Analysis of the Mechanism(s) of Immunological Tolerance to a Physiological Soluble Antigen in Transgenic Mice

by

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A Thesis submitted in conformity with the requirements for the degree of the Doctor of Philosophy, Graduate Department of the Institute of Medical Sciences, the University of Toronto

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Abstract

The mammalian immune system recognizes and responds to a vast array of foreign molecules, and its diversity is generated by V-(D)-J gene recombination during ontogeny. The central feature of the immune system is self-nonself discrimination, which results in immunological reactivity or tolerance (specific immunological hyporesponsiveness to a previously encountered antigen-Ag). Breakdown of self-tolerance may result in autoimmunity to the host’s own self-Ags or tissues. Recent studies have demonstrated that both central (thymus-dependent) and peripheral (extra-thymic) tolerance can operate in an Ag-specific manner by at least two major mechanisms, namely, clonal deletion (physical elimination of autoreactive lymphocytes) and clonal anergy (functional inactivation of autoreactive lymphocytes). A third mechanism, active suppression (functional inhibition of autoreactive lymphocytes), may also be of relevance, particularly for peripheral tolerance.

We previously characterized the T-cell receptor repertoire specific for different insulins in Balb/c mice and identified a minimal immunogenic peptide [A chain: 1-13]. These results prompted us to establish a transgenic (Tg) mouse model to study the mechanism(s) of tolerance to a soluble self-Ag under physiological conditions in vivo. These mice express the gene for a foreign Ag (beef insulin, BI, mutated from human insulin genomic DNA) under the regulatory control of its own promoter so that the Tg product is expressed in pancreatic β-islet cells. In this Tg mouse model, the functional expression of BI (range: $10^{-10}$-$10^{-11}$M) is regulated by the host's glucose/insulin homostasis, and is associated with a differential activation of BI-specific Th1/Th2 cells in vivo. These Tg mice are hyporesponsive to BI immunization at the level of both humoral and cell-mediated immune responses, mediated by Ag-specific T cells. Despite the level
of no detectable thymic expression of the Tg products. BI-specific hyporesponsiveness may still occur in mature thymocytes of the Tg mice, suggesting the operation of a thymic selection process to a peripheral soluble Ag. Exogenous IL-2 can restore responses in peripheral T cells in vitro, suggesting also the involvement of an "anergy-inducing" mechanism. Adoptive transfer of some BI-specific CD4\(^+\) Th2 cells from Tg mice into normal syngeneic Balb/c mice induced Ag-specific hyporesponsiveness (as determined by BI-specific ELISA). This in turn suggests an active suppression mechanism may be involved in the maintenance of peripheral self-tolerance. Using a transwell coculture system, it seems that the cytokine TGF-\(\beta\), not IL-4 or IL-10, may be involved in suppression (but not killing or apoptosis) of Ag-specific Th1 cells. This active suppression is exerted in a bystander (Ag-specific and effector-nonspecific) fashion in vivo and is associated with self-tolerance.

The work presented in this thesis significantly extends the earlier findings in other soluble-Ag Tg model systems in which one dominant mechanism has been reported to explain the tolerance observed. Our results suggest that, at least for soluble Ags expressed peripherally:

(1) self-tolerance can be accomplished both intra- and extra-thymically, and

(2) there are multiple levels of regulation for self-tolerance in vivo, operating in an Ag-specific fashion, including thymic selection, peripheral anergy and active suppression.

These results provide clear and important information which contribute to our understanding of the basic mechanism(s) involved in self-nonself discrimination in biological tolerance. Application of this information should improve our understanding and treatment of various diseases associated with dysregulation of immune responses, including autoimmunity, transplantation and perhaps malignancy.
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I would like to thank another member in my supervisory committee, Dr. Gary Levy, for his support and input during this period. I also would like to express my appreciation to many other individuals in Dr. Hozumi's laboratory for their friendship and sharing the scientific atmosphere which have strengthened my endeavour for this thesis work.

I am forever indebted to the dearest and unconditional support from my family, especially my wife, Wendy Hsieh. Without their support and love, it would not have been possible to go through this period and have completed this work.

I believe that having accomplished this thesis work is only the beginning of a new page for scientific life which is a mix of struggles and joys. However, the intellectual benefits and consequences are yet to come and forever.
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List of Abbreviations

Antigen: $Ag$
B cell receptor: $BCR$
T cell receptor: $TCR$
Immunoglobulin: $Ig$
Transgenic: $Tg$
Major histocompatibility complex: $MHC$
Antigen presenting cell: $APC$
Cytotoxic T cell: $Tc$
Helper T cell: $Th$
Antibody: $Ab$
Double positive thymocyte: $DP$
Single positive thymocyte: $SP$
Thymic epithelium: $TE$
Superantigen: $SAg$
Minor lymphocyte stimulating antigen: $Mls$
Staphylococcus enterotoxin B: $SEB$
Beef insulin: $BI$
Human insulin: $HI$
Mouse insulin: $MI$
Chicken ovalbumin: $OVA$
Complete Freund’s adjuvant: $CFA$
Adult thymectomy: $ATX$
Bone marrow reconstitution: $BMR$
Adoptive transfer: $AT$
Normal: $N$
Reverse transcription polymerase chain reaction: RT-PCR,
Enzyme-linked immunosorbent assay: ELISA
Lymph nodes: LN
Suppressor T cells: Ts
Experimental autoimmune encephalomyelitis: EAE
Antigen presentation: AP
Antigen presenting cells: APCs
Chapter I

Introduction

I. Background

The major function of the immune system is to recognize and respond to foreign molecules, in order to protect the host’s integrity. Lymphocytes are specialized antigen(Ag)-specific cells of the immune system which recognize Ags by cell surface receptors. B cell receptors (BCR) are membrane immunoglobulins (Igs) which recognize three-dimensional determinants of native proteins. In contrast, T cell receptors (TCRs) recognize processed peptides (8-25 amino acid long) contained within the peptide-binding-groove of heterodimeric proteins encoded by the "major histocompatibility complex" (MHC) on the surface of specialized Ag-presenting cells (APCs). In mammals, the primary lymphoid organs (bone marrow and thymus) are responsible for the development of immunocompetent cells from their precursors, whereas the secondary lymphoid organs (such as spleen, lymph nodes and tonsils) are responsible for the generation and regulation of immune responses (such as humoral and cell-mediated immunity) by host immunocompetent cells, upon the capture of foreign Ags.

BCR/TCR molecules are formed in lymphocyte precursors after rearrangement of V (variable), D (diversity), J (joining), and C (constant) gene segments. Functional rearrangement produces genes that encode either the disulphide-linked heavy and light chains of the Ig receptors on B cells or the disulphide-linked α and β TCR molecules (or γδ TCR). Since random recombination takes place separately in lymphocyte precursors, another process called "allelic exclusion" ensures that only one receptor specificity is generated per cell. The enormous
diversity of BCRs/TCRs constitutes the immune repertoire. The different lymphocyte lineages mediate different effector functions, including the secretion of Ag-specific antibodies (Ab) by B cells; destruction of virus (or bacteria)-infected cells by cytotoxic T cells (Tc); and secretion of different cytokines by T helper cells (Th). Binding of the TCR to peptide/MHC complex is assisted by the co-receptor molecules CD4 and CD8 on the surface of Th and Tc cells, respectively. The CD4 coreceptor binds to MHC class II and the CD8 coreceptor binds to MHC class I molecules. Engagement of TCR and CD4 by MHC class II/peptide or of TCR and CD8 by MHC class I/peptide plays a critical role in delivering at least two independent cell-activating signals to the TCR-associated CD3-complex proteins. The TCR:CD3 coreceptor is a multichain complex linked to multiple intra-cellular signalling pathways which determine activation of the lymphocytes. There are other accessory molecules on the cell surface which are also involved in the induction of proliferation, differentiation and/or effector functions of lymphocytes.

The principal developmental pathway for T cells expressing the αβ TCR molecule is in the thymus, although extra-thymic T cell differentiation also exists. As a result of the random generation of repertoire diversity, receptors are produced recognizing foreign and self-components of the host. There must be some "selective" mechanism(s) to regulate these self-reactive lymphocytes and, simultaneously, to diversify the recognition of foreign Ags presented by self-MHC molecules. During thymic ontogeny, it is believed that a given TCR recognizes the presenting self-polymorphic MHC molecules, thus imprinting the host's receptor repertoire (positive selection) for foreign Ags [1]. Developing T cells with receptors that can recognize self-peptides/MHC molecules are purged (negative selection) thus eliminate those cells with self-reactivity [2]. Thus, self-nonself discrimination is acquired during the development of T cells in thymus.
I-A. T cell development and its lineage commitment

The haematopoietic precursors that seed the thymus are CD3-CD4-CD8' thymocytes [also TCRαβPgp-1(CD44)IL-2Rα*(CD25)HSA*]. These cycling cells undergo TCR β chain rearrangement leading to cytoplasmic β-chain expression. This suppresses endogenous β-chain rearrangement and, after associating with gp33 molecules, produces an immature (pre)TCR/CD3 cell surface receptor complex. TCR β chain rearrangement also enhances TCR α-chain rearrangement and its expression [3]. Introducing a TCR β transgene (Tg) into rearrangement-deficient [RAG-1 & 2 (-/-) and SCID mutation] mice results in the promotion of thymocyte development [4]. Thus, the rearranged TCR β gene is essential for the regulation of allelic exclusion of TCR β chain and promotion of maturation and differentiation of thymocytes.

Thymocytes next turn off IL-2Rα expression, rearrange their TCR α locus, and begin to express CD4 and CD8 and low levels of surface TCR, making the transition from an IL-2Rα bearing CD4CD8' (double negative; DN) stage to a CD4+CD8+ (double positive; DP) stage. TCR-dependent selection within the thymus operates on this DP TCRlow phenotype.

It has been demonstrated, by adoptive transfer of DP TCRlow blast cells in vivo [5], that there are sequential quantal stages of TCR expression during DP thymocyte differentiation, which appear to be associated with positive, default and negative selection events in the thymus. Thus, in the CD4 differentiation pathway, DP TCRlow cells give rise to a transitional intermediate (CD4+CD8lowTCRmed) and then to CD4+TCRhigh cells. A parallel (CD4lowCD8+TCRmed) is also found on the pathway to CD8+TCRhigh cells. During this period, engagement of self-MHC by αβ+ TCR leads to the appearance of mature CD4+CD8' and CD4+CD8+ (single positive; SP) cells. However, the order and stage of positive and negative selections in thymus has been controversial, since the results are dependent upon the different experimental models used (see...
(i) Positive and negative selection:

Superantigens (SAgs) are potent immunostimulating Ags that bind to MHC molecules and particular TCR Vβ chains. They were originally termed the Mls (minor lymphocyte stimulatory) Ag, the templates for which were subsequently identified as the endogenous mouse mammary tumour virus (MMTV) integrants [6]. Positive and negative selection was examined in Vβ-defined thymic subsets in mice lacking or possessing cognate Mls. In Mls+ mice, DP TCRlow cells were neither enriched for nor depleted of, Mls-specific Vβ-specific thymocytes. It is the transitional intermediates (CD4highCD8lowTCRmed and CD4lowCD8highTCRmed) that were enriched in those Vβ types subject to positive selection. These cells continued to develop to SP thymocytes but during, or just following, the transition, particular Mls responding TCR Vβ types were deleted in the presence of the appropriate selecting self-MHC molecules. These data suggest that DP TCRlow blast cells give rise to TCRmed intermediates by positive selection, followed by the emergence of thymic TCRhigh cells, while negative selection prevents the production of self-Ag reactive TCRhigh cells. The significance of negative selection has also been clearly demonstrated for MHC class II I-E+, Vβ17a-reactive T cells, and for Vβ-TCR+ cells recognizing staphylococcal enterotoxin B (SEB) in normal mice. It is generally believed that thymocytes with specificity for self-Ags are present at the TCRlow stage but are eliminated before reaching high TCR expression.

In MHC class I Db, H-Y Ag-specific TCR Tg mice, von Boehmer et al. [4] showed that DP thymocytes were positively selected in female Db-positive mice and subsequently differentiated into CD4+CD8+ killer cells. However, in male mice, DP thymocytes committed
suicide when their TCR bound to H-Y Ag/D$^b$ MHC molecules. This result suggests that binding of the $\alpha\beta$ TCR to a thymic MHC molecule is essential for rescuing DP thymocytes from cell death and that the specificity of the $\alpha\beta$ TCR for either MHC class I or II molecules determines whether the rescued cells are of the CD4$^+$CD8$^+$ killer or CD4$^+$CD8$^-$ helper phenotypes, respectively. The general scheme of positive selection has now been established and extended to several different models where Tg mice expressing TCRs specific for different peptides presented by either class I or II MHC molecules have been used [7,8]. The consequences of positive selection of $\alpha\beta$ TCR include: 1) regulation of TCR $\alpha$-chain rearrangement 2) the termination of the further TCR rearrangement process, 3) the production of functionally mature effector precursors, and 4) the death of the majority of thymocytes by a default pathway (> 95% nonselected).

(ii) Models for positive and negative selection

It has been documented that peptide-MHC complexes are recognized by TCR during positive and negative selection. Therefore, peptides may be involved in producing and shaping the TCR repertoire. The crystal structure analysis of the murine MHC class I molecule revealed that a short peptide about 8-9 amino acids forms an integral part of the binding groove within the whole molecule [9], suggesting that TCR binds to MHC class I while contacting a peptide. Thus, a TCR recognizes a bimolecular surface, much of which is composed of the self-MHC molecule, with the peptide contributing important TCR contact residues in the centre. Similar observations were obtained in MHC class II crystallization studies [10], despite a longer peptide (about 13-23 amino acids) being embedded in the binding grooves.

More recent studies [11] have used a murine MHC class I K$^b$ "mutant" molecule with a
modification of the peptide-binding site outside the TCR contact residues. The results revealed that specific CD8+ T cells did not mature in the mutant thymus and that F1 mice (wild type plus mutant allele) could support the appearance of mature CD8+ T cells, suggesting that appropriate contact between peptide and TCR is important for positive selection. Similar results have been observed for positive selection of MHC class II-restricted CD4+ T cells. Using a gene-targeting strategy, it was demonstrated that there was very low level expression of MHC class I molecule and greatly reduced numbers of mature CD8+ T cells, in β2m(-/-) and TAP(-/-) mice [β2m and TAP-1 are protein involved in correct folding and expression of class I MHC molecule and peptide transport to the endoplasmic reticulum, respectively; ref 12 & 13]. Addition of exogenous β2m and peptides in thymic organ cultures significantly restored the class I expression and development of mature CD8+ T cells [12,13] in both cases. It was shown later that complex mixtures of peptides were very effective in restoring the normal TCR repertoire. However, it was also demonstrated recently by Ignatowicz et al. [14] using both MHC class II and invariant chain (Ii) -/- mutant mice, that a single peptide/MHC II ligand was able to select a relatively large number of cells in the mature CD4+ TCR repertoire. Many of these cells also reacted with allogenic MHC molecules bound to other self-peptides but were deleted due to high avidity with the same MHC bound to other self-peptides. These results suggest that a high proportion of the positively selected T cells specific for one peptide/MHC ligand are clonally deleted due to strong interactions with the same MHC molecule bound to other self-peptides. Thus, different arrays of self-peptides may be selected differentially depending on the individual TCR-peptide/MHC interactions. These data indicate an influence of specific peptide recognition on repertoire selection involving contact with TCR in the context of self-MHC molecules. There are three hypotheses proposed to explain the mechanism(s) mediating positive and negative selection. They
are described as follows:

(ii-1) The affinity hypothesis:

When T cells recognize self vs foreign Ags, the signals for positive and negative selection must presumably be recognized differently. One notion is that the affinity of the TCR for peptide/MHC determines whether positive or negative selection occurs. Briefly, positive selection involves low but significant affinity binding, while negative selection involves high affinity binding of peptide-MHC to the TCR. Consistent with the latter are findings by Lo et al. that positive selection was driven by lower affinity reactions than negative selection [15]. This quantitative hypothesis was tested recently using a Tg approach where the TCR remained constant and the expressed level of MHC or coreceptors was varied [16]. The density of MHC molecules was found to modulate the extent of positive selection of mature CD4+ T cells [16]. Additional Tg expression of the CD8 coreceptor improved positive selection of anti-HY TCR in female H-2b mice while efficient negative selection was observed in male mice [17]. Similar findings were also obtained in other MHC class I and class II TCR-Tg mice models [7,8].

Ashton-Rickardt et al. [18] examined the role of single peptides for positive selection in fetal thymic organ culture by crossing the TAP1 (-/-) mice [13] with lymphocytic choriomeningitis virus (LCMV) peptide (33-41)/H-2D^b determinant-specific TCR Tg mice. Addition of exogenous native peptide resulted in positive selection of CD8+ thymocytes. Low doses (< 3 uM) of agonistic peptide induced positive selection, while higher doses (> 30-300 uM) of the same peptide induced negative selection. Non-agonistic peptides were not tested in their study. Similar results were studied by Sebzda et al. using different concentrations of peptide
to mediate positive and negative selection in vitro [19]. The authors suggested that the number of TCR bound with peptide/MHC complexes determines the fate of the developing T cells. Note that the above assays do not provide measurement of the intrinsic affinity of the TCR/peptide/MHC complexes. The density of MHC, coreceptor or accessory molecules and the concentration of the peptides all presumably exert some influence on the ultimate fate of the T-cells undergoing selection.

