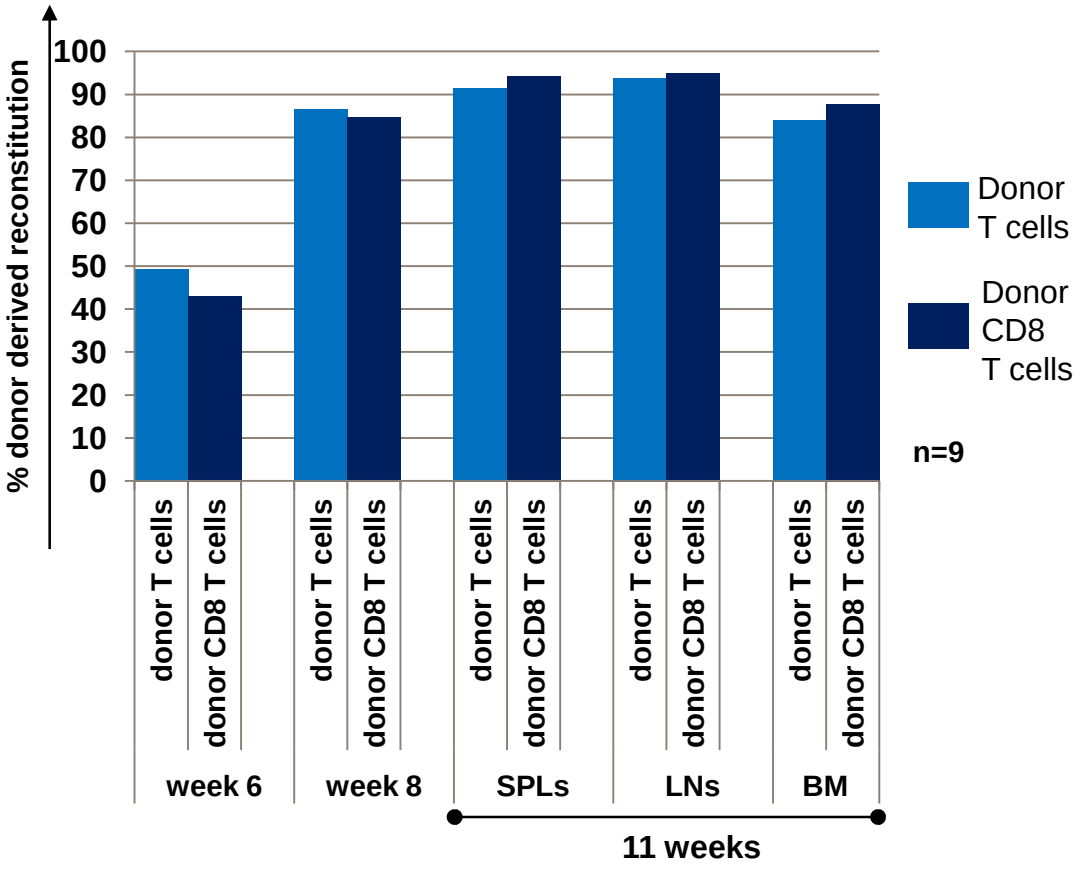


Figure 3.10 Donor-derived T reconstitution following transplantation of WT1 TCR transduced C57Bl/6 HSCs into A2Kb tg recipients. Lin-ve BM cells from C57Bl/6 recipients were lentivirally transduced with the genes for the hybrid WT1 TCR, prior to their transfer into 9.4Gy irradiated A2Kb recipients. Each recipient animal received $5x10^5$ cells. Peripheral blood samples taken at 6 and 8 weeks post-transplant, as well as splenocytes, LN and BM single cell suspensions harvested at 11 weeks post-transplant were stained with anti-CD45.1, anti-CD3 and anti-CD8 antibodies to monitor donor-derived T cell reconstitution. The chart summarises the results in 9 recipients, showing the mean percentage of donor derived to total T cells and CD8 T cells.

3.3.6 The class I restricted WT1-TCR is expressed in a proportion of donor derived CD8 T lymphocytes following transplant with WT1-TCR transduced HSCs

TCR gene transfer into HSCs allows the study of both thymic development and peripheral function of T cells expressing the particular TCR of interest, in an otherwise polyclonal T cell environment as only a small proportion of T cells will be expressing the introduced TCR. In this way this system is more physiologically relevant than a TCR transgenic mouse model. Nevertheless, as it was possible that WT1-specific T cells would undergo thymic deletion due to their specificity, it was important to first establish whether any WT1-specific T cells survived negative selection in the thymus and emerged in the periphery, prior to proceeding with further experiments. Furthermore, it was important to confirm that T cells expressing the HLA A2 restricted WT1-TCR, obeyed the long established rule that T cells expressing a class I restricted TCR commit to the CD8 T cell lineage during thymic development (Teh et al., 1988, Singer et al., 2008).

To answer these questions, TCR expression in splenocytes from A2Kb Tg mice receiving $5x10^5$ WT1-TCR transduced A2KbxCD45.1 HSCs, were assessed 11 weeks post-transplant. WT1-specific T cells were indeed detected in the periphery, suggesting that at least some of these T cells escape thymic deletion. As expected, the introduced TCR was only expressed in donor derived T cells and this is illustrated in the representative FACS histogram in figure 3.11A. The percentage of WT1-specific T cells in donor lymphocytes, donor T cells and donor CD8 T cells in the spleens of the same group of

transplant recipients, is shown in figure 3.11B. The mean value of WT1-specific T cells in the spleens of transplant recipients were 0.9% of donor lymphocytes, 3.3% of donor T cells and 9.6% of donor CD8 T cells, 11 weeks post transplant. For this analysis, splenocytes were stained with anti CD45.1, anti-CD3, anti CD8 murine antibodies, and human anti Vβ2 antibody was used to identify cells expressing the WT1-TCR. Analysis of the WT1 specific T cell development, phenotype and function in this group of mice was performed and is described in detail in Chapters 4 and 5.

Verifying that WT1-specific T cells were committed to the CD8 T cell lineage was done by staining splenocytes from the WT1-TCR td HSC transplant recipients with anti- human Vβ2, murine anti-CD4 and anti- CD8 antibodies. Representative FACS histograms showing CD4 and CD8 expression in Vβ2 positive and negative T cells are shown in figure 3.11C. It is obvious from the right panel histogram in this figure that CD8 expression in Vβ2 positive T cells is different from that of the whole CD8 T cell population. This phenotype was investigated in detail and is discussed in the following chapters.

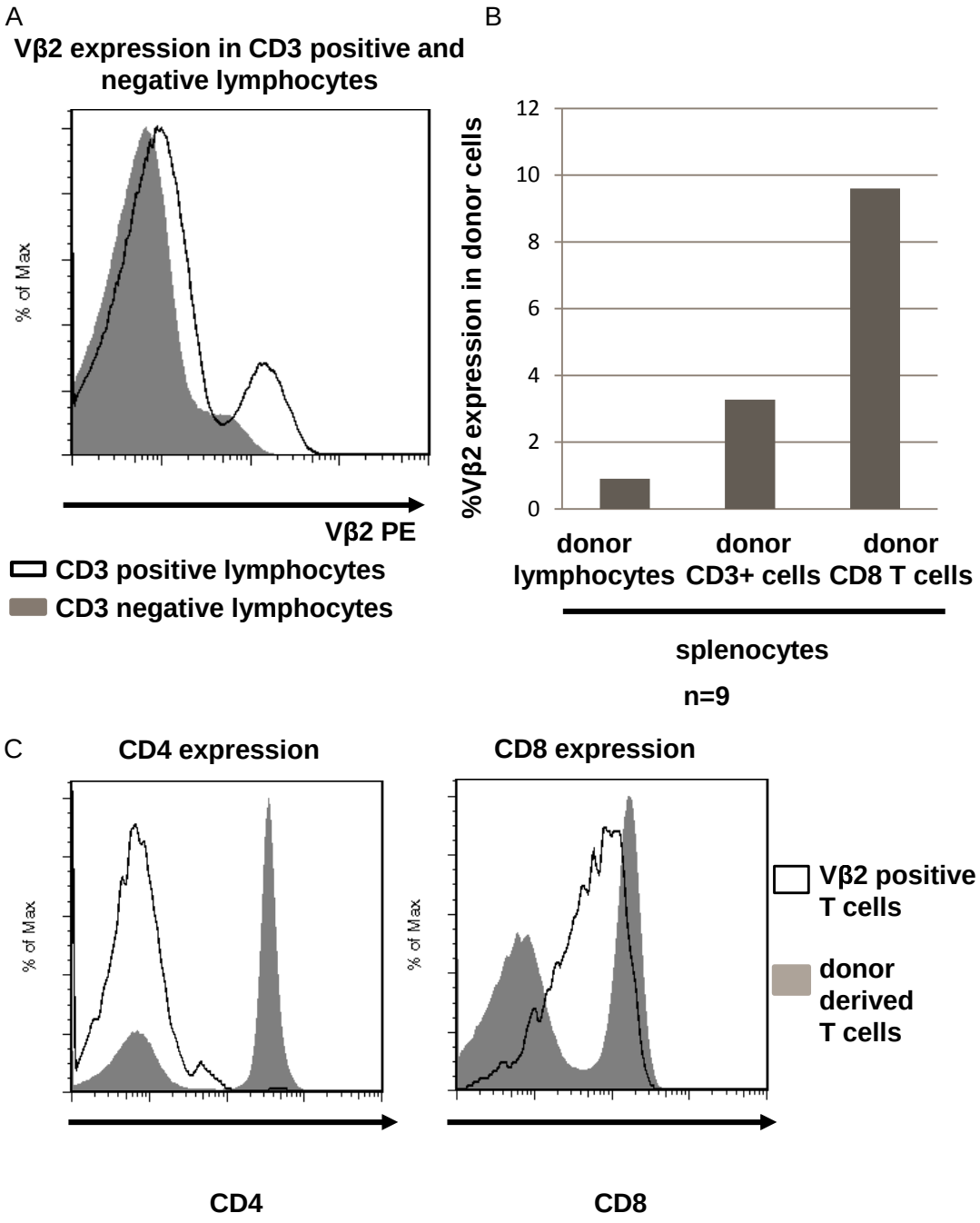


Figure 3.11 WT1 TCR is expressed in a proportion of donor derived CD8 T cells following transplant with WT1 TCR transduced HSCs. Recipient A2Kb tg mice were irradiated with 9.4Gy prior to receiving $5x10^5$ WT1 TCR transduced A2KbxCD45.1 HSCs. 11 weeks post-transplant the mice were sacrificed to study the in vivo generated WT1 specific T cells. n=9. (A) FACS histogram comparing Vβ2 expression in CD3 positive and CD3 negative lymphocytes in splenocytes from a representative transplant recipient. (B) Mean percentage of Vβ2 positive T cells in donor derived lymphocytes, T cells and CD8 T cells in transplant recipients. (C) CD4

Figure 3.11 (continued)

and CD8 expression profiles of Vβ2 positive and total donor derived T cells in a representative transplant recipient. Splenocytes were stained with murine anti CD45.1, anti CD3, anti CD4, anti CD8 and anti human Vβ2 antibodies prior to FACS analysis.

3.4 Discussion

In order to study the in vivo thymic and peripheral fate of high avidity, HLA A2-restricted WT1-specific T cells, an in vivo model of TCR td HSC transplants in A2Kb Tg mice recipients was utilised. The genes for the WT1 and LMP2 specific HLA A2 restricted TCRs were inserted in lentiviral vector plasmids and the constructs were validated in the 58-/- cell line. HSCs were enriched by the magnetic separation of Lin-ve murine BM cells. HSC transplant conditions were optimised to an irradiation dose of 9.4 Gy in two fractions prior to the transplant, and a cell dose of $5x10^5$ Lin-ve BM cells. Phenotypic and functional analysis of WT1/LMP2-specific T cells was performed at least 11 weeks post-transplant when donor reconstitution on all haematopoietic lineages was consistently over 90%. The appearance of WT1 specific CD8 T cells, in the periphery of WT1-TCR td HSC transplant A2Kb recipients not only confirmed that the experimental system is working, but also suggested that at least a proportion of WT1-specific T cells survive negative selection in the thymus and therefore emerge in the periphery. The development and function of WT1-specific T cells were subsequently studied in detail and these results are presented in the next chapters.

Chapter 4 – Results 2 - Thymic development of WT1 specific T cells and phenotypic analysis.

4.1 Introduction

Having confirmed that WT1 specific, CD8 T cells can be generated in vivo in our experimental system, this model was then used for the further study of the development, phenotype and function of these cells in the context of physiological WT1 Ag expression. It was already shown in the previous chapter that WT1-specific T cells emerged in the periphery and that while they were commited to the CD8 T cell lineage, the expression of the CD8 co-receptor on these cells differed from that of T cells expressing endogenous murine class-I restricted TCRs. This chapter is concerned with the phenotypic analysis of the WT1-specific T cells in the thymus and the periphery.

4.2 Results

4.2.1 WT1-specific T cells emerge in the periphery and exhibit CD3 downregulation

Purified lineage negative BM stem cells from A2Kb transgenic mice were transduced with the lentiviral WT1-TCR vector and transferred into A2Kb Tg mice that had received 9.4Gy irradiation. Recipient mice were tail bled 6 and 8 weeks post-transplant and blood samples were stained with CD45.1, CD3, CD4, CD8 and Vb2 antibodies and analysed by FACS. 11 weeks post-transplant the mice were sacrificed and their thymuses, spleens, LNs and BM were harvested for further phenotypic analysis and functional assays.

WT1-specific T cells were readily detected in tail bleed samples as well as in the spleen, LNs and BM of all A2Kb Tg mice that had received WT1-TCR td HSCs, but not in control A2Kb mice. Interestingly, as shown in the representative FACS plots in figure 4.1.A the level of CD3 expression in the Vβ2 positive T cell population appeared reduced in comparison to that of donor-derived T cells expressing endogenous murine TCRs. The CD3 molecule can be a marker of the level of TCR expression, as the cell surface expression of the two molecules is coupled. One possibility for the observed CD3/TCR downregulation in WT1-specific T cells was that this was due to low promoter activity of the lentiviral vector used to drive TCR expression. To exclude this, further experiments were performed. The genes for the WT1-TCR in the lentiviral construct were replaced with the genes for a hybrid, HLA-A0201-restricted TCR specific for a non-self peptide derived from the Latent Membrane Protein 2 (LMP2) of Epstein Barr virus (Hart et al., 2008). This TCR was also codon optimised and had an additional disulphide bond and was cloned into an identical vector backbone to the WT1-TCR. As in the WT1-TCR transplant, LMP2-specific T cells identified by staining with the human anti-Vβ13 antibody were readily detectable in the periphery of recipient mice that had received LMP2-TCR td HSCs and this is shown in the bottom row of figure 4.1A. However, in this case the levels of TCR/CD3 expression were similar to that of donor-derived T cells expressing endogenous TCRs. The bar chart in figure 4.1B summarises the CD3 MFI of WT1 and LMP2-specific T cells compared to that of the corresponding endogenous donor derived T cells in each experiment (Vβ2 –ve and Vβ13 –ve respectively). These results confirmed a statistically significant downregulation of TCR/CD3 expression in

WT1-specific T cells which appeared to be a feature of this self-reactive WT1-TCR but not of the non-self reactive LMP2-TCR that was used as a control.

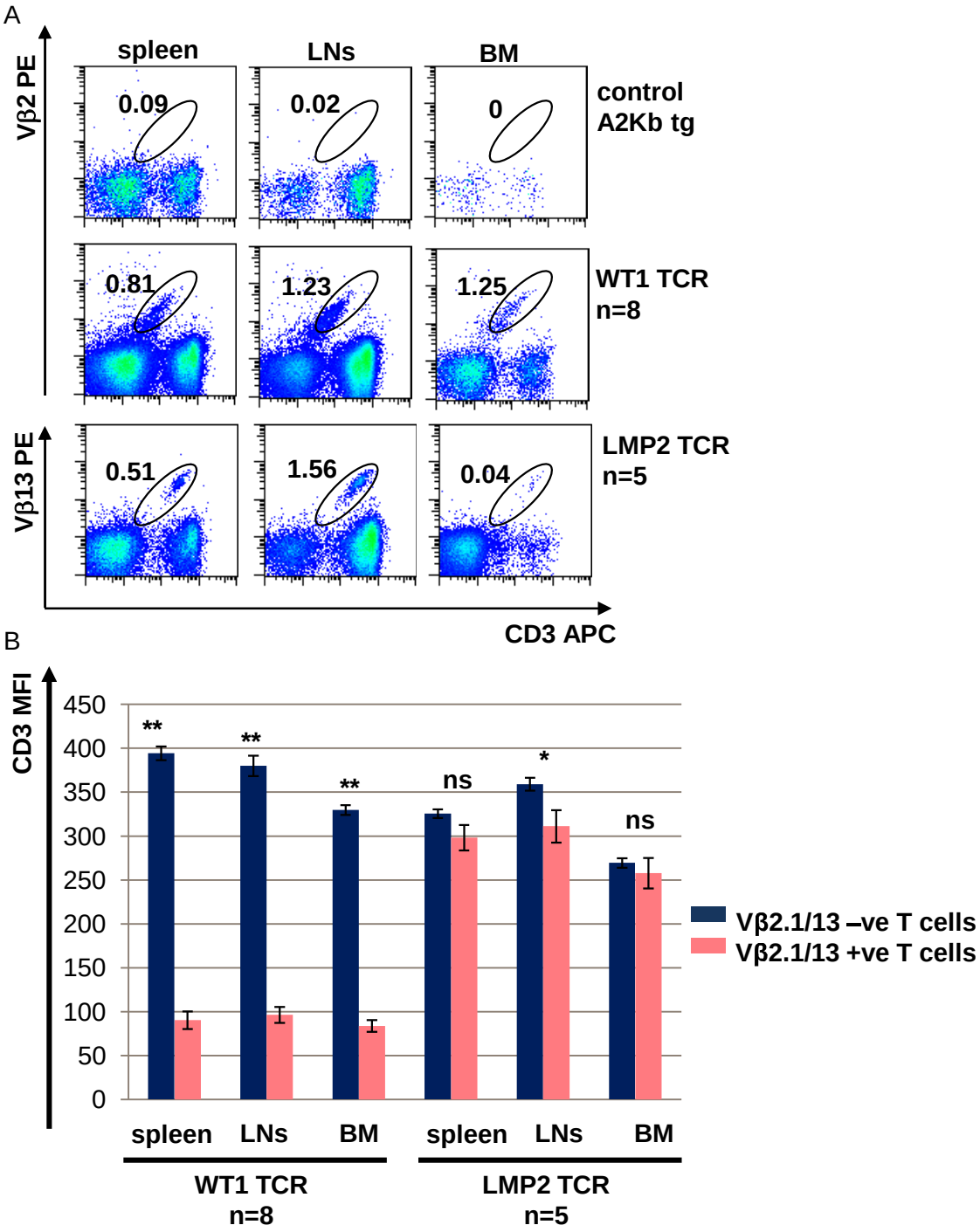


Figure 4.1 WT1 specific T cells emerge and persist in the periphery. (A) FACS analysis of peripheral T cells in spleen, LNs and BM of A2Kb Tg mice killed 11 weeks after transplantation with TCR transduced Lin-ve A2Kb BM stem cells. Mice received untransduced stem cells (top row), WT1 TCR transduced stem cells (middle row, n=8, or LMP2-TCR transduced stem cells (bottom row, n=5). Viable lymphocytes were stained with anti-CD3, and anti-human Vβ2.1 (WT1 TCR) and anti-human Vβ13 (LMP2 TCR) antibodies before FACS analysis. Percentages of Vβ2.1+ and Vβ13+ cells in total lymphocytes. (B) Cell surface CD3/TCR expression levels of the WT1

Figure 4.1 (continued)

TCR (self-reactive) and the LMP2 TCR (non-self-reactive) were compared with endogenous polyclonal T cells derived from transplanted stem cells (Vβ2.1 -ve and Vβ13-ve respectively). Data are mean ± SD of CD3 mean fluorescent intensity (MFI; n=8 mice for WT1 TCR and n=5 mice for LMP2 TCR).* p-value <0.05, **p-value<0.01, ns non significant, all based on 1-way ANOVA).

4.2.2 WT1-specific T cells escape negative selection, but CD3 downregulation takes place in the thymus

The presence of WT1-specific T cells in the periphery of A2Kb Tg mice that had received WT1-TCR td HSCs suggested that developing WT1-specific T cells can escape thymic deletion. The possibility that mature T cells along with HSCs were transduced with the introduced TCR genes is remote for two reasons. The transferred BM cells were enriched in HSCs by immunomagnetic lineage negative selection prior to their transduction removing most if not all T cells along with any other cells committed to other haematopoietic lineages. More importantly though, lentiviral transduction of murine T cells is known to be very difficult to achieve (Baumann et al., 2004, Tsurutani et al., 2007, Kerkar et al., 2011). Nevertheless, to confirm that WT1-specific T cells were generated through thymic development and to study this further, 11 weeks post-transplant thymic tissue was harvested from the A2Kb Tg mice that had received WT1-TCR td A2Kb HSC, stained with anti- CD3, CD4, CD8 and human Vβ2 antibodies and analysed by FACS.

CD4/CD8 thymocyte phenotyping as shown in representative FACS plots in figure 4.2A, was used to study WT1-TCR expression at the different stages of thymic T cell development. The WT1-TCR was expressed early on and throughout T cell development, first in immature double-negative (DN) thymocytes, then in the more mature double-positive (DP) population and consequently in the CD8 single positive (SP) T cells. As expected, only very few T cells expressing this HLA class I-restricted TCR went on to mature into CD4 SP T cells. This was consistent with the absence of such cells in the periphery of transplanted A2Kb Tg mice as discussed in chapter 4.

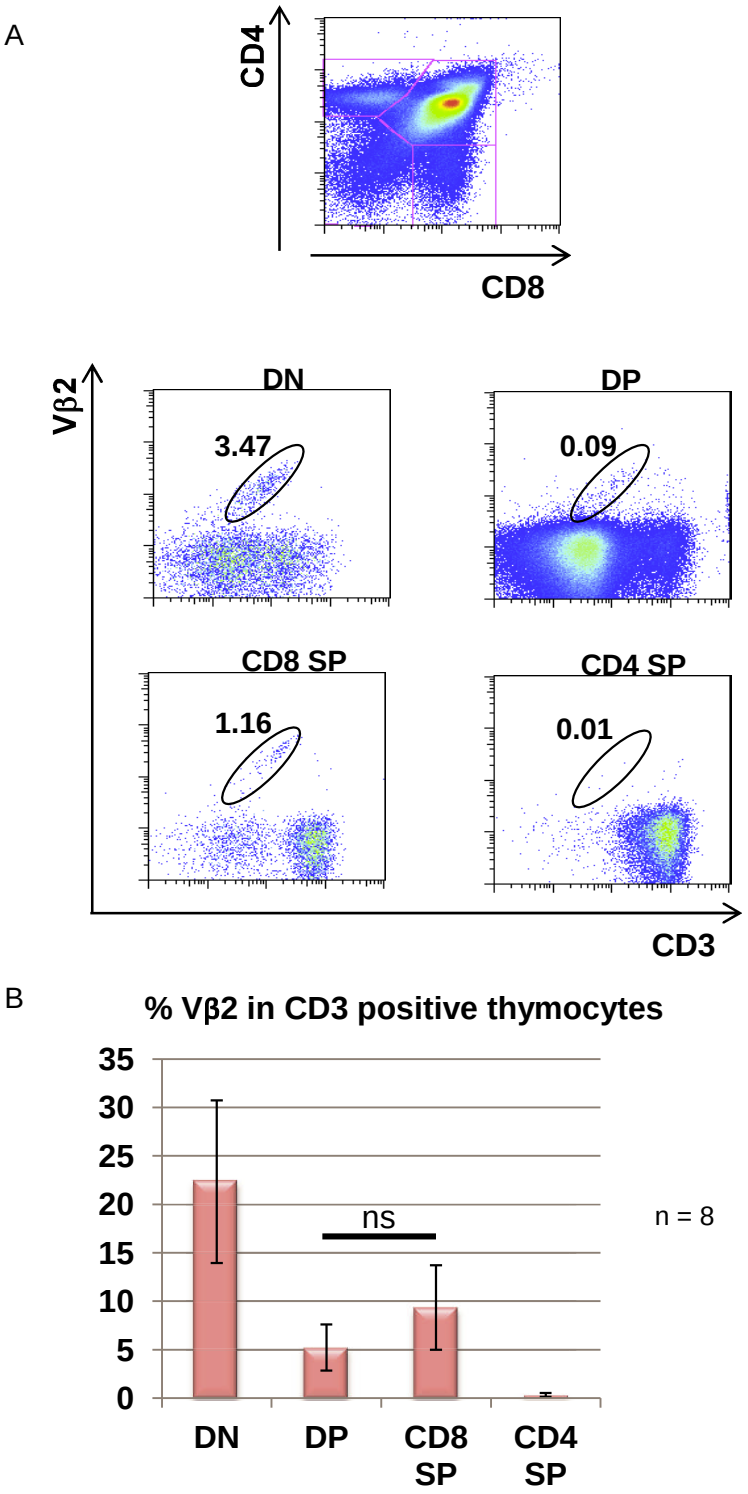


Figure 4.2 WT1 TCR are selected in the thymus. A. FACS analysis of thymocytes isolated from A2Kb mice transplanted with Lin-ve A2Kb stem cells transduced with the lentiviral WT1-TCR vector. Thymocytes were stained with antimurine CD3, CD4 and CD8 antibodies together with anti-human Vβ2.1 to detect the WT1 TCRβ chain. SP indicates single positive; DP, double positive; and DN, double negative. Representative plots from a total of 8 mice are shown. B. Percentage of CD3 positive

Figure 4.2 (continued)

thymocytes expressing the WT1 TCR at each stage of thymic development. Column bars represent mean percentage ±SEM. n=8.. ns indicates non-significant.

A summary of the frequencies of WT1-TCR-expressing thymocytes in the 8 recipients, at each stage of T cell development is shown in figure 4.2B. Similar frequencies of WT1-TCR expressing T cells in the DP and CD8 SP cells were noted, indicating that as CD4+CD8+ WT1-specific T cells progressed through thymic development to become CD8+ cells, they were not deleted by central tolerance mechanisms. Endogenous TCR$\alpha\beta$ complexes are not normally expressed before thymocytes reach the DP stage of T cell development. The presence of WT1-TCR expressing thymocytes in the DN stage and their relative high frequency compared to later stages of T cell development is most likely explained by the non-physiological early expression of the lentiviral TCR construct, a similar phenomenon to what is observed in TCR transgenic mice.

In order to delineate whether the noted TCR/CD3 downregulation in WT1-specific T cells (figure 4.1) takes place in the thymus or following the cells' exit in the periphery, the levels of TCR/CD3 expression in the spleen were compared to those in the thymus at the different stages in T cell development. This analysis, summarised in the bar chart in figure 4.3, showed that WT1-TCR/CD3 expression levels in the periphery were similar to those in the thymus and always lower than that of endogenous TCRs on other donor derived T cells. TCR/CD3 downregulation was evident as early as the DN stage, and was consequently thought to be a thymic event. Therefore, even though WT1-specific T cells are not deleted in the thymus, the observed TCR/CD3 downregulation taking place during thymic development may be the result of central tolerance. On the other hand, it is possible that WT1-specific T cells only manage to evade negative selection by downregulating their TCR early in their development. The prediction then would be that following their

thymic education, this self-specific T cell population will be poorly functional in the periphery. The phenotype and function of WT1-specific T cells in the periphery was studied in detail and these results are presented below.

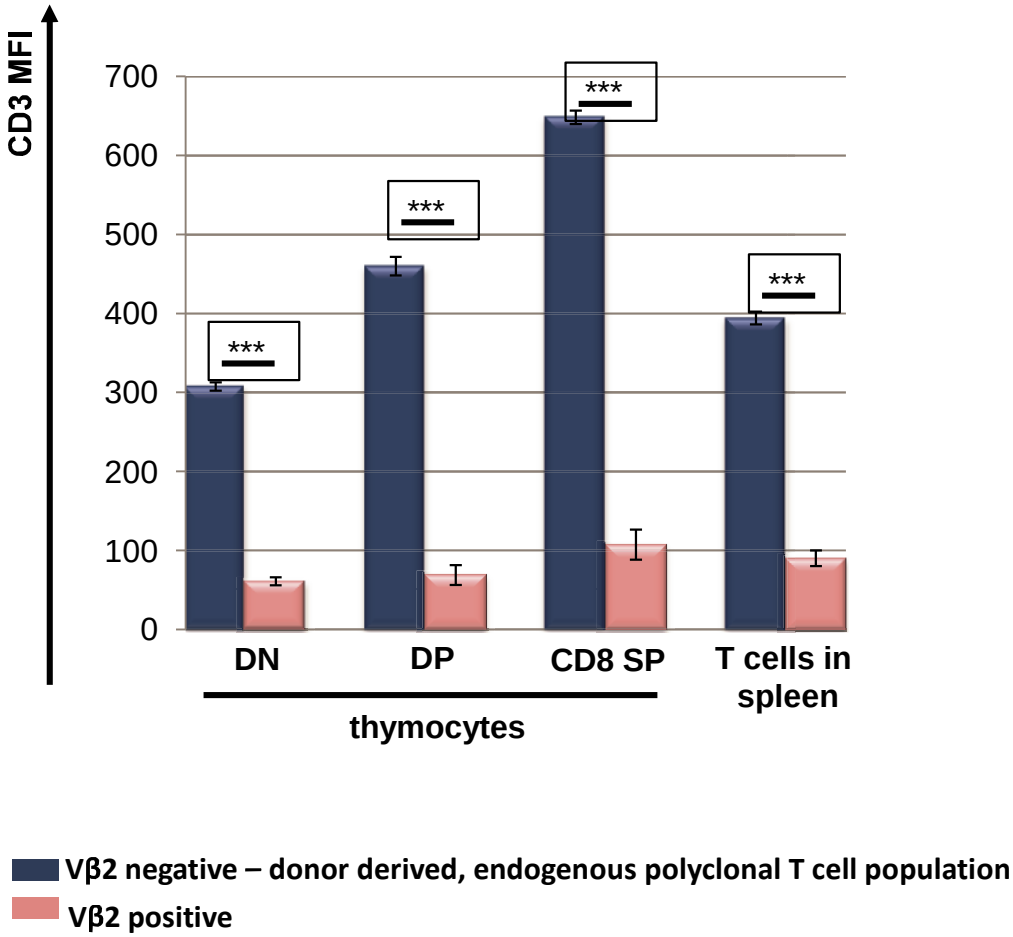


Figure 4.3 CD3/ WT1 TCR downregulation in WT1 specific T cells takes place in the thymus. Mean CD3/TCR expression levels of WT1 (Vβ2 positive) and endogenous polyclonal T cells ±SEM, at different stages of T cell development and in the periphery of transplant recipients (WT1 TCR td HSCs A2Kb→A2Kb, n=8). Single cell suspensions of thymocytes and splenocytes were stained with anti-CD45.1, anti-CD3, anti-CD4, anti-CD8 and human anti-Vβ2.1 antibodies to identify WT1 specific T cells and the donor-derived endogenous polyclonal T cell population at progressive stages of thymic T cell development and in the periphery. *** p=value < 0.001.

4.3 WT1-specific T cells differentiate into memory phenotype cells in the absence of vaccination

4.3.1 WT1-TCRhi and TCRlo subsets identified in the periphery

TCR and CD8 downregulation in the context of self-antigen are thought to be mechanisms of peripheral tolerance (Morahan et al., 1991, Rocha and von Boehmer, 1991, Schönrich et al., 1991). With CD3 being a surrogate marker for TCR expression levels, TCR downregulation was observed in WT1-specific T cells. Therefore, the levels of the CD8 co-receptor in these cells were also examined. A2Kb Tg mice receiving either WT1 or LMP2-TCR td HSC transplants were sacrificed at 11 weeks post-transplant and single cell splenocyte suspensions were stained with anti-CD45.1, anti-CD3, anti-CD8 and anti- human Vβ2.1 antibodies respectively, prior to FACS analysis. Figure 4.4A shows that the mean CD8 expression levels on Vβ2 positive cells detected in the spleen of WT1-TCR td HSC transplant recipients, were significantly lower than that of polyclonal donor derived CD8 T cells expressing endogenous murine TCRs.

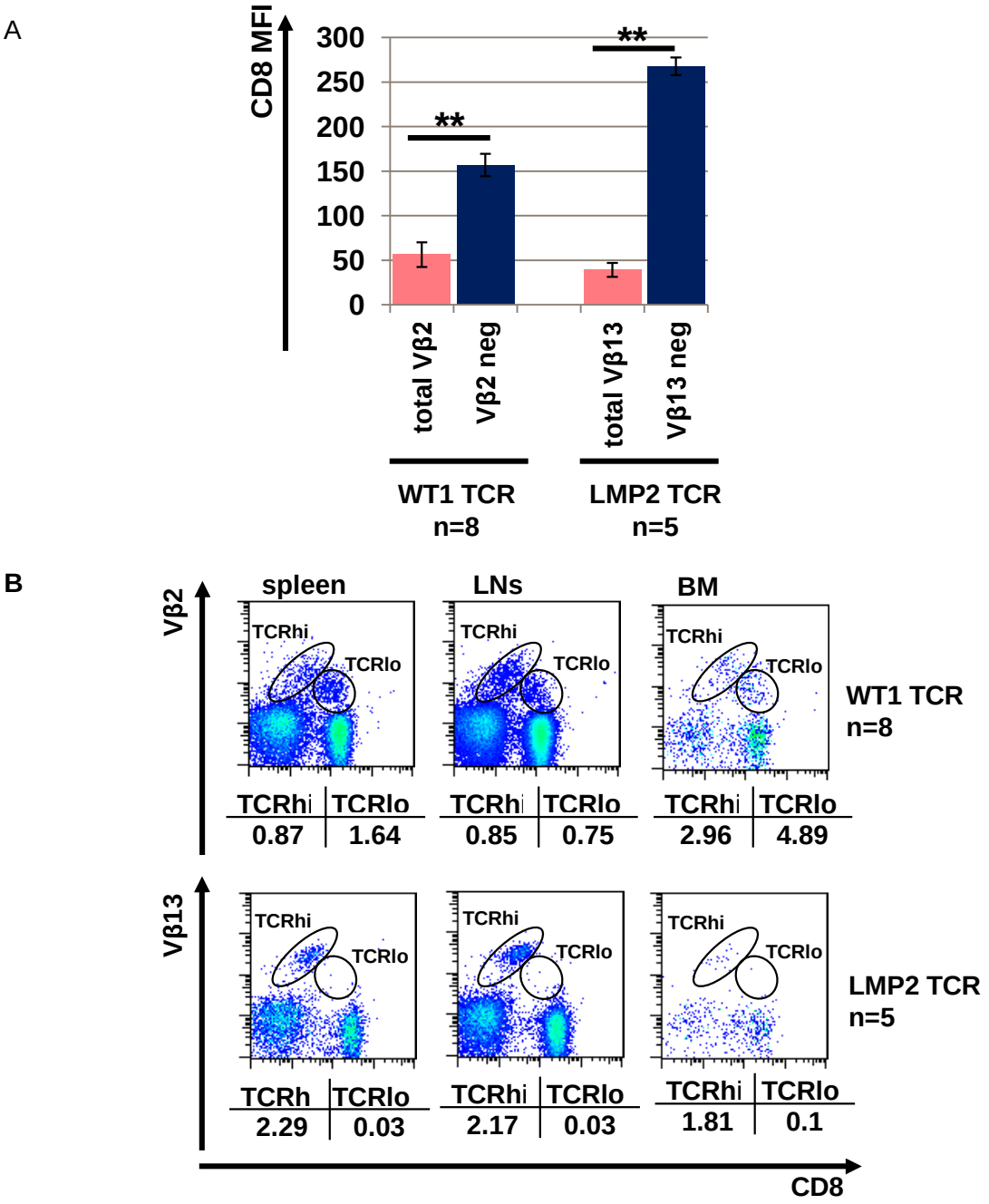


Figure 4.4 TCR down-modulation in WT1 specific T cells is associated with a relative CD8 upregulation. 5x10^5 WT1 or LMP2 TCR td A2Kb Lin-ve BM cells were transferred into 8 and 5 lethally irradiated A2Kb tg mice recipients respectively. 11 weeks post-transplant, transplant recipients were sacrificed and spleens, LNs and BM were harvested. Single cell suspensions from individual spleens were stained with anti-CD45.1, anti-CD3, anti-CD8 and anti-human Vβ2 or anti-human Vβ13 antibodies accordingly prior to FACS analysis. A. Column graph of the mean CD8 MFI in Vβ2 positive and Vβ2 negative donor-derived CD8 T cells and Vβ13 positive and Vβ13 negative donor-derived CD8 T cells in WT1 and LMP2 TCR HSC

Figure 4.4 (continued)

transplant recipients respectively. Error bars= ± SD. ** p-value< 0.01. B. Representative FACS plots of peripheral T cells in spleen, LNs and BM to examine TCR and CD8 modulation in WT1 and LMP2 specific T cells compared to endogenous polyclonal donor derived T cells. After gating on viable donor-derived CD3+ cells, percentages of TCRhi and TCRlo populations are indicated.

4.3.2 WT1-TCR downregulation is associated with a relative upregulation of the CD8 coreceptor

As LMP2-specific T cells also exhibited lower expression levels of the CD8 co-receptor than other donor derived T cells, it was thought that this phenomenon was related to the experimental system rather than to the WT1 self-antigen specificity. Surprisingly though, the representative FACS plots in figure 4.4B reveal that further TCR down-modulation in WT1-specific T cells was coupled with a relative upregulation of the CD8 molecule, giving rise to two WT1-specific T cell subsets. A TCRlo CD8hi (TCRlo) population and a TCRhi CD8lo (TCRhi) population of WT1-specific T cells were present in spleen, LNs and BM. On the other hand in LMP2-specific T cells a single TCRhi population was observed, suggesting that TCR downregulation and CD8 upregulation were likely to be related to the WT1 self-antigen specificity. These results are summarised in the column graph of figure 4.5A, where the mean CD8 MFI among the different Vβ2 and Vβ13 T cell populations is documented. The presence of a TCRlo population among WT1-specific T cells could potentially explain the observed CD3 downregulation among the total Vβ2 positive T cell population. However, comparing the mean CD3 MFI among TCRlo and hi WT1-specific T cells with that of endogenous CD8 T cells, as well as with LMP2-specific T cells confirms that instead both WT1 specific populations exhibit a marked CD3 downregulation that becomes more pronounced in WT1-TCRlo cells (figure 4.5B).

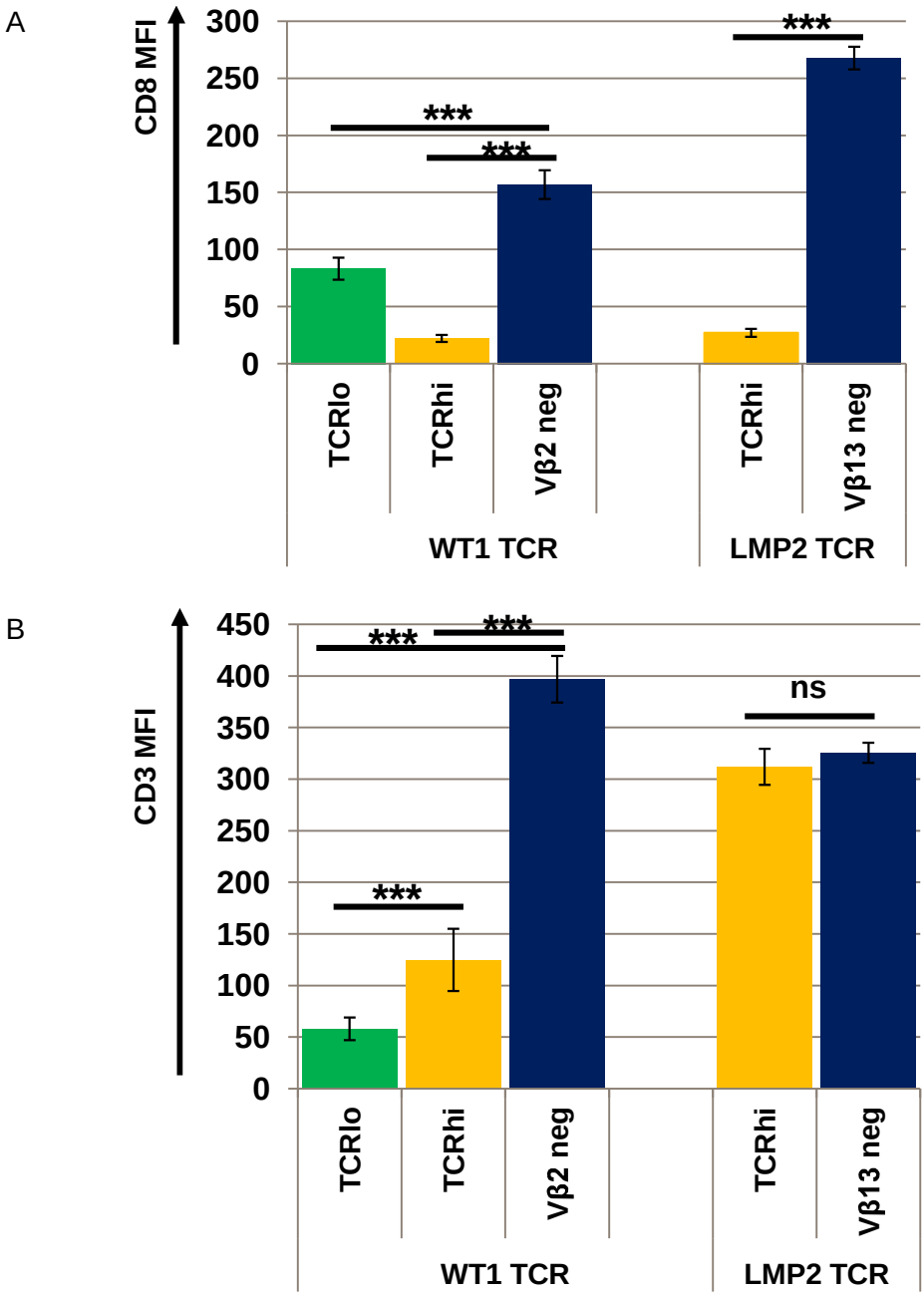


Figure 4.5 TCR down-modulation is observed in TCRhi and TCRlo WT1 specific T cell subsets. 5x10^5 WT1 or LMP2 TCR td A2Kb Lin-ve BM cells were transferred into 8 and 5 lethally irradiated A2Kb tg mice recipients respectively. 11 weeks post-transplant, transplant recipients were sacrificed and spleens, LNs and BM were harvested. Single cell suspensions from individual spleens were stained with anti-CD45.1, anti-CD3, anti-CD8 and anti-human Vβ2 or anti-human Vβ13 antibodies accordingly prior to FACS analysis. A. Column graph of mean CD8 MFI in WT1 TCRlo, WT1 TCR hi and Vβ2 negative donor CD8 T cells in the spleens of WT1 TCR HSC transplant recipients (n=8) and mean CD8 MFI in Vβ13 positive (TCRhi) and Vβ13 negative donor derived CD8 T cells in the spleens of LMP2 TCR

Figure 4.5 (continued)

HSC transplant recipients (n=5). B. Column graph of mean CD3 MFI in WT1 TCRlo, WT1 TCRhi and Vβ2 negative donor derived CD8 T cells in the spleens of WT1 TCR td HSC transplant recipients, and mean CD3 MFI in Vβ13 positive (TCR hi) and Vβ13 negative CD8 donor derived T cells in the spleens of LMP2 TCR td HSC transplant recipients. All mean MFIs are shown as mean ± SD. *** = p-value < 0.001, ns indicates non-significant.

4.3.3 WT1-specific T cells exhibit a memory phenotype in the absence of vaccination

Changes in TCR and CD8 levels have been associated with responses to antigen encounter, and subsequent activation or tolerance induction (Schönrich et al., 1991). For this reason the activation phenotype of WT1 and LMP2-specific T cells was examined by FACS analysis. As shown in figure 4.6, a large proportion of the WT1-specific T cells expressed high levels of CD44 and CD62L (range 28-69% of Vβ2 positive T cells, median 55.2% in LNs, A2Kb to A2Kb transplant), a phenotype normally associated with central memory T cells, while the activation phenotype of LMP2-specific T cells was similar to that of donor derived T cells expressing endogenous TCRs (control endogenous T cells) and consistent with a naive-like phenotype (CD44 low CD62L positive). Therefore, specificity against the WT1 self-antigen, but not against a foreign antigen was shown to drive the generation of memory phenotype T cells in the absence of vaccination.

4.3.4 WT1 specific T cell differentiation to central memory T cells is not dependent on WT1 Ag presentation by professional APCs

The survival of naive T cells is known to require tonic TCR stimulation by MHC molecules expressed on haematopoietic cells, whereas memory T cells are thought to be able to survive in the absence of this tonic triggering. However, the role of haematopoietic cells in the generation and maintenance of memory T cells in the absence of vaccination is not known and experiments were designed to study this. Lin-ve BM cells were isolated from C57Bl/6 donor mice and transduced with the genes for the WT1-TCR. These cells were then

transferred into myeloablated A2Kb recipients and allowed to reconstitute the haematopoietic system of the recipient animals with C57Bl6 derived cells, so that cross-presentation by professional APCs was not possible in this setting as donor BM derived cells did not express the hybrid A2Kb molecule. The cognate Ag of the WT1-TCR could only be presented directly by the few peripheral tissues expressing it at low levels. However, as C57Bl/6 BM progenitors, which are one of the few cell types physiologically expressing the WT1 Ag, lack the HLA A2 molecule, they were unable to present the pWT126 on the appropriate class I molecule for TCR recognition. The mice were allowed to reconstitute for 15-17 weeks and were then sacrificed and spleens, LNs and BM were harvested for phenotypic and functional analysis of WT1-specific T cells. The FACS plots in figure 4.6 demonstrate that CD44hi CD62Lhi WT1-specific T cells were still generated in the absence of A2Kb professional APCs (B6 to A2Kb transplant), implying that the development of Tcm WT1-specific T cells (CD44hi CD62Lhi) was not dependent on antigen presentation on haematopoietic cells.

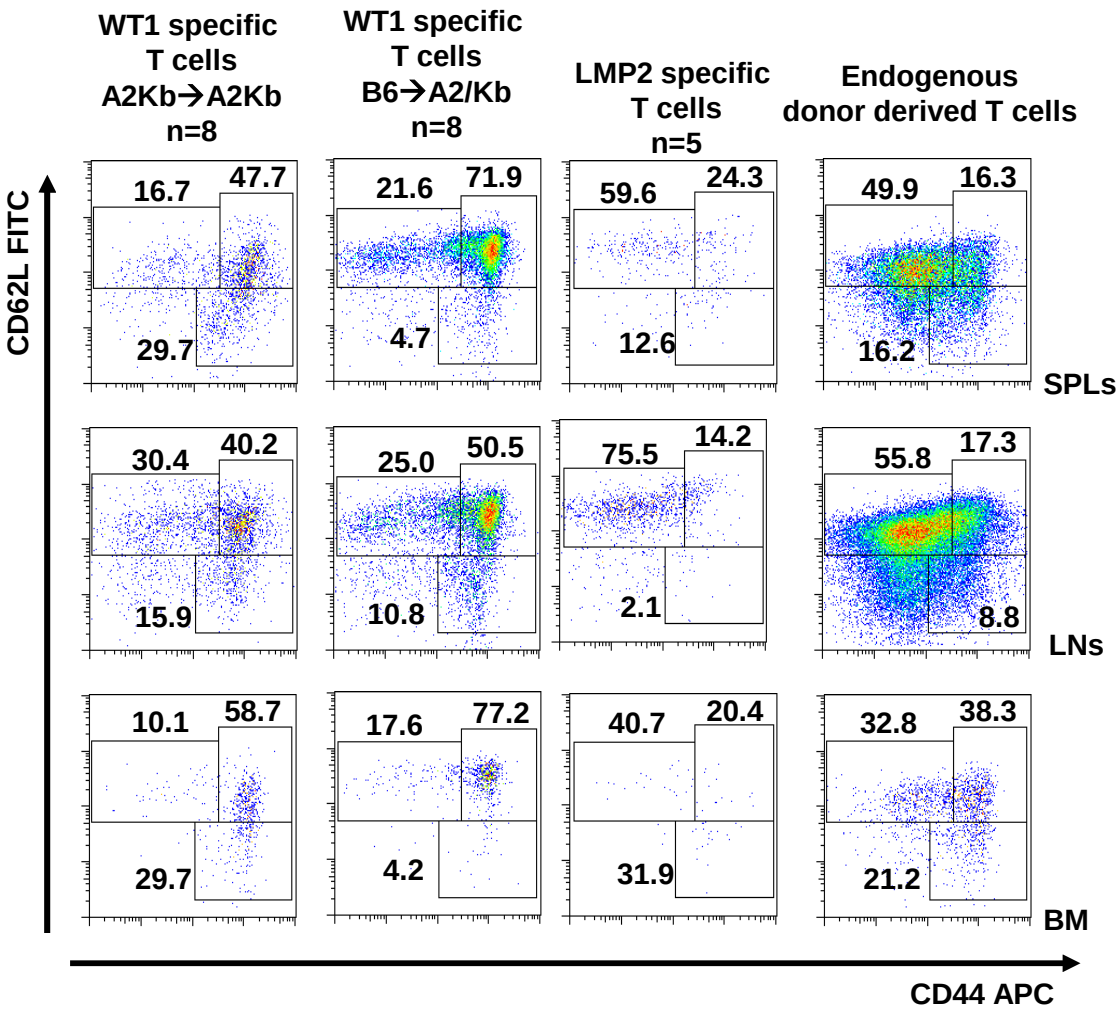


Figure 4.6 Peripheral WT1 specific T cells differentiate into memory phenotype, in the absence of vaccination. A2Kb lethally irradiated recipients received 5x105 WT1 or LMP2 TCR td HSCs, as specified. 1st, 3rd and 4th columns A2Kb donor Lin-ve BM cells . 2nd column C57Bl/6 donor Lin-ve BM cells. A2Kb and C57Bl/6 transplant recipients were sacrificed 11 and 15-17weeks post – transplant respectively. Spleens, LNs and BM were harvested and single cell suspensions were prepared and stained with hVβ2/ hVβ13, and murine CD8, CD44 and CD62L antibodies. Representative FACS plots of CD44 and CD62L expression on gated hVβ2+ve and hVβ13+ve T cells as well as gated endogenous donor derived peripheral T cells are shown. Percentages stated refer to the percentage of each indicated subset in hVβ2+ve, hVβ13+ve and endogenous donor derived T cells.

The results of the detailed analysis of the naive (CD44lo CD62Lhi), Tcm (CD44hi CD62Lhi) and Tem (CD44hi CD62Llo) T cell frequencies in the spleen of mice expressing the WT1-TCR or LMP2-TCR are shown in figure 4.7. While the majority of LMP2-specific T cells as expected were naive, significantly less WT1-specific T cells exhibited this phenotype ($p<0.001$). The opposite was true for Tcm T cells, as these were significantly more frequent in WT1-specific T cells than in LMP2-specific T cells ($p<0.001$). Interestingly, higher frequencies of Tem T cells were only noted in WT1-specific T cells generated in mice reconstituted with WT1-TCR td A2Kb stem cells, ($p<0.01$) but not in those reconstituted with WT1-TCR td B6 stem cells (ns, non-significant), alluding to the idea that antigen presentation by haematopoietic cells promoted the differentiation of WT1-specific T cells into Tem phenotype.

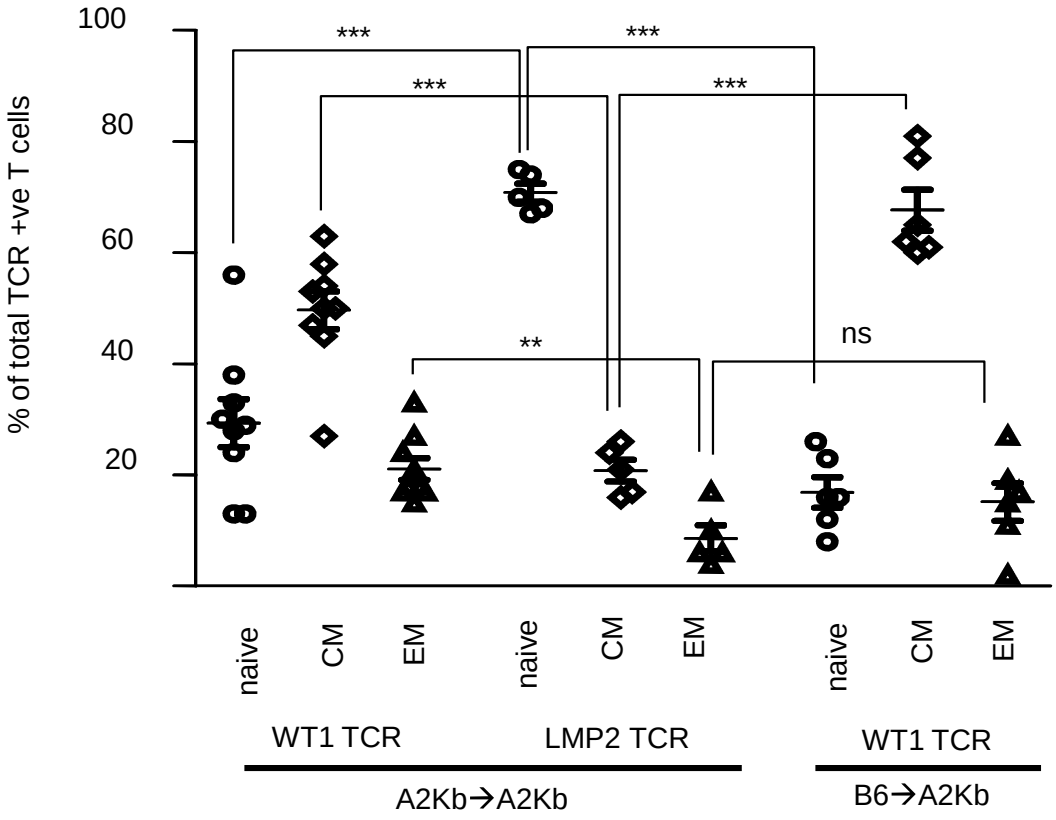


Figure 4.7 Antigen presentation by professional APCs promotes the differentiation of WT1 specific T cells into the EM phenotype. Summary data of naive (CD44lo, CD62Lhi), CM (CD44hi, CD62Lhi), and EM (CD44hi, CD62Llo) WT1- and LMP2-specific T cell frequencies in transplanted mice expressing the WT1-TCR or the LMP2 TCR. ***p <0.001, **p<0.01, ns non-significant. A2Kb → A2Kb or B6 → A2Kb as indicated in the figure. Data correspond to LN FACS plots.

Percentage donor chimerism in recipients receiving WT1-TCR td C57Bl/6 HSC transplants, at the time of this phenotypic analysis ranged between 92%-97%. Representative FACS plots, of single cell suspensions from spleen, LNs and BM of recipient animals stained with anti-CD3 and anti-CD45.1 antibodies, demonstrate that CD3 negative lymphocytes, including professional APCS, are in their great majority donor derived (figure 4.8A). The pooled data from all recipient animals (n=8) are summarised in the column graph in figure 4.8B. Nevertheless, the fact that complete donor reconstitution was never reached in the B6 to A2Kb transplants makes it possible that the very small number of residual A2Kb^{+} APCs contributed to, or was sufficient for, the development of central memory and effector memory phenotype cells in the mice transplanted with B6 stem cells. This would imply that such minimal cross-presentation of the pWT126 to WT1-specific T cells is enough to cause a major shift in the activation phenotype of the WT1 specific T cell population.

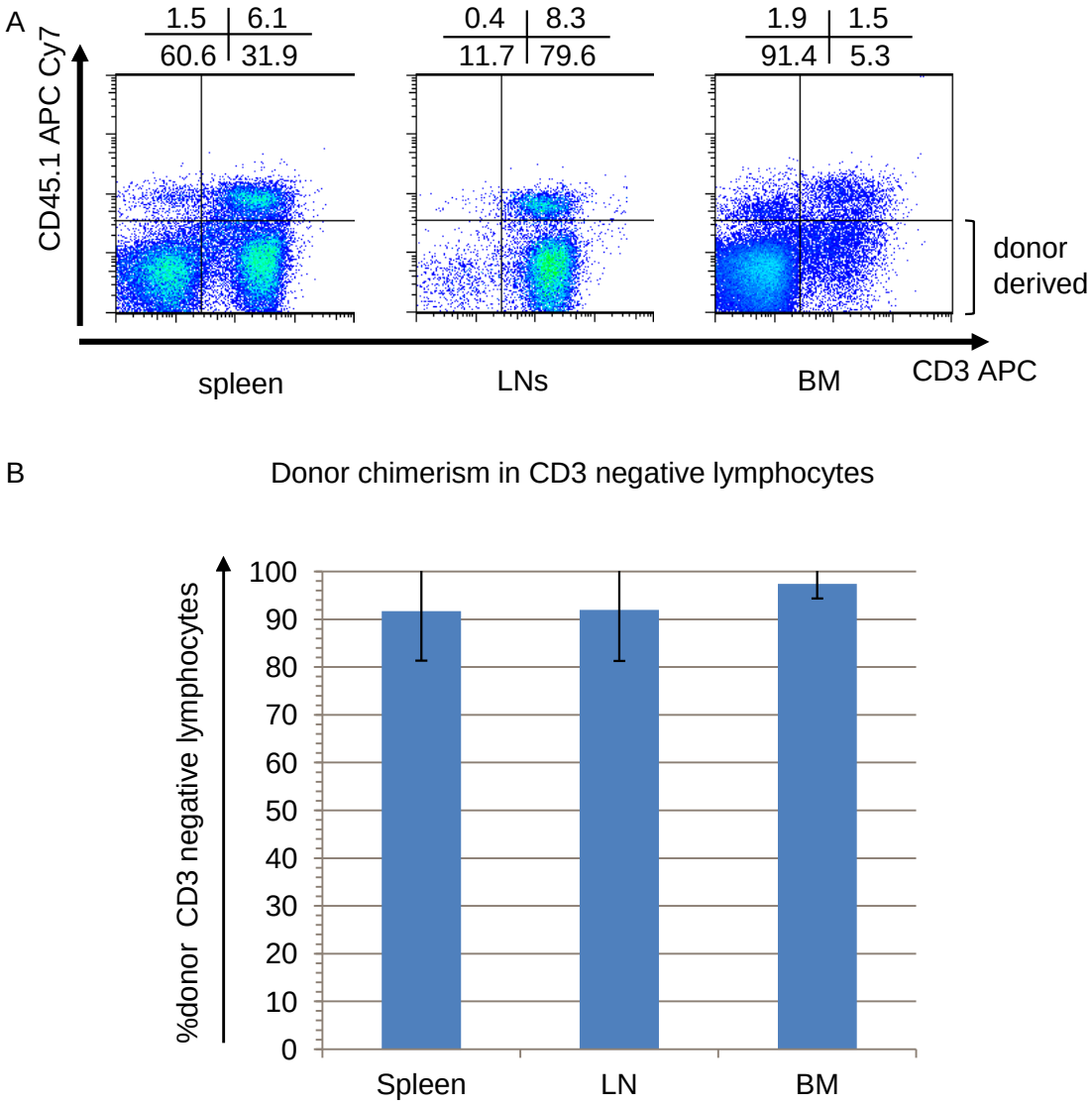


Figure 4.8 Donor Chimerism in C57Bl6, WT1 TCR HSC transplant recipients. Lethally irradiated A2KbxCD45.1 mice received 5x10^5 C57Bl/6 WT1 TCR td Lin-ve BM cells (n=8). 15-17 weeks post-transplant recipient mice were sacrificed and spleen, LNs and BM were harvested and single cell suspensions were prepared. Cells from individual organs were stained with murine anti-CD45.1 and anti-CD3 antibodies. A. Representative FACS plots of splenocytes, LNs and BM cells stained with above antibodies. Cells were gated on viable lymphocytes. Donor derived cells were CD45.1 negative. B. Donor chimerism in CD3 negative lymphocytes. Bars show mean donor chimerism ±SD.