To date, the strongest direct evidence correlating the affinity of a TCR and peptide/MHC ligand with the outcome of thymic selection comes from work of Alam et al. [20] in which the affinity of wild type ovalbumin peptide/H-2Kb complexes with the TCR was measured using a binding assay (via surface plasmon resonance; the BIAcore instrument). A weak affinity constant \( (6.5 \times 10^{-6} \text{M}) \), characterized by slow association \( (3100 \text{ M}^{-1}\text{S}^{-1}) \) and fast dissociation \( (0.02 \text{ S}^{-1}) \) rates was determined. Peptide variants can induce positive selection with different efficiencies in thymic organ culture [21]. The authors found a correlation between the affinity of the TCR/peptide/MHC interaction and the outcome of thymic selection and T-cell activation. The window of affinity selection resulting in positive selection vs negative selection was only 3-fold. These data suggest that small differences in the affinity between the TCR and peptide/MHC ligands are sufficient to alter the fate of the developing T cells. It has been shown recently that addition of a soluble CD8 molecule to soluble class I restricted TCR’s in the presence of the restricting element resulted in a measurable improvement in the \( K_{\text{diss}} \) rate for the TCR/peptide/MHC class I complex, suggesting CD8 is an active participant in the TCR recognition complex [22]. These results provide a mechanistic explanation for the quantitative affinity/avidity hypothesis. However, the roles of other players such as CD4 (for MHC class II), costimulatory molecules (B7/CD28) and adhesion molecules (LFA-1, ICAM-1) have not yet been
An alternate form of affinity hypothesis suggests that the nature of the affinity for TCR/peptide/MHC complex may change with the developmental stage in the thymus. There may be different triggering thresholds for developing thymocytes which mediate survival/maturational signals vs deletional/death signals. This would suggest that positive and negative selection occurs at distinct developmental stages [23]. The expressed level of the Bcl-2 (apoptosis-resistance protein) molecule seems to correlate with the susceptibility of developing thymocytes to deletion after positive selection in vivo [24]. Thus, the Bcl-2 level among SP thymocytes drops, leaving the cells more susceptible to deletional signals. Other data argue against this hypothesis, suggesting that the signals for selection are driven by avidity of the TCR-peptide/MHC interactions [4,23] as discussed previously. Some studies [25] have shown that the threshold for activation of mature T cells is higher than for thymic deletion, allowing a margin of safety in self tolerance achieved by negative selection. Thus, for immature thymocytes in the cortex, when peptide/MHC complexes in the microenvironment stimulate at a sub-optimal threshold to developing T cells, positive selection may occur. Afterwards, only high-avidity interactions trigger a response, and the response involved is cell death.

(ii-2) The altered peptide hypothesis:

Positive and negative selection may be mediated by different cell types in the thymic microenvironment. It was proposed that the thymic cortical epithelium might present a distinct repertoire of peptides which are not found elsewhere in the body, which in turn drive positive selection [26]. However, direct analysis of peptide bound to thymic MHC molecules and the finding of a MAb that detected self-peptide/MHC complexes on APCs but not thymic cortical
epithelium have not supported such a hypothesis. Recent studies have shown that MHC molecules on fibroblasts [27], or lymphoid cells [28] can drive positive selection in the thymic microenvironment. The fact that these epithelial cells or fibroblasts were able to mediate SAg-induced clonal deletion in-vitro is a strong argument against the altered peptide hypothesis. It has also been shown that thymocytes undergoing positive selection on epithelial cells can be deleted if their specific peptide was provided in large amounts [29]. This ruled out any simple difference in the signals delivered by APCs and also suggested that the different responses can not simply be a reflection of the developmental stage of the thymocyte itself.

(ii-3) The altered peptide ligands (APL) hypothesis:

There are some studies describing the use of peptide variants to partially activate or inhibit the activation cascades of T cell responses. TCR recognition of peptide variants was first reported in a CD4+ Th2 clone where a single amino acid variation in the native peptide resulted in a loss of T cell proliferation [30]. Some peptide variants act as competitive antagonists for T cell activation, suggesting that activation of the TCR is not simply an on/off switch [31]. The TCR can sense subtle changes in its ligand and respond differently on the basis of recognition of the variant peptide, defined as partial T-cell activation. Amino acid substitution at predicted TCR contact sites in an antigenic peptide alter its interaction without affecting peptide association with the relevant MHC [31] or competition for presentation by the MHC molecules. T-cell ligands can be classified as fully activating (agonist), suboptimally activating (partial agonist) or non-activating (antagonist). APLs are considered either partial agonists or antagonists, and are presumed to provide less or non-stimulatory signals associated with decreased affinity for TCR compared to the agonists [31].
Hogquist et al. [32] examined the role of peptide variants for positive selection in thymic organ cultures using the β2M(-/-) OVA(257-264)/H-2Kb-specific TCR-Tg mice. The results showed that antagonistic peptides gave positive selection while agonistic peptides gave negative selection, at all concentrations tested. One mixed (agonistic and antagonistic) peptide gave positive selection at low concentrations, where it acted as an antagonist, but negative selection when used at a higher, agonist, concentration. These data suggested that TCR binding to large numbers of peptide/MHC complexes drives positive selection, provided binding does not signal full activation, while binding and activation of mature T cells triggers deletion. These experimental systems [21,32] have also revealed a relationship between ligands that act as TCR antagonists for mature CTL and those that induce positive selection, consistent with the findings by Alam et al. discussed earlier [20]. These studies suggest that developing T cells in the thymus are sensitive to changes in their interaction with the selecting cells. Thus, it appears possible that there are two routes to positive selection, one involving a low level of complete TCR signals, and the other involving partial signals delivered by antagonistic peptides. The influence of multiple endogenous peptide variants (as APLs) on TCR repertoire development [33], if it exists in vivo, may thus offer an alternative explanation for thymic selection. However, analysis of the role of APLs in class II CD4+ T cell development gave rather conflicting results for positive and negative selection, compared to those of the class I CD8+ T cells [34,35]. Further analysis will be necessary to clarify the difference between CD4+ and CD8+ T cells interacting with peptide variants or antagonists.

(iii) The cell types mediating positive and negative selection

Cells in the thymic stroma can be divided into radiation-resistant epithelium and radiation-
sensitive cells (macrophages, dendritic cells and thymocytes). Bone marrow (BM) chimeric mice have been used to explore the role of different stromal elements in selection. In these chimeras, expression of donor vs host MHC alleles in the two thymic compartments differs after lethal irradiation and BM reconstitution in vivo (donor BM-derived cells; host-epithelium). T cell development in such chimeras or in thymus-grafts (haematopoietic lineages depleted) can be used to infer the cell type(s) responsible for positive and negative selection. Classic experiments showed that BM stem cells injected into irradiated allogenic or semiallogenic hosts (parent -- > F1 chimera) developed into T cells capable of responding to Ag presented by the MHC of the irradiated host, rather than that expressed by the BM-derived cells themselves. Analysis of chimeras engrafted with thymi depleted of BM-derived cells showed that it was the radioresistant TE that imprinted MHC restriction on developing thymocytes [36]. In class II I-E (/-) mice, when thymectomized, irradiated, BM-reconstituted mice were grafted with fetal deoxyguanosine (dGuo)-treated thymic lobes (to remove haematopoietic elements), the results showed that promotion of positively selected T cells (Vβ 17a+ T cells detected by MAbs) was dictated by the MHC haplotype of the TE [1]. Further studies using a tissue-specific promoter for Tg MHC class II I-E expression confirmed that the thymic cortical epithelium is the controlling cell type for positive selection [37]. There may be some additional influence of non-MHC background genes on positive selection, but the ligands and mechanisms involved are still unclear.

It has been suggested, in contrast, that BM-derived cells are more important for clonal deletion [15]. This was tested directly in the Vβ 17a/I-E system by analysis of BM chimeric mice constructed to express I-E on various cell types in the thymus [38]. Expression of I-E by BM-derived cells led to a significant elimination of Vβ 17a+ cells from the mature thymocyte pool. Interestingly, the interaction of TCR with the I-E complex on thymic medullary epithelium
also led to clonal elimination in this model [38]. Addition of purified dendritic cells to BM-depleted thymi led to induction of full tolerance [39]. In other models, T cells developing in H-2-different thymi depleted of BM-derived cells by dGuo treatment showed little or no tolerance to thymic-type H-2 Ags [38,39]. These data suggest that thymic negative selection is largely under the control of BM-derived cells.

TE has also been shown to induce a state of split tolerance [tolerance to class I CD8+ but not to class II CD4+ T cells; ref 40]. In such studies [41], T cells were tolerant to epithelial MHC molecules as assessed by the failure to reject skin grafts, but responded in in-vitro functional assays (CTL & MLR). Deletion could not be fully explained by contaminating haematopoietic cells [40]. One possibility is that spleen cells used as stimulators and targets in in-vitro functional assays expressed peptides that were not found on TE [42] and/or express higher levels of MHC molecules than the TE. The question of whether the TE can induce negative selection remains controversial. Since the expression of MHC on cortical epithelium is low, and the TCR density on the CD4+CD8+ T cells is also low, it is conceivable that epithelium-induced negative selection is inefficient (avidity effect). Thus, deletion in the thymus may instead require contact with BM-derived cells.

(iv) **Instructional vs stochastic model for CD4/CD8 lineage commitment**

It is not understood what mechanism(s) are responsible for how cells with class I or class II MHC-restricted TCRs become CD4+CD8+ cytotoxic killer and CD4+CD8- helper precursors in the thymus, respectively. Two hypotheses have been proposed to explain lineage commitment of CD4+ or CD8+ precursors. The first, called an "instructional" model, predicts that a class I MHC-specific TCR and CD8 coreceptor, coengaged by the same class I MHC molecule,
generates a different signal from a class II-MHC-engaged receptor complex, thus directing differentiation into either killer or helper cell precursors [43]. Positive selection would consist of a single recognition event at the DP stage, which drives maturation and directs the turn-off of the appropriate coreceptor (CD8 for MHC class II vs CD4 for MHC class I) subsequently followed by upregulation of TCR levels. The second model, a stochastic one, predicts that lineage commitment occurs stochastically by down-regulation of either CD4 or CD8 surface expression without regard for TCR specificity. A cell with a fit TCR-coreceptor combination (MHC class I/TCR and CD8, or class II/TCR and CD4) on binding to the appropriate MHC molecule, would be selected for survival and maturation [44], whereas cells with an un-fit TCR:coreceptor would die. Lineage commitment in this model occurs prior to, and independent of, positive selection.

Murine models using Tg-TCRs have shown that TCR specificity for MHC class I or class II is associated with coexpression of CD4 or CD8 molecule on maturing T cells [8,45], suggesting that lineage commitment and positive selection occur at the same stage and are driven by the same recognition event. Data in an anti-H-Y TCR Tg mice system favours the instructional model [4,17,43]. Alternatively, a stochastic/selective model could also explain the results of TCR Tg mice for lineage commitment [44]. Because MHC class I (or II) recognition requires CD8 (or CD4), thymocytes bearing class I-specific (or II-specific) TCR would undergo positive selection only if they have turned off CD4 (or CD8) and maintained CD8 (or CD4) expression. Based on the stochastic model, the failure of CD4⁺ class I-restricted TCR to mature is due to the lack of CD8 molecule. Constitutive expression of CD8 should thus rescue thymocytes bearing class I-specific T cells. A similar prediction occurs in regards to the rescue of class II TCR-bearing CD8⁺ T cells with constitutive expression of CD4 molecule. Data
obtained from experiments to test this hypothesis gave mixed results [46-48]. Tg CD8 expression dramatically improved the development of CD8-bearing class I-TCR lineage without any effect on CD4-bearing cells [46]. In contrast, CD8+ class II-restricted T cells were observed in a Tg CD4 and class II-restricted TCR [46]. Meanwhile, expression of a CD4 Tg in β2m mutant (MHC class I -/-) mice led to the appearance of a small number of mature CD8+ T cells [47]. However, the observed rescue efficiency was low unless the coreceptor Tg was highly overexpressed [48]. It was argued that the low number of rescued cells may reflect a minor developmental pathway which did not receive correct instruction. When the cytoplasmic tails of CD4 and CD8 were swapped, the maturing T cells with the fixed Tg TCR specificity were also altered, favouring the instructive hypothesis [49]. These results suggest that the instructional model is a more general pathway, but that stochastic commitment to the CD4 or CD8 lineage can occur for some class II-restricted T cells. Thus selection may follow an asymmetrical pathway for lineage commitment.

The results of intra-thymic transfer showed that transitional CD4+CD8b cells can re-express either CD4 or CD8 and give rise to SP thymocytes [50]. Moreover, in class I-deficient mice, only cells re-expressing CD4 were found among the CD4+CD8b intermediate population, whereas in double class I & II-deficient mice, reculture of DP thymocytes led to the appearance of cells expressing only CD4 molecules [51]. These data suggest that differentiating thymocytes may undergo an unexpected series of cyclic and asymmetric changes in coreceptor levels while progressing from the DP to SP lineages. A further understanding of the mechanism(s) involved in lineage commitment of CD4/CD8 precursors requires additional studies.

I-B. T cell receptor (TCR) structure and its activation
The TCR transduces the signals involved in the growth, differentiation and activation of the effector functions. Signalling via the TCR is followed by activation of a series of protein tyrosine kinases with the resultant phosphorylation of cellular proteins including enzymes that regulate lipid metabolism, GTP binding proteins, serine/threonine kinases and other transducing molecules. The TCR is composed of a glycosylated polymorphic heterodimer, either αβ or γδ, which is non-covalently associated with a non-polymorphic, membrane-bound, complex of proteins called CD3 complex. Both chains of the TCR have an amino-terminal variable domain with homology to immunoglobulin V regions, a constant domain with homology to immunoglobulin C regions, and a short hinge domain with a cysteine residue which forms the interchain disulphide bond. The hydrophobic transmembrane domain of the TCR contains some positively charged residues mediating interaction with appropriately charged polypeptides known as CD3 γ, δ, ε and ζ subunits [52], which are homologous to the Ig-α and Ig-β proteins of the BCR. The TCR αβ heterodimer recognizes Ag in a processed peptide form, bound and presented by the membrane-bound MHC molecules (class I or II molecules) displayed on the surface of APCs. Ag recognition by γδ TCR is more diverse and less well characterized and will not be discussed further.

Ligand-induced signalling via the TCR receptor complex is mediated by the cytoplasmic domains of the CD3 subunits γ, δ, ε and ζ-chain [52]. The intracellular protein of these molecules contain a common motif, termed an immunoglobulin receptor family tyrosine-based activation motif (ITAM), which is critical for TCR coupling to intracellular tyrosine kinases and subsequent TCR signalling cascade. An early phase of the biochemical response elicited by the TCR [53] has been shown to be the activation of protein tyrosine kinases (PTK). There are several PTKs associated with the TCR/CD3 complex including the src family kinases p59fyn and
A 70-kD PTK termed ZAP-70 is also critical for TCR signalling [52]. The initial events following TCR stimulation include activation of the src kinases, phosphorylation of the ITAMs, and the recruitment, tyrosine phosphorylation and activation of ZAP-70 [54]. This recruitment process is mediated by the SH2 domain of the ITAMs. ZAP-70 has two SH2 domains which bind preferentially to a doubly phosphorylated ITAM [54,55]. Tyrosine-phosphorylated ZAP-70 in turn interacts with the SH2 domains of other molecules such as Vav or the PTK p56\textit{Lck} [52], suggesting that it acts as an "adaptor" molecule. It has been well documented that during TCR activation, there are multiple signalling pathways activated including those involving inositol lipid metabolism, a GTPase-p21Ras pathway, PKC and calcium mobilization, and pathways involving \textit{Map} (mitogen-activated protein kinases) or \textit{Jun} kinases [52]. Differential involvement and/or regulation of downstream adaptor proteins by the ligand-induced TCR-signalling cascades may explain the pleiotropic effector functions following TCR-mediated signal transduction. Further, partial phosphorylation of the recruited ZAP-70 molecule has been shown to be associated with T cell anergy [56,57]. Such different signalling pathways could lead to different biological responses.

There is evidence that the TCRs undergo oligomerization during signal transduction [58]. Thus, T cells can be triggered by divalent Abs specific for TCR/CD3 determinants, but not by their monovalent Fab fragments [59]. Upon crosslinking of chimeric molecules (extracellular and transmembrane portion of TCR with cytoplasmic regions of CD3 \(\varepsilon\) or \(\delta\) chains), signals similar to those generated by the intact TCR were generated, suggesting that juxtaposition of CD3 subunits is required to promote cross-phosphorylation of PTKs associated with the TCR/CD3 complex [58]. However, TCR signalling via conformational changes was also proposed by Karjalainen [60] and Janeway [61] since the Fab fragment of one particular anti-TCR Ab can
induce the physical association of the TCR with CD4 and CD45 critical for T cell activation [62].

It is generally agreed that to trigger T-cell activation, two signals are required [63]. The first signal is mediated via the peptide/MHC ligands for the TCR/CD3 complex. The second so-called co-stimulatory signal, occurs via accessory molecules. Amongst these, the B7-1, B7-2/CD28, CTLA-4 have been most intensively studied (see below: peripheral tolerance section). Using an in-vitro model, it was shown that TCR occupancy in the absence of costimulation for activation resulted in clonal anergy [64,65]. The costimulatory pathway amplifies the signals necessary for autocrine/paracrine production of IL-2 for T cell proliferation. Lack (or blockade) of costimulation inhibits naive T cell activation, and may explain why certain APCs, such as resting B cells, can induce anergy in vitro or in vivo, and may promote transplantation tolerance [66].

I-C. Functional heterogeneity of T cells

Diversity in Th phenotypes was first demonstrated by Mosmann et al., who showed that murine CD4+ T cell clones could be classified into distinct populations based on their patterns of cytokine production [67]. These were termed Th1 and Th2 clones, with Th1 producing IL-2, IFN-γ and TNF-α, and Th2 producing IL-4, IL-5, IL-6, IL-9, IL-10, and IL-13 [68]. Recently, IL-10 has been shown to be produced by human Th1 cells, and activated macrophages [69]. Cytokines such as IL-3, GM-CSF, and TGF-β can be produced by both Th1 and Th2 cells. For individual T cells, other mixed patterns of cytokines may be produced [70]. Th1 cells are the principle effectors of cell-mediated immunity against intracellular micro-organisms and of delayed-type hypersensitivity (DTH) reactions [68]. IL-4, a cytokine released primarily by Th2
cells is critical for Ig class switching and production of IgE humoral Ab response, and another Th2 cytokine, IL-10, can inhibit APC functions in macrophages [71]. Th1 and Th2 cells may arise from uncommitted, naive common precursors [72]. Priming of naive CD4+ T cells in the presence of IL-12 induces the development of Th1 effector cells, while priming of naive CD4+ T cells in the presence of IL-4 yields Th2 effectors [73,74]. A recent study suggests that newly activated naive T cells express both Th1 and Th2 cytokines but rapidly extinguish the expression of inappropriate cytokine as they differentiate towards the Th1 or Th2 phenotype within the first 24-48 hrs [74].

The cytokines produced by each Th subset serve as autocrine growth factor to promote the differentiation of other naive T cells to that subset [75]. In addition, each subset produces cytokines that regulate the development of the other T cell population. For example, IL-12 produced by activated macrophages and dendritic cells is the principal Th1-inducing cytokine [73]. IFN-γ produced by Th1 cells amplifies Th1 development and inhibits the proliferation of Th2 cells [71,74,75]. IL-10 produced by Th2 cells can block the activation of Th1 cells [71]. In addition, IL-4 and IL-13, also produced by Th2 cells, can antagonize the macrophage-activating function of IFN-γ [75]. Thus, another function of Th1/Th2 cytokines is to cross-regulate the balance of immune responses. Similar observations have also been demonstrated in CD8+ T cells [76,77] and γδ T cells [78]. Th1-dominant immune responses are often associated with inflammation and tissue injury; for example, TNF-β and IFN-γ can recruit and activate inflammatory leukocytes associated with DTH. The cytokines IL-4 and TGF-β can suppress the proliferation and activation of leukocytes in acute or chronic inflammation including DTH [75].