4.3.5 WT1-TCRhi and TCRlo T cells exhibit different activation phenotypes

Since the TCRhi WT1-specific T cell subset exhibited a similar TCR/CD8 phenotype to that of LMP2-specific T cells, the idea that this subset would also have a similar activation phenotype to LMP2-specific T cells was also attractive. In this scenario, the TCRlo WT1-specific T cell subset would exclusively contribute to the Tcm WT1-specific population. To examine this, the activation phenotype of the TCRhi and TCRlo WT1-specific T cells was analysed and representative FACS plots of splenocytes are shown in figure 4.9A. Both in the presence and absence of professional antigen presentation (A2Kb to A2Kb and B6 to A2Kb transplants respectively) TCRlo cells were predominantly of Tcm phenotype (mean 67% and 84% respectively). In the case of TCRhi WT1-specific T cells in the presence of professional Ag presentation, a shift towards the memory phenotype was still observed with most cells being either Tcm (mean 34%) or Tem (mean 37%). In contrast, in the B6 to A2Kb setting the activation phenotype of TCRhi WT1-specific T cells follow a population distribution more similar to that of endogenous T cells, with most cells being naive (mean 53%).

These results suggested that while professional Ag presentation was not required to drive a proportion of WT1-specific T cells towards a Tcm phenotype, it did have an effect on further activating all WT1-specific T cells. Noting naive, Tem and Tcm cells within the TCRhi WT1-specific T cells in the A2Kb to A2Kb setting, while most TCRlo cells are Tcm, supports the hypothesis that chronic low level WT1 Ag encounter, results in WT1-TCR

triggering. T cell activation allows the WT1-specific T cells to progress from naive to memory as they downregulate their TCR and convert from TCRhi to TCRlo. Therefore, the TCRhi subset contains all 3 activation phenotypes (naive, Tem and Tcm) whereas the TCRlo subset being the most differentiated is mainly enriched in CM T cells.

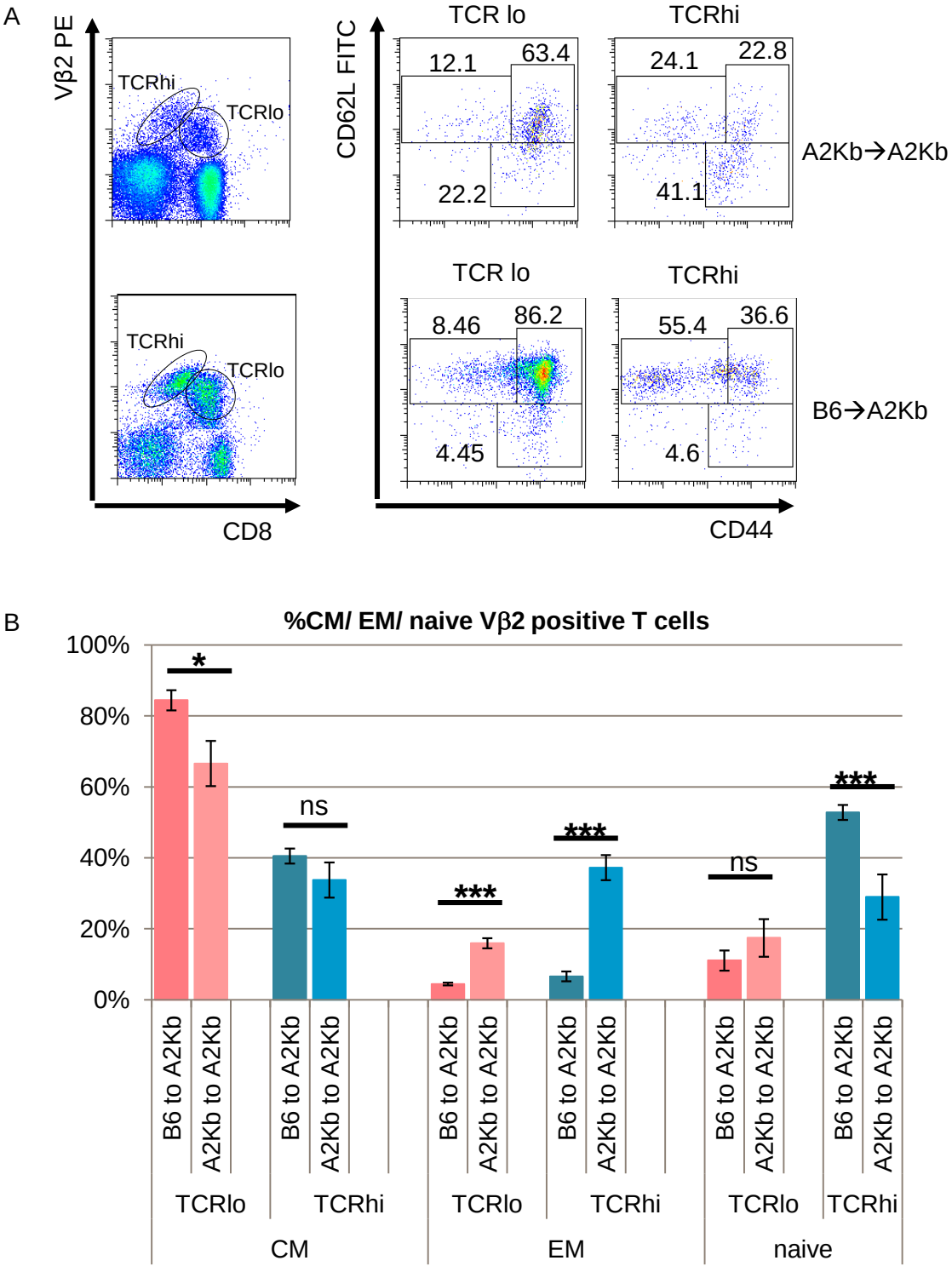


Figure 4.9 TCRlo WT1 specific T cells exhibit a CM activation phenotype, TCRhi WT1 specific T cells consist of naive, effector and CM T cells. A. CD44, CD62L phenotypic FACS analysis of gated WT1 TCRhi and WT1 TCRlo peripheral T cell populations, following antibody staining of splenocytes from transplant recipients receiving either A2Kb (top panel) or C57Bl/6 (bottom panel) WT1 TCR td HSCs. Single cell splenocyte suspension from individual recipients were stained with

Figure 4.9 (continued)

murine anti-CD45.1, anti-CD3, anti-CD8, anti CD-44, anti-CD62L and human anti-Vβ2. Representative FACS plots shown. B. Column graph summarising %of CM, EM and naive Vβ2 positive T cells in the TCRlo and TCRhi WT1 specific T cell subsets in transplant recipients receiving either C57Bl/6 or A2Kb WT1 TCR td HSC transplants. Data shown are mean ± SEM. A2Kb→A2Kb transplant n=8, B6→ A2Kb transplant n=8. *** p-values < 0.001, ** p-values <0.05, ns indicates non-significant.

4.3.6 WT1-specific T cells preferentially accumulate in the BM

The BM resident progenitor cells are one of the few cell types expressing low levels of the WT1 Ag in healthy subjects. In view of this, the frequency of WT1-specific T cells within the donor T cell compartment in the BM was compared to their frequency in spleen and LNs. The graph in figure 4.10A summarises the percentage frequency of WT1 and LMP2-specific T cells in these three organs and demonstrates the preferential accumulation of WT1-specific T cells in the BM compared to spleen and BM. As expected no enrichment of LMP2-specific T cells was observed in the BM.

The accumulation of WT1-specific T cells in the BM could also potentially be explained by the fact that the majority of these cells exhibited a memory phenotype. Being memory T cells it was possible that they were selectively recruited and proliferated at this site which is thought to be the niche of Tcm T cells. This scenario was further investigated in the A2Kb Tg mice reconstituted with WT1-TCR td C57Bl/6 HSCs, where direct and cross- presentation of the WT1 Ag could not contribute to the preferential accumulation of WT1-specific T cells in the BM. Indeed, despite the lack of Ag presentation, the BM was enriched in WT1-specific T cells in the B6 to A2Kb transplant recipients, suggesting that the BM environment plays a part in this phenomenon. The column graph in figure 4.10B shows the mean fold-enrichment of WT1-specific T cells in the BM compared to spleen and LNs in transplant recipients receiving A2Kb or C57Bl/6 HSCs, as indicated. In the presence of effective Ag presentation (A2Kb to A2Kb transplant) this enrichment is higher, but it only becomes statistically significant when the frequency of WT1-specific T cells in

the BM is compared to that in the LNs. Therefore, it was thought that the BM environment and WT1 Ag presentation were likely to have a cumulative effect on the preferential accumulation of WT1-specific T cells in the BM donor T cell compartment.

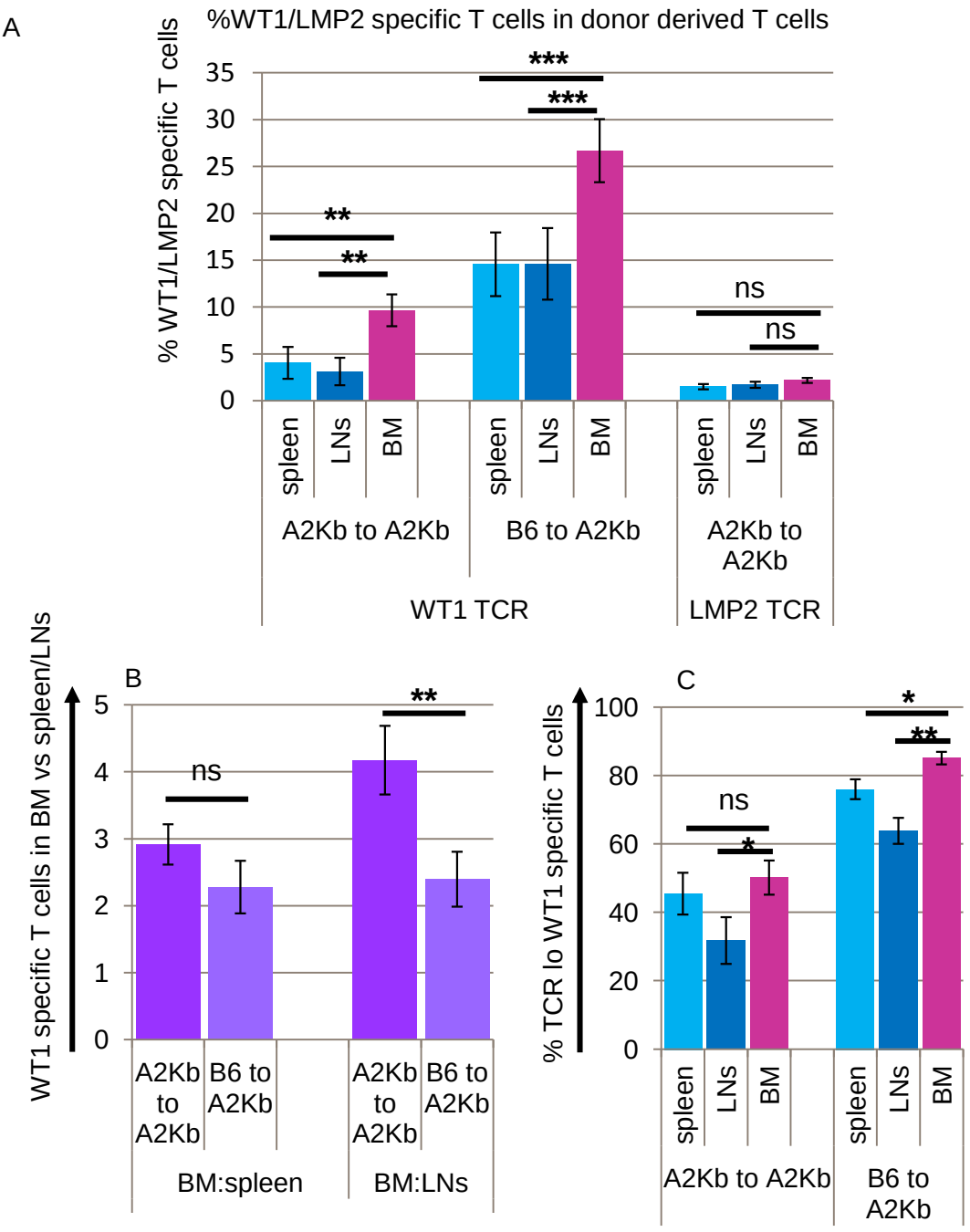


Figure 4.10 WT1 specific T cells preferrentially accumulate in the BM. A Column graph showing the mean frequency of WT1/LMP2 specific T cells in donor derived T cells in the spleens, LNs and BM of A2Kb and B6 HSC transplant recipients, as indicated. B. Column graph indicating the ratio %Vβ2 positive T cells in BM to %Vβ2 positive T cells in spleen and BM to LNs, in A2Kb to A2Kb and B6 to A2Kb WT1 TCR td HSC transplant recipients, as indicated. C. Column graph showing the % of TCRlo WT1 specific T cells among total WT1 specific T cells (TCRlo+TCRhi), in spleen, LNs and BM in A2Kb to A2Kb and B6 to A2Kb WT1 TCR td HSC transplant recipients, as indicated. For all A, B and C values shown are mean ± SEM. . ****p-value* < .001, ***p-value* < .01, *p-value<0.05, ns indicates not significant. WT1 TCR A2Kb to A2Kb HSC

Figure 4.10 (continued)

transplant $5x10^5$ Lin-ve BM cells, n=8. B6 to A2Kb HSC transplant 5x105 Linv-ve BM cells, n=8. LMP2 TCR A2Kb to A2Kb HSC transplant, 5x105, n=5. Spleens, LNs and BM were harvested from tranplant recipients 11 or 15-17 weeks post-transplant (A2Kb to A2Kb and B6 to A2Kb respectively) and single cell suspensions from individual organs were stained with murine anti-CD45.1, anti-CD3, anti-CD8, anti-CD44, anti-CD62L and human anti-Vβ2.1 and anti-Vβ13 identifying WT1 and LMP2 specific TCRs respectively. The stained samples were then analysed by FACS analysis.

4.3.7 WT1 Ag expression in BM progenitors and BM environment both contribute to the preferential accumulation of WT1-specific T cells in the BM

WT1-specific T cells exhibiting a Tcm phenotype, were mainly found in the TCRlo subset (figure 4.9). It was therefore expected that if the observed BM enrichment was to be mainly driven by the memory phenotype of WT1-specific T cells, then higher frequencies of TCRlo WT1-specific T cells would be noted in the BM rather than in the other organs. While this was indeed the case in the B6 to A2Kb setting, where WT1 Ag presentation cannot contribute to the phenomenon, the difference in the A2Kb to A2Kb transplant was minimal (figure 4.10c).

Even though TCRlo WT1-specific T cells which had a Tcm phenotype in their majority, were not significantly enriched in the BM compared to the periphery, what remained possible was that the TCRhi WT1-specific T cell subset in the BM shifted towards a Tcm phenotype. The FACS plots in figure 4.11A, show the activation phenotype of WT1-specific TCRhi T cells (left 3 plots) in spleen, LNs and BM of a representative A2Kb to A2Kb WT1-TCR td HSC transplant recipient. While a marked shift towards a memory profile is observed in WT1 specific TCRhi cells present in the BM, to a certain extent this is also the case for the donor derived endogenous polyclonal CD8 T cell population (far right plot). This was examined in more detail and the relative frequencies of Tcm WT1-specific T cells among total Vβ2 positive, TCRlo and TCRhi WT1-specific T cells was calculated based on the same FACS analysis as for figure 4.11A. These data, shown in the column graph of figure 4.11B illustrate that the

percentage of Tcm-phenotype WT1-TCRhi cells is significantly increased in the BM compared to spleen and LNs. Nevertheless, the increase in the proportion of Tcm T cells in the BM among WT1 specific TCRhi T cells is similar if not less than that observed among donor derived polyclonal CD8 T cells expressing an endogenous TCR repertoire. Therefore, as TCRhi WT1-specific T cells are enriched in Tcm phenotype T cells in keeping with a baseline increase of memory T cells present in the BM compared to the periphery, it can be concluded that this is unrelated to the WT1 specificity. On the other hand, it is possible to consider that among the endogenous T cell repertoire a certain percentage of T cell specificities will have encountered Ag (including self-Ag), been activated and eventually differentiated to Tcm T cells which then preferentially home to the BM. In a similar fashion, TCRhi, WT1-specific T cells also encounter their cognate Ag, resulting in a similar increase in the percentage of Tcm T cells among TCRhi and endogenous CD8 T cells being noticed in the BM.

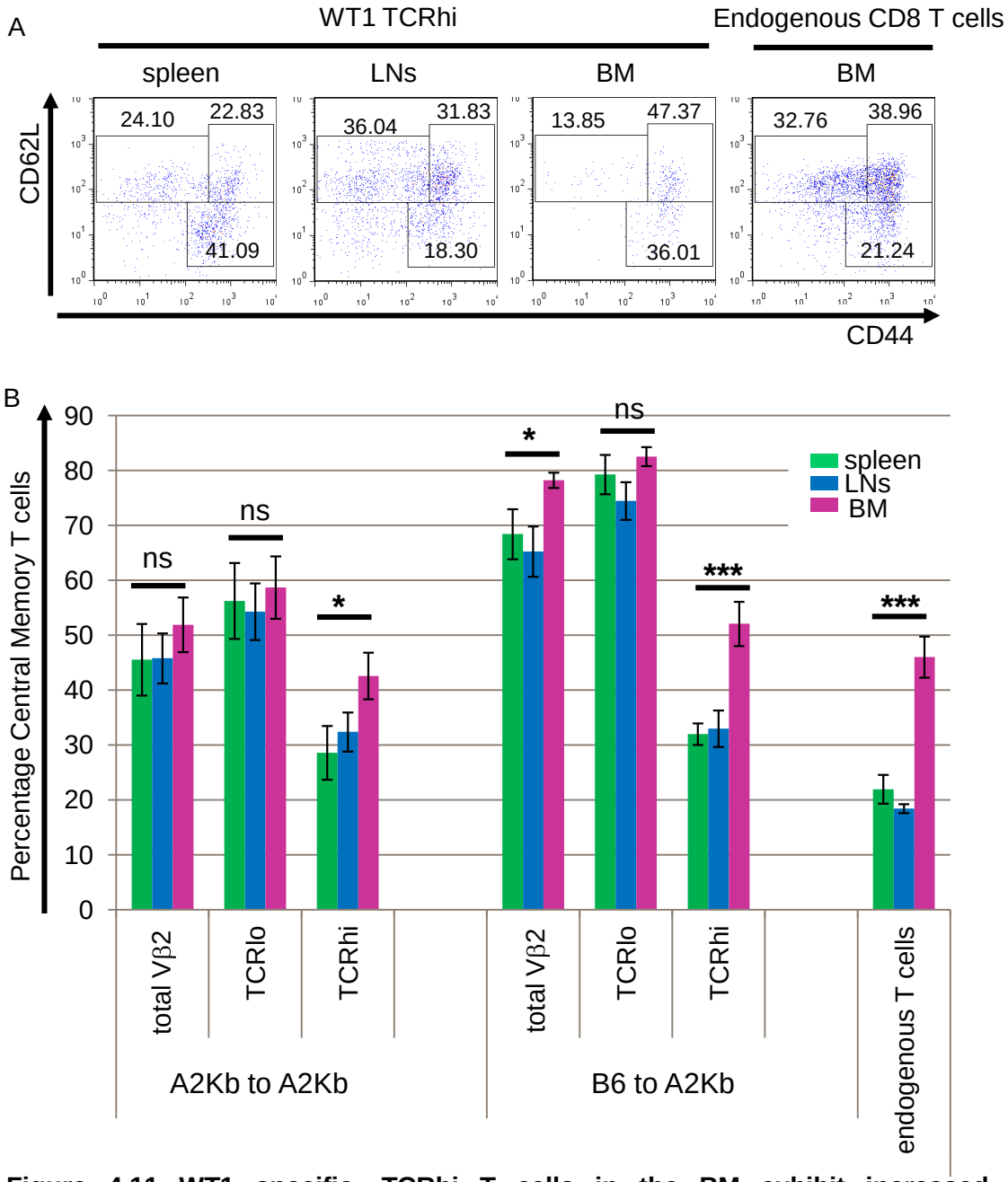


Figure 4.11 WT1 specific, TCRhi T cells in the BM exhibit increased frequencies of CM phenotype cells A. FACS analysis of the CD44, CD62L activation phenotype of gated WT1 specific TCR hi T cells, found in the spleen, LNs and BM of A2Kb to A2Kb WT1 TCR td HSC transplant recipients (left 3 plots) and donor derived CD8 T cells expressing endogenous TCRs (far right plot). Representative FACS plots shown.B. Column graph summarising the percentages of CM T cells (CD44, CD62L positive) in total Vβ2 specific T cells, as well as TCRlo and TCRhi WT1 specific T cells, in spleens, LNs and BM of A2Kb to A2Kb and B6 to A2Kb HSC transplant recipients, as indicated. The percentage of CM T cells among donor derived, CD8 T cells expressing a polyclonal endogenous TCR

Figure 4.11 (continued)

population in A2Kb to A2Kb transplant recipients is also shown (far right group). Values shown are mean ± SEM. ****p-value* < .001, ***p-value* < .01, *p-value<0.05, ns indicates not significant. FACS analysis was performed following antibody staining of single cell suspensions from individual spleens, LNs and BM of transplant recipients sacrificed at 11 and 15-17 weeks post transplant (A2Kb to A2Kb and B6 to A2Kb respectively), with murine anti-CD45.1, anti CD3, anti-CD8, anti-CD44, anti-CD62L and human anti-Vβ2.1 antibodies.

4.4 Discussion

In this chapter we present data showing that WT1-specific T cells escape negative selection, in mice expressing the WT1 Ag in physiological levels and tissue distribution. Instead of being deleted, WT1-specific T cells appeared to downregulate CD3 in the thymus. This event persisted in the periphery and was considered a surrogate marker for TCR downregulation. Interestingly, despite the expectation that the CD3/TCR downregulation would compromise the function of WT1-specific T cells in the periphery, our results suggest this not to be the case. The phenotypic analysis of WT1-specific T cells provided surprising evidence that these cells' specificity for the WT1 self-Ag mediated their activation and differentiation into Tem and Tcm-phenotype T cells. WT1-specific T cells were found to preferentially accumulate in the BM, and our data suggested that both Ag presentation at this site and the BM environment which is known to serve as a niche for Tcm T cells contributed to this enrichment.

Chapter 5 - Results 3 - Functional analysis of WT1-specific T cells

5.1 Introduction

The phenotypic analysis of WT1-specific T cells, presented in Chapter 4, showed that specificity for this TAA not only did not prohibit their development from thymocytes to mature T cells emerging in their periphery, but also drove their differentiation into Tem and Tcm cells. On the contrary, LMP2-specific T cells exhibited a phenotype similar to that of the endogenous polyclonal T cell repertoire, consisting mainly of naive T cells. Memory T cells, unlike naive T cells, are expected to exhibit rapid effector function upon antigenic stimulation. As both functional activity and phenotype are necessary to define a cellular subset, such analysis of the in vivo generated WT1-specific T cells in our experimental model was essential. This was also important to confirm that WT1-specific T cells were not tolerised, as even though this would disagree with their observed phenotype it was what was originally predicted due to their self-specificity.

5.2 Results

5.2.1 WT1-specific T cells display antigen specific in vivo cytotoxicity activity in the absence of vaccination

The killing activity of WT1-specific T cells was assessed by in vivo cytotoxicity assays. These were described in detail in Materials and Methods, Chapter 2. Briefly, A2Kb splenocytes were loaded with pWT126 or an irrelevant peptide, labelled with different concentrations of CFSE (CFSEhi and CFSElo

respectively) and mixed at an 1:1 ratio prior to being injected into mice that had been reconstituted with WT1-TCR td HSCs. 18hrs later, splenocytes of injected animals were harvested and analysed by FACS to identify CFSE-labelled cells. Figure 5.1A shows representative FACS histograms of CFSE labelled target cells persisting in the spleens of control and WT1-TCR td HSC recipients. Strong antigen-specific killing activity was observed both in mice that were transplanted with A2Kb or B6 WT1-TCR transduced HSCs and this is consistent with the presence of memory phenotype WT1-specific T cells identified in both experimental systems. The in vivo killing antigen specific activity of LMP2-specific T cells was also assessed using the LMP2 cognate antigen epitope (pCLG) in the same assay. Unlike WT1-specific T cells, LMP2-specific T cells did not exhibit any antigen-specific cytotoxicity. As LMP2-specific T cells had a naive phenotype, the fact that they did not exhibit antigen-specific cytotoxicity was justified and expected in the absence of any previous priming antigenic encounter. Primary human T cells transduced with the LMP2-TCR have previously been demonstrated to effectively kill and proliferate in an antigen specific manner (Hart et al., 2008) when activated rather than naive, and this further supports the notion that the in vivo antigen-specific killing activity of WT1-specific T cells relates to their memory phenotype. Figure 5.1B summarises the in vivo cytotoxicity assay data from all subjects in the WT1 and LMP2-TCR transplants.

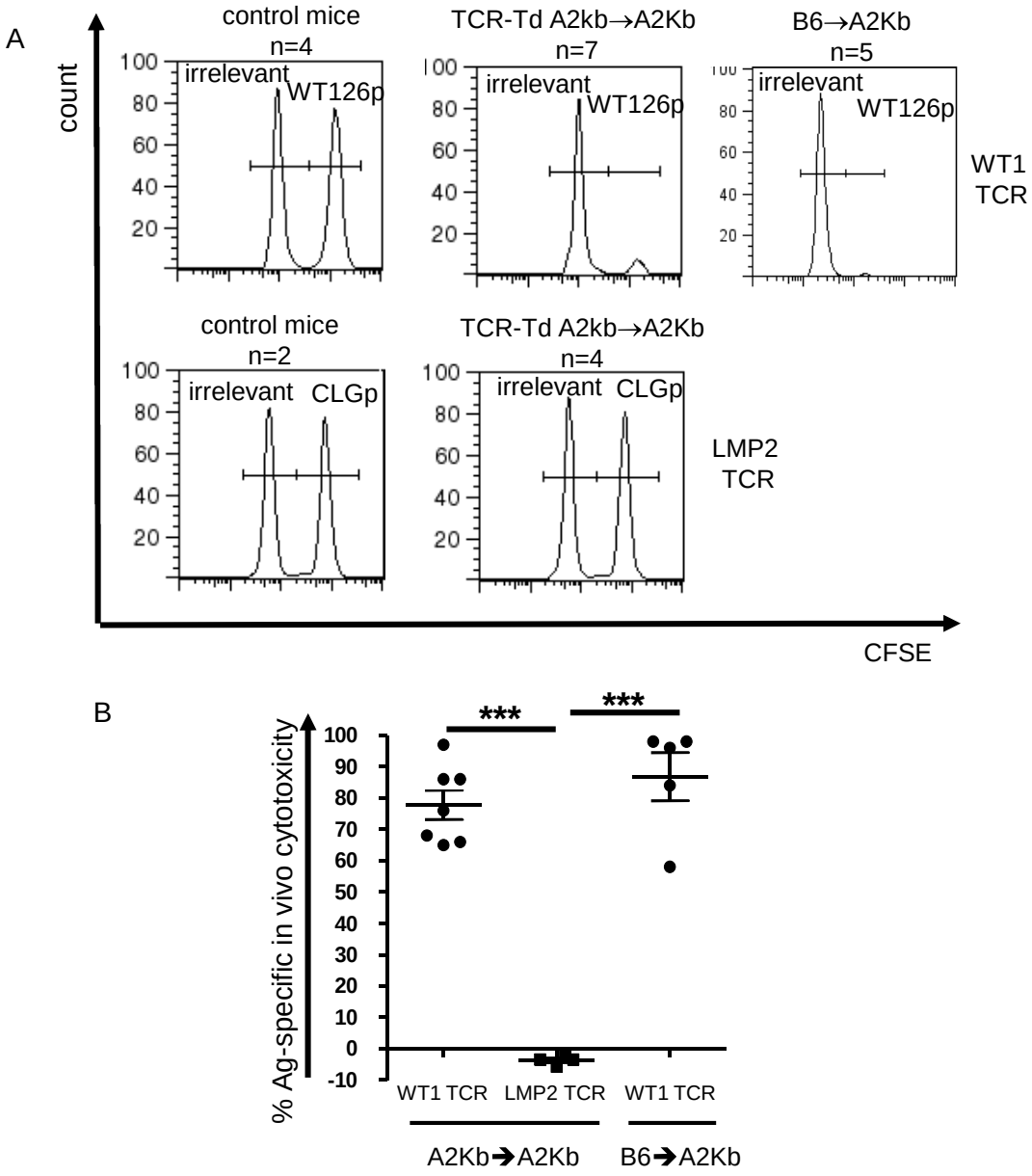


Figure 5.1 WT1 specific T cells exhibit antigen specific in vivo cytotoxicity in the absence of vaccination. In vivo cytotoxicity of CFSE labelled, peptide-loaded target cells. A2Kb Tg mice, 11 weeks after transfer of BM stem cells transduced with the WT1-TCR (top panel, n = 7 A2Kb → A2Kb, n = 5 B6 → A2Kb) or LMP2-TCR (bottom panel, n = 4 A2Kb → A2Kb) were intravenously injected with a 1:1 mix of relevant: irrelevant peptide-loaded A2Kb Tg splenocytes, differentially labeled with CFSE (WT1-TCR is specific for WT126 peptide, and LMP2-TCR is specific for CLG peptide). Control untreated A2Kb Tg mice were injected with CFSE-labeled peptide-loaded target cells. Eighteen hours later, splenocytes of injected animals were harvested and analyzed by FACS to identify CFSE-labeled cells. A. Representative plots showning gated CFSE labelled target cells, 18hrs post- injection.

Figure 5.1 (continued)

Top row shows target cells in control and WT1 TCR td HSC transplant recipients. Bottom row shows target cells in control and LMP2 TCR td HSC transplant recipients. WT126p loaded targets are cleared in mice containing WT1 specific T cells. No antigen specific killing is observed in LMP2 TCR td HSC transplant recipients. B Summary data of in vivo cytotoxicity assays are shown on the right. Percentage antigen-specific cytototoxicity was calculated as described in "In vivo cytotoxicity assays, materials and methods" ***$p < 0.001$.

5.2.2 WT1-specific T cells exhibit antigen specific ex-vivo proliferation in the absence of prior in vivo vaccination

The function of WT1-specific T cells was studied further with ex-vivo proliferation assays. A detailed description of these assays is given in Chapter 2, Materials and Methods. Briefly, splenocytes from TCR td HSC transplant recipients (A2Kb to A2Kb) were harvested 11 weeks post-transplant, labelled with CFSE and placed in culture. Relevant or irrelevant peptide was added to the wells and the cells were kept in culture, in the dark for 5 days. On day 5, the cells were stained with human anti-Vβ2/Vβ13 as appropriate and FACS analysed for CFSE dilution in Vβ positive and Vβ negative lymphocyte populations indicating proliferation.

The left panel in figure 5.2A shows a representative FACS histogram comparing the CFSE dilution in gated Vβ2 positive and Vβ2 negative lymphocytes in splenocytes of transplant recipients receiving WT1-TCR td HSCs. In all subjects, consistently more than 80% of the WT1-specific T cells present in the ex-vivo culture on day 5 had proliferated (figure 5.2B). This was an antigen-specific response as no proliferation was noted among the Vβ2 negative lymphocytes. In contrast to WT1-specific T cells, LMP2-specific T cells did not proliferate upon stimulation with their cognate antigen and this is illustrated in the right panel histogram of figure 5.2A. The dot plot in figure 5.2B summarises the results of these two experiments. Figure 5.2C shows representative CFSE dilution FACS plots of gated lymphocytes from the CFSE labelled splenocyte cultures of A2Kb WT1-TCR transplant recipients stimulated either with the pWT126 or an irrelevant peptide, confirming that no proliferation

was noticed in the presence of the latter. Interestingly, it appears that WT1-specific T cells expressing either high or low levels of the WT1-TCR, as identified by the human anti-Vβ2 antibody, proliferate. It is tempting to assume that these two populations correspond to the TCRlo and TCRhi WT1-specific T cells noted on FACS analysis directly ex-vivo and which exhibited different activation phenotypes (memory and naive respectively). While this remains a possibility, the fact that the two WT1 specific T cell subsets were stimulated ex-vivo with their cognate antigen together, in the same culture, makes it impossible to trace any subsequent phenotypic changes in each of the two WT1 specific T cell populations. What is reasonable to deduce from these data is that both TCRlo and TCRhi cells are able to proliferate in an antigen specific manner.

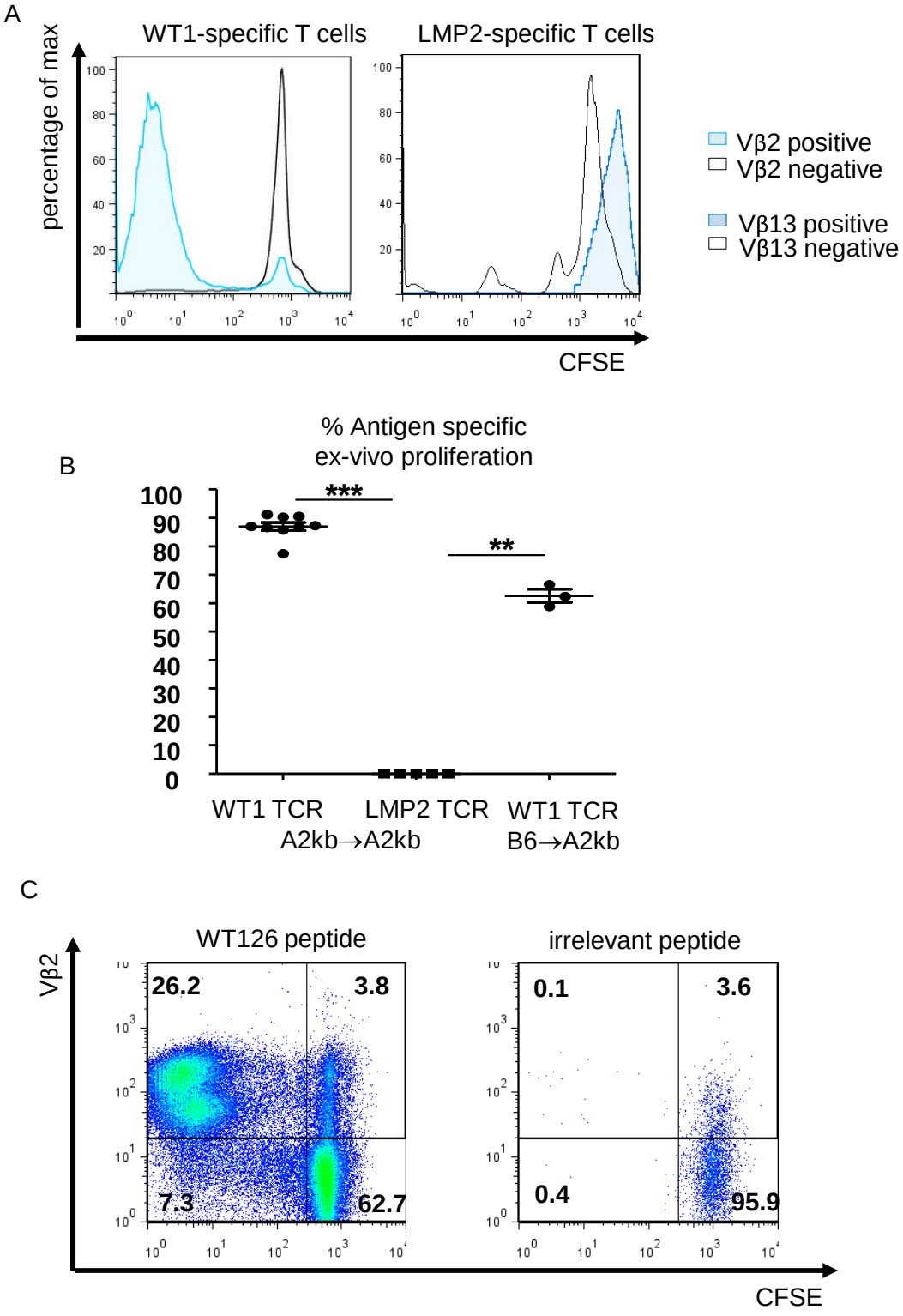


Figure 5.2 WT1 specific T cells exhibit antigen-specific ex-vivo proliferation. Ex vivo proliferation of splenocytes from mice previously transplanted with WT1-TCR and LMP2-TCR-transduced A2Kb or B6 BM stem cells. Splenocytes were stimulated for 5 days with 100μM relevant or irrelevant peptide. CFSE-labeled splenocytes were analyzed by FACS for CFSE dilution after anti–human Vβ2.1 or Vβ13 antibody

Figure 5.2 (continued)

staining. A. Representative FACS proliferation histograms of gated Vβ2 positive and negative lymphocytes (left) and Vβ13 positive and negative lymphocytes (right). Both A2Kb BM stem cell recipients. B. Summary data of ex-vivo proliferation assays using splenocytes harvested from mice transplanted with WT1-TCR (n=8 A2Kb→A2Kb, n = 3 B6 → A2Kb) and LMP2-TCR (n = 5 A2Kb → A2Kb) transduced BM stem cells **p-value < .01, ***p-value < .001. C. Representative FACS plots of gated, CFSE labelled lymphocytes from splenocytes of A2Kb WT1 TCR td HSC transplant recipients, on day 5 of ex-vivo proliferation assay. Left panel, cells stimulated with pWT126. Right panel, cells stimulated with irrelevant peptide.

5.2.5 WT1-specific T cells produce IL-2 and IFN-γ in an antigen specific manner

Antigen specific cytokine secretion was examined in WT1 and LMP2-specific T cells generated in mice that received WT1 or LMP2-TCR td A2Kb HSCs respectively. As described in chapter 2, materials and methods, IL-2 and IFN-γ production following the ex-vivo relevant and irrelevant peptide stimulation of splenocytes from WT1 and LMP2-TCR td HSC recipients, was measured by ELISA. The IL-2 and IFNγ ELISAs were performed on cell culture supernatant from each condition set up for the ex-vivo proliferation assays (section 5.2.2). As for the ex-vivo proliferation assay, cytokine secretion was measured on day 5 and the results were in agreement with the results of the killing and proliferation assays, figure 5.3A and 5.3B demonstrate that antigen specific IL2 and IFNγ production was only noted in the case of WT1-specific T cells being stimulated with their cognate peptide. LMP2-specific T cells did not however produce IL-2 or IFN-γ after stimulation with their cognate antigen, consistent with non-primed, naive T cells.

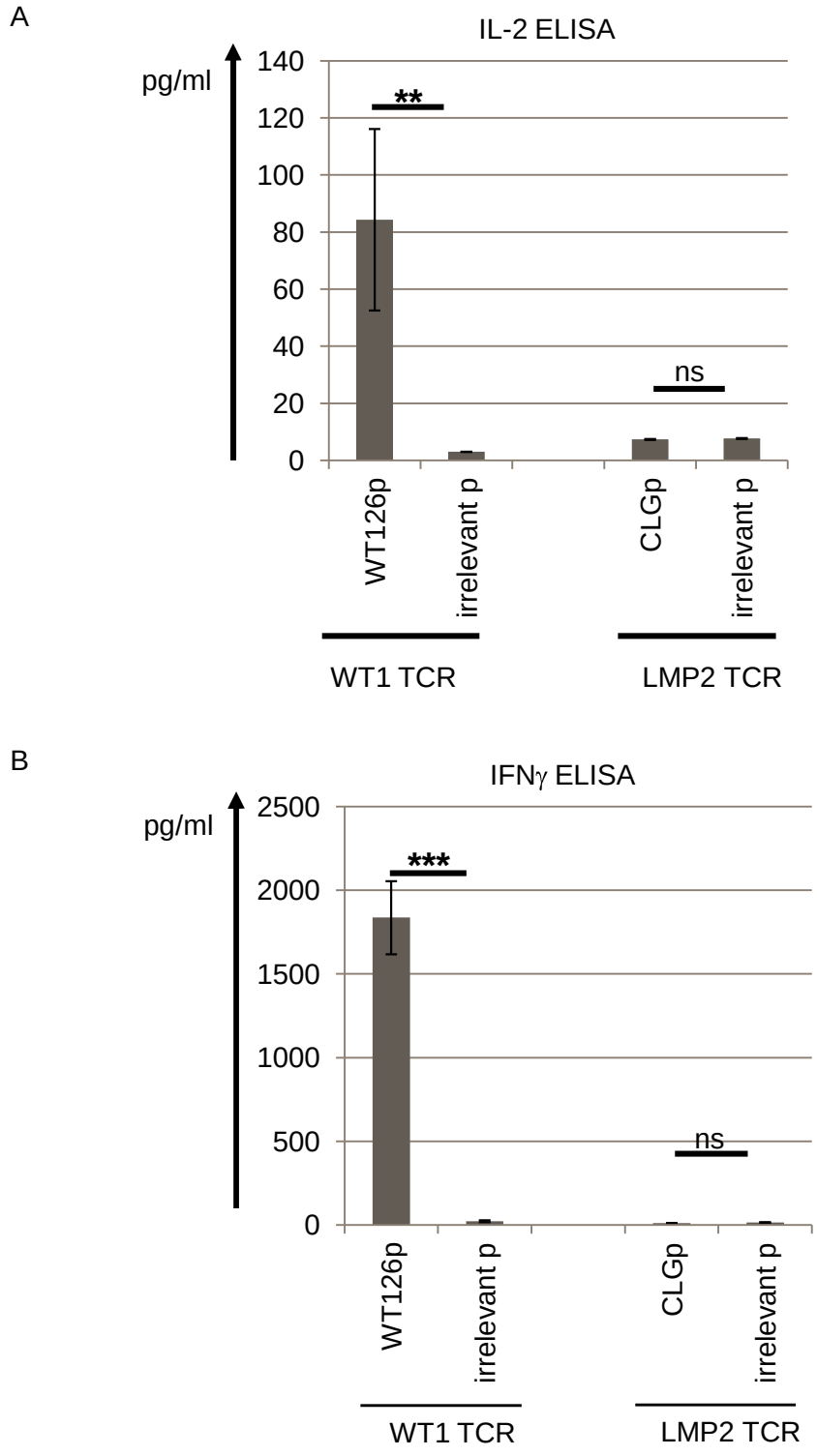


Figure 5.3 WT1 specific T cells show antigen-specific cytokine secretion. Splenocytes harvested from A2Kb mice transplanted with WT1-TCR-transduced (n=8) and LMP2-TCR-transduced (n=8) A2Kb BM stem cells were stimulated ex vivo with 100μM relevant or irrelevant peptide for 5 days. ELISAs were performed to detect antigen-specific IFN-γ and IL-2 secretion. ****p-value* < .001, ***p-value* < .01, ns indicates not significant.

5.2.6 WT1 specific T cell memory-phenotype subset exhibits functional activity without prior vaccination

The data described above demonstrate that the phenotypic differences between WT1 and LMP2-specific T cells described in chapter 5 were associated with equally dramatic differences in the functional activity of these two specificities in the absence of prior vaccination. Among WT1-specific T cells two phenotypically different subsets were observed, a TCRhi and a TCRlo subgroup. Interestingly, only TCRhi LMP2-specific T cells were noted and their activation phenotype was similar to the WT1-TCRhi T cells which were predominantly of naive phenotype. Due to this similarity, it was hypothesised that the function of WT1-TCRhi cells would be similar to that of LMP2-specific T cells, leaving the WT1-TCRlo subset responsible of the functional activity demonstrated by WT1-specific T cells (sections 5.2.1-5.2.5).

Ideally, the two WT1 specific T cell subsets would have been FACS sorted prior to ex-vivo proliferation and cytokine secretion assays in order to delineate their functional activity. However, due to the small cell numbers FACS sorting was technically difficult at the time the experiments were performed and an alternative approach was employed.

To further investigate the in vivo function of WT1 specific memory phenotype T cells, adoptive T cell transfer experiments were performed to generate mice lacking naive phenotype cells. T cells were purified from mice that were previously transplanted with WT1-TCR HSCs and then adoptively transferred (AT) into irradiated secondary A2Kb Tg recipients. The secondary recipients did not contain any WT1-TCR td HSCs, and were therefore unable to generate

“new”, WT1-specific T cells in the thymus. It was therefore expected that the naive phenotype WT1-specific T cells would eventually be lost whilst the memory phenotype WT1 specific T cells would persist. A schematic diagram of the experimental set up is shown in figure 5.4.

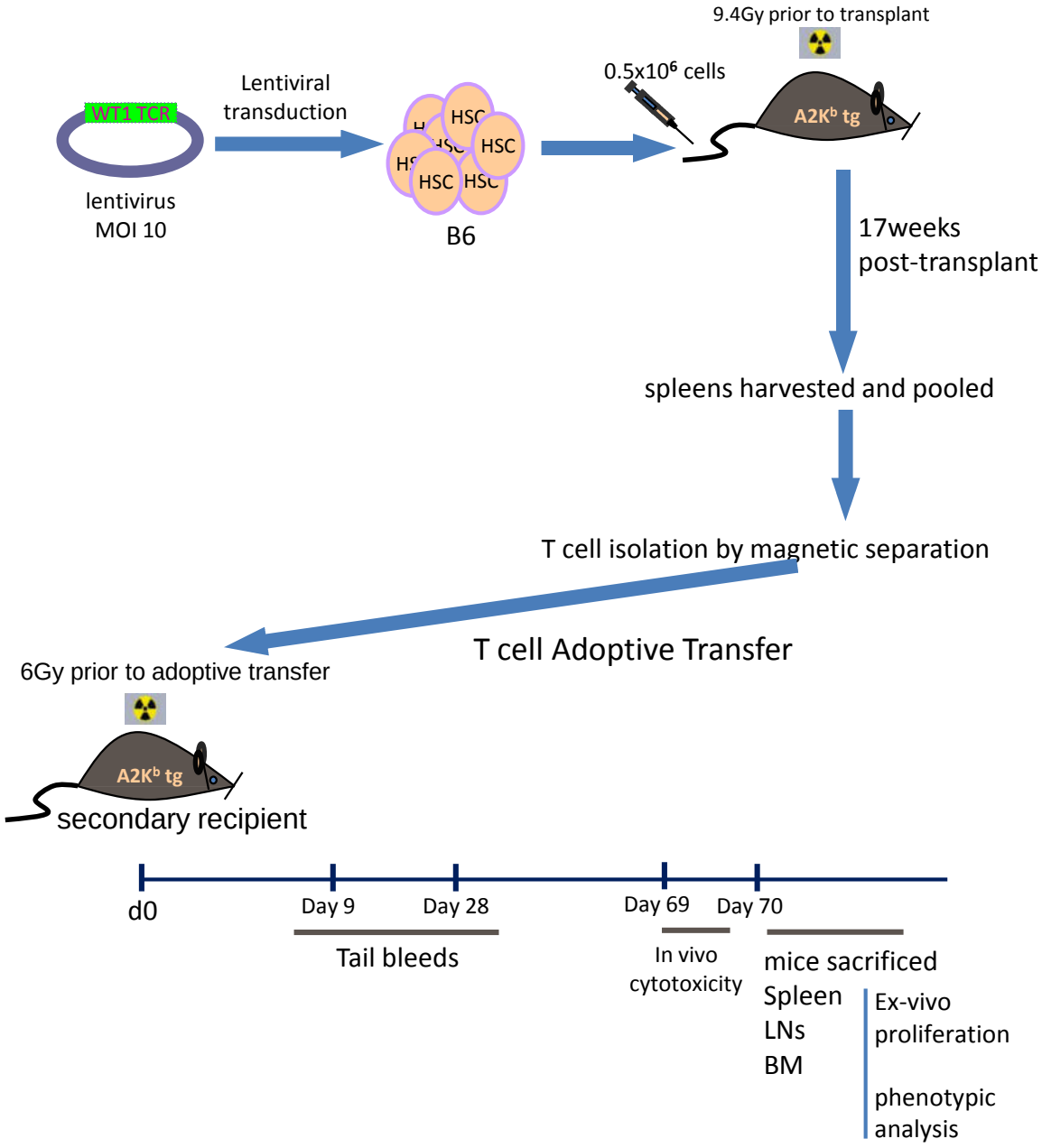


Figure 5.4 Schematic representation of WT1 specific T cell Adoptive Transfer Experiment. WT1 specific T cells were generated in vivo in primary HSC transplant recipients receiving WT1 TCR td , B6 BM stem cells. 11 weeks post-transplant the primary recipients were sacrificed and spleens were harvested. Single cell splenocyte suspensions from 3 primary recipients were pooled together and T cells were isolated by magnetic separation (pan-T cell isolation kit, Miltenyi Biotech). 4 secondary recipients received 5Gy of irradiation one day prior to the transfer. Each recipient received $3x10^6$ cells,. Tail bleeds were performed on day 9, and 28 post AT and on day 69 and 70 in vivo cytotoxicity assays were performed as described in materials and methods. Splenocytes from the secondary recipients were used in ex-

Figure 5.4 (continued)

vivo proliferation assays and phenotypic analysis of WT1 specific T cells. LNs and BM were also harvested for phenotypic analysis.

Tail bleeds were performed at day 9 and day 28 post-AT to monitor donor T cell and in particular WT1 specific T cell engraftment over time. The column graph in figure 5.5 shows that the mean percentage of WT1-specific T cells in the donor CD8 T cell compartment increased over time. While only 13.2% percent of the CD8 T cells transferred into secondary recipients were Vβ2 positive (day 0, cells were FACS analysed prior to AT), by day 9 their percentage among donor CD8 T cells increased to 28.4% and then to 37.9% on day 28 in peripheral blood samples. These percentages remained similar 10 weeks post-AT, in both spleens and LNs of secondary recipients. While the lymphopaenia-driven proliferation of transferred cells unquestionably contributes to the initial expansion of WT1-specific T cells along with all other donor T cell specificities, WT1-specific T cells exhibited improved engraftment. This may have been because WT1-specific T cells proliferated more in response to cognate antigen, in addition to surviving better due to their memory phenotype and function. As in the primary recipients some enrichment for WT1-specific T cells in the donor CD8 T cell compartment was observed in the BM compared to spleen and LNs (Chapter 4, section 4.2.6).

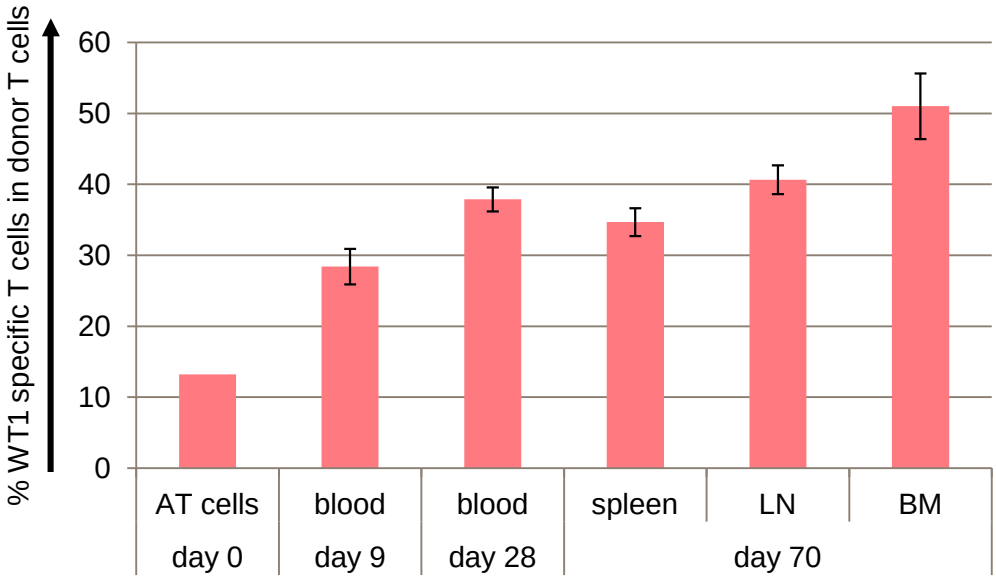


Figure 5.5 WT1 specific T cells display preferential engraftment in the A2Kb Adoptive Transfer recipients. Column graph summarising the percentage of WT1 specific T cells in the donor T cell compartment in secondary AT recipients (tail bleeds n=4, spleen, LNs and BM n=3). First column shows the percentage of WT1 specific T cells in the T cells used for AT, as these were FACS analysed prior to their transfer in the secondary recipients. Second and third columns show the percentage of WT1 specific T cells in donor T cells in peripheral blood samples taken on day 9 and day 28 post-transfer. Last 3 columns show the same percentages in the spleen, LNs and BM 70 days post-AT. Results shown are mean ± SEM.

The FACS plots in figure 5.6A look at the TCR/CD8 phenotype of the adoptively transferred cells, at the time of the transfer and 10 weeks later, confirming that whilst both TCRhi and TCRlo WT1-specific T cells were transferred into the secondary A2Kb recipients, only the latter were present at the time the functional assays were performed. The TCR/CD8 phenotype "switch" of the adoptively transferred WT1-specific T cells, was justified as the secondary recipients were unable to generate new naive WT1-specific T cells. It is likely that this was in part driven by the lymphopenic environment in which the Vβ2 positive and negative T cells were transferred. However, comparing the activation phenotype of WT1-specific T cells and Vβ2 negative, polyclonal donor T cells, revealed that the former converted to the memory phenotype more efficiently. The relevant representative FACS plots, illustrating the CD62L, CD44 phenotype of Vβ2 positive and negative cells in LNs, spleen and BM are shown in figure 5.6B. Consequently it was concluded that the WT1-self specificity contributed to the shift of WT1-TCRhi to TCRlo cells and naive to central memory phenotype, over and above the effects of homeostatic proliferation in a lymphopenic environment.

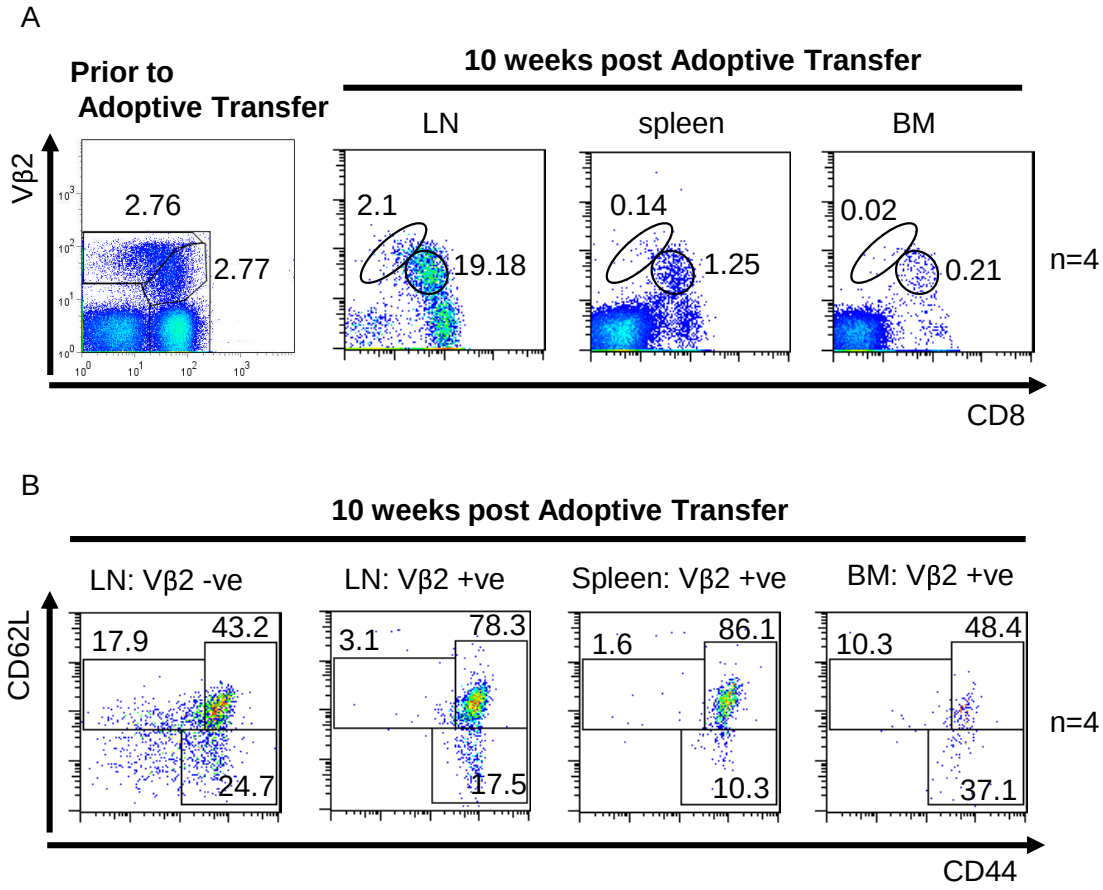


Figure 5.6 WT1-specific memory phenotype T cells display antigen specific effector function. T cells were purified from A2Kb**,** mice transplanted with WT1 TCR td B6 BM stem cells and used for adoptive transfer into irradiated secondary A2Kb Tg recipient mice. A. At 10 week post-AT, splenocytes, LNs and BM were harvested and stained with anti-human Vβ2 and anti-murine CD8 antibodies and FACS analysed to examine the TCR/CD8 phenotype of the adoptively transferred WT1 specific T cells. Far left plot shows the TCR/CD8 phenotype of the T cell, after magnetic separation and prior to AT. Remaining 3 plots show the TCR/CD8 phenotype of gated donor T cells in LNs, spleen and BM..B. 10 weeks post-AT cells the harvested splenocytes, LNs and BM were stained with anti-human Vβ2 and anti-murine CD8, CD44 and CD62L and FACS analysed to study the activation phenotype of Vβ2 positive and negative donor T cells at these sites. FACS plots show the CD44 CD62L phenotype of gated donor Vβ2 positive and negative T cells as indicated. Representative FACS plots are shown.

The phenotypic analysis of the WT1-specific T cells adoptively transferred into secondary recipients, confirmed that these mice were suitable to assess the functional activity of the TCRlo WT1 specific T cell subset. Therefore, in vivo cytotoxicity and ex-vivo proliferation assays were performed 10 weeks post-AT, using the same methods as in the primary recipients. The graph in figure 5.7A shows the results of in vivo cytotoxicity assay, which were comparable to the corresponding data obtained from the primary WT1 HSC transplant recipients. WT1 specific memory T cells, also exhibited antigen-specific ex-vivo proliferation as demonstrated by the representative histogram in figure 5.7B comparing CFSE dilution in gated Vβ2 positive and negative lymphocytes. The representative FACS plots in figure 5.7C. demonstrate marked CFSE dilution among the Vβ2 positive lymphocytes in the presence of the WT126 but not after stimulation with an irrelevant peptide.

These results clearly show that the CD44hi CD62Lhi memory phenotype T cell population was capable of displaying antigen-specific effector function without prior vaccination. When considered in the context of an observed lack of functional activity of LMP2-specific T cells which exhibited a TCRhi, CD44lo CD62Lneg naive phenotype, these results suggest that the TCRhi, naive phenotype WT1-specific T cells would not exhibit the rapid antigen specific functional activity shown by the TCRlo subset.

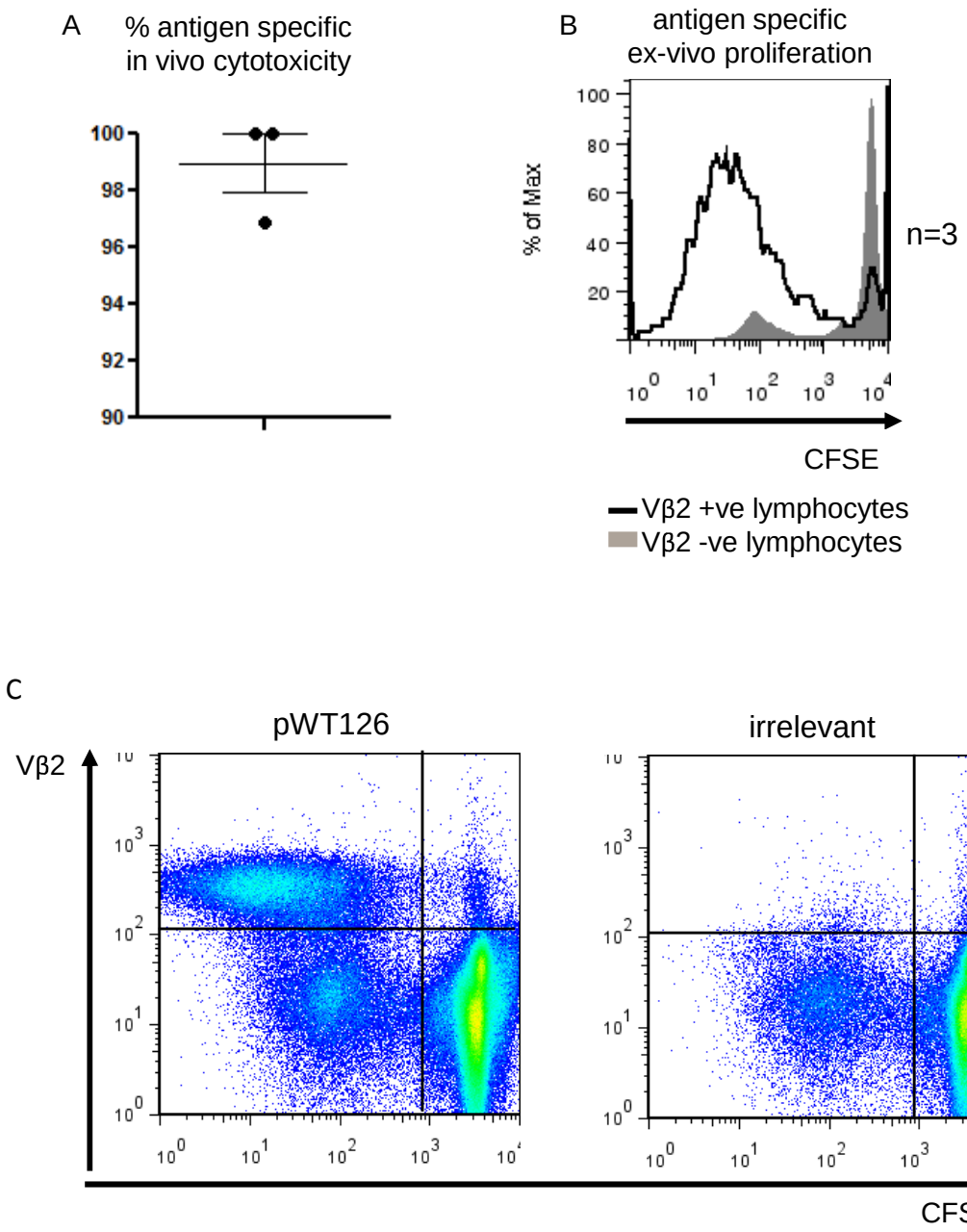


Figure 5.7 WT1 specific memory T cells exhibit antigen specific function. A. WT1 specific T cells show antigen specific in vivo cytotoxicity activity. On day 69 post-AT transfer, secondary A2Kb recipients (n=3) were injected with CFSE labelled peptide loaded target cells, using the same procedure as for primary recipients (section 6.1). Graph shows percentage antigen specific cytotoxicity calculated as described in materials and methods. B. Ex-vivo proliferation assay. On day 70 post-AT splenocytes were harvested from secondary recipients, CFSE labelled and placed in culture with 100μM of WT126 or an irrelevant peptide. 5 days later these cells were stained with human anti-Vβ2 antibodies to identify WT1 specific T cells.