No functional markers specific for Th1 and Th2 cells have been identified except the cytokine profiles. A recent study suggests that IL-12 receptors may be associated with priming
of naive T cells to Th1 cells, and are lost on differentiation to Th2 cells [79]. IL-12 can activate three main transcription factors, Stat1 (signal transducer and activator for transcription 1), Stat3 and Stat4 [80]. Stat 4 appears to be activated solely by IL-12, and animals with homologous gene deletion of either IL-12 or Stat4 produced markedly reduced Th1 responses [81,82]. IFN-γ can also promote Th1 differentiation, both by enhancing IL-12 production from activated macrophage or dendritic cells, and by maintaining the IL-12 receptor expression on CD4+ Th1 cells [81,82]. IL-4 promotes the differentiation of Th2 cells, probably through Stat6. There is a consensus Stat6-binding site present in the IL-4 promoter region. Homologous gene deletion of either IL-4 or Stat6 resulted in deficient Th2 responses [83,84]. T cells themselves can produce small amounts of IL-4 following initial activation so that continuous T cell stimulation may itself result in progressive IL-4 production to promote Th2 development. Despite these studies of cytokine-induced Stat pathways, the biochemical basis for Th1/Th2 development remains unclear. In addition, a small population of CD4+ NK1.1 cells was identified as another source for early IL-4 production in association with recognition carrying CD1 molecules [85]. The role of this pathway for in vivo Th2 responses is unknown.

Differential activation of Th1/Th2 cells has been associated with infectious diseases. Studies of cutaneous Leishmaniasis in inbred mice provided a clear demonstration that disease resistance and susceptibility are correlated with Th1 and Th2 immune responses, respectively [86]. In general, resistance to certain bacterial, fungal and protozoal infections is associated with production of macrophage-activating cytokines IFN-γ and TNF-α [77]. Similarly, in human tuberculoid and lepromatous leprosy, Th1 and/or Th2 cytokine productions have been implicated in different forms of the disease [77].
II. Models for studying self-tolerance

(1) Neonatal tolerance model:

The classic model of neonatally induced tolerance has provided significant information for the cellular basis of immunological tolerance.Billingham et al. [88] showed that lymphohaematopoietic cells (15 x 10^6 of bone marrow and spleen) from adult H-2 allogenic (AxB) F1 mice injected i.v. into newborn (<24 hr) mice of parental (A) strains produced tolerance. Surviving adult (A) animals (> 8 wks) challenged with allogenic skin grafts of the other (B) parental strain accepted skin grafts indefinitely. The tolerant mice rejected third-party (H-2 incompatible) skin allografts, indicating that tolerance was Ag-specific. These data suggested that introduction of foreign Ags into the immature immune system induced tolerance in Ag-reactive lymphocytes. It was hypothesized that clonal deletion of self-reactive lymphocytes was the cellular basis for the establishment of self tolerance [89].

Neonatal recipients (for B10.T and B10 strains) of semi-allogenic haematopoietic cells (class I or/and class II disparate) became chimeric in both thymic and peripheral lymphoid organs. Specific alloreactive T cells were depleted in the thymus and peripheral T cells as defined by MLR & lytic assays [90]. Maintenance of donor chimerism was required for persistent tolerance in the host and it was hypothesized that persisting chimeric APCs were associated with the maintenance of tolerance [91,92]. When donor chimeric cell numbers fell to insignificant levels, recovery of host alloreactive effector T cells resulted in the rejection of the skin graft [91].

When B10.AQR (I-E') and B10.T (I-E'; disparate at class II I-A & I-E only) neonates received F1 donor cells, the recipients became permanently tolerant of skin grafts [92].
defined by the lack of in-vitro reactivity (MLR). This tolerance could not be abolished by infusion of naive syngeneic T cells. Class II tolerance in this case was adoptively transferred with spleen and lymph node cells to naive syngeneic recipients, suggesting that active suppression was involved in maintaining tolerance. However, an in-vitro MLR response to I-E bearing stimulators could be restored when exogenous growth factors (IL-2) were added, implying that clonal anergy was also involved in maintaining the tolerance.

In using another strain combination (A.TL and A.TH; class II disparity alone), newborns that received F1 haematopoietic cells also became tolerant to a skin graft indefinitely but this time there was no chimerism and a vigorous in vitro MLR response occurred when host cells were stimulated with class II tolerogen-bearing cells [93]. Adoptive transfer of spleen and lymph node cells again confirmed the transfer of tolerance to naive mice suggesting a role for active suppression. Using limiting dilution analysis, primed, IL-4-producing, tolerogen-responsive T cells were amplified in association with the maintenance of tolerant [94] while the frequency of IL-2-producing T cells was comparable in both control and tolerant mice.

Taken together, these data suggest that when class I disparate skin grafts were accepted, clonal deletion and donor lymphoid chimerism is important for the maintenance of tolerance. For class II allogenic-Ag mediated neonatal tolerance, active suppression (or anergy) in association with priming of IL-4 producing Th2 cells, is important for self tolerance. These data are consistent with other reports by Dorsch and Roser [95] and Gorczynski and MacRae [96] using different models.

(2) Irradiation bone marrow chimera model:

A model of allogenic BM irradiation chimeras in which recipient mice were treated first
with total-body lethal irradiation and then reconstituted with allogenic donor BM cells has been explored for many years. If reconstitution was successful, the resulting chimeras were found to be tolerant of both donor and recipient Ags as assessed by in-vitro assays (MLR, CML) or by in-vivo transplant assays [97]. To overcome recipient resistance to donor BM engraftment, and the potential for graft-vs-host (GVH) disease (from the donor), elimination of host T and NK cells prior to BM reconstitution, and the removal of T cells (by MAbs) from the donor inoculum have been used.

In the BM chimeras, donor-derived BM cells repopulate the recipient's thymus and induce tolerance (in the host) to the donor Ags by a deletional mechanism in the thymus, presumably in a similar manner to self-tolerance in normal mice [see below (5) conventional mouse model]. Tolerance to recipient Ags is a function of expression of recipient Ags on the radio-resistant TE and other non-lymphoid tissues. Deletion of reactive cells is not complete and some form of intrathymic anergy has also been postulated [46,98].

MHC disparate allogenic BM chimeras may become immunologically compromised even after successful engraftment due to the positive selection of the T cells in the recipient thymus (by recipient MHC), resulting in a poor donor-APC/recipient-T cognate interaction in the periphery [99]. Alternative approaches which eliminate this immuno-incompetence have used reconstitution with a mixture of allogenic donor and syngeneic BM cells. The resultant mixed BM chimeras were tolerant to both sets of Ags, along with the presence of peripheral APCs expressing both sets of MHC Ags [100]. There was no GVHD observed even when T cells were not eliminated from the donor inoculum [101], implying that the mechanism responsible for tolerance is associated with thymic deletion. Use of fractionated irradiation to lymphoid organs (total lymphoid irradiation-TLI), a protocol which has been used clinically to treat
Hodgkin's disease, along with BM transplantation has also led to chimerism and tolerance induction [102].

In the field of transplantation tolerance, it has been suggested that the persistence of lympho-haematopoietic chimerism in recipients plays an important role in the long-term survival of organ allografts [103]. The issue of the importance of chimerism in transplantation tolerance is controversial [104].

(3) Thymic engraftment model:

Although the TE appears to be involved in positive selection of T-cells, the role of TE in tolerance induction has been controversial [105]. One of the experimental models to study the role of TE in tolerance induction involved the use of transplanting epithelial thymic rudiments prior to haematopoietic colonization into host embryo recipients at a early stage of development [106]. In rodents, well-dissected embryonic day-9 or-10 third and fourth pharyngeal pouches (day 6.5-8 in chickens) consist of an epithelium surrounded by a loose capsule of mesenchyme, which do not have significant influx of haematopoietic precursors. These TE can be engrafted into xenogenic, allogenic, athymic or thymectomized hosts to study the cellular mechanisms for tolerance in vivo. Host-derived T cells develop in the grafted thymus which is also colonized by other BM-derived cells in a similar fashion to normal animals [106]. When mouse fetal thymic lobes (day 14-15 gestation) were used for engraftment, they required certain treatments such as deoxyguanosine (dGuo) for 5 days in vitro [107], low-temperature culture (24°C) for 7-8 days [108], or lethal irradiation (1000R) to deplete BM-derived cells (dendritic cells, macrophages) before transplantation under the host's kidney capsules.

When quail embryo limb buds (or the bursa of Fabricius) were engrafted in xenogenic
chick embryos (day 4), acute rejection occurred after the maturation of the host's immune system [109]. Some degree of tolerance of the grafted wing was obtained in an allogenic situation, and tolerance to donor-type skin grafts could be induced [110]. In general, embryonic grafts of different tissues were accepted and full tolerance was established if the donor TE was also engrafted to the same recipients [109, 110]. When dGuo-treated donor fetal TE was engrafted into an allogenic intact (non-thymectomized) host environment, lymphocytes recovered from both the epithelial grafts and the spleen of the recipients responded to the donor MHC Ags in mixed leukocyte cultures [107], suggesting an inability of the TE to induce allo-tolerance. However, when the TE cultured was grafted into allogenic athymic nude mice, host-derived lymphocytes in the recipient spleen were found to be hyporesponsive to MHC Ags of the epithelial donor [108]. Using the same model system for transplanting dGuo-treated allogenic TE, it was shown later that T-cells were reactive to donor class I MHC Ags but tolerant to minor MHC Ags of the allogenic thymic donor presented in the context of recipient MHC expressed in BM-derived haematopoietic cells [111]. No MHC-haplotype specific suppressor cells were detected in this model.

There were concerns regarding the possibility that donor macrophages and/or dendritic cells remained in the thymic graft after the dGuo or low-temperature treatments. Either class II molecules on the TE grafts or molecules acquired by thymocytes [112] could thus induce the class II (not class I) tolerance in these chimeras, so-called "split tolerance". The tolerogenicity of the TE varies considerably depending on the Ag, the sub-population of T cells studied and the overall avidity of TCR-peptide/MHC complexes. CTL specific for allogenic (Balb/c) dGuo-TE grafted CBA nude mice were deleted [113], suggesting that under certain circumstances TE can induce tolerance to MHC class I Ags. However, in vitro functional (CTL assay) assays may
or may not correlate with the state of tolerance in vivo. One example for this was demonstrated in the case where C3H-derived embryonic TE was grafted into Balb/c nude mice. The repopulating mature T cells responded to donor lymphocytes in vitro (CTL assays) yet simultaneously accepted donor-specific skin grafts [114]. These data suggest that clonal deletion in the thymus may not be necessary to ensure in-vivo functional tolerance to tissue grafts. In fact, there are data to suggest that dGuo-treated TE can delete high but not low affinity CD8+ T cells in vivo [113] and that tolerance can be induced by a peripheral mechanism such as anergy [106] in embryonic allo-grafts [110].

(4) Superantigen model:

T cells recognize processed peptide Ags bound to MHC molecules by their heterodimeric TCR. Other forms of interaction involving so called superantigen (SAg) can occur to engage specific TCR Vβ families in association with certain MHC molecules and trigger T cell responses. The SAg ligands binding at high frequency to responding T cells (exceeding 15 - 20 % of TCR repertoire) were initially defined as Mls Ags [115]. Cells expressing TCR Vβ6.8.1.9 reacted to Mls-1* expressing cells in vitro, and T cells obtained from Mls-1 expressing mice were specifically deleted of responding Vβ cells in the thymus [115]. Mls is presented to immature thymocytes in the context of a MHC class II molecule, but the ability of different MHC molecules to present Mls does not appear to be uniform; thus, deletion can occur in some haplotypes but not in others expressing the same Mls haplotype (such as H-2k). The first genetic linkage between the Mls SAgS and the mammary tumour provirus (MTV) integrants was discovered for the Eto-1 Ag. This interacts with Vβ5 & 11 expressing T cells, and the encoded gene was linked to the endogenous MMTV-9 (Mtv-9) locus on chromosome 12 [116]. B cells
are potent presenters of Mls SAGs [117], particularly after stimulation with the mitogen lipopolysaccharide (LPS). It appears that Mls SAGs are not expressed by TE, BM-derived macrophages and thymic DC [117]. However, Mls SAGs can be transferred between these cells so that Mls-mediated negative selection can occur in vivo [115, 117].

Adoptive transfer of Mls-expressing splenocytes, B cells or CD8+ T cells into Mls' mice resulted in three-phases of an immune response [118, 119]. Initially, there was a clonal expansion of Mls-reactive TCR Vβ cells (CD4+ T cells) followed by a subsequent clonal deletion phase [119]. Not all T cells expressing Mls-reactive Vβs were deleted. The remaining T cells became hyporesponsive (or anergic) to activation signals delivered by TCR crosslinking or Mls SAGs. This particular anergy state was reversed in vivo when the tolerogen was removed [114, 120].

Bacterial toxins such as staphylococcal enterotoxins (SE) and the toxic shock syndrome toxin (TSST) bind to MHC class II molecules of mice and human with estimated binding affinity (10^4-10^7M) and activate TCR Vβ gene segments [121, 122], in a similar fashion to endogenous Mls SAGs. Injection of these bacterial toxins into neonatal mice led to clonal deletion of thymocytes expressing toxin-specific Vβ molecules [121]. In adult mice, i.v. injection of SEB also resulted in the deletion of CD4+ Vβ8+ T cells followed by Ag-specific inactivation (anergy) in the peripheral lymphoid organs [123].

The dominant TCR Vβ gene usage for SAGs in humans remains less clear. Several studies in human immunodeficiency virus (HIV; ref 124) and rheumatoid arthritis [125] have implicated SAg-like phenomenon as reflected by unique Vβ-specific deletion in some AIDS patients [124]. Again, these data are somewhat controversial [126].

(5) Conventional mouse model:
The study of tolerance mechanism(s) important for self-Ags in conventional mouse strains has benefited from the development of MAbs and Tg technology. Clonal deletion was first shown by the absence of MHC class II (I-E) reactive T-cells in I-E+ mice [2]. For example, Vβ17a+ T cells which recognized I-E molecules (MAb KJ23a) were absent in the peripheral lymphoid tissues of I-E+ mouse strain (B19.Q, B10.BR) but were present in high numbers in I-E- strain (SWR). Examination of the TCR Vβ chain expression in the thymocytes showed that clonal deletion occurred during thymocyte development from the DP to SP stage. Deletion was also demonstrated in normal F1 (Vβ17a+, I-E- x Vβ17a+, I-E+) mice. Experiments with irradiation BM chimera indicated that MHC molecules expressed on BM-derived cells but not TE induced self tolerance [127].

von Boehmer et al. used Tg mice expressing a TCR (Vβ8.2 identified by MAb F23.1) that recognized the murine self male Ag (H-Y) in the context of MHC class I (Db) molecules [4]. Expression of the Tg H-Y specific TCR in male mice also expressing H-2Db resulted in deletion of DP and SP (CD4-CD8+) thymocytes. The resulting peripheral mature T cells were hyporesponsive to H-Y Ags suggesting that self-tolerance had been induced. Several Ag-Tg and TCR-Tg systems including allogenic MHC class I and II molecules, viral Ags, and foreign protein Ags have been used to examine the underlying mechanisms of self-tolerance. It has been suggested that the mechanism responsible for self-tolerance may vary depending on the amount of Ag expression; the site of expression; the timing of Ag expression; the level of costimulation; the APC involved for lymphocyte activation; the accessory molecules involved; the available ligand density (such as MHC molecules) on the cell surface; and the overall avidity of TCR/peptide/MHC complexes. More detailed discussion follows in the section below entitled "Mechanism of Self-Tolerance".
(6) Other adult tolerance model:

Protocols have been developed to induce adult tolerance towards allogenic Ags in murine systems. These include cyclophosphamide(CP)-induced tolerance, and anti-lymphocyte serum (ATS) or MAb-induced tolerance. In one such study, adult mice were given i.v. injections of allogenic spleen cells followed 2 days later by i.p. injection of 200 mg/Kg CP. Long-lasting Ag-specific skin allograft tolerance was seen across non H-2 barriers of many strain combinations, but not with fully allogenic mice. Different mechanisms of tolerance were involved at separate stages in this model [128-130].

During the first stage after infusion of allogenic cells and CP treatment, clonal deletion [130] occurred in the peripheral tissues (CTL assay), but not in the thymus. Four weeks later, Tc cells were identifiable in the peripheral lymphoid organs, although they failed to respond to allo-Ags in vitro, suggesting that anergy [129] may be involved in tolerance at this stage. If the donor cells were irradiated before CP treatment, no persistent chimerism was found and deletion of allo-reactive T cells was incomplete. In addition, a significant allo-response was seen in vitro, suggesting the peripheral chimerism may be associated with the maintenance of tolerance to allogenic skin graft [131]. Before the 4th to 5th week, tolerance was not transferable to naive animals. However, at the 3rd to 4th month, tolerance was transferable to syngeneic naive recipients using spleen and lymph node cells, suggesting that a suppressive mechanism [128] had emerged to downregulate tolerogen-specific allo-reactivity. These findings were comparable to those of the neonatal tolerance model.

Since, the thymus is the primary site in which central tolerance occurs, direct injection of donor cells into the thymus has also been used as a mechanism for tolerance induction. The original approach used pancreatic islets as the donor tissue [132] and was followed by the use
of spleen cells, BM and kidney cells [133,134].

Monaco & Russell developed an technique involving treatment of the recipients with ATS followed by infusion of donor bone marrow cells [135] to achieve adult tolerance in rodents. This was associated with some degree of lymphoid chimerism. A more recent protocol involves the transfer of donor BM cells and the use of MAb to modulate the activity of peripheral CD4⁺ and CD8⁺ T cells. This resulted in promotion of tissue engraftment and tolerance induction by anergy or/and suppression mechanisms [136]. When MAbs directed against CD4 or CD8 T cells were used in an allogenic skin graft model [137], circulating donor CD4⁺ Th2 cells were shown to be responsible for transferring the allograft tolerance to the naive host, suggesting an active suppression mechanism was involved in maintaining the tolerance to tissue grafts in these particular models.