Figure 5.7 (continued)

Representative FACS histogram showing CFSE dilution in gated Vβ2 positive and negative lymphocytes on day 5. C. Ex-vivo proliferation assay. Representative FACS plots show gated lymphocytes from splenocyte cell cultures from AT recipients, containing either the pWT126 (left panel) or an irrelevant peptide (right panel).

5.2.7 WT1-specific T cells do not cause any autoimmune damage in tissues physiologically expressing low levels of the WT1 antigen

Since WT1 specificity did not correlate with thymic deletion or peripheral T cell tolerance, and in fact a subset of WT1 specific T cells was driven to differentiate towards a fully functional, memory phenotype population, investigating whether these cells were able to cause autoimmune damage was relevant. Two sites of WT1 Ag expression in healthy tissues are the BM progenitor cells and the renal podocytes of kidneys. Bone Marrow and kidney from A2Kb recipient mice that received WT1-TCR td HSCs were harvested and analysed by histopathology to detect autoimmune damage relating to T cell tissue infiltration. The same organs were harvested from mice that received LMP2-TCR td HSCs and stained in the same way to control for any tissue damage caused by the experimental conditions. Histological analysis performed by Dr. Emma Morris revealed no autoimmune damage or T cell infiltration in the tissues examined, irrespective of whether these were harvested from WT1- or LMP2-TCR td HSC transplant recipients (data not shown).

In view of the preferential enrichment of WT1-specific T cells in the BM of transplanted animals, it was important to further investigate any potential damage on the stem/progenitor BM cells known to be expressing the WT1 Ag, To do this BM was harvested from WT1-TCR transplanted and control animals and transferred into myeloablated secondary recipients in order to monitor their long-term haematopoietic reconstitution. Tail bleeds were performed at 3, 5, 8 and 12 weeks post-transplant and the blood samples obtained were

stained with murine anti-CD11c, anti-B220 and anti-CD3 antibodies to identify cells of the granulocyte, B cell and T cell lineages respectively and murine anti-CD45.1 to distinguish between donor and recipient derived cells. The results obtained are summarised in the column graph in figure 5.8. This graph shows the percentage of donor derived cells in gated granulocytes, B cells and T cells at the indicated time points. The dark blue columns represent the percentage donor reconstitution in the secondary recipients receiving a BM transplant from control, untreated animals (n=5), whereas the light blue columns represent the same data obtained from secondary recipients receiving BM cells from the primary recipients of the WT1-TCR td A2Kb HSC transplants. The graph demonstrates that the presence of WT1-specific T cells in the BM did not impair the ability of BM stem/progenitor cells for haematopoietic reconstitution. However, unlike donor derived B cell and granulocyte reconstitution, T cell engraftment was significantly slower in the experimental mice compared to the control. It is possible that the altered T cell reconstitution kinetics was a consequence of the lentiviral vector-driven WT1-TCR expression in developing thymocytes. The best control for this would have been a serial BM transplant from primary recipients of LMP2-TCR td HSC transplant.

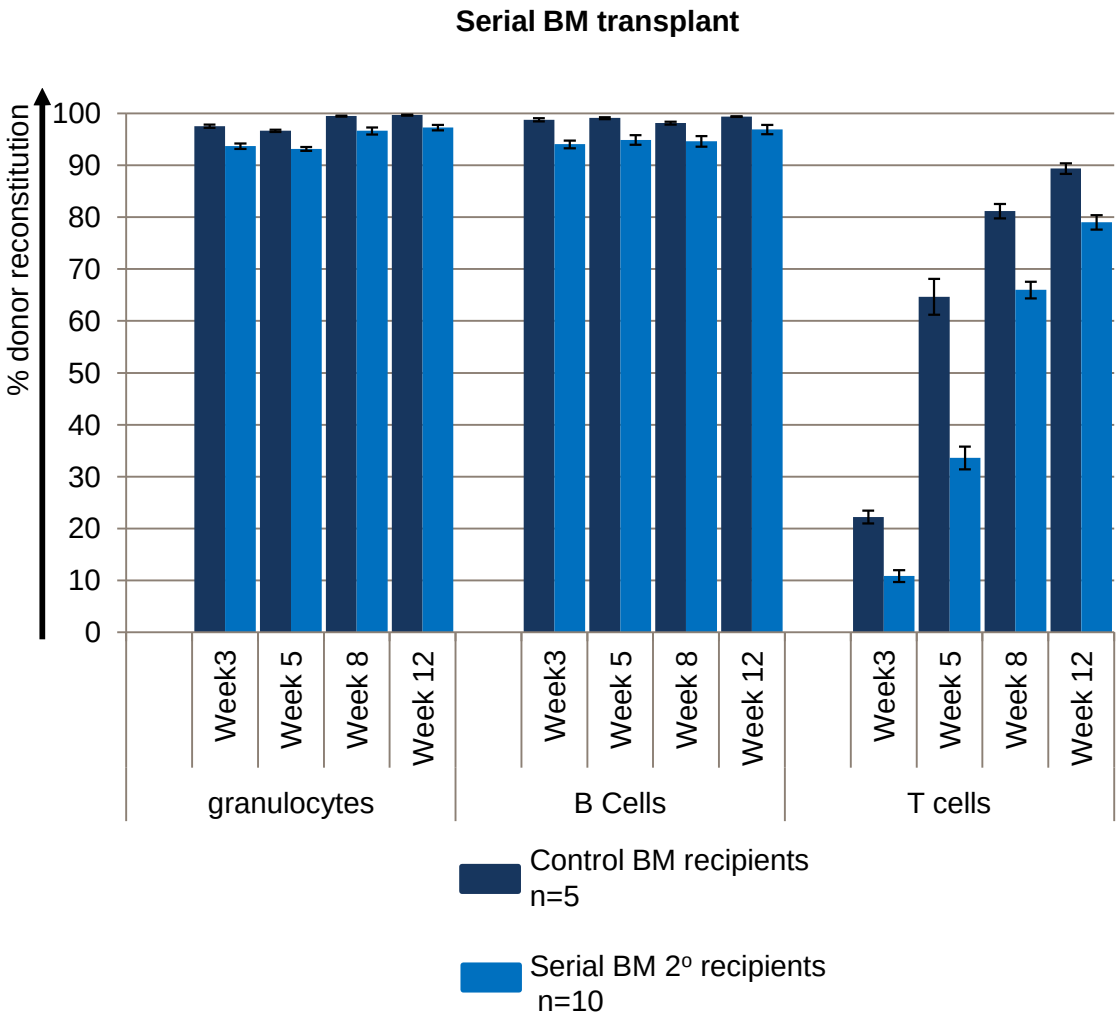


Figure 5.8 WT1 specific T cells accumulating in the BM do not impair stem cell function. Hematopoietic engraftment in secondary C57Bl/6 recipients (CD45.2) after transplantation of BM cells from A2Kb Tg mice that were previously transplanted with A2Kb BM stem cells (CD45.1) transduced with the WT1-TCR (n = 10). Peripheral blood of secondary recipients was stained with antimurine CD45.1, CD3, B220, and CD11b to identify donor hematopoietic cells, donor T cells, donor B cells and donor granulocytes, respectively. The peripheral blood analysis was done at weeks 3, 4, 8, and 12 after second transplantation. Control C57Bl/6 recipients received BM stem cells from untreated A2Kb (CD45.1) Tg mice (n = 5). Percentage of donor-derived cells are shown after gating on total granulocytes, B cells, and T cells, respectively. **p-value* < .05, ***p-value* < .01, ****p-value* < .001. ns indicates not significant.

5.3 Discussion

In this chapter, the functional activity of WT1-specific T cells was analysed. In agreement with their phenotype, these cells exhibited antigen-specific in vivo killing and ex-vivo proliferation and cytokine secretion. This was clearly related to their activation phenotype rather than simply their specificity, as LMP2-specific T cells which in another study were shown to have antigen-specific functional activity when activated (Hart et al., 2008), had a naive phenotype in our system and did not respond to their cognate Ag in the absence of prior Ag exposure.

The use of AT experiments from the primary WT1-TCR td HSC transplant recipients, to secondary A2Kb recipients allowed us to study the functional activity of Tcm, TCRlo WT1-specific T cells in particular. The results obtained confirmed that this population indeed had antigen-specific functional capacity. However, in the absence of any data from functional assays on TCRhi WT1-specific T cells we can only speculate that this population did not contribute to the functional activity of the WT1-specific T cells in the primary transplant recipients.

Finally, despite their surprising phenotype and function, WT1-specific T cells did not appear to cause any autoimmune damage at sites of low level physiological WT1 Ag expression.

This work and its implications are discussed in detail in the following chapter.

Chapter 6 - Discussion

In this project, TCR gene transfer into HSCs was employed to investigate the thymic development and peripheral function of T cells specific for the TAA WT1. WT1 has been the target of many T cell based immunotherapies, including vaccination approaches, WT1-specific T cell AT and TCR gene transfer and is regarded a very attractive target for such therapies for a number of reasons. WT1 is overexpressed in many tumours and haematological malignancies but only expressed at low levels in a few healthy tissues. Thus, WT1-specific immunotherapies can be applied to the treatment of a variety of cancers. Furthermore, studies have shown that WT1 expression in transformed cells is not only involved in oncogenesis (Yamagami et al., 1996, Algar et al., 1996), but also in tumour metastasis (Barbolina et al., 2008). This makes it unlikely that WT1 expression will be switched off as a tumour evasion mechanism.

While many of the WT1-specific T cell therapies are already being tested in clinical trials, the very fact that WT1 is a self-Ag raises questions around the issues of T cell tolerance and autoimmunity. In order to study these two opposing phenomena, an in vivo humanised murine model was developed. Murine BM cells were enriched for HSCs and transduced with the genes for the high avidity, HLA-A2 allorestricted WT1-TCR. Transferring these cells into myeloablated HLA A2 Tg recipients provided T cell progenitors that expressed the introduced TCR on the cell surface when in the thymus, following assembly with the CD3 molecule. The WT1 Ag expression pattern in mouse is similar to that seen in humans and the cognate Ag for this particular WT1-TCR, the

pWT126, is identical in the two species. Therefore in the HLA A2 Tg recipients, high-avidity WT1-specific T cells would be expected to go through thymic development - possibly be susceptible to central tolerance mechanisms - and subsequently circulate in the periphery of a mouse exhibiting a WT1 Ag expression pattern similar to that seen in humans.

6.1 TCR td HSC transplants preferred over TCR Tg mice for the in vivo study of WT1-specific T cells

Utilising TCR td HSC transplants recipients instead of TCR Tg mice was preferred as in this setting, the introduced gene inserts at different, distinct sites in each cell. While there is still a bias towards certain sites, the fact that the transgene does not insert at a single genomic site prevents founder effects that cannot be excluded in transgenic mice (Bettini et al., 2012). Another important advantage of this model was that the WT1-TCR expressing T cells were expected to represent only a proportion of the whole T cell repertoire. As the focus of this study was to elucidate the effect of physiological self-Ag expression on the phenotype and function of high avidity WT1-specific T cells, it was important that this was not the only T cell specificity generated. It was therefore possible to compare the development and differentiation of the WT1-specific T cells to that of the endogenous polyclonal T cell repertoire. Furthermore, Leitao et al have shown that inter- as well as intra-clonal T cell competition for p:MHC complexes shapes both the naive and activated/memory T cell pool as it affects the relative expansion of one specificity over another (Leitão et al., 2009). Therefore in order to strengthen the argument that any effects observed among WT1-specific T cells were

driven by specificity for their cognate self-Ag rather than by homeostatic expansion, it was desirable to study the fate of these cells in the presence of a polyclonal T cell population competing for self p:MHC complexes possibly including the pWT126:MHC complex. Indeed, in our experimental model both WT1 and LMP2-specific T cells formed less than 10% of total CD8 T cells.

Importantly, this experimental system also provided us with the means to study the effect of WT1 Ag expression on WT1-specific T cells in the absence of BM-derived, professional APCs. Experiments addressing this issue were carried out by transducing B6 HSCs with the genes for the WT1-TCR and subsequently transferring them into lethally irradiated A2Kb Tg mice. In this way, the haematopoietic system, including professional APCs, was reconstituted with B6 cells unable to present the pWT126 on HLA A2. At the same time the A2Kb thymic epithelial cells of the recipient animals, being radio-resistant could still mediate the thymic development of the HLA-A2 restricted WT1-specific T cells.

Haematopoietic stem cells can rescue lethally irradiated recipients by fully reconstituting haematopoietic function (Rekers et al., 1950, Lorenz et al., 1951). As they also have the ability to self-renew, they can provide long-term haematopoietic reconstitution. In mice these cells are found in the Lin-ve Sca1+ve BM compartment (Uchida and Weissman, 1992). Therefore, BM cells were enriched for HSCs by magnetic separation. Lin-ve BM cells, found in the untouched negatively selected fraction of the magnetic separation, were used in all experiments. Long-term donor derived haematopoietic reconstitution was achieved both in the primary HSC transplants, as well as in serial BM

transplant experiments where lethally irradiated syngeneic recipients received BM from the primary HSC transplant recipients. This verified that HSCs were present in the small number of Lin-ve, lentivirally transduced Lin-ve BM cells.

Donor haematopoietic reconstitution in transplant recipients receiving WT1-TCR td HSCs was confirmed and the final steps in setting up the experimental model involved demonstrating WT1-TCR cell surface expression to be exclusively found on T cells. Despite lacking a regulatory element dictating transgene expression to the CD8 T cell lineage, this was expected because TCR surface expression is dependent on the correct assembly of the TCR-CD3 multimeric complex. Since CD3 gene expression is only switched on in developing thymocytes, the introduced TCR can only appear on the surface of T cells. Indeed FACS analysis of CD3 positive and negative lymphocytes in WT1-TCR HSC transplant recipients showed that human Vβ2 expression - used to identify WT1-TCR expression - was only present in CD3 positive lymphocytes. Since the WT1-TCR is a class I restricted TCR, WT1-specific T cells generated in this experimental system committed to the CD8 T cell lineage. LMP2-specific T cells, generated in the same way using the class I restricted, LMP2 specific TCR genes, followed the same pattern.

6.2 WT1-specific T cells escape thymic deletion

The emergence of WT1-specific T cells into the periphery of WT1-TCR td HSC transplant recipients indicated that physiologic low level WT1 Ag expression in a few tissues was not linked to complete thymic deletion of developing thymocytes bearing this specificity. Analysis of developing thymocytes, showed similar frequencies of CD3 positive cells expressing the WT1-TCR at the DP

and CD8 SP stages of T cell development, excluding the possibility that a percentage of WT1 specific developing T cells could be the subject of negative selection.

Thymic expression of the WT1 Ag has been reported in some studies. In our lab high levels of WT1 expression have previously been observed only in the mesothelial lining but not in the epithelial stroma of the thymus. Therefore, if this self-Ag is not expressed in the epithelial thymic compartments mediating negative selection, WT1 specific thymocytes would be expected to go through thymic development unaffected by central tolerance. However, while the WT1 expression pattern in adult life is limited to renal podocytes, testicular Sertoli cells, ovarian granulosa cells and a small subset of haematopoietic progenitors, it is still possible that WT1 may be expressed in the thymus. More than a decade ago, Klein et al using the inducible inflammatory protein CRP which is only expressed in the presence of inflammation, demonstrated that the thymic expression of peripheral self-Ags was independent of the time at which they are expressed in the periphery (Klein et al., 1998). With this in mind and the fact that WT1 is extensively expressed in embryonic life, it would be reasonable to expect that WT1 will be one of the many peripheral Ags expressed in the thymus with the purpose of shaping a non-autoreactive T cell repertoire. Furthermore, in a detailed and definitive study looking at PGE in the different cell subsets of the thymus, Derbinski et al showed that this was a property of mTECs and any particular peripheral Ag was only expressed in 100-1000 mTECs per thymus while not all mTECs exhibited PGE (Derbinski et al., 2001, Derbinski et al., 2008). Therefore, it is possible that WT1 is only expressed in the thymus at low levels in a few mTECs but is not readily

detectable. Nevertheless, such a low-level expression profile in the thymus may still be adequate to induce central tolerance against WT1 specific thymocytes.

Interestingly, even though WT1-specific T cells were not susceptible to thymic deletion, CD3 downregulation was observed in this cell population in comparison to endogenous donor-derived T cells. Since LMP2-specific T cells, generated in control LMP2-TCR td HSCs transplant experiments displayed CD3 expression levels similar to the endogenous T cells, this was not an experimental artefact related to the lentiviral transduction. As TCR surface expression is coupled to that of the CD3 molecule, the CD3 MFI was considered to be a surrogate marker for TCR expression levels. Back in the early 1990s when it was still thought that the expression of self-Ags in the thymus was only derived from either thymic proteins or Ags circulating in the bloodstream carried by professional APCs, Schonrich et al described TCR downregulation as a mechanism of peripheral T cell tolerance. So it was perhaps somewhat surprising that the CD3/TCR downregulation observed in WT1-specific T cells in our project proved to be a thymic event persisting in the periphery rather than only a peripheral event. In their study, Schonrich et al used a double Tg model where the Tg TCR was specific for an experimental-Ag expressed under a brain-restricted promoter. While they confirmed that this Ag was not expressed in the thymus by PCR and Southern Blotting on cDNA from whole thymus, it remains possible that low level Ag expression in only a small number of mTECs was present but not detected (Schönrich et al., 1991).

Regardless of whether TCR downregulation takes place in the periphery or the thymus, it is a well accepted tolerance mechanism. For this reason, the observed CD3/TCR downregulation in WT1-specific T cells in our experiments was likely to be either a central tolerance process alternative to clonal deletion, or the mechanism by which these cells managed to evade negative selection in the thymus. With these two possibilities essentially being flip sides of the same coin, it was predicted that following their thymic education, WT1-specific T cells would be poorly functional in the periphery.

6.3 WT1-specific T cells spontaneously differentiate to memory T cells in the absence of vaccination

On this basis, demonstrating that WT1-specific T cells were not anergic but instead differentiated spontaneously into fully functional memory phenotype T cells was not anticipated. Some indirect evidence has been provided by a number of publications that central, as well as peripheral tolerance to WT1-specific T cells is incomplete. Firstly WT1-specific T cells have been detected in both healthy volunteers and leukaemia patients (Rezvani et al., 2003, Scheibenbogen et al., 2002, Rezvani et al., 2005), as well as breast cancer patients (Gillmore et al., 2006). WT1-specific T cells identified in leukaemia patients showed an activated phenotype and antigen-specific IFNγ secretion in response to peptide stimulation and were of greater frequency and magnitude than in healthy controls suggesting that the WT1 TAA is immunogenic rather than tolerogenic. Consistent with the presence of naturally occurring WT1-specific T cells in the human T cell repertoire, WT1 peptide and DNA vaccination trials have resulted in both an immunological WT1 T cell response

and a transient clinical response in a proportion of vaccinated patients (Oka et al., 2004, Keilholz et al., 2009, Rezvani et al., 2008).

The classical paradigm of memory T cell development dictates that naive T cells respond to their cognate Ag and co-stimulation, expanding to form a large Ag-specific effector population, which following antigen clearance contracts. The small number of memory T cells surviving is able to mount an immune response effectively and faster upon a subsequent antigen exposure. Nevertheless, it is now well accepted that memory T cells can also be generated in lymphopenic conditions, driven by homeostatic proliferation (Cho et al., 2000, Goldrath et al., 2000). The current understanding of this phenomenon is that TCR interactions with self p:MHC complexes together with exposure to IL-7 under steady-state conditions provide survival signals to naive T cells. In lymphopenia these signals become mitogenic and promote naive T cell proliferation and differentiation into memory T cells, in the absence of exposure to cognate Ag (Boyman et al., 2009). The development of WT1 specific memory T cells in our system could clearly not be justified by the classic mechanism of memory T cell development, as apart from some limited WT1 self-Ag availability, signals 2 and 3 necessary for T cell activation were absent. On the contrary, LIP-driven memory T cell development does require p:MHC- TCR interaction but not co-stimulation, therefore making this process worth considering as an explanation for the appearance of WT1-specific memory T cells in our model.

In our system WT1-specific T cells were initially generated in a lymphopenic environment, as the WT1-TCR td HSC transplant recipients were lethally

irradiated prior to the transplant. However, the phenotype and function of WT1-specific T cells was studied after haematopoietic reconstitution was achieved in the transplant recipients. At that time around 20% of the endogenous T cell specificities exhibited a memory, CD44hi CD62Lhi phenotype and a similar percentage of LMP2-specific memory T cells were noted in control, LMP2-TCR td, HSC transplant recipients. These results were in agreement with other studies reporting that 15-20% of total CD8 T cells in unimmunised, steady-state mice are comprised of memory phenotype cells, present even in mice housed under germ-free conditions (Haluszczak et al., 2009, Huang et al., 2005). In fact, Haluszczak et al isolated foreign-antigen specific CD8 T cells from unprimed mice and showed that 10-30% of these very rare T cell specificities exhibited a memory phenotype. Having controlled for and excluded the possibility that this was the result of exposure to their corresponding foreign antigens they also showed that these so called “Virtual Memory”, antigen-specific T cells had the phenotypic signature of CD8 T cells undergoing homeostatic proliferation. Similar to this study, a proportion of WT1 and LMP2-specific T cells as well as endogenous CD8 T cells exhibit a memory phenotype in non-lymphopenic hosts. However, the percentage of central memory cells in WT1-specific T cells was much higher (mean of 8 recipients= 50% in A2Kb to A2Kb transplant, mean of 8 recipients=70% in B6 to A2Kb transplant), suggesting that even if the initial lymphopenic conditions contributed to the formation of this memory population the self-Ag specificity had an effect over and above that of LIP-driven memory development. Staining the WT1-specific T cells with an antibody against α4 integrin, a molecule Haluszczak et al elegantly showed, can be used to discriminate memory T

cells generated by antigen priming (α4 integrin high) from those developed in response to homeostatic proliferation (α4 integrin low), can in the future be utilised to provide more information on the stimulus driving development of the memory WT1 specific T cell subset.

Kieper et al have shown that a higher TCR affinity for self p:MHC complexes gives naive T cells a competitive advantage for homeostatic proliferation both in lymphopenic and T cell-sufficient conditions (Kieper et al., 2004). The affinity of this interaction is a separate parameter from the TCR affinity for its cognate Ag, and it was determined by the expression levels of the CD5 molecule (Azzam et al., 1998) on the different transgenic specificities used. Relating these findings to our model in which high-affinity WT1-specific T cells developed and circulated in subjects expressing their cognate Ag, can perhaps explain our findings. The hypothesis would be that in our system specificity for the self-Ag WT1 resulted in the preferential homeostatic proliferation of WT1-specific T cells which consequently drove their differentiation into memory T cells. On adoptively transferring WT1 specific and endogenous polyclonal T cells from the primary WT1-TCR td HSC transplant recipients to secondary lymphodepleted A2Kb recipients, the percentage of WT1-specific T cells among total donor T cells increased 3-fold in the first 4 weeks post-AT. In this lymphopenic environment, WT1-specific T cells also converted more efficiently to a memory phenotype than the endogenous specificities undergoing LIP-driven memory differentiation, suggesting again that this self antigen-specificity contributed to the observed memory phenotype differentiation. Labelling the WT1 and endogenous polyclonal T cells with CFSE prior to their transfer into secondary recipients would have allowed us to directly assess the hypothesis

that the WT1 specificity conferred a proliferative advantage in lymphopenic conditions similar to that observed by Kieper et al. Without such direct evidence, it is not possible to distinguish between a proliferative or a survival advantage of WT1-specific T cells, mediating the cells' improved engraftment.

The initial period of lymphopenia, induced by irradiation prior to the TCR td HSC transplants performed in this study can be considered to have an impact similar to that of neonatal lymphopenia. Min et al demonstrated that around 20% of adoptively transferred T cells in newborn mice undergo LIP-driven memory phenotype development (Min et al., 2003). These findings are in agreement with our observations in LMP2 and endogenous polyclonal specificities, but also with the results described by Haluzsczak et al, mentioned above.

The observed WT1-specific memory development in the absence of immunisation is similar to the previously described memory T cell development driven by homeostatic proliferation in the context of irradiation, neonatal lymphopenia, or even steady-state homeostasis in adult subjects. However, the similarity is more evident in the phenotypic profile of LMP2-specific and endogenous polyclonal T cells, rather than with that of WT1-specific T cells. Our data indicate that the spontaneous differentiation and persistence of WT1 specific memory T cells, is over and above any background memory T cell development driven by homeostatic processes. Our hypothesis, illustrated in figure 6.1 is that the chronic stimulation by self-Ag triggers WT1 T cell memory development.

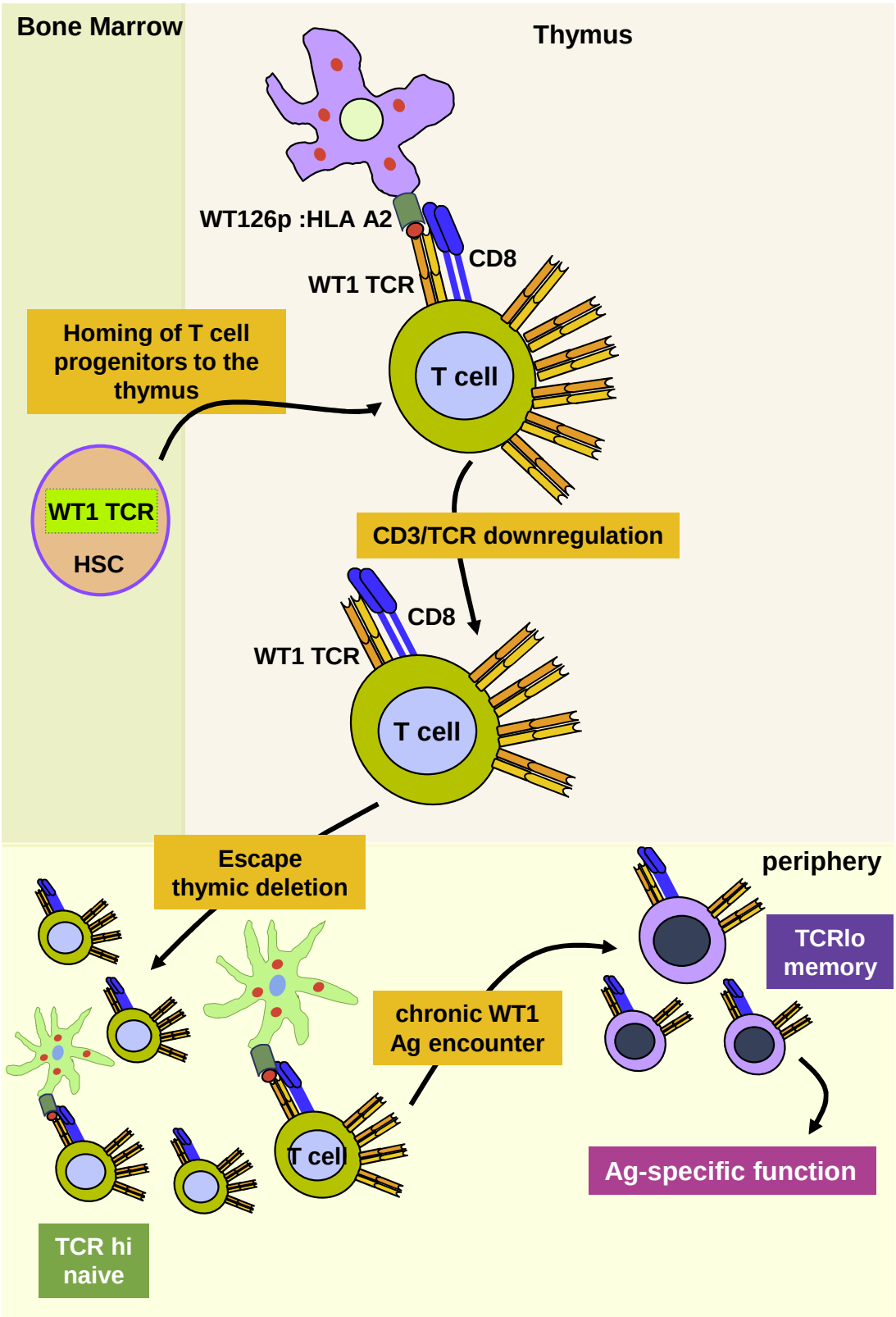


Figure 6.1 Schematic diagram illustrating the TCR triggering hypothesis. WT1 specific T cells escape negative selection but demonstrate a relative CD3 downregulation compared to endogenous developing thymocytes. In the periphery the chronic, low level WT1 Ag presentation results in the activation of a proportion of

Figure 6.1 (continued)

WT1-specific T cells which downmodulate their TCR further. TCR triggering in response to chronic Ag encounter seems to be driving the WT1 specific T cells to differentiate into fully functional memory T cells. As the thymus continuously feeds the periphery with new, naive TCRhi WT1 specific T cells , TCRhi and TCRlo WT1 specific T cell subsets co-exist in the periphery.