III. Studies concerning the mechanism(s) for immunological tolerance

In general, immunological tolerance has several features:

1) Antigen-specificity is usually maintained.

2) It is an acquired process during development.

3) Immature lymphocytes are most susceptible to tolerance induction, and

4) Tolerance can be induced in mature lymphocytes to foreign antigens under specific conditions.

Recent studies have demonstrated that both central (thymus-dependent) and peripheral (extra-thymic) tolerance can be mediated, in an Ag-specific manner, by at least two distinct mechanisms, clonal deletion and clonal anergy. A third mechanism "active suppression", defined as the functional inhibition of self-reactive lymphocytes, may be of relevance in peripheral
tolerance. Breakdown of self tolerance results in autoimmunity or self-destruction to the host's own self-Ags or tissues.

The thymus is the major site of T cell tolerance induction. However, peripheral tolerance mechanisms also exist [138] probably to serve as a fail-safe mechanism for preventing autoimmunity. This is necessary since, (i) central tolerance may be a leaky event [2,23,139]. (ii) there exist many tissue-specific self Ags in the periphery not expressed within or not circulating to the thymus. It is not clear how tolerance is induced or maintained to developmentally regulated self Ags [140,141].

**Mechanisms of self tolerance:**

(1) **Central tolerance**

In the thymus, immature thymocytes contact a variety of peptide/MHC complexes and, go through the complex processes of differentiation and selection [4,7,105], eliminating potentially damaging self reactive cells, while retaining the ability to recognize foreign antigens in the context of self-MHC molecules. Intrathymic deletion of self-reactive T cells has been demonstrated in several model systems (see above p.20-29: models for studying self tolerance) including conventional mouse model (I-E reactive Vβ17a⁺ TCR and anti-H-Y TCR-Tg; see p.26-27) and SAg model (Mls and SEB; see above p.25-26).

In studies comparing the TCR β-chain Tg and αβ-chain Tg (recognizing Mls-2⁺ & -3⁺) mice, Berg et al. reported that deletion of DP thymocytes only occurred in αβ Tg mice and deletion of mature SP thymocytes occurred in both β and αβ Tg mice [142]. It was suggested that higher surface expression of TCRs (both α and β genes) on immature thymocytes allowed
deletion of most DP as well as more mature SP thymocytes. In contrast, for TCR β-chain Tg mice, developing thymocytes followed a normal differentiation course awaiting endogenous rearrangement of the α-chain locus for successful expression and, therefore, deleted only the more mature SP thymocytes [142]. A more complex model using TCR(αβ-chain)-Tg mice of dual Ag-specificity (lymphocytic choriomeningitis virus-LCMV and Mls-1a) was investigated [23]. In LCMV-carrier Tg mice, there were reduced numbers of DP thymocytes and peripheral CD8+ T cells. By contrast, clonal deletion was observed in both mature SP thymocytes and peripheral T cells of the Mls-1a+ mice. These results suggested that 1) clonal deletion had not necessarily occurred at only one stage of T cell development, 2) the mechanism(s) of tolerance induction involving the same TCR can vary with the Ag used, and 3) the stage at which deletion occurred could be a function of the density and affinity of the TCR [23].

Most of the model systems studied showed that thymic deletion was not complete, suggesting that it may be a leaky process and autoreactive T cells do still exist in the periphery. A subsequent experiment using TCR(Vβ8.1)-Tg mice (recognizing Mls-1a Ag) showed that a significant fraction of the CD4+ peripheral T cells that survived thymic deletion were hyporesponsive to further stimuli (anti-TCR/CD3 Abs, mitogens and SAgs) in vitro [139]. These data suggest that Mls-1a-reactive cells were subject to two mechanisms of tolerance, clonal deletion in the thymus and clonal anergy in the periphery. One hypothesis to explain these findings is that high avidity clones are clonally deleted in the thymus, whereas lower avidity clones are inactivated and therefore hyporesponsive in the periphery [23,139].

A more recent study [143] using the same TCR αβ-Tg mouse model (recognizing LCMV and Mls-1a), demonstrated that while mature Tg-T cells were hyporesponsive to a self Mls-1a. CD8+ T cells could still mount immune responses to conventional peptide Ags or alternative
SAGs in vivo. The results suggested that low-affinity self-tolerant T cells remained functionally competent for high-affinity foreign Ags, and thus maintained an ability to eliminate foreign pathogens in vivo. The reasons for the different results from the Kawal et al. [143] & Blackman et al. [139] studies regarding the function of peripheral tolerant T cells, remains unclear.

In addition to deletion, thymic inactivation (or central anergy) is implicated in self tolerance. The mechanism for this was investigated in Tg mice with tissue-specific expression of MHC class II I-E [144] in pancreatic islets or the exocrine pancreas with the fate of Vβ17 and Vβ5 bearing T cells being followed. A reduced mixed lymphocyte reaction (MLR) was observed using either thymocytes or splenocytes and anti-TCR Ab to stimulate non-deleted mature T cells. Peripheral tolerance could originate in the thymus possibly due to the transportation of MHC class II I-E molecules back to the thymus [156]. Irradiation-bone-marrow chimeras (parent --> F1 BM reconstitution) were generated to assess the role of the thymic epithelium (TE) in tolerance induction [98]. In these studies, the fate of Vβ 17+ (I-E reactive) and Vβ 6+ (Mls-1* reactive) T cells were followed by MAbs. Only limited deletion of Vβ 17+ and Vβ 6+ T cells (10-25% and < 50% of the normal control, respectively) was seen, suggesting that tolerance was achieved by clonal anergy. Further support for this hypothesis came from the inability to stimulate Vβ 17+ and Vβ 6+ mature SP splenocytes from the chimeras by MAbs. Both mature thymocytes and peripheral T cells manifested comparable levels of tolerance. Thus, central tolerance could also result from specific clonal anergy [98].

It was hypothesized [98,145] that the TE might not provide sufficient co-stimulation for full T cell activation, so that anergy occurs by default. The TE does express MHC molecules and can present autologous haemoglobin to T cell hybridomas, but does not activate T cell clones [145,146]. Anergy induction by TE may thus be connected with an inability of TE to function
as a source of competent APCs. Many self-Ags reach the thymus in soluble form, or can be captured and presented by circulating or resident thymic APCs in situ. Previous experiments demonstrated that i.v. administered myoglobin could be presented by class II-expressing dendritic cells in the thymus, within 15 minutes [147]. In a complement (C5) sufficient mice model [148], T cell immunity or tolerance resulted from the self(C5)-peptide/MHC class II presentation and activation of the thymocytes or T cell clones [148]. Therefore, peripheral Ags may require a thymic microenvironment for tolerance induction in some conditions.

(2) Peripheral tolerance

Several groups have generated tissue-specific Ag expression models to study the nature of peripheral tolerance and have demonstrated non-deletional tolerance to Tg-Ags in vivo without lymphocytic infiltration in target tissues, suggesting the usefulness of this approach to study tolerance to normal tissue-specific Ags.

Studies in MHC-Tg mice where foreign class I or class II genes were expressed selectively in pancreatic β-cells or hepatocytes of the liver [139,149], have demonstrated systemic tolerance to the MHC-Ags without lymphocytic infiltration in the target organs. There was a higher incidence of diabetes but not autoimmune insulitis in some Tg models [150,151]. It was considered that diabetes resulted from MHC overexpression in the pancreatic β-islets as a result of: 1) Tg competition for regulatory factors with inhibition of insulin secretion [151] or 2) class II protein binding insulin molecules. Inappropriately high level MHC expression may itself interfere with insulin secretion [152].

Using Tg mice carrying the MHC class II I-A^d [151,153] gene expressed in the pancreatic acinar cells, under the regulatory control of rat elastase promoter-REP, or in β-islet cells under
the regulatory control of a rat insulin promoter-RIP, a T cell proliferation assay (MLR) demonstrated significant allo-reactivity against cells bearing the I-A^d allele. However, functional tolerance to the Tg pancreatic I-A^d seemed to exist in vivo [151,153] since there was a lack of lymphocytic infiltration. Thus, T cell tolerance in vivo was mediated by a non-deletional mechanism. Tg mice carrying the MHC class II I-E molecules under the regulatory control of REP & RIP, all demonstrated a weak response using T cells from the thymus and the periphery with no signs of autoreactivity in pancreatic islets or exocrine pancreas [139,144]. The explanation for this preservation of reactivity to MHC class II Ags in vitro is not clear, although it has been speculated that the different expression pattern of the different constructs used may result in the difference observed. Alternatively, there may be an intrinsically stronger reactivity to I-A than I-E molecules [154]. When these Class II I-E Tg mice were backcrossed to appropriate I-E^- strains (to become homozygous for Vβ 17a^+ and Vβ 5^+ TCR genes in SJL/J and C57BL/6, respectively), there was no significant difference in the frequency of Vβ 17a^- and Vβ 5^- bearing T cells (measured by MAbs) between RIP-I-E & REP-I-E Tg mice and I-E^- non-Tg controls [139], suggesting that functional tolerance in vivo was not caused by clonal deletion.

In mice with a Tg MHC class I H-2K^b under the control of different tissue-specific promoters (metallothionine promoter, or rat insulin promoter-RIP), an inability to reject H-2K^b skin grafts, as well as reduced allo-reactivity of Tg splenocytes (in vitro assays), was observed. There was a vigorous thymocyte reactivity to H-2K^b targets, suggesting a post-thymic nature for the tolerogenic mechanism [149,150]. To follow the fate of CD8^+ (H-2K^b allele-specific) T cells, class I K^b Tg mice (driven by different tissue-specific promoters, including glial fibrillary acid protein-GFAP, keratin IV and albumin) were mated with anti-K^b TCR Tg mice to generate double Tg mice. Peripheral tolerance can be mediated by several distinct mechanisms including:
receptor down-regulation (of TCR); clonal anergy; and possibly clonal deletion of CD8$^+$ T cells [155,156]. Similar double Tg (RIP-H-2k$^+$ Tg x anti-H-2k$^+$ TCR) mice were mated with RIP-IL-2 Tg mice (all carrying RIP promoters). Rapid onset of diabetes was seen only in the triple-Tg mice [157], suggesting that these non-deleted but tolerant T cells, in the presence of high local concentrations of IL-2, can trigger autoimmune diabetes.

Presentation of Ag by cells that lack competent APC function (like pancreatic β-islets) can paralyse Ag-reactive lymphocytes, reflecting the absence of a requisite second costimulatory signal by APCs [65,144,145,149,154]. These conditions result in low avidity T cell:APC interactions and anergy induction and/or suboptimal triggering with low level IL-2 production for autocrine stimulation [158]. Many protocols have been used to produce T cell anergy in vitro and in vivo, including the use of chemically modified spleen cells [158]; purified MHC class II on planar membranes [159]; IFN-γ treated class II$^+$ keratinocytes [160]; and Ag presentation by non-professional APCs [161]. The study of non-transformed, cloned T cell lines has shown that TCR:CD3-engagement in the absence of costimulation is insufficient to induce optimal IL-2 production and results in long-lasting anergy [158,159,161]. However, anergized MHC class I-restricted CD8$^+$ clone can still maintain their effector functions after delivery of a tolerogenic stimulus [162], suggesting that anergy is focused primarily on preventing autocrine proliferation after Ag exposure.

Costimulation that prevents anergy induction and promotes optimal IL-2 production (and regulation of apoptosis) can be mediated by the interaction of CD28 on the T cells with molecules of the B7 family on APCs [161,163]. Other costimulatory ligand pairs, including ICAMs/LFA-1 [164], VLA-4/VCAM-1 [165], CD40/gp39(CD40L) [166] may also display these functions. Both CD28 and B7 belong to the immunoglobulin superfamily. IL-2 production by
naive T cells stimulated with Ag and APCs or LPS is markedly augmented if B7/CD28 interaction occurs [167]. By contrast, APCs that lack B7 are poor inducers of IL-2 production and a competitive inhibitor of the B7-CD28 interaction (CTLA-4-Ig) blocks IL-2 production [168]. More recent studies suggest that the different B7 family molecules (B7-1, B7-2; CD80, CD86, respectively) may transduce different signals to naive T cells [169-171]. MAbs to B7-1 inhibit the development of murine EAE [171], whereas anti-B7-2 MAbs treatment made the disease worse [184]. Yet, CTLA-4, a CD28 homologue expressed on activated lymphocytes which binds both B7-1 and B7-2 [172] with greater avidity than CD28, can apparently participate in mediating a negative signal to downregulate optimal T cell activation [173] by inhibiting CD28-dependent IL-2 production [174]. In vivo studies showed that treatment with CTLA-4-Ig prevented cardiac allograft rejection [175] and resulted in long-term tolerance to xenogenic islet grafts in mice [176]. A similar treatment suppressed T-cell dependent Ab production in mice [177]. The requirement for CD28/B7 interaction to stimulate T cell optimal reactivity may explain why small, resting B cells are poor APCs [66]. It has recently been shown that the ability of anergic T cells to induce B cells to proliferate is impaired as a result of lack of functional expression of CD40 ligand [178], which is known to deliver critical B cell activation signals. In a corollary fashion, in the absence of CD40 signal, B cells were tolerogenic for a induction of T cell response [179].

Anergy induction in CD4+ Th1 cells results in altered early and/or late signalling events culminating in a block in IL-2 transcription [180-182]. Although unable to produce IL-2 in vitro, anergic Th clones proliferate in response to exogenous IL-2 under some conditions [183]. On the contrary, anergic CD4 T cells tolerized by SEB, Mls-1a or peptide Ags in vivo, are resistant to recovery by exogenous IL-2 [184]. Why these different results are seen with in-vivo vs in-
vitro studies is not clear. The possibilities include: 1) differential expression of high-affinity IL-2R on cloned Th cells in vitro and normal T cells in vivo, 2) different methods used for anergy induction other than Ag presentation on costimulator-incompetent APCs, and 3) a more complex microenvironment involved in vivo to trigger anergic normal T cells.

Allen et al. [185] proposed an APL hypothesis (see p.10-11) suggesting that the fate of T cells upon activation depends upon the quality of peptides recognized [30,185]. Recent studies demonstrate that a failure to activate Zap-70 kinase and unique pattern of phosphorylation of TCR-ζ chain may be associated with qualitatively different signalling by APLs [56,57]. Addition of anti-CD28 or B7-expressing transfectants failed to prevent the induction of hyporesponsiveness which suggested that "partial signalling" may not be equivalent to anergy induction based on a two-signal model [63-65].

Another mechanism for peripheral T cell tolerance, activation-induced cell death (AICD), results from Fas and FasL interactions [186]. This may serve as an important mechanism in controlling the size of the peripheral repertoire and maintaining tolerance to self-Ags. The phenomenon was first delineated in inbred mice bearing lpr/lpr (Fas) or gld/gld (Fas-L) mutations [187], both of which develop systemic autoimmune disorders. Phenotypically similar diseases exist in human due to defects in Fas expression or function [188]. By comparing the Ag-specific immune responses of the Fas⁺ and Fas⁻ TCR-Tg (cytochrome C-specific) mice under defined conditions, Perijs et al. were able to demonstrate that there are two independent forms of apoptosis for peripheral mature T cells [189]. Naive T cells undergo apoptosis unless they receive both a TCR-mediated stimulus and costimulation. The protection mediated by costimulation is due to Bcl-x₁ (an apoptosis-resistance molecule) gene up-regulation. AICD induced in recently activated T cells by Fas-FasL interactions is not prevented by CD28-mediated
costimulation (of IL-2 & Bcl-x). It is suggested that Fas:Fas-L mediated AICD is important in maintaining peripheral tolerance to widely disseminated and abundant self-Ags which are presented by competent APCs. Recent studies have extended this notion by studying unresponsiveness in immune-privileged sites (anterior chamber of the eye, CNS and testis). These were considered to represent instances of anatomical barriers and/or lack of efferent lymphatics, capable of accepting allo (or xeno)-genic organ grafts in vivo [190]. Expression of FasL in the restricted tissues (transcripts found in Sertoli cells of the testis, corneal epithelium, iris, and retina of the eye), led to suicide of Ag-activated lymphocytes in vivo, and provide an alternative explanation for immune privilege [191]. Both TNF-α/FasL (for example for CD8+ T cells) and TNFR/Fas (for example for CD4+ T cells) interactions play important roles in AICD for the maintenance of peripheral T cell homeostasis in response to foreign Ag [239,240]. It has recently been shown that Tg expression of FasL (RIP promoter) in NOD mice resulted in a higher rate of spontaneous diabetes associated with upregulation of Fas by pancreatic β-cells. This suggests that Fas:FasL-mediated apoptosis of β-cells may be involved in the autoimmunity of diabetes [241]. SAg may potentially activate certain autoreactive TCR-Vβ+ T cells to trigger destruction of the pancreatic β-cells in type 1 diabetes [242].

Tolerance vs immunity following Ag challenge can be strongly influenced by the "route" through which the foreign Ag enters the host. i.v. injection of protein Ag favours tolerance induction [192], whereas s.c. injection favours an immune response [193,194]. The mechanism(s) involved in tolerance following i.v. injection is not understood. One hypothesis suggested that preferential encounter of Ag with specific cell types (B cells) may result in subsequent induction of peripheral anergy [66,195]. i.v. injection of a high-dose (1 mg) of aqueous protein Ag (TNP-OVA conjugate, deaggregated human γ-globulins) can induce Ag-
specific hypo-responsiveness of Th1 cells while a Th2 (IL-4-producing) cell persisted, capable of immune regulation in vivo [195]. Recent studies also suggest that preferential activation of Ag-specific CD4* (or CD8*) Th2 cells was induced when continuous s.c. administration of a low dose of soluble Ag hen egg lysozyme (or a topical allergen: oxazolone or picryl chloride) was given [196,197]. The results were consistent with the notion that type-II cytokine (IL-4 & IL-10) expression can be observed in an Ag-specific hypo-responsive T cell population [30,198,199]. Hosken et al. have also shown that either high (> 10 uM) or low (< 0.05 uM) doses of Ag-peptides can result in the development of Th2 phenotypes in Ag-specific primary cultures (OVA-specific TCR-Tg T cells) by modulating endogenous IL-4 production [200].

It has been known for some time that T cells are sensitive to different doses of Ag administered. Very low or very high doses of Ag may induce specific hypo-responsive states with regard to as low-zone vs high-zone tolerance, respectively [201,202]. Tolerant T cells may become more hypo-responsive by further downregulating their TCRs when more Ag is given [203] in a multi-step process [204]. It is clear that Th1 and Th2 cells are triggered differently by soluble Ags. The consequences of the different cytokine profile produced in the local micro-environment may dictate subsequent immune responsiveness or tolerance [190,205,2061], as documented in disease models such as leprosy [77] and parasitic infections [86,87]. In these diseases, IL-4 and IL-10 expression were found associated with suppressed cell-mediated immunity, whereas IFN-γ expression was found in more reactive lesions.

In summary, there are several mechanisms involved in peripheral tolerance, including deletion, AICD, anergy, and the differential activation of Th1/Th2 cells. The nature and dosage of the peptide (Ag); the route of Ag administration; the type and function of APCs involved; the local cytokine milieu; and the level of costimulation may each contribute to determine the
outcome of T-cell activation. More recently, Matzinger and colleagues have formulated an alternative hypothesis to the central role of self-nonself discrimination in the evolution of immune system [243,244]. This hypothesis suggests the key signal discrimination is for danger vs nondanger [245]. However, there is no convincing evidence to date that the prediction and observations formulated using their model are necessarily different from the traditional self-nonself models described above. Accordingly, the discussion in this thesis takes as its central underlying hypothesis, a major role for self-nonself discrimination in the development of tolerance vs immunity.

(3) Active Immunosuppression

While controversial, there is also evidence that specific suppressor T cells may play an active role in self tolerance by inhibiting the activity of other T cells. In non-obese diabetic (NOD) mice, destruction of pancreatic β-islets can be prevented by injecting islet-specific autoreactive T lymphocytes [207], suggesting that these cells can suppress the activity of auto-aggressive T cells in an Ag-specific manner. This reversal of autoimmunity can be transferred by T cells, so called suppressor T cells (Ts) or regulatory T cells. Traditionally, CD8+ T cells have been shown to suppress immune responses and are referred to as cytotoxic/ suppressor T cells. In the murine experimental autoimmune encephalo-myelitis (EAE) model, elimination of CD8 T cells by Ab treatment or by homologous gene deletion leads to a more severe form of disease [208].

Gershon et al. first demonstrated that transfer of T cells from mice made hyporesponsive to sheep red blood cells (S-RBCs), as defined by a reduced anti-SRBCs Ab response, could suppress the response of naive mice to the same Ag, but not to horse RBCs or unrelated Ags
Purified CD8+ T cells from the tolerant mice had the suppressive activity. The underlying mechanism(s) for the inhibition of immune response by Ts in vivo is not known. Neonatal mice rendered tolerant of allogenic skin grafts by injection of semi-allogenic bone marrow cells \[88\] also contained cells which could adoptively transfer specific tolerance to naive adult recipients \[95,96\], again suggesting an active Ag-specific suppression.

CD8+ T cell-mediated suppression was suggested to be responsible for the unresponsiveness to pork insulin (PI) in H-2b (C57BL/6) mice, which fail to produce IgG Ab after PI challenge. These mice do make Ab to beef insulin (BI; two amino acids different from PI). Removing CD8+ T cells from the primed T cell population revealed that primed CD4+ T cells help B cells to make Ab response to both PI and (self)-mouse insulin (MI). Addition of CD8+ T cells back to the CD4+ T cells inhibited the CD4 T-cell response to MI and PI \[210\] but not to the BI. This result suggested that the inability to respond to PI in such mice was due to CD8+ T cells that suppressed the response to MI (self), maintaining self-tolerance, and producing a cross-reactive tolerance to PI but not BI.

Other experimental models use an oral tolerance protocol to model host protection from autoimmune attack. Oral feeding of Ag elicits a local immune response in mucosal-associated lymphoid tissues. Subsequent delivery of the same Ag by a systemic route led to depressed immune responses. Thus, susceptible mice are protected from autoimmune diabetes by orally administered insulin. Mice are similarly more resistant to EAE when myelin basic protein (MBP) is given orally \[211,212\]. EAE is usually induced by inflammatory Th1 cells that produce IL-2 and IFN-γ in the host CNS after MBP immunization. When these mice are fed MBP, CD8+ T cells producing IL-4 and TGF-β are found in the brain tissue \[213\]. More recent studies suggested that both CD4 and CD8 T-cell subsets can mediate active suppression of autoimmunity...
following a feeding regimen in rat and murine models [205.214]. It is still not clear why and how the feeding of Ag leads to the production of such (suppressor) cells, which appear to be Th2-like in phenotype. However, the protection observed appears to be tissue-specific rather than Ag-specific. It has been shown [211] that when Lewis rats were fed OVA (chicken ovalbumin) and then immunized with MBP/CFA plus OVA given at separate subcutaneous sites, protection from EAE is observed. Lewis rats fed OVA and then immunized with MBP/CFA in the absence of OVA were not protected. Feeding bovine serum albumin (BSA) or keyhole limpet haemocyanin (KLH) did not suppress EAE in rats immunized with MBP/CFA plus OVA. These results suggested that while interaction may be Ag-specific, an effector non-specific, bystander suppression may be an important mechanism(s) for Ag-driven peripheral tolerance after oral administration of Ag [206.211]. Antiserum specific for TGF-β administered in vivo abrogated the protective effect of oral tolerance to MBP in EAE animals, whereas injection of the same Ab to non-tolerized animals resulted in an increase in both the duration and severity of EAE [215]. These data suggested that oral feeding induced TGF-β-producing T cells which are involved in regulating protection vs destruction of the self-Ags or tissues.

Different doses of Ag administered orally have been studied in animal murine and rat models. Low-doses (1 mg) of Ag (MBP or HEL) induced tolerance, characterized by an Ag-driven active suppression (as assayed by in vitro T-cell proliferation) with increased secretion of TGF-β and IL-4. Higher doses (5-20 mg) of Ag induced a tolerant state characterized by anergy with little or no active suppression and increased secretion of IL-4 [216]. Further, using OVA-specific TCR-Tg mice, it was shown that when very high (up to 500 mg) doses of Ags were fed there was clonal deletion of both Th1 and Th2 cells (in Payer's patches) following an initial activation in vivo. T cells that secreted TGF-β were resistant to deletion [217].
Collectively, these data suggested that oral administration of Ag can induce tolerance by active suppression, clonal anergy and deletion of both Ag-specific Th1 and Th2 cells. Thus, different mechanisms are involved in preventing autoimmune reactivity in the host, depending on the amount of Ag encountered by the mucosal immune system [214,216].

Immunoregulation may be mediated by regulatory T cell-produced cytokines such as IL-4 [94], IL-10, or TGF-β which inhibit the type-I cytokine-producing Th1 effectors. A similar phenomenon has been implicated in transplantation rejection [198,199], peripheral tolerance to self [218,219], and in some cases of parasite infection such as Leishmania [86,87]. In an adult murine allograft model [137,220], prolonged skin graft acceptance was mediated by CD4+ T cells in the tolerant host and adoptive transfer of suppression was a function of both the number of suppressor T cells transferred and IL-4 produced [220].

Using an in-vitro model and an anergic Th1 clone (specific for influenza haemagglutinin peptide) or an allogenic skin-graft model [220,221], it was suggested that Ag-specific T cells that have been rendered anergic may suppress other T cells of the same specificity by different mechanisms. These include: 1) competition for ligands (Ags) for costimulation presented by APCs, and/or for cytokine IL-2, and 2) preferential secretion of cytokine IL-4 by the non-tolerant population as a consequence of these mechanisms. Zhang et al. [217] showed in an Ag-specific adoptive transfer system where they followed the fate of T cells after Ag-specific activation in vivo, that a small proportion of residual Ag-specific hyporesponsive T cells escaped apoptosis and produced Th2 cytokines. These cells were able to inhibit the proliferation of other Ag-specific T cells, suggesting that some Ag-specific hyporesponsive lymphocytes may be involved in the maintenance of tolerance in vivo [217]. Whether these remaining T cells secret cytokine TGF-β as described by Weiner et al [206,211-216] in the oral tolerance model, remains
to be explored.

IV. The beef insulin transgenic mice model

(1) The background of beef insulin transgenic mice

In order to study the mechanism(s) of tolerance towards soluble self-Ags expressed under physiological conditions in vivo, we have generated Tg mice carrying the gene for beef insulin (BI) under the regulatory control of the insulin promoter [222]. Tg products are expressed in a tissue-specific manner.

Insulin is derived from the proteolytic cleavage of proinsulin, generating equimolar amounts of mature insulin A and B chains and a connecting peptide (C-peptide). Insulin homeostasis is regulated by glucose, amino acids and fatty acids. Glucose homeostasis is achieved by insulin and insulin receptor binding at the cell surface via the carboxyl terminus of the insulin B-chain (23-27 residues). Insulin is a heterodimeric molecule linked by two interchain disulphide bonds between the 21mer A-chain and 30-mer B-chain (Cys7-Cys7 & Cys20-Cys19; Fig 1). A third cystine bond is found within the A-chain (Cys6-Cys11) described as the A-loop region. It is primarily the A-loop region (A8-10) which displays the most significant differences between insulins from different species [beef-BI, sheep-SI, equine-EI, pork-PI; Fig 2A] while the B-chains share identical sequences. Human insulin (HI) sequences are identical to PI except in the B-chain (B 30) amino acids (Thr in HI; Ala in PI). Immunological responses to species-specific isoforms is dependent upon the MHC genetic background of the responder [Fig 2B; ref 223]. Balb/c (H-2b) mice are high responders to all insulin isoforms including BI, SI, PI and EI [223]. The differences in the A-loop sequences provide an excellent model system to investigate the
mechanism(s) involved in the interaction of the TCR/peptide/MHC trimolecular complex. The nature of the immunogenicity of the insulin molecule has been controversial [224], especially in regards to the epitope region required for MHC class II binding and TCR recognition.

Previous work from our lab has contributed towards an understanding of the TCR/peptide/MHC interaction by developing an insulin specific T cell recognition system [225,226]. These studies have characterized the TCR repertoire recognizing insulin peptides and identified both TCR epitopes and MHC class II (I-A\(^d\)) epitope/agonotope regions using a collection of insulin analogs and truncated peptides. Our lab is currently collaborating with the Scripps Research Institute to study the TCR/peptide/MHC class II (I-A\(^d\)) interaction, using X-ray crystallography and computer molecular modelling techniques. The information derived from these studies will further contribute to an understanding of tolerance mechanisms and autoimmunity.

We chose BI as a model Ag in transgenic mice for the following reasons: 1) insulin is a well known, physiological soluble self-Ag which is highly conserved among mammalian species. 2) a large collection of insulin-reactive hybridomas have already been generated and analyzed for TCR repertoires in Balb/c strain [I-A\(^d\) restricted]. 3) insulin A chain residues 1-13 represent the minimal immunogenic peptide identified, and 4) commercial reagents (anti-human C-peptide Ab) are available to follow the insulin secretion pattern in vivo.

(2) The establishment of BI-Tg mice

Establishment of BI-Tg mouse lines has been described [227]. Briefly, a 12.5 Kb human genomic insulin gene [228] was obtained from W. Rutter (University of California, SF). A 4.5 Kb EcoR I-Sac I fragment [containing 2.6 Kb of 5' flanking region including the tissue specific
human insulin promoter (including several transcriptional regulator: GG-11, CRE, CT-11, GC-11, GAGA motifs and polymorphic region). 1.4 Kb of human insulin gene and 0.5 Kb of 3' franking region; Fig 3] was subcloned into a 2.7 Kb pUC12 vector. To construct the BI gene, the insulin A loop region (A₈ & A₁₀) was mutated from Thr=ACC (A₈) and Ile=ATC (A₁₀) into Ala=GCC (A₈) and Val=GTC (A₁₀) using a site-directed mutagenesis technique. The HI B chain (B30-Thr) was not mutated because it was non-immunogenic in Balb/c mice [I-A₄ restricted, 226]. The mutated 4.5 Kb EcoR I-Sac I fragment was injected into fertilized embryos (Balb/c mice) for the generation of founder mice using the standard micro-injection technique. Three (E14, E16, E18) mice were identified as Tg founders after screening of the mouse tail genomic DNA samples by Southern blot with the HI-specific probe [non-hybridizing with mouse insulin gene, 243]. All three founders were crossed with normal Balb/c mice. The E14 founder did not transmit the BI transgene to its offspring; the others (E16 & E18) transmitted the BI transgene successfully and were bred to generate homozygous BI-Tg mice. The F3 to F6 generations of these homozygous BI-Tg mice were used in current study, which stably produced detectable amounts of BI (see below) and manifested no other growth or cellular abnormality. The expression of the Tg was examined and confirmed by Northern blot analysis [227] using a 158 bp human insulin cDNA probe which did not cross-hybridize to mouse insulin mRNAs.

These Tg mice responded to a glucose shock test [by over-night fasting and i.p. injection of glucose (2mg/g body weight)] with a 3-fold increase of BI expression in the serum (Chapter III, Fig 1) within 18 ± 2 min of glucose injection. This suggests preservation of the "normal glucose homeostasis". A human C-peptide specific radioimmunoassay demonstrated that there was some 3-3.5 ng/ml of the BI in the urine [227] and about 0.23-0.65 ng/ml in the serum (Chapter III, Fig 1). The mean basal level of BI secretion in nonmanipulated Tg mice was
approximately 0.18 ± 0.05 ng/ml (Chapter III, Fig 1). Tissue-specific protein expression in Tg mice was confirmed using immunohistochemical staining with anti-human C-peptide Ab. As shown by semi-quantitative RT-PCR analysis, transcription of the Tg was "restricted" to pancreatic β-islets (Chapter III, Fig 2). Overall, the transgene was estimated to be responsible for <30% of the total insulin expression in these mice [222,229]. We concluded therefore, that tolerance generated by functional expression of BI in the serum may mimic physiological self tolerance in vivo and overexpression of insulin may result in insulin resistance [230].

(3) Comparison of BI-Tg mice with other models

Whitely et al. & Poindexter et al. have generated an outbred line of Tg mice carrying genomic human insulin (HI) gene under the control of it's own promoter [231,232]. These mice were backcrossed to Balb/c mice to study the HI TCR repertoire and tolerance. Their results showed: 1) no restricted TCR gene usage in normal and Tg mice to HI, ii) no auto-Ab against HI in Tg mice, and iii) the existence of low-affinity CD4+ T cells (by comparing the reactivity pattern of HI hybridomas) in HI-Tg mice. They suggested that HI-specific hyporesponsiveness could be mediated by a peripheral mechanism (possibly by "anergy"). The origin of T cells proliferating to HI could not be determined due to the fact that their reactivity to a dominant immunogenic peptide was not examined [232,233]. The detailed mechanism of self tolerance was not clearly elucidated.

Our previous studies [225,226] have shown: 1) that A(1-13) was the minimal immunogenic peptide (I-A^d restricted), 2) the MHC II epitopes/ agretopes and TCR contact residues for insulin in this model, 3) that TCR V-(D)-J junctional regions (CDR3 equivalent) were involved in the Ag recognition in both TCR α & β chains which contribute to antigenic
reactivity in BI-reactive hybridomas, and 4) that there was a "restricted" TCR Vα & Vβ gene usage in BI & PI-derived T hybrids in contrast to the HI-derived TCR repertoire [233,225,226]. It has been speculated that the HI and PI TCR repertoires may indeed be different from those of BI, SI and EI, due to the fact that they differ in the A-loop sequence to the indicated isoforms, and that the majority of the T cell hybridomas generated from control non-HI Tg mice recognized HI and PI but not BI [231,233]. Thus, the TCR repertoire for HI may be diverse [233].

There are several soluble Ags which have been chosen for use in Tg mice to study the mechanism(s) of self tolerance. These include hen egg lysozyme (HEL), HI, F liver protein and complement C5 protein [148,234-238]. The "neo" self-Ag, HEL cDNA was placed under the control of a housekeeping gene promoter (hydroxymethyl glutary Co-A reductase), resulting in a differential amounts of Tg expression (from 0.5 ng/ml to 1500 ng/ml; ref 246). Both T and B cells were tolerized when the HEL serum concentration was high, whereas, only T cells were tolerized when the HEL concentration was low (less than 0.5-2ng/ml; around 10^{-10}M; ref 234-236). The mechanism of tolerance in T cells was also dependent upon the serum HEL concentration. Higher doses induced a deletional event [234,236] and lower doses induced anergy [235,237]. Anergy was suggested to be involved in the mechanism for self tolerance since a sufficient Ag stimulus could partially restore the HEL-specific responses in vitro, and a full recovery of HEL-specific T cell clonal proliferation could be achieved in extended long-term cultures [237]. In the case of F liver protein (10^{-8}M) and complement C5 protein (10^{-7}M) Tg models, central deletion in the thymus was shown to be important for tolerance as determined using thymic organ cultures in vitro and C5-TCR Tg mice in vivo [148,238]. Collectively, there was no one dominant mechanism responsible for the self tolerance described in these models.
(4) **Hypothesis and introduction of the thesis project**

It has been clearly demonstrated that T cells can be rendered hyporesponsive [201,202,234,235] and the Ag dosage may be associated with different levels of T cell hyporesponsiveness seen [203,216]. In our Tg mouse model, BI expression is regulated by the host's glucose/insulin homeostasis. Therefore, tolerance generated from such a functional expression of BI in the serum may mimic the normal physiological condition. Our hypothesis was that, if the Tg mice expressed BI predominantly, if not solely, in the periphery, they would be hyporesponsive to BI challenge (defined by Ab response and T cell proliferation). The goal of the project described in this thesis was to elucidate the mechanism(s) involved in self tolerance towards a physiological soluble Ag, BI. Specifically, the following questions were addressed:

1) how is tolerance to a tissue-specific self Ag (BI) maintained in the periphery? and 
2) does the thymus play a role in the establishment of self tolerance towards a physiological soluble Ag (BI)?