To unequivocally test this hypothesis, ideally we would allow the development of WT1-specific T cells in A2Kb mice, as already described in this thesis, and subsequently FACS sort naive WT1-specific T cells to transfer them in 4 different groups of mice. Two of these groups of mice would consist of A2Kb, WT1 knockout animals and the other two groups would consist of A2Kb, as those used throughout most experiments presented in this thesis. The other two groups would also be standard A2Kb, or WT1 knockout A2Kb, but they would also be lymphodepleted with 6Gy of irradiation prior to the AT. With this experimental setup we will be able to ask whether naive WT1-specific T cells are driven to functional memory cells by the interaction with their cognate Ag, both in a lymphopenic and in a T-cell sufficient, steady-state environment. Additional control experiments where polyclonal endogenous, or LMP2 specific naive T cells are adoptively transferred would be useful to assess the hypothesised proliferative and differentiation advantage that naive WT1-specific T cells have in the presence of WT1 Ag. Unfortunately, performing these experiment is not possible as WT1 knockout mice as well as inducible tissue-specific WT1 knockout animals are not viable beyond term (Martinez-Estrada et al., 2010).

A less optimal experiment could be to follow the above setup replacing WT1 knockout A2Kb Tg mice with WT B6 animals. These mice express WT1 and can process and present the pWT126 for which WT1-specific T cells are specific. However, regardless of this antigenic epitope being available in B6 mice, pWT126 will not be presented on the appropriate class I molecule in order to be recognised by T cells expressing the human HLA A2 restricted, WT1-TCR. In this way, the effect that the WT1 Ag has on naive WT1-specific T

cells can be obliterated simply because these T cells will not be able to recognise the Ag. However, the limitation of this experiment, is that unlike using WT1 knockout A2Kb Tg mice, this system cannot be used to exclude the possibility that cross-reactivity with other epitopes presented by the A2Kb molecules drives WT1-specific T cell differentiation into memory T cells rather than encounters with WT1 Ag.

6.4 Memory WT1-specific T cells are produced in the absence of professional antigen presentation

Interestingly, a memory population was present in the periphery of A2Kb recipients receiving WT1-TCR td HSC from B6 donors. In this setting the pWT126 presentation on the HLA A2 molecule is limited to the few non-professional APCs, mainly renal podocytes, which express the antigen at low levels. It is therefore possible that chronic, direct presentation by these cells is enough to mediate T cell memory differentiation. Even in the presence of A2Kb APCs, these cells are able to present the pWT126 epitope but they do so in the absence of co-stimulatory signals. Consequently, whether the WT1 Ag is cross-presented by professional APCs or directly-presented by peripheral tissue cells might not make a difference to the T cell differentiation profile instructed by the high avidity WT1-TCR interaction with its cognate Ag. Of course, this scenario requires that WT1-specific T cells reach the sites were tissue cells express the WT1 Ag when they circulate in the periphery.

Another explanation for observing memory T cells in the periphery of B6 to A2Kb transplant recipients may be that WT1 memory differentiation takes place in the thymus prior to their emergence in the periphery. In the B6 to

A2Kb WT1-TCR td HSC transplants the haematopoietic system is reconstituted with B6 cells unable to present pWT126 on HLA A2, but the recipients' HLA A2 thymic epithelial cells are radioresistant and persist. Therefore they can still mediate the thymic development of the A2-restricted WT1-specific T cells and can also potentially mediate presentation of the WT1 Ag through promiscuous gene expression. All WT1-specific T cells showed marked TCR downregulation compared to both the endogenous polyclonal population and LMP2-specific T cells, as measured by CD3 surface expression levels, and this was shown to be a thymic event persisting in the periphery. However, in the periphery phenotypic analysis of the WT1-TCR and CD8 co-receptor expression revealed 2 distinct subsets of WT1-specific T cells. Memory phenotype WT1-specific T cells mainly belonged to the subset exhibiting the lowest levels of WT1-TCR, named TCR lo. The other subset exhibited relatively higher levels of the WT1-TCR, was named TCRhi, and looked similar to the endogenous polyclonal population. This further TCR downregulation in the WT1-TCRlo T cells was accompanied by a relative upregulation of the CD8 co-receptor in comparison to the WT1-TCRhi T cells. Our hypothesis, as illustrated in figure 6.1, is that WT1-specific T cells encounter the WT1 in the context of HLA A2 in the thymus and this leads to a certain level of TCR/CD3 downregulation. However, WT1-specific T cells escape thymic deletion and emerge from the thymus as naive, TCRhi cells. Further, chronic, low-level WT1 Ag encounter in the periphery subsequently causes further TCR downregulation and mediates their differentiation into fully functional, memory T cells. An experimental limitation remains that it was not possible to distinguish the two WT1 specific populations (TCRhiCD8lo and

TCRloCD8hi) among developing thymocytes in the same way they were identified in the periphery, as the CD8 expression varies at different stages of thymocyte development. Therefore we were unable to answer the question whether the thymus only produced naive, TCRhi CD8 low WT1-specific T cells as expected.

The only reports of memory phenotype CD8 T cells being generated in the thymus concern the so-called bystander memory CD8 T cells which arise in response to IL-4 (Weinreich et al., 2010). This phenomenon is prominent in wild type Balb/c mice, shown to contain increased frequencies of IL-4 secreting NKT cells, but not in B6 mice (Lai et al., 2011). It is therefore relevant that all mice used in our experiments were on a B6 background. The group that reported the existence of Virtual Memory T cells - mentioned above - also studied bystander memory T cells. “Virtual Memory” T cells, like memory WT1-specific T cells in our study, formed only a proportion of each specificity examined. Their site of production, as in our model, was also uncertain until this year, when Akue et al were able to clarify that these cells, were not produced in the thymus, but in the periphery during the neonatal period (Akue et al., 2012). These authors noticed a peak with a subsequent drop and a plateau in “Virtual Memory” T cell frequencies before mice were 4 weeks old. Therefore, in order to more directly address the possibility that WT1 specific T cell memory differentiation was triggered by irradiation-induced LIP, CD44 – CD62L phenotypic analysis of WT1-specific T cells in the periphery as well as in the thymus at 1, 2, 3 and 4 weeks post-transplant in addition to those already performed at 11 weeks when haematopoietic reconstitution was achieved, would have been very relevant.

A third explanation about the appearance of WT1 specific memory T cells in B6 to A2Kb transplant recipients can simply be that since donor chimerism was only nearly complete in these animals, professional WT1 Ag presentation is provided by the few remaining A2Kb APCs. Although possible, such a scenario implies that within this 2-8% of A2Kb APCs persisting in the B6 to A2Kb transplant recipients, enough cells would cross-present the pWT126 to induce the differentiation of similar percentages of WT1-specific T cells to a memory phenotype, as observed when all APCs express HLA A2 (A2Kb to A2Kb transplant). Even though memory WT1-specific T cells were detected in both A2Kb to A2Kb and B6 to A2Kb HSC transplants, the proportions of Tcm and Tem subgroups was not identical. Lower percentages of Tem WT1-specific T cells were noted in B6 to A2Kb transplant suggesting that WT1 Ag presentation by professional APCs was needed to observe the Tem population. It seems unlikely that the difference in the activation phenotype of WT1-specific T cells in the two settings can be explained by different numbers of APCs able to present the pWT126 on HLA A2 rather than an all or nothing effect.

6.5 WT 1 specific T cells accumulate in the BM

Intriguingly, WT1-specific T cells appeared to accumulate in the BM both in A2Kb to A2Kb and B6 to A2Kb transplant recipients. The BM is one of the few sites were the WT1 Ag is expressed at low levels, under physiological circumstances. While in the former setting this phenomenon could be driven by both direct Ag presentation on BM progenitor cells expressing WT1 and cross-presentation by professional A2Kb APCs, neither of these cell types could

mediate Ag presentation in the latter setting. This initially suggested that the BM enrichment of WT1-specific T cells had to be driven by an antigen-independent mechanism.

In 2005 Mazo et al showed that Tcm T cells home preferentially to and are retained in the murine BM and consequently are the predominant subset among CD8 T cells at this site (Mazo et al., 2005). In the same year, another two groups showed that memory T cell, known to proliferate in response to homeostatic cues and independently of antigenic encounter, do so more efficiently in the BM (Parretta et al., 2005, Becker et al., 2005). It was therefore hypothesised that preferential homing and proliferation of the Tcm WT1-specific T cells in the BM could be a reason for the increased frequencies of WT1-specific T cells in this organ in comparison to the periphery. However, our results did not offer a straightforward answer. WT1-specific TCRlo cells, shown to mainly consist of Tcm, were not enriched in the BM compared to the periphery. Neither did, WT1-specific Tcm cells among either TCRhi or TCRlo WT1-specific T cells, in the A2Kb to A2Kb transplant setting. However, in B6 to A2Kb transplant recipients a small difference was observed both in the percentages of WT1-TCRlo cells and Tcm WT1 T cells in general, perhaps suggesting that in the absence of professional WT1 Ag presentation, Tcm WT1 T cells are more likely to remain in their homeostatic niche rather than circulate in the periphery. On the other hand, in the A2Kb to A2Kb transplants effective direct-presentation and cross-presentation of the WT1 Ag in the BM transforms what is generally an environment of preferential accumulation of Tcm T cells to one that is simply another site of Ag presentation. Consequently as Ag is presented in BM and periphery, the percentage distribution of WT1-specific T

cells into naive, Tcm and Tem phenotypes becomes broadly similar among the two sites.

Our data could not provide a conclusive explanation for the preferential accumulation of WT1-specific T cells in the BM. Additional experiments will be needed to clarify this and these are described in section 6.8, Future Work.

6.6 TCRlo WT1-specific T cells exhibit antigen-specific function in the absence of vaccination

WT1-specific T cells not only spontaneously differentiated to memory phenotype T cells, but also exhibited antigen specific proliferation, cytokine secretion and killing activity. As WT1-TCRlo cells in their majority had a Tcm phenotype, whereas WT1-TCRhi cells composed of naive, Tem and Tcm phenotype cells, it was expected that the functional activity of these two WT1 specific T cell subsets would prove to be different in terms of magnitude and timing with TCR lo cells responding better and faster.

AT of T cells, containing WT1-TCRhi, WT1-TCR lo and endogenous T cells, into lymphodepleted secondary recipients, allowed us to study the function of the TCR low, Tcm phenotype WT1-specific T cells, as at 10 weeks post-AT all WT1-specific T cells converted to this phenotype. There was evidence that their subsequent differentiation was driven by their Ag-specificity over and above the effects of the lymphopenic environment in the irradiated secondary recipients. The cells showed strong antigen specific in vivo cytotoxicity activity and ex-vivo proliferation, clearly suggesting that TCR lo, Tcm WT1-specific T cells were capable of antigen specific function. However, demonstrating the

same functional activity in sorted TCR lo, Tcm WT1-specific T cells transferred to non-irradiated A2Kb recipients would indisputably confirm this hypothesis.

6.7 WT1-specific T cells are fully functional but do not cause any autoimmune damage

Despite the functional activity of WT1-specific T cells, these cells did not appear to cause any autoimmune damage in tissues expressing the WT1 Ag at low levels. Earlier work by our group demonstrated that the human T cell clones from which the genes for this particular WT1-TCR were cloned and subsequently used for WT1-TCR gene transfer in this and other projects, effectively killed leukaemic stem cells but did not damage healthy, human CD34+ stem cells also expressing the WT1 Ag. Furthermore, numerous WT1 vaccination trials showing a range of WT1 specific T cell responses have been performed, but so far no report of autoimmune damage caused by WT1-specific T cells has been made. Unlike the WT1-specific T cells in our model, the WT1-specific T cells expanded by vaccination are likely to be of low avidity and their frequencies even lower than those achieved here by the WT1-TCR, lentiviral transduction of HSCs prior to the HSC transplants.

In a recent study, Falkenburg et al isolated WT1-specific T cells that were either self-HLA A2- restricted or allo-HLA A2 restricted (from HLA A2 positive and negative individuals respectively) and were able to show that only the allorestricted specificities recognised the WT1 epitope used in a high avidity interaction. In contrast, all self-restricted WT1 specificities were of low avidity supporting the theory that high avidity self-restricted WT1 specificities are generally deleted in the thymus (Falkenburg et al., 2011). In our case, even

though the WT1-TCR used was generated from the allorestricted repertoire, developing thymocytes expressing it went through thymic development before emerging in the periphery as WT1-specific T cells. Therefore, in this experimental system the allorestricted TCR was used to model self-restricted, high avidity T cells liable to central and peripheral tolerance. Demonstrating that a particular high-avidity WT1 specific T cell specificity, can escape negative selection without its function being compromised by peripheral tolerance mechanisms, even when as shown by Falkenburg et al this is not a common event, is encouraging for vaccination trials targeting TAAs.

Once experiments are performed to clarify the hypothesised differences in the antigen-specific functional activity of TCRhi and TCRlo WT1-specific T cells, our model can be used to optimise vaccination protocols in order to preferentially expand the WT1 specific subset that will be most useful in the clinical setting. One limitation of our model is the non-physiological frequency of high avidity WT1-specific T cells. It may therefore be possible to use primary WT1-TCR td HSC transplant recipients to generate naturally occurring-like WT1-specific T cells that develop in the thymus and do not undergo any in vitro manipulation as mature T cells and subsequently adoptively transfer the required numbers and cell subsets into secondary non-irradiated A2Kb recipients, in order to setup more physiologically-relevant experimental conditions.

The results produced in this study are also relevant to the WT1-TCR gene therapy clinical trial which has recently opened to recruitment. In this trial the genes for the same WT1-TCR used in this project will be transferred to

autologous T cells harvested from patients with poor prognosis Acute Myeloid Leukaemia. The TCR gene-modified T cells will then be returned to the patients after lymphodepletion and immunological and clinical responses will be monitored. As discussed already, our results provide additional reassurance that such high avidity WT1-specific T cells are unlikely to cause autoimmune toxicity in the clinical trial patients. This is particularly important since targeting healthy CD34+ HSCs and renal podocytes, that express this Ag, can potentially result in myelosuppresion and renal failure respectively.

Ex-vivo proliferation and cytokine secretion assays were performed by stimulating splenocytes from WT1-TCR td HSC transplant recipients containing WT1-specific T cells with A2Kb splenocytes loaded either with the pWT126 or an irrelevant HLA-A2 presented peptide as a negative control. A simple way to test for cross-reactivity of WT1-specific T cells would be to add an additional control condition where no exogenous peptide would be loaded on A2Kb target splenocytes. These cells will then be able to present endogenously processed epitopes to WT1-specific T cells. A proliferative, or cytokine secretion response under these conditions would indicate that the WT1-specific T cells are able to also recognise other epitopes on HLA A2. Despite the lack of direct evidence, the very fact that WT1-specific T cells in our model, expressing an allorestricted, high avidity TCR on their cell surface, did escape central tolerance in an HLA A2 thymic environment allows us to speculate that they should not exhibit any promiscuous off-target recognition. Of course the limitation of this idea then becomes that while no cross-reactivity might be exhibited by WT1-specific T cells present in A2Kb transgenic mice this remains a possibility in the human system.

6.8 Future work

Demonstrating that high avidity, WT1-specific T cells going through thymic development in subjects expressing physiological, low levels of the WT1 TAA, emerge from the thymus, differentiate into memory T cells and are fully functional in an Ag-specific manner, was unexpected. Having confirmed that these high avidity WT1-specific T cells were not susceptible to central or peripheral tolerance mechanisms we attempted to characterise their phenotype and function in detail and gain an understanding into what drives their differentiation into fully functional memory T cells.

Currently 3 main questions remain unanswered and can be addressed in future experiments, as described below.

6.8.1 Delineating the function of TCRhi and TCRlo WT1-specific T cells

TCRhi and lo, WT1-specific T cells together were shown to kill, proliferate and secrete cytokines in an antigen-specific manner. The AT experiments described in section 5.2.6, suggested that TCRlo, Tcm phenotype WT1-specific T cells are capable of this functional activity. However, to unequivocally test if there are any differences in the functional capacity of the two WT1 specific T cell subsets it is necessary to isolate each group from the spleens of WT1-TCR td HSC A2Kb to A2Kb transplant recipients by FACS sorting.

The two cell populations can then be used in ex-vivo proliferation and cytokine secretion assays as they have been performed with non-sorted WT1-specific T cells. To test the in vivo cytotoxicity activity of the two subsets, it would be

necessary to adoptively transfer each FACS-sorted subset in non-irradiated A2Kb recipients and then proceed with the same assay as the one performed in primary WT1-TCR td HSC transplant recipients. It is likely that in the AT recipients the WT1-TCRhi cells will respond to the physiological low level WT1 Ag presentation, downregulate their TCR and differentiated into Tcm T cells giving rise to a TCRlo population as the one seen in primary transplant recipients. To avoid this, the in vivo cytotoxicity assay should be performed the same day the T cells are transferred into secondary recipients. While conceptually this is a simple experiment, the technical details of T cell numbers to be transferred into each secondary recipient, in order to see a response to this assay, would have to be optimised. Acquiring enough cells from each WT1 specific T cell subset to obtain statistically significant results, most likely would require large numbers of primary WT1-TCR td HSC transplant recipients.

6.8.2 Are TCR lo, memory WT1-specific T cells derived from TCRhi WT1-specific T cells in the periphery

The AT experiments, in which bulk WT1-specific T cells were transferred in lymphodepleted secondary recipients, indicated that TCRhi T cells, consisting mainly of naive T cells, differentiate to memory T cells and downregulate their TCR to become WT1-TCRlo cells. Nevertheless, this does not necessarily reflect the events that lead to the generation of the TCRlo, memory T cell population in the primary WT1-TCR td HSC transplant recipients. It was mentioned earlier that FACS analysing thymocytes for the presence of TCRhi and lo WT1-specific T cells was not possible due to the differential expression

of the CD8 coreceptor, which is used to identify the two populations, at different stages in T cell development.

An alternative experiment that could provide us with valuable information, would involve the AT of FACS sorted TCRhi WT1-specific T cells into non-irradiated A2Kb secondary recipients. Observing the conversion of naive, TCRhi WT1-specific T cells into memory T cells and their simultaneous conversion to a TCRlo population in the steady-state would provide more direct evidence supporting the hypothesis that the chronic antigenic stimulation of naive TCRhi, WT1-specific T cells triggers their differentiation into memory T cells in the absence of vaccination (figure 6.1).

6.8.3 What drives the accumulation of WT1-specific T cells in the BM? Antigen presentation, the BM environment being a niche for Tcm, or a combination of both?

Both in the presence and absence of professional APCs (A2Kb to A2Kb and B6 to A2Kb transplant recipients respectively) WT1-specific T cells preferentially accumulate in the BM over the spleen and LNs. Even though the phenotype of the WT1-specific T cells in these 3 different sites was analysed extensively, our results did not provide us with a conclusive answer as to what drives this phenomenon and further experiments are required.

To clarify whether the accumulation of WT1-specific T cells in the BM is the result of increased proliferation in the BM, homing to this organ, or a combination of both, it is necessary to assess cell division at this site and in the periphery. Employing the techniques used by Becker et al, active cell division can be assessed by combining surface staining to identify naive and memory

WT1-specific T cells with intracellular staining with PI to obtain a snapshot of DNA synthesis in the 3 different organs studied(Becker et al., 2005). Combining these results with in vivo BrdU incorporation assays as another measure of proliferation in spleen, LNs and BM over a fixed amount of time can offer an accurate description of the site at which most proliferation is taking place at the time of the assay (PI staining) and which site contains the cells that have proliferated the most, irrespective of where this proliferation has taken place (BrdU assays).

In addition AT experiments with FACS-sorted, naive, Tcm and Tem WT1-specific T cells that would be CFSE labelled prior to their transfer into non-irradiated A2Kb transgenic recipients could inform us on whether naive WT1-specific T cells divide and differentiate in the periphery and subsequently migrate in the BM as Tcm T cells, or whether this differentiation happens locally in the BM. To confidently distinguish whether this accumulation relates to antigen presentation in the BM or to the memory character of WT1-specific T cells these experiments, CFSE labelled, sorted WT1-specific naive and memory cells should be adoptively transferred into non-irradiated A2Kb and B6 recipients so that their rate and site of proliferation, as well as migration profile from the periphery to the BM in the presence and absence of WT1 Ag presentation on HLA A2 can be compared. Of course, ideally we would use WT1 knockout A2Kb Tg mice rather than B6 mice as secondary recipients. In WT1 knockouts the WT1 Ag would be truly absent instead of simply not presented on the appropriate class I molecule for recognition by the hybrid WT1-TCR. Unfortunately WT1 knockout mice are embryonic lethal.

6.8.4 The role of WT1-specific T cells in tumour protection and clearance

In this PhD project, high avidity WT1-specific T cells developing in the context of physiological WT1 Ag expression were shown to have a full armoury of functional properties. Testing these T cells in tumour challenge experiments, will allow us to ask whether they can also integrate their killing, proliferation and cytokine secretion activities to successfully eradicate or contain a tumour. This will clearly be a much more difficult task, as tumour cells are able to proliferate, organise themselves in vivo and in most cases establish an immunosuppressive environment within the tumour site. Therefore, the recruitment of WT1-specific T cells to the tumour, the rate at which they can kill transformed, multiplying cells and their ability to maintain effector function within the tumour microenvironment will be assessed in tumour challenge experiments. In such experiments, it would be interesting to delineate the contribution of TCRlo and TCRhi cells in controlling tumour growth. It would be even more interesting to investigate whether the presence of an establishing tumour causes any changes in the activation profile of circulating and tumour-homing WT1-specific T cells and assessing whether ways to encourage or avoid such changes to suit a therapeutic scenario can be devised.

Nevertheless, establishing an appropriate WT1 expressing-tumour challenge model in relation to the project presented here will be complicated. The first requirement would be to establish a murine tumour cell line that expresses the WT1 Ag, can endogenously process it and subsequently present the pWT126 on the HLA A2 molecule. Preliminary work has been performed towards establishing such a cell line, however time limitations did not permit the

completion of this work during this PhD. Following the generation of an appropriate tumour cell line the second requirement would be to optimise the tumour cell numbers required to establish a tumour burden that would allow a difference to be observed among untreated control mice, and mice containing WT1-specific T cells. Once these requirements are met, the simplest experimental setup would be to harvest splenocytes from primary WT1-TCR td HSC transplant recipients and FACS sort WT1-specific T cells from these samples, to adoptively transfer in tumour bearing, A2Kb Tg mice.

Once the experimental conditions are optimised, the TCRhi and low WT1-specific T cells can be sorted and transferred separately into tumour bearing mice. This will allow us to assess their function separately but also track any phenotypic changes among each subset. WT1 specific T cell subsets could also be CFSE labelled and their tumour-specific proliferation and migration profiles studied.

The above tumour challenge experiments would answer questions around the function of WT1 specific TCRlo and TCRhi subsets individually and together, within the tumour microenvironment. In addition, tumour challenge experiments could also be performed to establish a clinically relevant WT1 vaccination model. In such a model, the tumour cell line would be injected in primary WT1-TCR td HSC transplant recipients that will already contain fully functional memory WT1-specific T cells as well as naive WT1 specific T cells. These conditions will mimic the hypothetical, clinical scenario of high-affinity, functional WT1 specific T cells being present in a patient. Different WT1 vaccination strategies can subsequently be tested, to identify the protocols that

would be most successful in enhancing tumour protection and tumour clearance. Of course protection from a not yet established tumour is a very different scenario, from tumour clearance. This model can allow the study of both situations in relation to a WT1-specific antitumour response. It can be used to ask what breaks tumour protection; is it just a matter of WT1-specific cell numbers or is it mediated by a phenotypic and functional change among WT1-specific T cells. It can also be used to ask whether the “naturally occurring” WT1-specific T cells that failed to prevent the establishment of the experimental tumour can be made effective to clear the tumour by vaccination.

In order for such experiments to be clinically relevant, the initial frequencies of WT1-specific T cells in the WT1 TCR td HSC transplant recipients will have to be reduced substantially. Reducing the MOI of the lentivirus with which the HSC are transduced prior to the transplant would reduce the percentage of WT1 specific T cells present in the periphery, but it might also change the expression levels of the WT1 TCR on individual T cells. To avoid this complication, HSCs would be transduced with the same MOI as the one used in this project (MOI 10), but the HSCs transferred into the transplant recipients will consist on untreated, untransduced HSCs to which a small number of transduced HSCs will be added. In this way it will be possible to control the frequency of WT1-specific T cells without affecting the WT1-TCR expression levels.

6.9 Conclusions

The unexpected finding that high avidity WT1-specific T cells can escape central and peripheral tolerance in the context of physiological WT1-Ag

expression and spontaneously differentiate into fully functional, memory T cells, can be used to optimise WT1 T cell-based tumour immunotherapies. Dissecting the role of each WT1 specific T cell subset in tumour protection and clearance will be critical before using this model as a platform to test different strategies to promote the development and persistence of the most desirable WT1-specific populations.

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Specificity for the tumor-associated self-antigen WT1 drives the development of fully functional memory T cells in the absence of vaccination

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Recently, vaccines against the Wilms Tumor antigen 1 (WT1) have been tested in cancer patients. However, it is currently not known whether physiologic levels of WT1 expression in stem and progenitor cells of normal tissue result in the deletion or tolerance induction of WT1-specific T cells. Here, we used an human leukocyte antigen-transgenic murine model to study the fate of human leukocyte antigen class-I restricted, WT1-specific T cells in the thymus and in the periphery. Thymocytes expressing a WT1-specific T-cell receptor derived from high avidity human CD8 T cells were positively selected into the single-positive CD8 population. In the periphery, T cells specific for the WT1 antigen differentiated into CD44-high memory phenotype cells, whereas T cells specific for a non–self-viral antigen retained a CD44^{low} naive phenotype. Only the WT1-specific T cells, but not the virus-specific T cells, displayed rapid antigen-specific effector function without prior vaccination. Despite long-term persistence of WT1-specific memory T cells, the animals did not develop autoimmunity, and the function of hematopoietic stem and progenitor cells was unimpaired. This is the first demonstration that specificity for a tumor-associated self-antigen may drive differentiation of functionally competent memory T cells. (*Blood*. 2011;117(25): 6813-6824)

Introduction

The Wilms Tumor antigen 1 (WT1) is an attractive target for immunotherapy of leukemia and solid tumors as it is expressed at high levels in many malignancies, whereas progenitor and stem cell populations express only low levels of the WT1 transcription factor.[1-5] In acute myeloid leukemia (AML), high WT1 levels are associated with poor prognosis, and the quantitative measurement of WT1 RNA transcripts is now widely accepted as a sensitive molecular marker for monitoring minimal residual disease in patients undergoing chemotherapy or transplantation.[6]

In the past few years, vaccination with WT1 peptides has been tested as a treatment option for various malignancies, including myelodysplasia and leukemia.[7,8] In these studies clinical responses were observed in 60% to 74% of evaluable patients (including stable disease and reduced expression of tumor markers), but correlation with the detection of immunologic responses in peripheral blood was variable. Studies in breast cancer patients showed that WT1-specific T cells were undetectable in peripheral blood, although present in tumor-draining lymph nodes (LNs).[9] Therefore, failure to detect WT1-sepcific T cells in the blood of cancer patients might be the result of selective migration to the site of tumor growth.

It is possible that low-level WT1 expression in normal progenitor cells may result in the central deletion of high avidity WT1-specific T cells or induce unresponsiveness by peripheral tolerance mechanisms. However, there is good evidence indicating that central and peripheral tolerance to WT1 is incomplete. First, vaccination in humans can induce self-restricted WT1-specific T-cell responses in some patients, although their frequency is generally low.[10] In addition, WT1-specific T cells were detectable in leukemia patients after allogeneic stem cell transplantation and their detection correlated with low risk of leukemia relapse.[11] Finally, WT1-specific T cells can be detected in the peripheral blood of healthy persons.[12-17]

It is currently unclear to what extent self-antigen expression in normal tissue shapes the phenotype and functional activity of WT1-specific T cells. For example, recent studies examining the in vivo generation of "natural" memory phenotype T cells have suggested that self-antigen might trigger naive T cells to differentiate into memory phenotype cells.[18-20] In this study, we designed murine model experiments to analyze the thymic development of HLA-A0201–restricted, WT1-specific T cells and to determine the phenotype and function of these cells in the periphery.

Previously, we have used the allo-restricted strategy to isolate HLA-A0201–restricted, pWT126-specific T cells from healthy persons.[21,22] High avidity T-cell lines were established and used to isolate the genes encoding the α- and β-chains of a pWT126-specific TCR. Retroviral TCR gene transfer readily converted human peripheral blood T cells into high avidity, pWT126-specific CTL,[23] which were able to recognize and kill CD34$^+$ cells of leukemia patients but not normal CD34$^+$ hematopoietic progenitor/stem cells. Furthermore, adoptive therapy with TCR-gene transduced T cells resulted in the elimination of autologous leukemia cells in the xenogenic NOD/SCID model.[24]

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In this study, we transferred the WT1-TCR gene into purified murine lineage-negative hematopoietic stem cell (HSCs). Transplantation of the gene-modified stem cells into HLA-A0201 transgenic recipients allowed us to study the phenotype and function of TCR-expressing cells in the thymus and periphery. Surprisingly, WT1-specific T cells were not impaired by central or peripheral tolerance but differentiated instead into memory phenotype T cells able to display antigen-specific effector function. Thus, self-specificity for a tumor-associated antigen can contribute to the establishment of natural memory phenotype T cells.[18,19] The implications of these findings for WT1-based vaccination strategies are discussed.

Methods

Mice

C57BL/6 mice were purchased from Charles River Laboratories or the Comparative Biology Unit of University College London, Royal Free Campus. HLA-A2Kb transgenic (A2Kb Tg) mice on a C57Bl/6 background were a kind gift from Theobald M (University Medical Center, Utrecht, The Netherlands). C57Bl/6 CD45.1 and A2KbxCD45.1 Tg mice were bred and maintained in the Comparative Biology Unit of University College London. All animals were housed in pathogen-free conditions in individually ventilated cages and were kept in accordance with the University and United Kingdom Home Office regulations. All procedures were performed aseptically, and irradiated animals received 2.5% enrofloxacin (Baytril, Bayer) in their water 1 week before irradiation and for 2 weeks after irradiation. Donor mice were 7 to 10 weeks old at the time of bone marrow (BM) harvest. Recipient mice were 12 weeks old at transplantation and received 9.4 Gy irradiation in 2 divided doses at day −2 and day 0. Secondary adoptive transfer recipients were sublethally irradiated (5 Gy) 4 hours before T-cell transfer.

Lentiviral vector constructs

A pSIN second-generation lentiviral vector, containing a spleen focus forming virus LTR promoter and the HIV-1 central polypurine tract cis-active element, was modified for this study. Both the codon-optimized hybrid HLA-A*0201-restricted WT1-specific TCR and the latent membrane protein 2 (LMP2)–specific TCR genes have been described before.[24,25] The WT1-TCR uses the Vβ2.1 and Vα1.5 TCR chains and is specific for the WT126 peptide, RMFPNAPYL. The LMP2-TCR uses the Vβ13.1 and Vα3.1 TCR chains and is specific for the CLG peptide, CLGGLLTMV. The hybrid TCR sequences contain murine constant and human variable region sequences. An additional disulphide bond between the α- and β-chains was created by the introduction of 2 additional cysteine residues in the α- and β-chain constant regions. The genes for the TCR α- and β-chains cloned into the lentiviral vector were separated by a porcine tsechovirus self-cleaving 2A sequence to optimize expression of both TCR α- and β-chain genes. The leader sequence was derived from the pMP71 retroviral vector, and the full-length woodchuck hepatitis virus post-transcriptional regulatory element was truncated to prevent encoding of the oncogenic protein X.[26]

Lentivirus preparation

Human embryonic kidney 293T cells were used to produce WT1-TCR and LMP2-TCR lentiviral particles. A total of 2×10^6 293T packaging cells were cultured in T150cm tissue-culture flasks in Iscove modified Dulbecco medium supplemented with 10% heat-inactivated FCS (Sigma-Aldrich), 1% penicillin and streptomycin (Pen/Strep), and 1% L-glutamine, for 24 hours at 37°C, 5% CO_2. To produce the appropriate lentiviral particles, the 293T cells were cotransfected with a plasmid encoding either WT1-TCR or LMP2-TCR genes, the pMD.G plasmid encoding the vesicular stomatitis virus glycoprotein envelope, and the pCMVΔ8.91 plasmid that encodes genes necessary for the production of functional viral particles. The Fugene6 transfection kit (Roche Diagnostics) was used for packaging cell transfection as per the manufacturer's protocol. Twenty-four hours later, the transfection medium was replaced with fresh IMDM. The lentiviral supernatant was harvested 48 hours later, concentrated 100 times by ultracentrifugation, before resuspension in StemSpam medium (StemCell Technologies) and stored at −80°C until required for transduction. Before storage, serial dilutions of each lentiviral supernatant were added to 1 mL of 5×10^5 $58^{-/-}$ cells, which were cultured in RPMI medium supplemented with 10% FCS, 1% Pen/Strep and 1% L-glutamine, to titrate the multiplicity of infection (MOI). An MOI 10 was used to transduce Lin^- BM cells in all experiments.

HSC isolation, infection, and transfer

Whole BM was harvested from 7- to 10-week-old donor mice (C57BL/6 or A2Kb Tg as specified in each experiment). Briefly, femurs, tibias, and pelvic bones were removed and the BM was harvested by flushing it with cold RPMI medium containing 1% Pen/Strep with a syringe equipped with a 25-gauge needle. Uncommitted BM progenitors, highly enriched in HSCs, were negatively selected using either the lineage cell depletion kit for mouse (Miltenyi Biotec) or the Hematopoietic Progenitor Enrichment kit (StemCell Technologies) following the manufacturer's protocols. The $lineage^-$ selected cells (Lin^- BM cells) were placed in culture at 1×10^6 cells/mL in StemSpan medium (StemCell Technologies) containing 1% Pen/Strep, 100 ng/mL murine stem cell factor, 100 ng/mL human Fms-like tyrosine kinase 3, 100 ng/mL human IL-11, and 20 ng/mL murine IL-3. All cytokines were purchased from PeproTech. The Lin^- BM cells were transduced with the appropriate lentiviral particles at an MOI of 10. At 18 to 24 hours after transduction, the transduced Lin^- BM cells were harvested, washed, and resuspended in RPMI medium containing 1% Pen/Strep at 2.5×10^6 cells/mL. Lethally irradiated recipients received 5×10^5 Lin^- BM cells/each by tail vein injections. Tail bleeds were performed at weeks 5, 7, and 9 after transplantation. Recipient mice were killed 11 weeks after transplantation, and thymus, spleen, LNs, and BM were harvested for phenotypic analysis and functional characterization of T cells.

Adoptive T-cell transfer into secondary recipients

Lentiviral TCR-transduced C57BL/6 Lin^- BM cells were transferred into lethally irradiated A2KbxCD45.1 Tg recipients as described in "HSC isolation, infection and transfer." Seventeen weeks after transplantation the mice were killed, and their spleens were harvested. Untouched CD3 T-cell selection was performed on splenocytes pooled from 5 primary recipients, using a mouse pan-T cell isolation kit (Miltenyi Biotec). A total of 3×10^6 T cells were transferred to secondary A2KbxCD45.1 recipients that were sublethally irradiated (5 Gy) 1 day before the transfer. Tail bleeds were performed at day 9 and day 28 after adoptive transfer. On days 69 and 70, in vivo cytotoxicity assays (See "In vivo cytotoxicity assays") were performed. Splenocytes from the secondary recipients were used in ex-vivo proliferation assays (See "Ex vivo proliferation assays").

Serial HSC transplants

BM cells from primary WT1-TCR HSC transplant recipients (A2KbxCD45.1 to A2Kb transplant) were pooled together and then transferred into lethally irradiated (9.4 Gy) C57BL/6 mice (5×10^6 cells/recipient). Tail bleeds were performed at 3, 5, 8, and 12 weeks after transplantation. Peripheral blood was stained with anti-CD45.1 allophycocyanin-Cy7 (APC-Cy7), CD3 fluorescein isothiocyanate (FITC), B220 phycoerythrin-Cy5 (PE-Cy5), and CD11b APC antibodies to monitor the reconstitution kinetics of all hematopoietic lineages. BM cells from untreated A2KbxCD45.1 Tg mice were used to reconstitute lethally irradiated C57BL/6 mice as a control for donor-derived hematopoietic reconstitution.

Antibodies and FACS analysis

The following fluorescently labeled anti–murine antibodies (BD Bioscience) were used in the study: CD45.1 APC Cy7, CD3 APC, CD3 FITC,

CD4 FITC, CD8a PE Cy5, CD44 APC, CD62L FITC, B220 PE Cy5, CD11b APC, and streptavidin APC. Anti–human Vβ2.1 PE, Vβ2.1 biotin, and Vβ13 were purchased from Immunotech. An LSR II cytometer (BD Bioscience) was used for flow cytometric analysis, and data were analyzed using FlowJo Version 7 software (TreeStar).

In vivo cytotoxicity assays

Splenocytes from female A2Kb Tg mice were peptide loaded with 100μM of either relevant peptide (WT1-TCR WT126p, LMP2-TCR CLGp) or an irrelevant HLA-A0201 presented peptide before labeling with 1.5μM carboxyfluorescein diacetate succinimidyl ester (CFSE; $CFSE^{high}$) or 0.15μM CFSE ($CFSE^{low}$), respectively, for 5 minutes at 37°C. The cells were then washed once in ice-cold RPMI with 8% FCS and twice with ice-cold PBS. Labeled cells were mixed at a 1:1 ratio, relevant: irrelevant targets and a total of 10×10^6 mixed cells were injected per mouse. Eighteen hours later, splenocytes of injected animals were analyzed by flow cytometry to identify CFSE-labeled cells. Control untreated A2Kb Tg mice were injected with labeled target cells, and in vivo cytotoxicity was calculated as previously described. Percentage antigen-specific cytotoxicity was determined using the following formula: $[1 - ((A \div B)/(C \div D))] \times 100$, where A = mean number of relevant peptide-loaded splenocytes at 18 hours in experimental mice; B = mean number of irrelevant peptide-loaded splenocytes at 18 hours in experimental mice; C = mean number of relevant peptide-loaded splenocytes at 18 hours in control mice; and D = mean number of irrelevant peptide-loaded splenocytes at 18 hours in control mice.

Ex vivo proliferation assays

Splenocytes from mice that had been transplanted with WT1 or LMP2-TCR-transduced HSCs, or mice that had received an adoptive transfer of T cells from primary transplant recipients, were labeled with 1.5μM CFSE as in "In vivo cytotoxicity assays" and placed in culture at 1×10^6 cells/mL. CFSE-labeled splenocytes were stimulated with 100μM of relevant (WT126p/CLGp) or irrelevant peptide for 5 days before FACS analysis for CFSE dilution after anti–human Vβ2 or Vβ13 antibody staining. In the case of A2Kb Tg mice that received C57BL/6 TCR-transduced HSC cells, professional antigen-presenting cells were almost completely replaced with donor C57BL/6 BM-derived antigen-presenting cells. Therefore, to ensure adequate antigen presentation, splenocytes were harvested from A2Kb Tg mice, peptide loaded with relevant/irrelevant peptides (100μM), and added to the cell cultures at a 1:1 ratio to the splenocytes from the experimental mice. The final cell concentration was 1×10^6 cells/mL.

ELISA

Supernatants from the ex vivo proliferation assays were harvested on day 5 and stored at −20°C. IL-2 and IFN-γ production after peptide stimulation was determined by performing ELISA on these supernatants using the BD OptEIA mouse IL-2 and IFN-γ ELISA sets, as per the manufacturer's protocol.

Statistical analysis

Statistical analysis was performed using GraphPad Prism software Version 5.0, and Microsoft Excel.

Results

TCR gene transfer into stem cells to study the development of HLA-restricted, WT1-specific T cells

Previous studies have shown that retroviral TCR gene transfer into HSCs can serve to analyze the development and function of antigen-specific T cells in the thymus and periphery.[27,28] In these studies, retroviral vectors were used to infect bulk BM cells followed by transfer into myeloablated recipients. We found that lentiviral vectors efficiently transduced purified, lineage-negative stem cells that efficiently reconstituted conditioned recipients and developed into mature T cells. Hence, we assembled a lentiviral vector containing the HLA-A0201-restricted TCR specific for the WT1-derived peptide pWT126 (Figure 1A). The human constant regions of the TCR-α and -β genes were replaced with murine sequences to facilitate expression in murine cells.[29] The constant regions were further modified to encode a cysteine residue to improve the pairing of the introduced TCR-α and -β chains (Figure 1B).[30] HSCs were transduced with the WT1-TCR lentiviral construct before transplantation into myeloablated transgenic A2Kb mice (Figure 1D). The transgenic mice expressed hybrid HLA-A0201 molecules consisting of human α1 and α2 domains, which together form the peptide binding groove required for antigen presentation (Figure 1C). The α3 domain was of murine origin to facilitate appropriate interaction between murine CD8 coreceptors and the hybrid HLA molecule. Importantly, both human and murine WT1 proteins are highly conserved, and the HLA-A0201 presented pWT126 peptide is identical between the 2 species. It has been demonstrated that murine cells expressing murine WT1 can generate the pWT126 for major histocompatibility complex (MHC) presentation and T-cell recognition.[31] Further, the endogenous WT1 expression pattern in normal tissues is very similar in humans and mice.[32] Thus, the HLA transgenic host provides an in vivo model to investigate how physiologic WT1 expression affects the development and function of HLA-restricted, WT1-specific T cells.

WT1-specific T cells are selected in the thymus

Purified Lin^- BM stem cells from A2Kb transgenic mice were transduced with the lentiviral WT1-TCR vector. Using CD4/CD8 phenotyping and staining with antihuman Vβ antibodies, we found expression of the WT1-TCR in immature double-negative thymocytes and also in the more mature double-positive population (Figure 2A) in A2Kb transplant recipients. As expected, the HLA class I-restricted TCR was selected into the CD8 single-positive thymocyte population, with little TCR expression in CD4 single-positive cells. We observed similar frequencies of TCR-expressing cells in the $CD4^+CD8^+$ population and $CD8^+$ cells (Figure 2B), suggesting no preferential loss of WT1-specific T cells at the double-positive to single-positive stage of thymic selection. The relative high frequency of WT1-TCR-expressing thymocytes in the double-negative population is most probably the result of nonphysiologic early expression of the lentiviral TCR construct, before the expression of endogenous TCR-α/β heterodimers. Together, the thymus data were compatible with positive selection of WT1-specific T cells at the double-positive to single-positive stage.

WT1-specific T cells persist in the periphery and differentiate into memory phenotype cells without vaccination

Next, we explored whether TCR-expressing cells were detectable in the periphery of transplanted mice (A2Kb → A2Kb). Mature T cells expressing the WT1-TCR were readily detectable in the spleen, LNs, and BM (Figure 3A). As expected, the WT1-TCR was expressed in peripheral $CD8^+$ T cells and not in $CD4^+$ cells (Figure 3B). Anti-CD3 antibodies were used to measure the level of TCR/CD3 complex expression in all T cells derived from the transplanted stem cell population. There was a substantial reduction in the level of CD3 expression in the WT1-TCR^+ T cells compared with the WT1-TCR^- T cells (Figure 3C). Low expression of the WT1-TCR could be the result of the self-specificity of this TCR, or alternatively it could be the result of low promoter

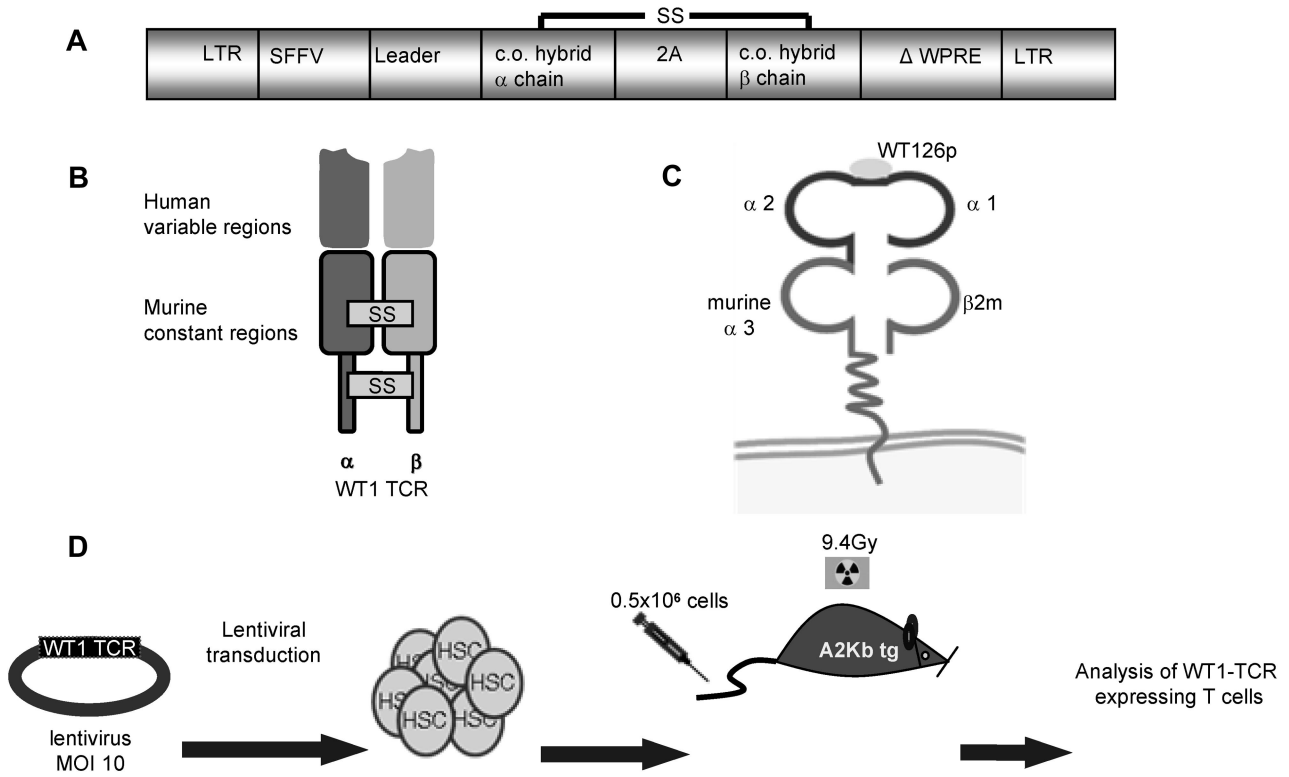

Figure 1. Schematic representations. (A) Schematic representation of the pSIN second-generation lentiviral vector encoding the codon-optimized, murinized hybrid HLA-A0201–restricted pWT126-specific Cys-1 modified TCR α- and β-chain genes (or the HLA-A0201–restricted LMP2-specific Cys1 modified TCR chains) separated by a self-cleaving porcine tsechovirus 2A sequence. SFFV indicates spleen-forming focus virus; LTR, long terminal repeat; and WPRE, woodchuck hepatitis virus posttranscriptional regulatory element. (B) Schematic representation of the Cys-1 modified, codon-optimized hybrid TCR containing murine constant regions (black outline) and human variable regions. (C) Schematic representation of the hybrid HLA-A2Kb molecule expressed in HLA-A2Kb (A2Kb) transgenic mice. The hybrid MHC class I molecules in A2Kb mice contain human alpha1 and alpha2 domains that interact with the human HLA-A2–restricted TCR, fused to a murine alpha3 domain, which facilitates interaction with the murine CD8 coreceptor. (D) Schematic representation of TCR-transduced HSC transplantation. Lineage$^-$ BM stem cells (HSCs) were transduced with lentivirus at an MOI of 10. Each lethally irradiated recipient received 5×10^5 bulk transduced HSCs.

activity of the lentiviral vector driving TCR expression. We therefore replaced the WT1-TCR variable gene segments in the lentiviral vector with the variable region gene segments of an HLA-A0201–restricted TCR specific for a non–self-peptide derived from the LMP2 of Epstein Barr virus.[25] Transplantation experiments with transduced stem cells indicated that mature T cells expressing the LMP2-TCR were readily detectable in the spleen, LNs, and BM of transplanted mice (Figure 3A). As expected, LMP2-TCR expression was limited to CD8$^+$ T cells and not seen in CD4$^+$ T cells (Figure 3B). Importantly, the level of CD3 expression in the LMP2-TCR$^+$ T cells was similar to that seen in control T cells expressing endogenous TCR (Figure 3C). This indicated that the reduced level of TCR/CD3 expression was not caused by poor expression from the lentiviral vector but was a feature of the self-reactive WT1-TCR and not of the non–self-reactive LMP2-TCR.

In previous transgenic models, the down-modulation of TCR and CD8 was described as an important mechanism of peripheral tolerance induction.[33] Unexpectedly, TCR down-modulation in WT1-specific T cells was associated with an up-regulation of CD8 coreceptor expression (Figure 3D). The down-modulation of TCR and up-regulation of CD8 coreceptors were only seen in WT1-specific T cells and not in T cells specific for the non–self-LMP2 antigen (Figure 3D).

Differences in TCR and CD8 expression between WT1 and LMP2-specific T cells correlated with differences in their activation status. The expression of the CD44 activation marker in LMP2-specific (TCR$^+$) T cells was similar to that seen in endogenous control (TCR$^-$) T cells (Figure 4A). In contrast, a large proportion of the WT1-specific T cells expressed high levels of CD44 and CD62L, a phenotype normally associated with central memory T cells (Figure 4A).

Previous studies indicated that survival of naive T cells required tonic TCR stimulation by MHC molecules expressed on hematopoietic cells, whereas memory T-cell survival did not require tonic triggering by hematopoietic cells.[34] However, the role of hematopoietic cells in the generation of memory phenotype T cells in the absence of vaccination has not yet been explored. Hence, we analyzed whether the generation and maintenance of WT1-specific memory phenotype T cells was dependent on antigen-presentation by hematopoietic cells. Stem cells from normal B6 mice were transduced with the WT1-TCR lentiviral vector and transplanted into HLA-A2Kb transgenic B6 recipients. In the transplanted mice, hematopoietic cells, including professional antigen-presenting cells, were unable to present antigen to the HLA-A0201–restricted WT1-TCR. Analysis of WT1-specific T cells in these mice showed that the development of CD44^{high}CD62L^{high} memory phenotype cells was not dependent on antigen presentation by hematopoietic cells (Figure 4A bottom row).

A more detailed analysis of the naive (CD44^{low}CD62L^{high}), central memory (CD44^{high}CD62L^{high}), and effector memory (CD44^{high}CD62L^{ow}) T-cell frequencies in mice expressing WT1-TCR or LMP2-TCR showed that the WT1-TCR–expressing population contained significantly less naive phenotype T cells

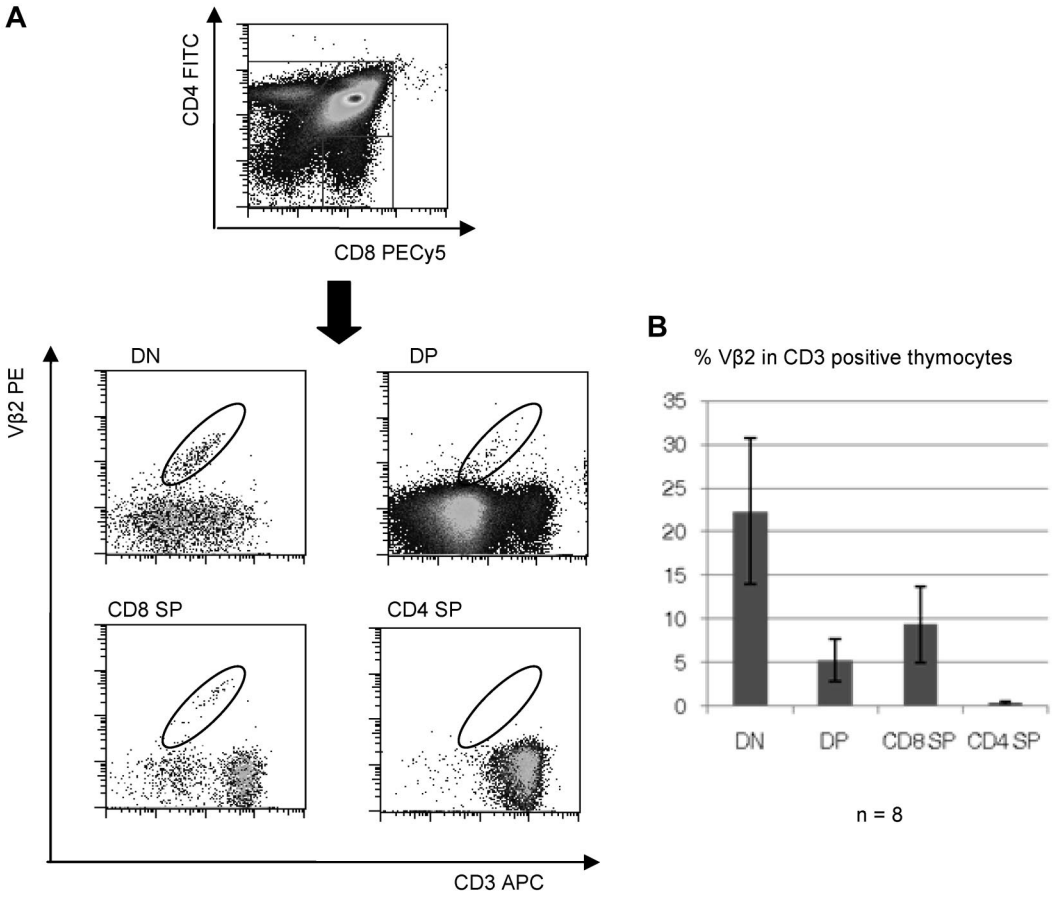

Figure 2. WT1 specific T cells are selected in the thymus. (A) FACS analysis of thymocytes isolated from A2Kb mice transplanted with lin$^-$ A2Kb BM stem cells transduced with the lentiviral WT1-TCR vector. Thymocytes were stained with antimurine CD3, CD4, and CD8 antibodies together with anti–human Vβ2.1 to detect the WT1-TCR β chain. SP indicates single positive; DP, double positive; and DN, double negative. Representative plots from a total of 8 mice are shown. (B) Percentage of murine thymocytes expressing the WT1-TCR as detected by anti–human Vβ2.1 antibody at each stage of thymic selection was determined by FACS analysis after gating on DN, DP, or SP populations. A total of 8 transplanted A2Kb → A2Kb mice were analyzed.

compared with LMP2-TCR–expressing population ($P < .001$; Figure 4B). In contrast, central memory cells were significantly more frequent in the WT1-TCR–expressing population ($P < .001$) in both recipients of TCR-Td A2Kb and TCR-Td B6 stem cells. An increased frequency of effector memory T cells in the WT1-TCR–expressing population (compared with the frequency of LMP2-TCR–expressing effector memory T cells) was only observed in mice reconstituted with A2Kb stem cells ($P < .05$) but not those reconstituted with B6 stem cells (P = not significant; Figure 4B). Therefore, antigen presentation by hematopoietic cells promoted the differentiation of WT1-specific T cells into effector memory phenotype cells. At the time of the phenotypic analysis, the percentage donor chimerism of the CD3$^-$ cells (including antigen-presenting cells) in the B6 → A2Kb transplant recipients showed between 92% and 97% donor cells (data not shown). It is therefore possible that the small number of residual A2Kb$^+$ antigen-presenting cells contributed to, or was sufficient for, the development of central memory and effector memory phenotype cells in the mice transplanted with B6 stem cells.

Within the WT1-specific T cells, the TCR-low population contained primarily memory phenotype cells, whereas the TCR-high population contained both naive and memory phenotype T cells (Figure 4C). Together, these observations suggested that naive phenotype T cells differentiated into memory phenotype cells by a mechanism that involved TCR triggering, which resulted in TCR down-modulation and up-regulation of the CD44 activation marker. It is possible that the lymphopenic environment of transplanted mice facilitates the generation of WT1-specific memory T cells, although this environment does not promote LMP2-specific memory T-cell development.

WT1-specific T cells display antigen-specific effector function without vaccination

We explored whether WT1-specific T cells, and more specifically the memory phenotype T cells, were able to display antigen-specific effector function. In vivo cytotoxicity assays revealed strong antigen-specific killing activity in A2Kb mice transplanted with WT1-TCR–transduced A2Kb stem cells or B6 stem cells (Figure 5A). In contrast, no antigen-specific killing was observed in mice transplanted with LMP2-TCR–transduced stem cells, although the number of peripheral T cells expressing the LMP2-TCR or WT1-TCR was similar (Figure 5A). Ex vivo, the WT1-specific T cells isolated from both A2Kb → A2Kb and B6 → A2Kb transplanted mice displayed antigen-specific proliferation (Figure 5B), IL-2 and IFN-γ production (Figure 5C, B6 → A2Kb not shown), whereas LMP2-specific T cells displayed no antigen-specific functions (Figure 5B-C). These data indicated that the phenotypic differences between WT1-specific and LMP2-specific T cells correlated with the ability of WT1-specific T cells to display antigen-specific effector function without prior vaccination.

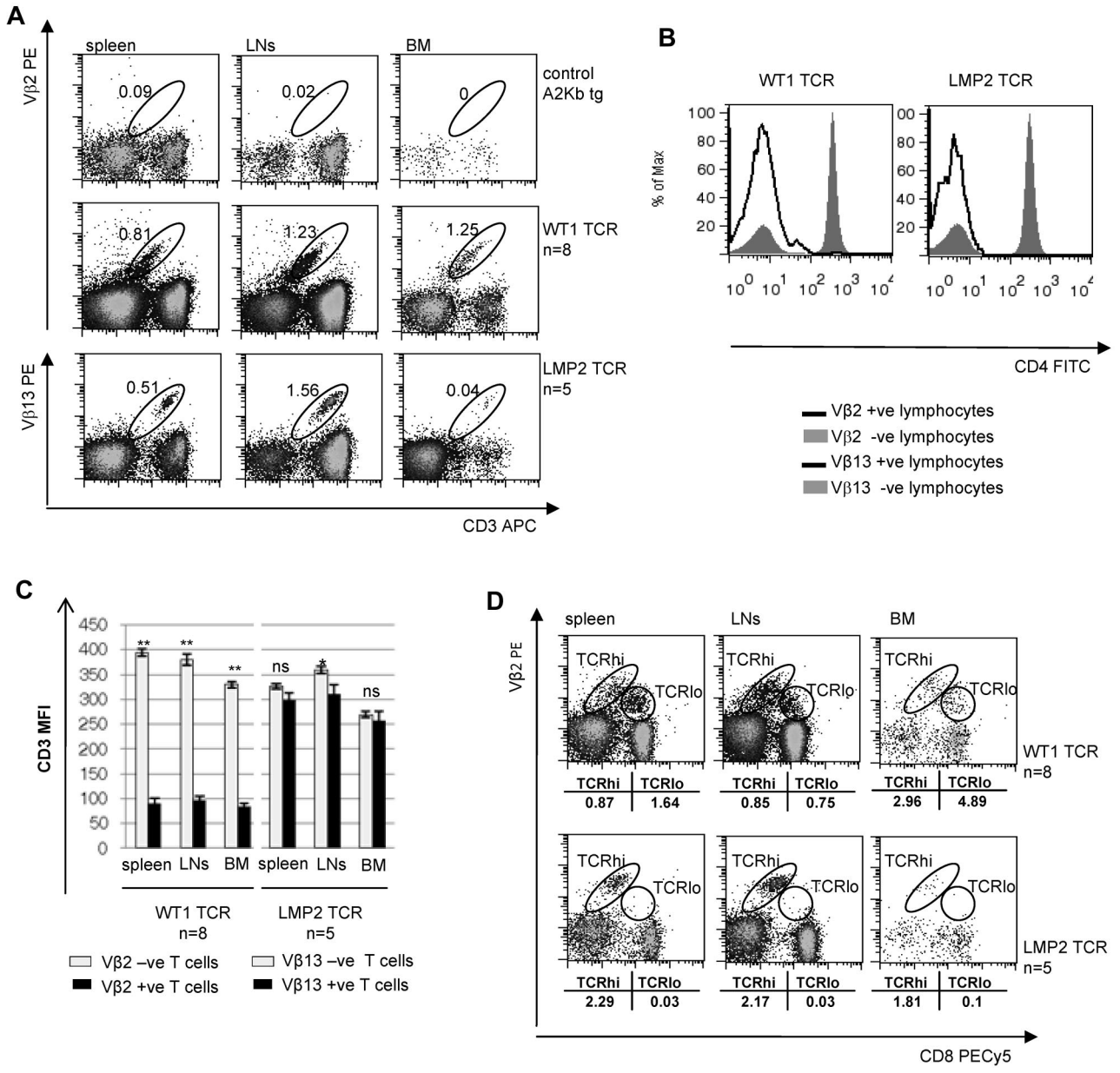

Figure 3. WT1 specific T cells persist in the periphery. (A) FACS analysis of peripheral T cells in spleen, LNs, and BM of A2Kb Tg mice killed 11 weeks after transplantation with TCR-transduced Lin$^-$ A2Kb BM stem cells. Mice received untransduced stem cells (top row), WT1-TCR-transduced stem cells (middle row, n = 8), or LMP2-TCR–transduced stem cells (bottom row, n = 5). Viable lymphocytes were stained with anti-CD3, and anti–human Vβ2.1 (WT1-TCR) and anti–human Vβ13 (LMP2-TCR) antibodies before FACS analysis. Percentages of Vβ2.1$^+$ and Vβ13$^+$ cells in total CD3$^+$ cells are indicated. (B) FACS analysis of splenocytes isolated from A2Kb Tg mice transplanted with Lin$^-$ A2Kb BM stem cells transduced with the lentiviral WT1-TCR or LMP2-TCR vector. Splenocytes were stained with anti–murine CD3, CD4, and CD8 antibodies together with anti–human Vβ2.1 and anti–human Vβ13. (C) Cell surface CD3/TCR complex expression levels in peripheral T cells were determined by FACS analysis after staining with anti-CD3, and anti–human Vβ2.1 and anti–human Vβ13 antibodies. CD3/TCR expression levels of the WT1-TCR (self-reactive) and the LMP2-TCR (non–self-reactive) were compared with endogenous polyclonal T cells derived from transplanted stem cells (Vβ2.1$^-$ and Vβ13$^-$, respectively). Data are mean ± SD of CD3 mean fluorescence intensity (MFI; n = 8 mice for WT1-TCR and n = 5 mice for LMP2-TCR). *$P < 0.05$, 1-way ANOVA. **$P < .01$, 1-way ANOVA. (D) Modulation of TCR and/or CD8 expression was determined by FACS analysis of peripheral T cells of spleen, LNs, and BM stained with anti-CD8, and anti–human Vβ2.1 (WT1-TCR, n = 8) and anti–human Vβ13 (LMP2-TCR, n = 5) antibodies. After gating on viable CD3$^+$ cells, percentages of TCR^{hi} and TCR^{lo} populations are indicated. All recipient mice were A2Kb and received TCR-Td A2Kb stem cells.