The results of these studies are described in chapter II-IV, where it is argued that, there are several levels of regulation for self tolerance. All operate in an Ag-specific fashion and include thymic selection, peripheral anergy and active suppression. All may contribute to the induction and/or maintenance of self tolerance to the soluble self-Ag, BI in the current Tg model.
Insulin A chain

\[
\text{S} - 1 - 2 - 3 - 4 - 5 - 6 - 7 - 8 - 9 - 10 - 11 - 12 - 13 - 14 - 15 - 16 - 17 - 18 - 19 - 20 - 21
\]
\[-\text{Gly-Ile-Val-Glu-Gln-Cys-Ala-Ser-Val-Cys-Ser-Leu-Tyr-Glu-Leu-Glu-Asn-Tyr-Cys-Asn}\]

Insulin B chain

\[
\]

Figure 1: Amino acid sequence of beef insulin

Figure 2A & 2B:
Different insulin A-chain sequences among mammalian isofroms and immunogenecity in mice with different MHC haplotypes

<table>
<thead>
<tr>
<th>Species</th>
<th>A-chain</th>
<th>Antibody Responses</th>
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<td>Asp</td>
<td>A4</td>
</tr>
<tr>
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<td>Glu</td>
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<td>Equine</td>
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</table>
4.5 kb BI TRANSGENE FRAGMENT
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Chapter II
Evidence for Th2-like T cell-mediated suppression of antibody responses in transgenic, beef insulin tolerant mice


Yen-Tung Teng, Reginald M. Gorczynski, Shinji Iwasaki, Douglas B. Williams

Nobumichi Hozumi
Summary

Clonal deletion, anergy and suppression have all been considered mechanisms of immunological tolerance. Although adoptive transfer of immunosuppression has been shown to occur in the periphery, particularly for transplantation tolerance, it has proven difficult to characterize this phenomenon further, due to the lack of suppressor T cell clones. In order to characterize tolerance towards a physiological soluble antigen, we constructed beef insulin (BI) transgenic (Tg) Balb/c (H-2d) mice, in which the BI transgene is expressed in pancreatic β-cells. These Tg mice were tolerant to BI immunization at the level of both humoral and cell-mediated immune responses. Adoptive transfer of splenocytes from Tg mice into normal syngeneic Balb/c mice demonstrated that the reduction in antibody production is regulated by transferred T cells. The cytokine profile of T cell clones obtained after in vitro selection demonstrated dominant Th1-like clones from normal non-Tg mice and dominant Th2-like clones from Tg mice. Some Th2-like clones (CD4+) from Tg mice produced significant suppression of antibody production after adoptive transfer into normal syngeneic Balb/c mice. These data confirm the existence of Th2-like regulatory T cells in vivo in a model of peripheral tolerance to a physiological soluble antigen as a potential mechanism for self tolerance.
The central feature of the immune system is self-nonself discrimination. Biological phenomena generated from this feature are immunological reactivity or tolerance. Recent studies have demonstrated that both central and peripheral tolerance can be modulated, in an antigen (Ag)-specific manner, by at least two distinct mechanisms, clonal deletion [1-3] and clonal anergy [4-6]. A third mechanism called active suppression [7-10] has been suggested only in peripheral tolerance. Breakdown of tolerance could result in autoimmunity to self-Ags.

The mechanisms involved in the establishment and maintenance of self tolerance are not clearly understood. In order to elucidate further the mechanisms of tolerance towards soluble self-Ags under physiological condition in vivo, we have generated a transgenic (Tg) mouse model carrying the gene for a foreign Ag (beef insulin, BI) under the regulatory control of an insulin promoter so that the Tg product is expressed in the pancreatic \( \beta \)-cells.

The BI construct used for microinjection in Balb/c (H-2\(^d\)) mice was derived from a human insulin (HI) genomic DNA with mutations of the A-chain residues Thr\(^8\) (into Ala\(^8\)) and Ile\(^10\) (into Val\(^10\)). HI B-chain (Thr\(^30\)) was not mutated because it was not immunogenic [11]. The BI transgene was expressed in pancreatic \( \beta \)-cells and not in the thymus as detected by RT-PCR (data not shown). The level of BI protein expression detected by HI C-peptide specific radioimmunoassay was similar to that of the HI-Tg mice described by Selden et al [12].

To examine the immune responses of the BI-Tg mice to BI challenge in vivo and in
vitra, two assays were used, an ELISA (enzyme-linked immunosorbent assay) and an in-vitro bulk T cell proliferation assay. The results from these experiments showed (in Figure 1 & 2) that there was a significant reduction in both the anti-BI Ab response (at least 100x less by IgG titres) and in-vitro bulk T cell proliferation (4-5 fold reduction) in the BI-Tg mice. compared to those of normal Balb/c mice. The Ab response and in-vitro bulk T-cell proliferation to a third party control Ag (chicken ovalbumin, OVA) was comparable in the BI-Tg to that seen in normal Balb/c mice. Thus, the immunological hyporesponsiveness observed in BI-Tg mice was Ag-specific. Neither lymphocytic infiltration into the pancreatic β-cells nor any other cellular abnormality or immunodeficiency has ever been observed in BI-Tg mice. In summary, Ag-specific hyporesponsiveness in BI-Tg mice is manifest at the level of both humoral and cell-mediated immunity in peripheral lymphoid organs. Thus, peripheral tolerance is induced and/or maintained in the Tg mice. A collaborative experiment (J. Wither in N. Hozumi's Lab) using a plaque-forming-assay suggested that B cells were not tolerant to BI in vivo, consistent with other studies [33, 51]. B cells require higher dose of Ag to be tolerized [52] and B cells were not tolerized in H1-Tg mice [33, 51]. Other evidence suggesting that B cells were not tolerant to BI in our current model came from an adoptive transfer study (in Fig 4) in which hyporesponsiveness (defined by reduced anti-BI Ab response) was adoptively transferred by splenic T cells from BI-Tg mice after BI immunization and not by B cells.

In brief, the results described in this paper suggest that CD4+ Th2 regulatory T cells may play a key role in maintaining self-nonself discrimination in peripheral lymphoid organs.
2 Materials and Methods

2.1 Mice

Normal Balb/c mice were purchased from the Charles River Laboratory (Montreal, Quebec) or the Jackson Laboratory (Bar Harbor, ME). Three Balb/c BI-Tg founder mice (screened by southern hybridization) were produced from microinjection, one founder did not transmit the transgene to its offspring; the other two (E16 & E18) were bred to generate homozygous BI-Tg mice. The homozygous BI-Tg mice used in this study were the F3 to F6 generations of one line E16 which stably produced significant amounts of BI and manifested no other growth and cellular abnormality. Importantly, tolerance to BI is maintained throughout the entire life span of the animal. Minimal amounts (0.18 ± 0.05 ng/ml) of BI was detected in the serum of Tg mice. In addition, we have shown the anticipated specific up-regulation of BI transcription in these mice after glucose shock. By RT-PCR and autoradiography, transcription of BI in Tg mice was restricted to pancreatic tissues (see Chapter 3, Figure 2: p140).

2.2 Reagents and tissue culture

BI and ovalbumin (OVA) were purchased from Sigma (MO). Complete Freund’s adjuvant (CFA) from Gibco BRL (ON). mIL-2 & mIL-4 from Boehringer Mannheim (Quebec). mIFN-γ from Serotec (London, UK). mIL-10 from Preprotech (North Carolina). Anti-mIL-2 & mIL-4 Abs, anti-mouse Thy1.2, CD3, CD4 & CD8 MAbs and Low-Tox
rabbit complement from Cedarlane (ON). Hybridomas 14.8 for anti-mouse B220 Ab, 11B11 (anti-IL-4 Ab) and S4B6-1 (anti-IL-2 Ab) from ATCC. Goat anti-mouse IgG & IgM Abs from Cappel Co (PA). All the tissue cultures were performed in the complete RPMI 1640 plus 5% fetal calf serum.

2.3 Adult thymectomy (ATX), bone marrow reconstitution (BMR), adoptive transfer (AT), BI immunization and bulk lymphocyte stimulation in vitro

Adult 4-6 weeks old homozygous BI-Tg (3 to 5 per group) mice were thymectomized (ATX). Three to four weeks later, they were lethally irradiated (950 rads) and reconstituted with 5 x 10^6 T cell depleted bone marrow cells (3-5 ug/ 5-10 x 10^6 cells/ml anti-thy1.2 MAb plus rabbit Low-Tox complement 1:12 dilution) as described [13]. FACS analysis showed ≤ 1% CD4+/CD8+ T cells after depletion. Four weeks later, 20 x 10^6 splenocytes from unprimed normal syngeneic (Balb/c) mice were injected intravenously into ATX-BMR Tg mice. After 3 weeks, mice were immunized with BI (100ug) in complete Freund's adjuvant (CFA, 1:1 ratio) in the foot-pad. The draining lymph nodes (LN) were collected 10 to 14 days later and cultured with BI and irradiated (3000R) syngeneic splenocytes. Bulk T cell proliferation (5 x 10^5 LN cells/well) was assessed (plus 10^6 splenocytes/well) after 3 days of culture with addition of ^3H-thymidine (1 uCi/well) for the last 18-22 hours.

2.4 B or T cell depletion, adoptive transfer and ELISA

Adult 8-10 weeks old homozygous BI-Tg or normal mice (3 to 5 per group) were immunized with BI (in CFA) 14 days prior to the preparation of total splenocytes. RBC-lysed
total splenocyte populations are used as follow: (i) total splenocyte pool, (ii) B-cell depleted, (iii) T-cell depleted. Complement-dependent cytotoxic antibodies were used for B cell (cocktail of goat anti-mouse IgG & Ig M, & purified anti-B220 MoAbs) and T cell (cocktail of anti-CD3 or CD4, CD8 and anti-thy1.2 MoAbs) depletion [14]. This procedure was repeated twice. FACS analysis showed ≥ 97% FITC-CD3+cell and ≥ 98% FITC-B220+ cell after B cell or T cell depletion, respectively. 20 x 10⁶ cells were injected via the lateral tail vein into 10-12 week old syngeneic non-Tg normal Balb/c mice. All recipients were immunized with BI in CFA on the day of cell transfer. Two weeks later, serum was collected from individual mice in each group for analysis of BI-specific Ab by ELISA as described [15]. Dilutions of the serum samples were 1:10 and 1:100.

2.5 Cloning of T cells and cytokine bioassays

To generate BI-specific clones, normal non-Tg and BI-Tg mice were immunized with BI in CFA. After 10-14 days, the draining lymph nodes were collected and LN cells were cultured with BI (20uM) and T-cell depleted, irradiated (2000R) syngeneic naive splenocytes (5 x 10⁶/ml, feeder cells). LN cells were restimulated 14 days later and LN blasts were put into 96-well plates and cloned by limiting dilution in the presence of BI (20uM), feeder cells (prepared as above) and IL-2 (5U/ml) as described [16]. Wells exhibiting proliferation were restimulated and expanded by passage every 12-14 days with BI (20uM), T-cell depleted irradiated (2000R) syngeneic splenocytes (5 x 10⁶ cells/ml) and IL-2(5-10U/ml) in 24-well plates. Three independent clonings were performed. All T cell clones were stable for 4-6 months in vitro after cloning as defined by phenotype and function (cytokine production). To
analyze the cytokine production profile of T cell clones. 10^3 resting T cells were stimulated with BI and irradiated (3000R) syngeneic splenocytes. Culture supernants were collected at 48 hours for IL-4 & IFN-γ and at 72 hours for IL-2 & IL-10. Cytokine activities were determined by bioassays as follow: IL-2 by CTLL-2 cell line [17], IL-4 by CT.4S cell line [18], IFN-γ by WEHI-279 cell line [19]. IL-10 activity was indirectly assayed using inhibition of IL-2 production from antigen-pulsed APC restimulation of Balb/c, KLH-specific, IL-2 producing, Th1 cell line as described [17]. All studies included a standard cytokine titration curve (commercially available recombinant cytokines) so that the activity in the supernants could be expressed as U/ml.

2.6 Adoptive transfer of T cell clones

12 to 14 days after restimulation of Th2 T cell clones (CDC35, P3X, P4X, P5X, P6X, P9X, P10X, P14X, P22X, P24X as described in Table 1) in vitro, cells from the clones were centrifuged over Ficoll gradient (1500 rpm, 20 min, Sorvall RT6000), washed twice in PBS and two different concentrations of cells (2 x 10^6, 2 x 10^3) in 500 ul PBS were injected i.v. into 2 to 3 syngeneic normal non-Tg Balb/c mice per group. A control group (N) of mice received PBS injection only. All recipients were immunized i.p. with BI or OVA in CFA. Serum was collected 14 days after immunization for Ag-specific ELISA as described. The Th2-like clone, CDC35, was kindly provided by Dr. David Parker, University of Massachusetts, Worcester.

2.7 Statistical analysis
The paired student t-test was used to compare the differences in Figure 1-6; analysis of variance (one -way; F-test) was used to compare the cytokine profile between the N non-Tg and BI-Tg T cell clones.

3 Results

3.1 Antigen-specific tolerance is maintained (or induced) peripherally in the BI-Tg mice

To demonstrate that tolerance occurring in BI-Tg mice operated in the periphery without thymic involvement, we performed the following experiments. Adult thymectomized (ATX), bone marrow reconstituted (BMR) mice were used in which transplantation of normal non-Tg or Tg splenocytes to Tg mice were studied. As shown in Fig 3, cells from the N --> Tg group demonstrated BI-specific reduced T cell proliferation compared to cells from the control animals (N --> N). Cells from Tg --> Tg and N --> Tgx (see Figure 3 legends) also showed Ag-specific reduced proliferation. These data suggest that for the peripherally expressed, soluble Ag, BI, the thymus is not required for either the induction or maintenance of tolerance. In other studies, where Tg cells were adoptively transferred to ATX-BMR normal mice, the BI response detected was a function of the cells transferred (see below, Fig 4-6) suggesting an active suppression by Tg cells.

3.2 Adoptively transferred tolerance defined by the reduced Ab production is mediated by splenic T cells from BI-Tg mice

To investigate whether the reduced Ab production seen in Tg mice could be
adoptively transferred to normal non-Tg mice. we transferred splenocytes with and without B or T cell depletion from Tg mice into normal syngeneic Balb/c mice. The results in Fig 4 show that when T cells were depleted from the donor population, no suppression of Ab production was detected. In contrast, suppression of Ab production persisted when B cells were depleted. This suggests that the Tg T cells are responsible for the suppression of Ab production in the normal recipients.

3.3 Different cytokine profiles are generated by T cell clones from normal, non-Tg and BI-Tg mice

It has been suggested that differential activation of Th1 and Th2 cells may be important features of the process(es) by which tolerance is induced and/or maintained [20-22]. Weiner et al. [23] demonstrated "suppressor Th2-like T cell clones" after oral tolerance induction in murine autoimmune experimental acute encephalomyelitis (EAE) model. There is a large body of work examining differential Th1/Th2 activation in transplantation tolerance [17,24,25] and in immunoregulation of chronic parasitic infection [26]. We examined the cytokine production profile of T cells from the normal and Tg mice obtained after cloning in vitro with BI (3 independent clonings). Table 1 (p.104) clearly demonstrates the dominant (10/12 clones) Th1 cytokine profile (IL-2^high^, IFN-γ^high^, IL-4^low^, IL-10^low^) in T cell clones from N mice and dominant (11/12 clones) Th2 cytokine profile (IL-4^high^, IL-10^high^, IL-2^low^, IFN-γ^low^) in T cell clones from Tg mice.

3.4 Some CD4^+^, Th2 T cell clones can adoptively transfer the BI-specific tolerance defined
by the reduced Ab production

We tested whether T cell clones obtained from Tg mice would transfer the BI-specific tolerance (decreased Ab production) into normal non-Tg syngeneic Balb/c mice. As demonstrated in Fig 5, 4 out of 9 T cell clones tested were able to suppress significantly BI-specific Ab production after adoptive transfer (2 x 10^6 cells/tail i.v. injection). A well-characterized Th2 clone (CDC35, normal rabbit Igγ-specific and I-A^d restricted) was used as a control to document Ag-specificity in the immunosuppression from these Th2-like clones (Fig 5). FACS analysis of 3 Th2-like clones (P4X, P6X, P24X) demonstrated that they were TCR_{aβ}^+,CD4^+ phenotype. When 2 x 10^3 cells from the clones were tested no suppressive effect was seen after adoptive transfer (Fig 6).

4 Discussions

Previously, we have characterized TCR repertoires specific for insulins in Balb/c mice and identified a minimal immunogenic peptide (11,27). These results prompted us to establish BI-Tg to understand the mechanism of self tolerance towards a soluble Ag under physiological conditions in vivo. In this model, Tg-BI expression is regulated by the host's glucose/insulin homeostasis as demonstrated by an approximately three-fold increase of serum BI expression from base line (.65 ± .07 vs .23 ± .06 ng/ml, respectively) after a standard glucose shock test (Chapter 3, Figure 1: p139). The basal level of BI secretion in Tg mice was measured about 0.18 ± 0.05 ng/ml by human C-peptide specific radioimmunoassay (see
It has been demonstrated that different Ag concentrations may have a profound influence on the levels (such as high zone or low zone tolerance, [ref 28-29]; suppression or anergy, [ref 30]) of tolerance [31]. Therefore, tolerance generated from functional expression of BI in the serum may mimic the physiological situations in vivo to investigate its mechanism(s). Over-expression of insulins may result in the development of insulin resistance in the mice [32].

Our findings significantly extend observations in the earlier report by Whitely et al. [33] using a HI-Tg mice model in which it was suggested that HI-specific tolerance could be mediated by a peripheral mechanism. The mechanism(s) for tolerance induction or/maintenance remains unclear. The results of our ATX-BMR-AT experiment suggest that BI-specific tolerance is induced (or maintained) in the periphery, although it does not completely rule out a possible role of the thymus in deleting reactive T cells for endogenous soluble Ags. Tolerance may be maintained, at least in part, by the Ag-specific, CD4\(^{+}\), Th2 regulatory T cells. We demonstrated elsewhere that soluble Ag-induced, class II-restricted, CD8\(^{+}\) and CD4\(^{+}\) CTLs could mediate Ag-specific immunosuppression of antigen presenting cells (APC), such as B cells, in vitro [34]. The data in this report again present evidence for a CD4\(^{+}\) regulatory T cell population.

Specific suppression mediated by MHC class I-restricted [7,35-37] or class II-restricted [38] CD8\(^{+}\) T cells has been reported. MHC class I-restricted CD8\(^{+}\) suppressor (Ts) may play a significant role in some cases of genetic nonresponsiveness [15,39]. It has been suggested by Sercarz and Krzych that Ts and T helper cells may recognize different determinants on protein antigens [40]. Previously, we identified the A(1-13) of BI A-chain as
the dominant immunogenic peptide in H-2d mice [11]. Whether the Th2 regulatory T cell clones described above recognize the same determinants of the BI molecule as other Th1-like clones is currently under investigation.

The dominant Th2 cytokine profile generated from BI-Tg mice is in accordance with the findings from a similar system using mice transgenic for hen egg lysozyme [16]. These data are consistent with the following hypothesis: (i) Th1 and Th2 cells are triggered differently by soluble Ags as shown by other reports [20-22] and (ii) the T cell population remaining after tolerance induction is heterogenous and may not be completely anergic. There was no correlation between the relative ability of the Ag-specific tolerance and the level of IL-4/IL-10 expression by those clones (Table 1 & Fig 5). There are several possible explanations for such observations. There may be at least two Th2 populations, the one being specific Ts and the other being regular (cytokine producing) Th2 helper cells. Alternatively, a different molecule (for example TGF-β, [ref 23] ) from those assayed here, could mediate the suppression. Another mechanism, but by no means, the last alternative, suggests that unique suppressor epitope recognized by some of the Th2 clones described here may be critical to adoptive transfer of tolerance.

Ag-specific bystander immunosuppression has been explored as one mechanism to explain peripheral tolerance after the oral administration of soluble antigen (oral tolerance, ref [41]) which usually lasts about 6-8 weeks [42]. Weiner et al. clearly demonstrated that cytokine TGF-β and possibly IL-4 production by CD8+ (in rats) or CD4+ (in mice) Th2-like T cells (or Ts) may protect the host from developing EAE [23, 41-43]. The human Ts populations have been studied in a chronic infectious disease, leprosy. The cytokine IL-4
produced by leprosy-specific CD4+ or CD8+ Th2 T cells, alone or with others, may suppress the Ag-specific cell-mediated immunity required for protection [44-45]. A recent study done by Scott et al. [46] also suggests the existence of such Ag-specific Th2 (or Th0-like) regulatory T cells which may suppress a spontaneous autoimmune disease (diabetic insulitis).

Gershon et al showed that T cell-mediated "infectious" immunological tolerance to sheep erythrocytes in naive mice was antigen-specific [47]. The underlying mechanism that achieves inhibition of humoral Ab production by CD4+ clones in vivo is not yet known. One hypothesis suggests that the cytokine production profile in the local microenvironment dictates responsiveness or tolerance [23, 41-45, 48-49]. Alternatively, cell-cell interaction may be critical for suppression directly [50], or for induction of the cells/factors which regulate the suppressive phenomenon. Our data do suggest, however, that CD4+ Th2 regulatory T cells are important in the maintenance of tolerance towards self-antigen, and may play a key role in the prevention of autoimmune disease.
References


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Table 1: Cytokine profiles of T cell clones derived from BI-Tg and normal (N) non-Tg Balb/c mice

<table>
<thead>
<tr>
<th>Clone</th>
<th>Source</th>
<th>IL-2 (U/ml)</th>
<th>IFN-γ (U/ml)</th>
<th>IL-4 (U/ml)</th>
<th>IL-10 (U/ml)</th>
<th>Th-like</th>
</tr>
</thead>
<tbody>
<tr>
<td>TP1</td>
<td>N non-Tg</td>
<td>3.4±0.9</td>
<td>54.2±7.5*</td>
<td>1.4±0.3</td>
<td>&lt;1.5</td>
<td>Th1</td>
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<tr>
<td>TP2</td>
<td>N non-Tg</td>
<td>1.9±0.4</td>
<td>3.2±0.6</td>
<td>8.3±1.1*</td>
<td>3.7±1.8*</td>
<td>Th2</td>
</tr>
<tr>
<td>TP3</td>
<td>N non-Tg</td>
<td>1.2±0.3</td>
<td>2.4±0.4</td>
<td>7.5±0.6*</td>
<td>2.4±1.5*</td>
<td>Th2</td>
</tr>
<tr>
<td>TP4</td>
<td>N non-Tg</td>
<td>10.2±1.5*</td>
<td>34.5±2.8*</td>
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<td>Th1</td>
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<tr>
<td>TP5</td>
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</tr>
<tr>
<td>TP6</td>
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<td>17.5±3.7*</td>
<td>67.4±8.2*</td>
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<td>Th1</td>
</tr>
<tr>
<td>TP8</td>
<td>N non-Tg</td>
<td>18.4±4.1*</td>
<td>32.7±4.4*</td>
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</tr>
<tr>
<td>TP9</td>
<td>N non-Tg</td>
<td>24.6±4.3*</td>
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<td>Th1</td>
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<td>TP13</td>
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<td>18.7±6.2*</td>
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</tr>
<tr>
<td>TP14</td>
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<td>11.3±2.4*</td>
<td>20.5±2.3*</td>
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<tr>
<td>TP16</td>
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<tr>
<td>P3X</td>
<td>BI-Tg</td>
<td>2.4±0.3</td>
<td>1.9±0.3</td>
<td>7.8±0.8*</td>
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</tr>
<tr>
<td>P4X</td>
<td>BI-Tg</td>
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<td>1.5±0.4</td>
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</tr>
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<td>P6X</td>
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<td>12.5±2.8*</td>
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<td>Th2</td>
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<td>P23X</td>
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<tr>
<td>P24X</td>
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<td>3.3±0.7</td>
<td>9.8±0.9*</td>
<td>5.8±1.5*</td>
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</tbody>
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ND: not determined,
*: significant cytokine production, p < 0.01, analysis of variance
Figure 1

ELISA

O.D value

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<th>OVA</th>
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</tr>
<tr>
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<tr>
<td>N</td>
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Figure 2

In vitro T-cell Proliferation

[Graph showing CPM vs. [uM] with different concentrations and symbols for N-BI, Tg-BI, N-OVA, and Tg-OVA]
Figure 3
Figure 5

Relative O.D. 405 nm

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</tr>
<tr>
<td>P4X</td>
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<td>0.8</td>
</tr>
<tr>
<td>P6X</td>
<td>0.3</td>
<td>0.4</td>
</tr>
<tr>
<td>P24X</td>
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<tr>
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<td>0.2</td>
<td>0.2</td>
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Figure legends:

Figure 1: There is a significant reduction (p<0.0008; paired t-test) of the anti-BI Ab response in Tg mice. 4-5 normal Balb/c (N) vs. Tg (heterozygous and homozygous mice: hetero-Tg & homo-Tg) mice per group were immunized i.p. with BI (50 or 100 ug) or OVA in CFA (1:1 ratio), and serum samples were collected at day 12-14 for ELISA analysis. For the base line, non-immunized normal Balb/c (pre-imm N) vs. BI-Tg mice (pre-imm Tg) were included. Figure 1 shows the representative data from six independent experiments. Error bars denote SD.

Figure 2: There is a significant reduction (p< 0.007; paired t-test) of the BI-specific T-cell proliferation in Tg mice. 4-5 normal Balb/c (N) and BI-Tg (Tg) mice per group were immunized with BI (50 or 100 ug) or OVA in CFA (1:1 ratio) in the foot-pad and the draining LN cells were collected at day 10 to 12 post-immunization. Bulk T-cell proliferation was assessed by adding 5 x 10^5 draining LN cells/well with BI or OVA in the presence of 3000-rads irradiated (10^9 cells/well) naive syngeneic splenocytes in culture for three days with the addition of [^3H]thymidine (1uCi/well) for the last 18-22 hrs. Figure 2 shows the representative data from five independent experiments. Error bars denote SD.

Figure 3: The thymus is not involved in tolerance to BI in Tg mice. 3-5 ATX-BMR Tg mice received 20 x 10^6 naive syngeneic normal splenocytes as described in Materials and Methods (N--> Tg). A control group (N-->Tgx) received the same treatment as N-->Tg
but no adoptive transfer of splenocytes was given. A positive control (N-->N) consisted of thymectomized normal syngeneic non-Tg mice, followed by BMR and AT. A negative control (Tg-->Tg) consisted of thymectomized Tg mice, followed by BMR and adoptive transfer of total splenocytes from Tg mice. All animals were immunized with BI and subsequent T cell proliferation to BI was assessed in vitro. Proliferation to a control Ag, OVA, was also examined. Peak values (not shown in Fig. 3) after immunization with a control Ag (OVA) for these groups (N-->N , Tg-->Tg, N-->Tg, N-->Tgx) were 54827 ± 4284, 48953 ± 3720, 44796 ± 5860, 6905 ± 1470, respectively. Figure 3 shows the representative data from two independent experiments. The values of N-->Tg group (BI) were significantly higher than those of the other groups (p < 0.0001, paired t test). Error bars denote S.D..

Figure 4: Splenic T cells from BI-Tg mice are responsible for the reduction of antibody production after adoptive transfer into syngeneic normal non-Tg Balb/c mice. Primed Tg splenocytes were prepared as follow (i) total splenocyte pool (Tg->N), (ii) B-cell depleted (Tg-B-->N), (iii) T-cell depleted (Tg-T-->N) for adoptive transfer as described in Materials and Methods. For a control group (N-->N), equivalent numbers of splenocytes were prepared 14 days after immunization of normal, non-Tg mice with BI. The control Ag used OVA. Figure 4 shows the representative ELISA data from three independent experiments. The values of Tg-T-->N (BI) were significantly lower than those of the other groups (p < 0.001, paired t test). Error bars denote S.D..
Figure 5: Four Th2 T cell clones are able to suppress antibody production after adoptive transfer into syngeneic normal non-Tg Balb/c mice. Nine Th2-like T cell clones, CDC35 (2 x 10^6 cells in 500ul PBS) or PBS only were adoptively transferred into normal Balb/c mice as described in Material and Methods. Two Th1 clones TP-5 & TP-9 were also used as controls and the results (not shown in Fig. 5) were 0.92 ± 0.08 and 1.05 ± 0.12, respectively. Figure 5 shows the representative ELISA data from three independent experiments. The values of P3X, P4X, P6X and P24X (BI immunization) were significantly lower than those of the N and CDC35 controls (p < 0.001, paired t test). Error bars denote S.D..

Figure 6: There is no significant suppression of BI-specific antibody production after adoptive transfer of 100-fold less (2 x 10^3) BI-specific clones into normal syngeneic Balb/c mice. Th2 clones P3X, P4X, P6X, P24X and a control Th1 clone TP9 (Table 1) were used for adoptive transfer as described in Material and Methods. Figure 6 shows representative ELISA data from two independent experiments. P > 0.01, paired t test. Error bars denote S.D..
Chapter III
Multiple levels of regulation for self tolerance in beef insulin transgenic mice


Yen-Tung. Teng, Douglas B. Williams, Nobumichi Hozumi,

and Reginald M. Gorczynski
Summary

To characterize the mechanism(s) of tolerance towards soluble self-antigens (Ags), beef insulin (BI) transgenic (Tg) mice were generated in which the transgene was expressed in pancreatic β-cells. Our previous data showed that: i) Ag-specific tolerance can be induced and/or maintained in peripheral T-cells in thymectomized BI-Tg mice and ii) CD4+ Th2 regulatory T-cells are involved in maintaining peripheral tolerance (as measured by anti-BI antibody response). In this paper, we have further characterized the relationship of low levels of BI expression (10^{-10}-10^{-11} M) on Th1/Th2 activation. In addition, we have explored intrathymic events associated with tolerance to self Ags not expressed in the thymus and/or to circulating self Ags. Limiting dilution analysis showed there was a significantly higher frequency of BI-specific Th2 cells in Tg mice with correspondingly higher frequency of Th1 cells in non-Tg mice. While there was no transgene expression in the thymus (by RT-PCR), independent studies showed that BI can be processed and presented in the Tg thymus which correlated with the Ag-specific hyporesponsiveness of mature thymocytes detected in vitro. High dose rIL-2 (150 U/ml) was able to restore in vitro peripheral T-cell response of Tg mice to levels comparable to the non-Tg control. Collectively, our data suggest that: i) there is a differential activation of BI-specific Th1/Th2 cells in vivo in the presence of low Ag concentration, ii) the thymus may play a role in self tolerance to Ags whose expression in adults is restricted to the periphery; and iii) multiple levels of regulation such as thymic selection, peripheral anergy and active suppression may be involved in tolerance to BI in BI-Tg mice.
Introduction

Negative selection of potentially harmful auto-reactive T cells is important for the induction or maintenance of self-tolerance [1-3]. Breakdown of self-tolerance may result in autoimmunity to the host's own self-Ags or tissues. Examination of a number of model systems suggests that both central and peripheral tolerance can operate in an Ag-specific manner by at least two major mechanisms, namely clonal deletion and clonal anergy [1-11]. A third mechanism, active suppression [12-16], may be of relevance in peripheral tolerance. Previously, we characterized the T cell receptor repertoire specific for insulin in Balb/c (H-2<sup>d</sup>) mice and identified a minimal immunogenic peptide [17-18]. In order to elucidate further the mechanism(s) of tolerance towards physiological soluble self-Ags in vivo, we generated a transgenic (Tg) mouse model in Balb/c mice, in which mice expressed the gene for a foreign Ag (beef insulin, BI, mutated from human insulin genomic DNA) under the regulatory control of its own promoter so that the Tg product is expressed in pancreatic β-cells [16]. These Tg mice were tolerant to BI immunization as assessed by humoral and cell-mediated immune responses [16,19]. Our previous data showed that: i) Ag-specific tolerance was induced and/or maintained peripherally in the BI-Tg mice, and ii) CD4<sup>+</sup> Th2 regulatory T cells were important to suppress the antibody (Ab) response for the maintenance of self tolerance in the periphery [16].

It remains unclear whether the thymus is involved in the induction of tolerance to an Ag, BI, not expected to be expressed in the thymus in this BI-Tg model. There are reports which demonstrate that i.v. & i.p. injection of foreign Ags not expressed in the thymus [20,21], and/or circulating blood-borne self-Ags [22, 23] leads to functional presentation of foreign or self/la-
molecule complexes in the thymus in vivo [24]. Ag presentation in these cases can be accomplished by circulating antigen presenting cells [APCs; such as macrophages; 24] and/or medullary thymic epithelial cells [20,25-26] which may activate T cells for the induction of central tolerance.

It was shown by previous T cell cloning experiments that there were dominant Th1 (10/12) clones and dominant Th2 (11/12) clones from our normal non-Tg and BI-Tg mice, respectively [16]. In the current paper, we investigated the functional influence of Tg (BI) expression on the activation of T helper cells and addressed the question of whether central tolerance occurs in our BI-Tg mice system. The results described below suggest that there is a differential activation of Th1/Th2 cells in the non-Tg and BI-Tg T cells, and that for an extra-thymically expressed soluble Ag, BI, central tolerance can indeed take place.

2 Materials and Methods

2.1 Mice

Normal Balb/c mice were purchased from the Charles River Laboratory (Montreal, Quebec) or the Jackson Laboratory (Bar Harbor, ME). The BI construct used for microinjection in Balb/c (H-2d) mice was derived from a human insulin (HI) genomic DNA with mutations of A-chain residues (Thr8 into Ala8; Ile10 into Val10). HI B-chain (Thr30) was not mutated because it was not immunogenic [18]. The BI-Tg homozygous mice (E16 & E18 lines) used in this study were F3 to F6 generations which stably produced significant amounts of BI detected in the serum
as described previously [16].

2.2 Reagents and tissue culture

BI and ovalbumin (OVA) were purchased from Sigma (MO). Complete Freund's adjuvant (CFA) from Gibco BRL (ON). mIL-2 from Boehringer Mannheim (Quebec). Anti-mIL-4 Ab and Low-Tox rabbit complement from Cedarlane (ON). Hybridoma J11d.2 for anti-mouse HSA Ab from ATCC and anti-HSA mAb from (Pharmingen,CA). Insulin C-peptide radioimmunoassay kit was purchased from Cedarlane (ON). All tissue culture was performed in complete RPMI 1640 medium with 10% fetal calf serum.

2.3 Insulin C-peptide radioimmunoassay (RIA)

Normal (N) non-Tg Balb/c mice and BI-Tg mice (3 to 5 per group per 10 min measurement) were fasted overnight (14-16 hrs) and were then given glucose by i.p. injection (2 mg/g body-weight). Serum samples were collected every 10 min for an hour. Unmanipulated N and Tg mice were used as the baseline controls. The anti-human C-peptide antibody used for RIA has no cross-reactivity with mouse C-peptide according to the manufacturer and Selden et al.[27]. Serum HI C-peptide (125I labelled) levels were measured using a commercially available kit (Cedarlane, ONT) under the conditions recommended by the manufacturer.

2.4 RNA isolation, reverse transcription polymerase chain reaction (RT-PCR) and autoradiography

Thymus and pancreatic tissue were obtained from N non-Tg and Tg mice. Fetal day 18
(by vaginal plugs). newborn day 1, neonate week 1 and adult animals were used. 10 x 10^6 cells were lysed in 5 ml of guanidinium buffer (4M guanidinium isothiocyanate /0.1M Tris-HCL/0.01M EDTA/0.5% sarcosyl/0.1M 2-mercaptoethanol). After phenol & chloroform extraction, the lysate was layered on a 5.7 M CsCl gradient and centrifuged at 10,000 g at 25°C for 60 min in a Sorvall RC-5B rotor (DuPont). The RNA pellet was resuspended in RNase inhibitor (20U, Boehringer Mannheim) and RNase-free DNase I (BMC) for 20 min at 37°C. The cDNA was prepared by a reaction mixture (10 ul) for 60 min at 37°C, which contained 1 ug RNA, 0.4 mM deoxy-nucleotide triphosphate, 20U RNase inhibitor, 0.2 ug oligodeoxy-thymidylate, 0.1M DTT, 1x reverse transcriptase reaction buffer (Pharmacia), and 20U MMLV reverse transcriptase (Pharmacia). cDNA was heated to 95°C for 10 min and then chilled on ice. An aliquot (200 ng) of cDNA sample was used for a 50 ul PCR reaction mixture. which contained 5 ul of 10x PCR reaction buffer (Pharmacia), 0.2 mM deoxynucleotide triphosphate, 2.5U Taq polymerase (Cetus), and 20 pmole of 5' and 3' primers. Reaction mixtures were amplified in a DNA thermocycler (Perkin Elmer) for 30 cycles. Each cycle consisted of denaturation at 94°C for 30 sec, annealing at 55°C for 30 sec. and extension at 72°C for 1 min. The following primers were used: BI-5':32p labelled-GGGCCCTGGTGCGAGCGAG, BI-3':CCTTATTTTCGGGAACCTTG; MI-5':32p labelled-AGGCCCCGGGAGCTGGA, MI-3':GGTCGAGTGGGAGACGT. The control used for PCR was β-actin (5':32p labelled-AATGGATGACGATATCGCT, 3':ATGAGGTAGTCTGTCAGG). All end-labelled 5' primers used for PCR had comparable levels of specific activity after purification with ethanol precipitation. An aliquot (25 ul) of the PCR product was then electrophoresed in 12% of polyacrylamide gel and then subjected to overnight (14-18 hrs) exposure for autoradiography.
For semi-quantitative PCR, aliquot (200 ng) of Tg pancreas cDNA samples or 10x serial dilutions mixed with a normal tissue (liver) cDNA (200 ng) sample were amplified with the same PCR conditions described above and the same or prolonged exposure time.