To test the in vivo function of WT1-specific memory phenotype T cells, cell transfer experiments were performed to generate mice lacking naive phenotype cells. T cells were purified from A2Kb mice transplanted with WT1-TCR-transduced B6 stem cells and then adoptively transferred into irradiated secondary A2Kb-transgenic recipients. The secondary recipients were unable to generate "new" WT1-specific T cells in the thymus, which was expected to result in the loss of naive phenotype cells and in the persistence of memory phenotype T cells. The analysis of LNs, spleen, and BM of the secondary recipients 10 weeks after transfer demonstrated a loss of the CD44^{low}CD62L^{high} naive phenotype T cells with high TCR expression level and persistence of CD44^{high}CD62L^{high} memory phenotype T cells expressing reduced TCR and elevated CD8 (Figure 6A-B). In transiently lymphopenic hosts, the WT1-specific T cells converted more efficiently into memory phenotype cells than the WT1-TCR negative control

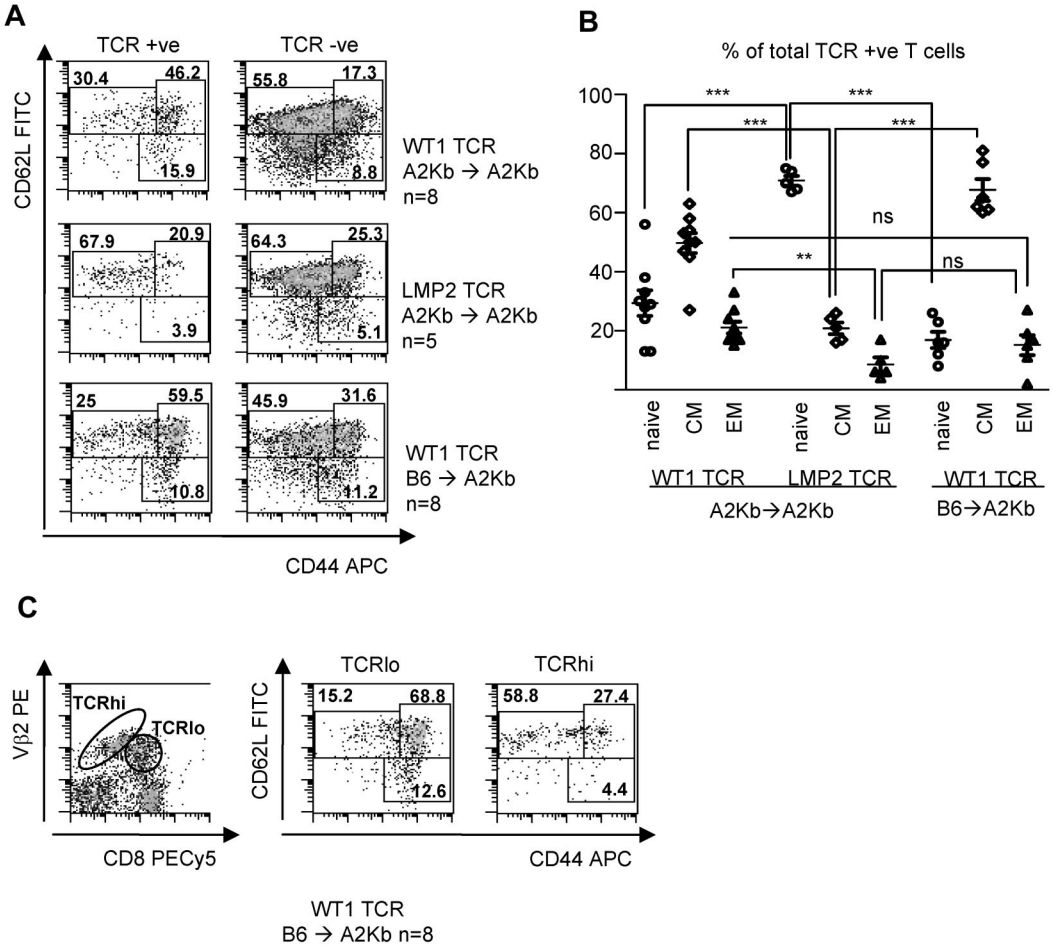

Figure 4. Peripheral WT1-specific T cells differentiate into memory T cells without vaccination. (A) CD44 and CD62L expression on gated TCR-expressing (TCR^+) and gated TCR^- peripheral T cells as determined by FACS analysis after staining with anti–murine CD44 and CD62L antibodies. Similar staining patterns were observed for both splenocytes and LN (shown here). (Top and middle panels) Resentative plots from A2Kb Tg mice transplanted with A2Kb stem cells transduced with the WT1-TCR (top panel, n = 8) or the LMP2-TCR (middle panel, n = 5). (Bottom panel) Representative plots from A2Kb Tg mice transplanted with B6-derived stem cells transduced with the WT1-TCR (n = 8). (B) Summary data of naive ($CD44^{low}$, $CD62L^{high}$), CM ($CD44^{high}$, $CD62L^{high}$), and EM ($CD44^{high}$, $CD62L^{low}$) T-cell frequencies in transplanted mice expressing WT1-TCR or LMP2-TCR. ***$P < .001$. **$P < .01$. A2Kb → A2Kb or B6 → A2Kb as indicated in the figure. (C) Phenotypic analysis of gated $WT1\text{-}TCR^{hi}$ and $WT1\text{-}TCR^{lo}$ peripheral T-cell populations in A2Kb recipients after transplantation with TCR-Td B6 stem cells, after staining with anti–murine CD44, CD62L, and CD8 antibodies. ns indicates not significant.

T cells expressing endogenous TCRs (Figure 6B left panel). Cytotoxicity experiments in the secondary hosts demonstrated efficient WT1-specific effector function, which correlated with robust WT1-specific proliferation ex vivo (Figure 6C). This clearly demonstrated that the $CD44^{high}/CD62L^{high}$ memory phenotype T cells were capable of displaying antigen-specific effector function without prior vaccination.

WT1-specific T cells accumulate in the BM without impairing stem cell function

Considering that BM resident stem/progenitor cells express low levels of WT1, we explored whether WT1-specific T cells accumulated in the BM of mice transplanted with A2Kb stem cells. Analysis of the donor T-cell compartment showed an increased percentage of WT1-specific T cells in the BM compared with LNs and spleen (Figure 7A). In contrast, analysis of LMP2-TCR transplanted mice demonstrated no enrichment of LMP2-specific T cells in the BM compared with LNs and spleen of these mice (Figure 7A). It was possible that the accumulation of WT1-specific T cells was the result of selective recruitment and proliferation of memory phenotype T cells to the BM, as shown in other studies.[35] However, analysis of WT1-specific T cells showed that the relative frequency of naive and memory phenotype cells was similar in BM compared with spleen and LNs (data not shown). This suggests that the observed accumulation in BM of WT1-specific T cells, but not LMP2-specific T cells, was primarily driven by the specificity and not the memory phenotype of WT1-T cells.

Finally, we examined whether the presence of WT1-specific T cells resulted in damage to the stem/progenitor cells in the BM of transplanted mice. BM was harvested from WT1-TCR transplanted mice and from control mice, followed by transplantation into myeloablated secondary recipients to measure long-term reconstitution of hematopoiesis. Analysis revealed that both myeloid and lymphoid lineages were efficiently reconstituted by BM isolated from donors transplanted with WT1-TCR–transduced stem cells, demonstrating that WT1-specific T cells did not impair the functional competence of the hematopoietic progenitor/stem cells (Figure 7B). However, T-cell engraftment was slower in the experimental mice compared with control mice ($P < .01$). It is possible that lentiviral vector-driven TCR expression in developing thymocytes alters the kinetics of T-cell repertoire selection and T-cell reconstitution in the thymus of transplanted mice.

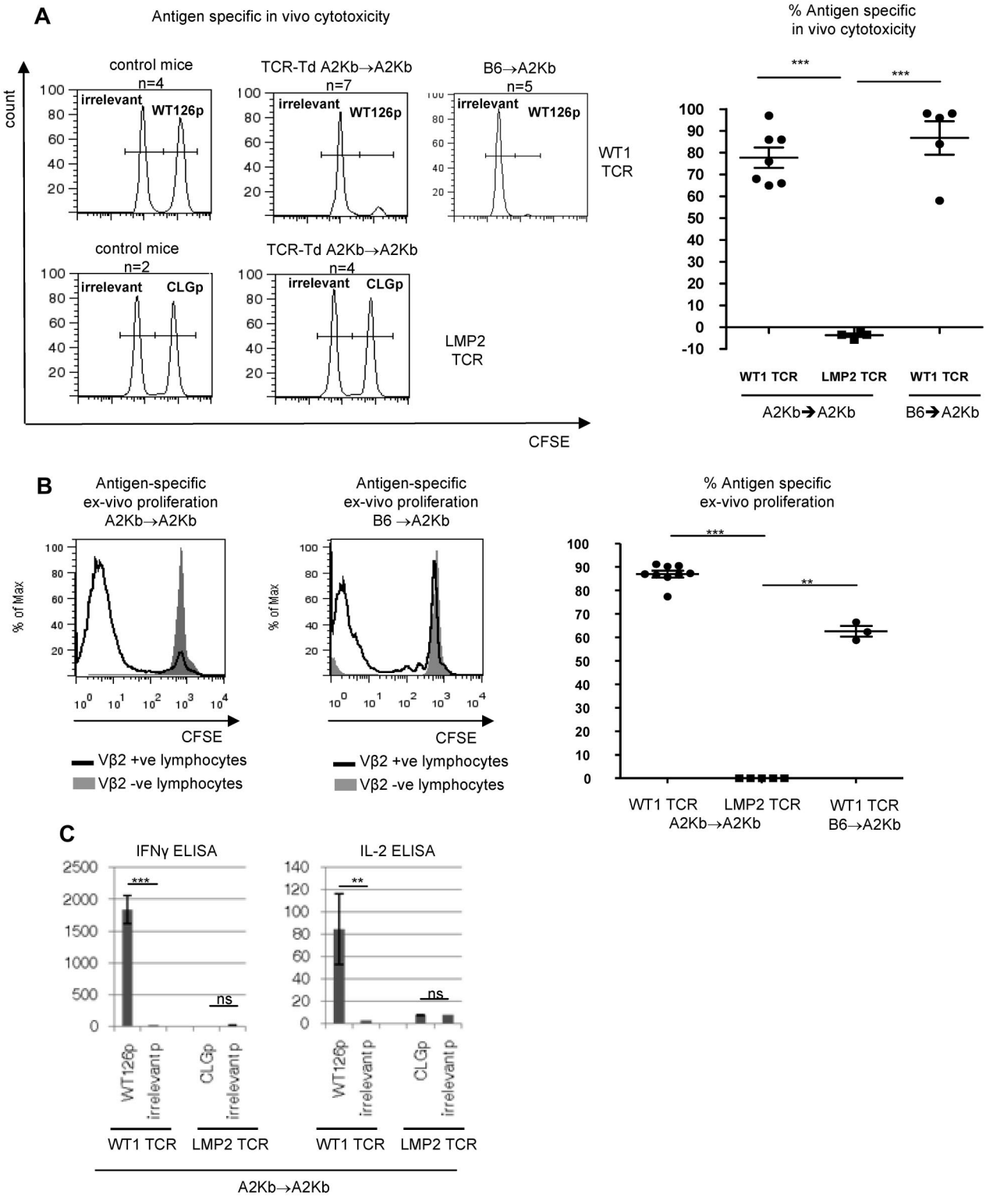

Figure 5. WT1 specific T cells display peptide-specific effector function. (A) In vivo cytotoxicity of CFSE-labeled peptide-loaded target cells. A2Kb Tg mice, 11 weeks after transfer of BM stem cells transduced with the WT1-TCR (top panel, n = 7 A2Kb → A2Kb, n = 5 B6 → A2Kb) or LMP2-TCR (bottom panel, n = 4 A2Kb → A2Kb) were intravenously injected with a 1:1 mix of relevant: irrelevant peptide-loaded A2Kb Tg splenocytes, differentially labeled with CFSE (WT1-TCR is specific for WT126 peptide, and LMP2-TCR is specific for CLG peptide). Eighteen hours later, splenocytes of injected animals were harvested and analyzed by FACS to identify CFSE-labeled cells. Representative plots are shown. Control untreated A2Kb Tg mice were injected with CFSE-labeled peptide-loaded target cells. Summary data of in vivo cytotoxicity assays are shown on the right. Percentage antigen-specific cytototoxicity was calculated as described in "In vivo cytotoxicity assays." ***$P < .001$. (B) Ex vivo proliferation of splenocytes from mice previously transplanted with WT1-TCR and LMP2-TCR-transduced A2Kb or B6 BM stem cells. Splenocytes were stimulated for 5 days with 100μM relevant or irrelevant peptide. CFSE-labeled splenocytes were analyzed by FACS for CFSE dilution after anti–human Vβ2.1 or Vβ13 antibody staining. A representative plot of pWT126-specific proliferation is shown on the left. Summary data of ex vivo proliferation assays using T cells harvested from mice transplanted with WT1-TCR (n = 8 A2Kb → A2Kb, n = 3 B6 → A2Kb) and LMP2-TCR (n = 5 A2Kb → A2Kb) transduced BM stem cells is shown on the right. **$P < .01$, ***$P < .001$. (C) Splenocytes harvested from A2Kb mice transplanted with WT1-TCR and LMP2-TCR-transduced A2Kb BM stem cells were stimulated ex vivo with 100μM relevant or irrelevant peptide for 5 days. ELISAs were performed to detect antigen-specific IFN-γ and IL-2 secretion. ns indicates not significant.

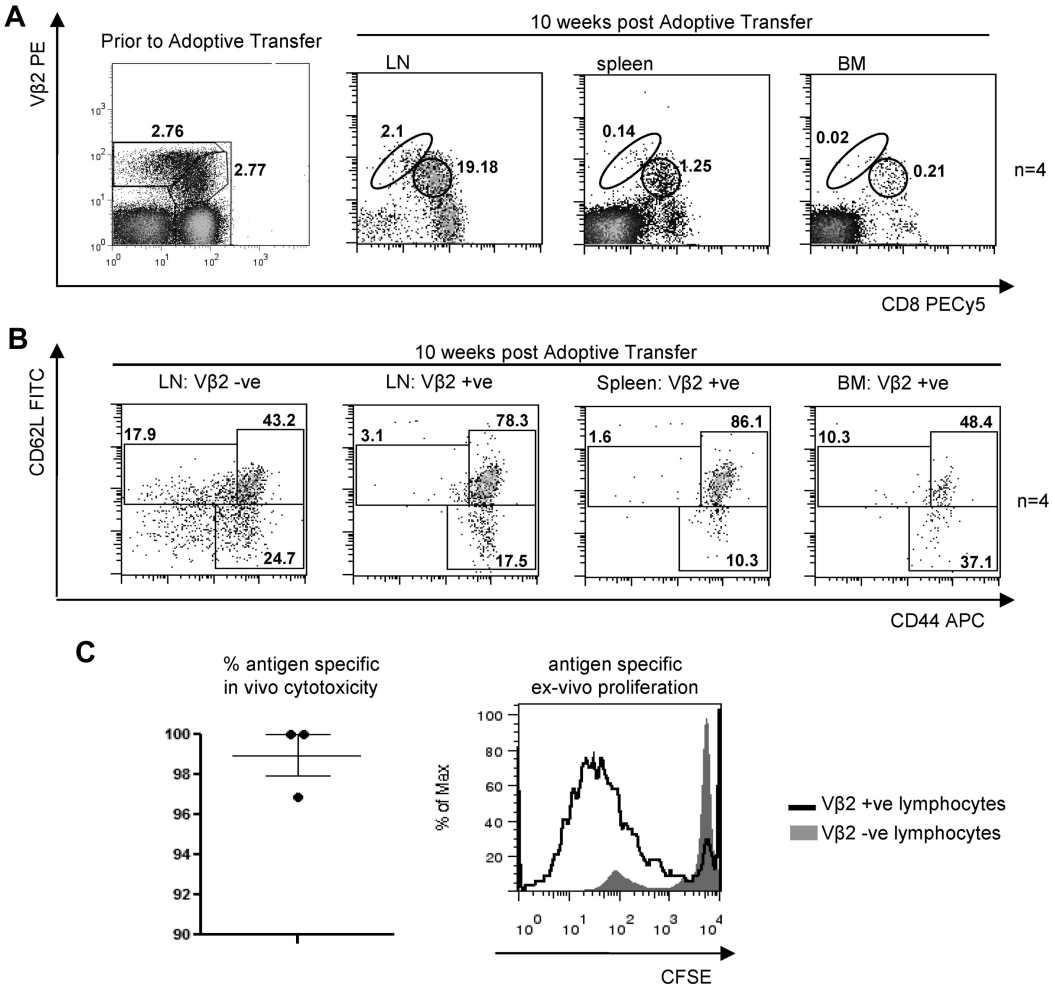

Figure 6. WT1-specific memory phenotype T cells display antigen specific effector function. (A) T cells were purified from A2Kb mice transplanted with WT1-TCR-transduced B6 BM stem cells and used for adoptive transfer into irradiated secondary A2Kb Tg recipient mice (n = 4). At 10 weeks after transfer, CD8/TCR expression of the transferred T cells was determined by FACS analysis of splenocytes, LNs, and BM after staining with antimurine CD8 and antihuman Vβ2.1 antibodies, respectively. (Left panel) The TCR and CD8 profile of the T cells before transfer. (Right panel) The profile 10 weeks after transfer. Percentage of TCR^{hi} and TCR^{lo} populations are shown after gating on total $CD3^+$ cells. (B) Analysis of the naive/memory phenotype of gated WT1-TCR^+ ($V\beta2.1^+$) T cells from spleen, LNs, and BM 10 weeks after adoptive transfer was determined by FACS analysis after staining with anti-CD44 and CD62L antibodies. (Left panel) The naive/memory phenotype of gated WT1-TCR^- T cells in LNs. (C) In vivo cytotoxicity experiments were performed in A2Kb Tg secondary recipients 10 weeks after adoptive transfer of T cells. Mean percentage killing of CFSE-labeled relevant peptide-loaded splenocytes from 3 mice is shown on the left. Ex vivo proliferation of WT1-TCR^+ and WT1-TCR^- T cells after stimulation with relevant peptide was determined by FACS analysis for CFSE dilution after staining with antihuman Vβ2.1 antibodies.

Discussion

We have used TCR gene transfer into HSCs to analyze the thymic development and peripheral function of T cells specific for a WT1 peptide presented by HLA-A0201. In this approach, populations of stem cells carrying distinct gene insertions give rise to mature T cells, thus avoiding possible founder effects of transgenic mice because of insertion of the transgene at a single genomic site. Further, in this model, the TCR-expressing cells represent only a small proportion of the whole T-cell repertoire providing an environment of polyclonal competition that contributes to the survival and maintenance of the peripheral T-cell pool.[36]

The WT1-specific TCR used was derived from high avidity human allorestricted $CD8^+$ T cells that efficiently recognize WT1-expressing human leukemia cells without impairing the function of human hematopoietic stem/progenitor cells.[23] In this study, the TCR was not deleted in the thymus but successfully selected into the single-positive CD8 lineage. Although some studies have demonstrated thymic WT1 expression, we found that the expression was high in the mesothelial lining but not in the epithelial stroma of the thymus, in agreement with previously described WT1 expression in cells with dual mesenchymal and epithelial properties, such as mesothelium, renal podocytes, and testicular Sertoli cells.[37] Our data indicate that physiologic WT1 expression does not prevent selection of a high-avidity WT1-specific human TCR.

Unexpectedly, in our study, WT1-specific T cells did not display signs of self-antigen–induced tolerance but instead differentiated spontaneously into fully functional memory phenotype T cells. Previous studies have clearly demonstrated that lymphopenia-induced proliferation can result in the differentiation of naive T cells into memory phenotype T cells,[38,39] and such cells can display antigen-specific effector function. However, this effect was transient, as antigen-specific effector function was only detectable during a time period of 12 to 31 days after T-cell transfer into

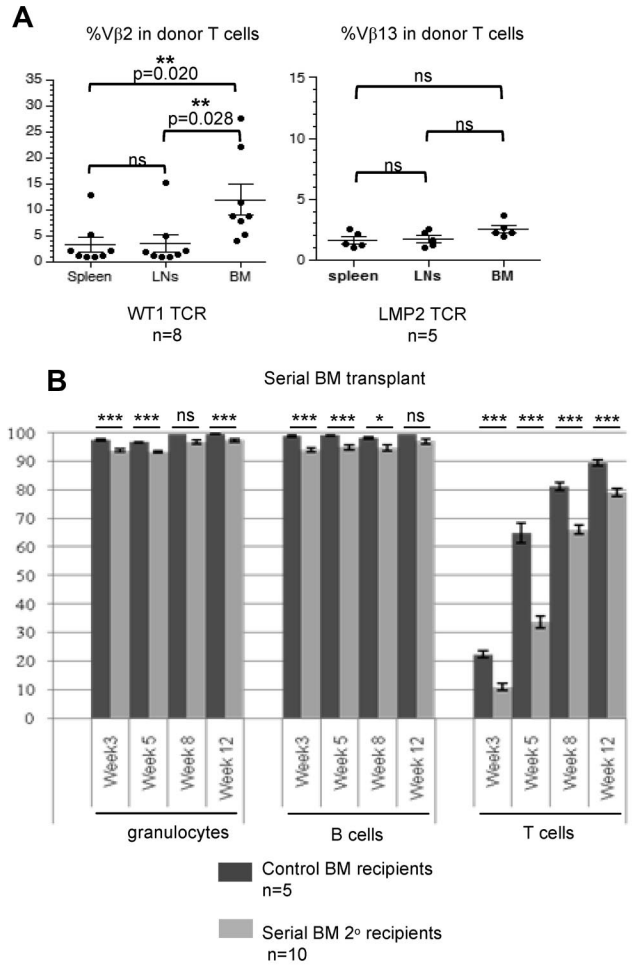

Figure 7. WT1 specific T cells accumulate in the BM without impairing stem cell function. (A) Donor T cells (CD45.1$^-$) were analyzed in the spleen, LNs, and BM of A2Kb Tg mice transplanted with BM stem cells transduced with the WT1-TCR (n = 8) or the LMP2-TCR (n = 5) at 12 weeks after transplantation. The percentage of CD45.1$^-$ donor T cells expressing Vβ2.1 (WT1-TCR) or Vβ13 (LMP2-TCR) was determined by FACS analysis. (B) Hematopoietic engraftment in secondary C57Bl/6 recipients (CD45.2) after transplantation of BM cells from A2Kb Tg mice that were previously transplanted with A2Kb BM stem cells (CD45.1) transduced with the WT1-TCR (n = 10). Peripheral blood of secondary recipients was stained with antimurine CD45.1, CD3, B220, and CD11b to identify donor hematopoietic cells, donor T cells, donor B cells and donor granulocytes, respectively. The peripheral blood analysis was done at weeks 3, 4, 8, and 12 after second transplantation. Control C57Bl/6 recipients received BM stem cells from untreated A2Kb (CD45.1) Tg mice (n = 5). Percentage of donor-derived cells are shown after gating on total granulocytes, B cells, and T cells, respectively. *$P < .05$, **$P < .01$, ***$P < .001$. ns indicates not significant.

lymphopenic hosts.[39] Although there are some similarities between our model and lymphopenia-induced memory phenotype induction, there are also important differences. In our stem cell transplantation model, thymic development generates a polyclonal T-cell population that reconstitutes the peripheral compartment. In this situation, the majority of non–WT1-specific T cells do not develop a memory phenotype, whereas a large proportion of the WT1-specifc T cells differentiate into memory T cells that maintain the ability to display antigen-specific effector function for several months after the initial stem cell transplantation. The reconstitution of a lymphopenic periphery after stem cell transplantation is similar to the generation of a peripheral T-cell repertoire in newborn mice, where adoptive T-cell transfer has previously demonstrated that lymophopenia-induced proliferation promoted memory phenotype development in approximately 20% of a transferred polyclonal T-cell population.[40] It was also shown that 2 monospecific TCR transgenic T-cell populations did not proliferate in the newborn hosts, suggesting that these TCRs, such as the TCRs of 80% of the polyclonal T cells, were not stimulated sufficiently by endogenous MHC/peptide ligands to trigger proliferation and memory development. These findings in neonates are very similar to our findings of T-cell reconstitution after stem cell transplantation. However, we found that the self-specific WT1-TCR was more effective than the polyclonal endogenous TCRs or the non–self-specific LMP2-TCR in differentiating into memory phenotype cells.

Our data are compatible with the proposal that chronic stimulation by self-antigen triggered the spontaneous differentiation of WT1-specific T cells into persisting memory cells. Unfortunately, both WT1 knockout mice as well as inducible tissue-specific knockout animals are not viable.[41] Thus, it is not possible to perform stem cell transplantation experiments in a WT1-deficient host to unequivocally prove that WT1 is the self-antigen driving the generation of central memory T cells.

Although specificity for self-antigen has been suggested to play a role in the generation of natural central memory phenotype cells, experimental evidence to support this has not yet been described.[18] A population of memory phenotype T cells was found in germ-free mice, suggesting that exposure to microbial antigens is not essential for their development.[19,20] Typically, the number of natural memory phenotype T cells increase with age,[18] which is similar to our observation that over time the frequency of WT1-specific T cells increased relative to the frequency of WT1-negative T cells (data not shown). A recent report demonstrated that, although natural memory phenotype T cells rapidly up-regulated CD69 on encountering peptide antigen in vivo, this did not correlate with in vivo cytokine production.[19] The lack of detectable effector function led the authors to propose the term "virtual memory cells." In contrast, the WT1-specific T cells were not "virtual memory cells" as they readily displayed in vivo cytotoxicity. A possible explanation for this difference is that the cytotoxic effector function is more readily triggered than in vivo cytokine production. Alternatively, it is possible that the natural memory phenotype population contains less differentiated virtual memory cells as well as more differentiated T cells capable of full antigen-specific effector function. It is tempting to speculate that the generation of virtual memory T cells is primarily driven by homeostatic proliferation, as suggested by Haluszczak et al,[19] whereas the generation of more differentiated memory T cells involves additionally TCR stimulation, as suggested by the observed TCR down-modulation in WT1-specific memory T cells.

The analysis of TCR-β usage showed that naive and natural memory phenotype T cells in young mice used similar TCR-β variable gene segments.[40] Our study extends this observation by demonstrating that T cells expressing identical TCR sequences can be present in both compartments. The data presented here are supported by a recent study in healthy human volunteers showing that both the naive and memory phenotype compartments contained WT1-specific T cells.[15] In patients with WT1-expressing malignancies, the frequency of naive phenotype cells was reduced, whereas memory phenotype T cells were increased. Whether this shift was the result of priming and differentiation of naive T cells or to the stimulation and expansion of memory phenotype T cells is currently not clear. The HLA transgenic model described here provides an opportunity to dissect which of the WT1-specific T-cell populations responds more effectively when encountering WT1-expressing malignancies or WT1-containing vaccines. It is conceivable that the antigen dose and the costimulatory signals for

the productive vaccination of naive T cells are different from the conditions required for optimal triggering and expansion of the memory phenotype T cells. Thus far, immune responses triggered by WT1 peptide vaccination were often transient and weaned soon after the vaccination.[10]

The transgenic murine model will help to define optimal conditions for WT1-directed T cell immunotherapies including vaccination with the aim of efficiently priming naive T cells while at the same time supporting the expansion and persistence memory phenotype T cells.

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Authorship

Contribution: E.C.M. and H.J.S. designed the experiments, interpreted results, and wrote the manuscript; C.P. designed and performed experiments and contributed to writing the manuscript; S.-A.X., A.H., C.V., M.P., F.F.-A., and B.F. assisted with and performed selected experiments; J.K. provided experimental data for the revision manuscript; and R.C. contributed to experimental design and writing the manuscript.

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