2.5 Limiting dilution analysis (LDA)

LDA was used to quantitate the number of BI-specific IFN-γ & IL-4-secreting lymphocytes in draining lymphoid populations. The limiting dilution culture conditions were as described previously [27]. Briefly, dilutions of draining lymph node cells [purified CD4+ T cells, as described in the previous paper using complement-dependent cytotoxic elimination; 16] were added to 96-well U-bottom microtiter plates, using 96 replicates per dilution (50, 500, 2500, 12500, 50000). Cells were pooled from 3-4 mice per group 7 days post-immunization. BI was added to all wells (2 uM) with 5 x 10^4 irradiated (3000 Rad) syngeneic splenocytes as feeder cells. After 24-30 hrs of incubation, culture supernatants were collected and frozen at -70° for further analysis. Background controls were constructed as the following: 1) Ag plus the feeder cells without the responder cells, 2) Ag plus the responder cells without the feeder cells. Later, 10^4 IL-4-dependent CT.4S cell lines were cultured with the supernatants (undiluted) for 22 hrs to assess IL-4 induced proliferation. [3H] thymidine (1 uCi/well) was added for the last 14-16 hrs. Cultures were harvested using an automatic harvester and counted by liquid scintillation. The cut-off was computed as the mean plus 3 SD of control values. Minimal estimates of the cytokine-secreting cell frequency were calculated by computer using X^2 minimization [28] as described previously [29-30]. The analysis yielded a minimal frequency estimate (1/f), the 95% confidence limits of the frequency estimate, and a X^2 estimate of probability. Frequency was
expressed as number of BI-reactive Th1/Th2 cells per 10^6 LN CD4+ T cells. The detection of IFN-γ producing lymphocytes was performed by using culture supernatants (undiluted) and an IFN-γ (detection and capture) ELISA minikit system (Pharmigen, CA). The frequency of responding cells was again calculated as described above.

2.6 Antigen presentation (AP) assay prepared from N non-Tg and BI-Tg thymi and IL-2 functional assay

Thymi of 4-6 weeks old, over-night fasted and glucose shocked (see Materials and Methods 2.3) N non-Tg and BI-Tg mice were prepared, using animals which had been immunized (7, 5, and 3 days) i.p. with BI (100 μg in CFA, 1:1 ratio) or non-immunized. Bulk thymic suspensions were irradiated (1000 rads) and cultured (2 x 10^6 cells/well) in the presence of BI reactive T cell hybridomas (I-A^d restricted) B73 and SI38 [17-18]. 5000 cells/well were incubated with or without exogenously added BI for 36 hours. Supernatants (100 ul/well) were taken and frozen at -70°C. An IL-2 sensitive cell line CTLL-2 was used to detect the IL-2 level in the thawed supernatants (undiluted) as described elsewhere [31].

2.7 In vitro thymocyte (and mature lymphocyte) proliferation assay

Thymi of 4-6 weeks old N non-Tg and BI-Tg mice which had been previously (7 and 10 days) immunized i.p. with BI (or control Ag, OVA; 100μg in CFA, 1:1 ratio) were prepared. A complement-dependent cytotoxic antibody (anti-HSA, J11d.2) was used to deplete immature (double negative and double positive) thymocytes. 3-5 μg MAb of anti-HSA (J11d.2) or supernatants were used for 5-10 x 10^6 cells/ml with 1:12 dilution of rabbit complement [16,32].
This procedure was repeated twice. FACS analysis showed ≤ 5% FITC-HSA⁺ cells after depletion. Single positive mature thymocytes (10⁶ cells /well) were cultured with BI (or OVA) and irradiated (2000 rads) syngeneic splenocytes (10⁶ cells/well). Bulk thymocyte proliferation was assessed after 2 days of culture with addition of [³H]-thymidine (1 uCi/well) for the last 12-14 hours. Proliferation in cultures of mature T lymphocytes (10⁶ draining LN cells/well, 7 days post-immunization in BI + CFA) was assessed in the presence or absence of exogenous mouse rIL-2 (150 U/ml). After 54 hrs of culture [³H] thymidine was added for the last 18-22 hrs as described elsewhere [16].

2.8 Statistical analysis

X² minimization was used to compute the differences for minimal frequency estimate and the estimate of probability between the N non-Tg and BI-Tg groups (in Figure 3). The paired student t--test was used to compared the differences in Figure 4-6.

3 Results

3.1 Functional up-regulation of serum BI expression in Tg mice after glucose shock test

Due to the equimolar amounts of insulin and C-peptide stored and co-released after physiological stimuli (such as hyperglycaemia), measurement of HIC-peptide levels can be used to monitor BI expression in Tg mice. Our results (Fig 1) showed a three-fold increase of BI secretion (at 18 ± 2 min) in BI-Tg mice after a standard glucose shock test compared to that of
N non-Tg mice (Fig 1). Interestingly, the level of BI protein expression detected by HI C-peptide specific RIA was similar to that of HI-Tg described by Selden et al.[27]. Meanwhile, the mean basal level of BI secretion in unmanipulated Tg mice was approximately $0.18 \pm 0.05$ ng/ml (about $6 \times 10^{-11}$ M; Fig 1, pre-imm Tg).

3.2 Tissue-specific expression of BI detected in the Tg pancreatic tissues and not in the thymus by RT-PCR

To discriminate between transgenic BI and endogenous MI transcriptional products, we designed the BI and MI specific 5' and 3' primers so that the size of the products was different. Besides the BI expression in the pancreas, both E16 and E18 lines transcribed the MI gene indicating that endogenous MI expression was not disrupted in Tg mice (Fig 2, lane 2-5). There was no detectable expression of BI in any of the thymic tissues examined (fetal day 18, day 1 new-born, neonate week one and adult thymi; Fig 2, lane 7-12), although a significant expression of MI was detected in new-born (day 1) thymi of both N non-Tg and BI-Tg mice (Fig 2, lane 13-14). Results of semi-quantitative PCR showed the limit of sensitivity for dilution was a $10^3$-fold dilution of cDNA. (Fig 2, lower panel). There was also no detectable expression of BI molecules in other tissues (kidney, spleen and liver) of the Tg mice as demonstrated by RT-PCR.

3.3 There is a significantly higher frequency of BI-specific Th2 cells in BI-Tg mice as assessed by LDA

LDA was employed to estimate the frequency of BI-specific, functional Th1 (IFN-γ
producing) & Th2 (IL-4 producing) cells activated after immunization (Fig 3). The estimated frequency (1/f x 10^6) of BI-specific Th1 cells was 81.6 ± 8.8 (S.E.M.) v.s. 27.4 ± 4.6 (per 10^6 cells) for normal non-Tg and BI-Tg mice, respectively. The estimated frequency (1/f x 10^6) of BI-specific Th2 cells was 78.2 ± 6.7 v.s. 19.8 ± 2.6 (per 10^6 cells) for BI-Tg and normal non-Tg mice, respectively. \( \chi^2 \) analysis indicated significant differences in both groups (p < 0.015; p < 0.004) suggesting a differential activation of BI-specific Th1/Th2 cells in normal non-Tg and Tg mice in vivo.

3.4 Peripherally expressed BI can be presented in the thymus of the Tg mice

To investigate whether soluble BI can be presented as a functional Ag in the thymus of the Tg mice, "total" thymic suspensions were used as APCs. Cells were prepared from N non-Tg and BI-Tg mice which had been immunized previously (7, 5, and 3 days prior to sacrifice) or non-immunized. The results of analysis of IL-2 production from stimulated BI-specific lines by Ag-pulsed APCs suggested that thymic APCs prepared from N non-Tg and BI-Tg mice can present BI and activate BI-specific hybridoma B73 (I-A^d restricted) in the presence of exogenous BI in vitro, with or without previous BI immunization (Fig 4). There was no statistical difference for stimulation of IL-2 production between N non-Tg and BI-Tg thymic APCs when exogenous BI was added in cultures (P > 0.05). The results obtained from the other BI-specific SI38 hybridoma were similar to B73 (data not shown). Interestingly, thymic APCs prepared from naive, over-night fasted and glucose shocked BI-Tg mice induced significantly higher (4-6 fold) amount of IL-2 in the "absence" of any exogenously added BI in vitro, compared to those of N non-Tg mice (Fig 4). Comparable levels of IL-2 were also detected from the thymic preparations
of immunized N non-Tg mice. There was no significant difference in IL-2 production in our AP assay system in terms of the time (day 3, 5, 7 days) post-immunization at which APC function in thymic preparations was assayed. Background levels of IL-2 were produced by unstimulated T cell hybridomas alone (data not shown in Figure 4) which were comparable to those of unpulsed thymic APCs of the N non-Tg mice.

3.5 BI-specific hypo-responsiveness occurs in the mature thymocytes of the Tg mice

Since BI molecules are present in the Tg thymus (Fig 4), we tested whether central tolerance to BI occurred as defined by intrathymic hypo-responsiveness. N non-Tg and Tg mice were immunized i.p. with BI or control Ag-OVA (100 ug in CFA, 1:1 ratio) before in vitro stimulation. The data obtained from in vitro proliferation of mature (single positive) thymocytes showed BI-specific hypo-responsiveness in Tg mice (Fig 5). There was no significant difference in OVA-specific proliferation induced by N non-Tg and BI-Tg mature thymocytes (Fig 5). This result suggested that thymic selection to a peripheral soluble Ag, BI, may have occurred in Tg mice.

Importantly, addition of mouse rIL-2 (100 U/ml) restored significantly the BI-specific in vitro peripheral T cell proliferation [19]. Higher dosage (150 U/ml) of rIL-2 was able to restore in vitro peripheral T cell proliferation of the Tg mice to levels comparable with the non-Tg control mice (Fig 6). These data suggest that peripheral T cell tolerance can be mediated partly, in vitro, by anergic mechanism.
4 Discussion

In this Tg mouse model, BI expression was regulated by the host's glucose/insulin homeostasis after a standard glucose shock test as demonstrated in Fig 1. A minimal constant level of BI secretion was measured at 0.18 ± 0.05 ng/ml (about 6 x 10⁻¹¹ M). Therefore, tolerance generated from functional expression of BI in the serum may mimic normal physiology and different Ag concentration may have significant influence on the levels of tolerance [33-36].

It has been demonstrated that Th1 and Th2 cells are triggered differently by soluble Ags [37-39]. One of the hypotheses suggest that the cytokine production profile in the local environment dictates responsiveness or tolerance [16, 40-44]. It was recently demonstrated, by Hosken et al., that high (> 10 uM) and low (< 0.05 uM) doses of Ag peptide can result in the distinctive development of Th2 phenotypes in Ag-specific primary cultures by modulating the endogenous IL-4 production [45]. Our data were consistent with these findings in that there was a significantly higher frequency of Th2 cells in the BI-Tg mice, in contrast to a higher frequency of Th1 cells in normal non-Tg control (Fig 3). Similar differential activation of Th cells was also observed, when we compared the frequency of BI-specific IL-2 producing Th1 cells (data not shown). However, the mechanism(s) responsible for such differential activation other than endogenous cytokine production in vivo, require further investigation.

We showed earlier that antigen-specific tolerance was induced and/or maintained peripherally in BI-Tg mice by suppressor T cells with a type 2 cytokine profile [16]. In this paper, we have performed experiments to investigate the possible role of the thymus in the BI-Tg model. We found no detectable expression of Tg-BI molecules from various thymic preparations.
by semi-quantitative RT-PCR (Fig 2; upper & lower panels), although there was a significant expression of endogenous MI in neonatal day one thymi, similar to the findings of Jolicoeur et al [46]. There are, at least, three possibilities for these latter findings. Differences between BI and MI expression in the developing thymus [46] could be due to (i) transcriptional competition, (ii) different 5' flanking regions for the insulin used for the constructs of microinjection [47] or (iii) different chromosomal location. As well, tissue-specific expression of BI molecules in pancreatic β-cells was confirmed by immunohistochemistry (using anti-human C-peptide Ab, data not shown).

We used APCs prepared from "total" thymic tissue rather than particular thymic APC subsets isolated by enzymatic digestion and Mab selection, to activate BI-specific hybridomas [B73 & S138, 17-18] in the "absence" of exogenous BI. There was a significant and comparable activation of T cells (Fig 4) characterized by the amount of IL-2 detected. Thymic APCs from N non-Tg mice immunized with BI (in CFA) also induced activation of T hybridomas which recognized BI in the context of MHC class II I-A^d molecules (Fig 4). However, Ag-specific hypo-responsiveness to BI occurred in mature (HSA-, single positive) thymocytes (Fig 5) obtained from Tg mice. It is not clear which cell type is responsible for the thymic tolerance in vivo, although thymic medullary epithelial cells or bone marrow-derived class II-positive APCs have been suggested to mediate deletion [20-21,25-26]. It has been shown that i.p. immunization can result in primed cells in the thymus. One possible model to explain thymic tolerance to the peripherally expressed BI in Tg mice suggests that circulating APCs bind and present BI after APCs gain access to the thymus. Alternatively, circulating BI reaches APCs in the thymus for negative selection. It has been suggested in another Tg model [32] that peripherally expressed
MHC class II I-E molecule (from the exocrine pancreas) was transported back to the thymus to induce central tolerance. Our results do not pin-point the maturational stage (double positive or single positive) at which tolerance occurred. However, our results suggest that presentation of self BI/class II complexes in the thymus of Tg mice in vivo may explain why mature thymocytes are tolerant to BI immunization. It is difficult to determine the mechanism for tolerance in the thymus (whether it is clonal deletion or anergy that mediates the thymic selection) since there are no discrete markers for BI-specific T cells in this Ag-Tg mice model.

Consistent with the above hypothesis, we have found that dominantly expressed TCR gene pairs analyzed in normal Balb/c mice were absent in the BI-Tg mice repertoire. The characteristics of the BI-specific TCR selected in Tg mice were distinctively different from N non-Tg mice [19], suggesting that repertoire selection may have occurred in the Tg thymus. It is possible that peripheral tolerant lymphocytes may re-circulate through the thymus and themselves contribute to the BI-specific hyporesponsiveness. This remains a topic for further investigation.

It has been previously described that a peripheral soluble Ag, such as F liver protein, can produce central tolerance at low concentration (10⁻⁸ M) using an in vitro thymic organ culture system, indicating that thymic selection can be induced by a soluble protein [48]. Another example is the C5 protein. A recent study by Zal et al. [23] using TCR Tg mice suggested that negative selection occurred late in thymic ontogeny (at the single positive thymocyte stage) in the presence of circulating complement C5 protein (average serum level of 10⁻⁷ M). This phenotypic difference from that reported elsewhere [49] may be due to a delay in contact with self-antigen presentation under physiological conditions in this case.
Our findings [including ref 16,19] significantly extend observations in the other studies using soluble Ag Tg systems [50-52], where the contributions of central tolerance and active suppression were not defined. In these studies [including our present findings in Fig 6, 19], anergy was demonstrated to be involved in peripheral tolerance. In BI-Tg mice, Tg peripheral T cells that can be activated in vitro may be of lower affinity [requiring higher Ag dose for activation, 19] and/or anergic [19,52]. Anergy can be inferred, in our model, from the fact that in-vitro BI-specific T cell proliferation in Tg mice can be significantly restored by exogenous IL-2, in a dose-dependent and dominant epitope-dependent fashion [Fig 6, and ref 19]. BI-specific suppressor Th2 cells [16] do not proliferate well to exogenous IL-2 (see Appendix: Figure 1: p209). Thus, the addition of exogenous IL-2 may allow expansion of some, not all, anergic T cells that proliferate poorly in the absence of IL-2 [16], which is consistent with lower frequency of Th1 cells detected in Tg mice (Fig 3). Anergy may prevent autocrine proliferation in vivo.

It has been suggested that T cells are sensitive to high zone and low zone tolerance [33] depending on the threshold of avidity of TCR for Ags [53-54]. Peptide concentration was suggested to range from $10^{-6}-10^{-12}$ M for negative selection in one model [55]. Tolerant T cells may acquire "deeper levels" of unresponsiveness after downregulation of TCRs [32] which may require a multi-step process [56]. In our case, the estimated BI serum concentration was between $10^{-10}-10^{-11}$ M (0.18-0.65 ng/ml) which correlated with BI-specific hypo-responsiveness in the thymus in vivo (Fig 1.4.5).

Our Tg mice were tolerant to BI immunization at the level of both humoral and cell-mediated immune responses [16,19]. We presented data suggesting that peripheral tolerance is
also regulated by BI-specific regulatory Th2 cells [16]. More recent experiments have shown that a concurrent suppression of the OVA-Ab response when OVA (third-party Ag) is co-immunized with the BI (in CFA) in the BI-Tg mice or normal Balb/c mice adoptively transferred with BI-specific suppressive Th2 clones [16], suggesting a by-stander (Ag-specific and effector-nonspecific) suppressive mechanism involved in the maintenance of peripheral tolerance (see Chapter 4: Figure 5 & 6). These data are consistent with previous findings, by Weiner et al. [40-44], in which Ag-driven by-stander suppression of the clinical scores of the experimental autoimmune encephalomyelitis can be induced by "oral tolerance" in animal models.

In summary, our data suggest that there may be several levels of regulations for self-tolerance, operating in an Ag-specific fashion, including thymic selection, peripheral anergy and suppressive mechanisms [16]. These mechanisms involved in inducing hyporesponsiveness in potentially harmful T cells are likely coordinated in some fashion in the host. Whether various levels of regulation are the result of qualitatively or/and quantitatively different signals remains to be explored. However, our data suggest that, at least for soluble Ags expressed peripherally, self tolerance can be accomplished both intra and extra-thymically.
5 References


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Figure 1

- pre-imm N
- pre-imm Tg
- test-N
- test-Tg

Human C-peptide (ng/ml)

Time (min) post Glucose-shock
Figure 3

**BI-specific IFN-gamma secretion**

**BI-specific IL-4 secretion**
Figure 4

The graph shows the CPM (counts per minute) as a function of Bi-(uM) concentration. Different lines represent different conditions:
- N
- Tg
- N-no Ag
- Tg-no Ag
- N-imm
- Tg-imm

The x-axis represents the Bi- concentration (uM) ranging from 0 to 30, and the y-axis represents the CPM ranging from 0 to 120,000.
Figure 6

The graph shows the CPM (counts per minute) response to different concentrations of BL (umol) across various conditions: Normal, Tg, rIL-2 + N, and rIL-2 + Tg.
Figure legends:

**Figure 1:** Functional up-regulation of BI secretion in Tg mice after glucose shock test. Insulin C-peptide RIA shows a three-fold increase of BI secretion (0.23 ± 0.06 ng/ml v.s. 0.65 ± 0.07 ng/ml; at 18 ± 2 min) in BI-Tg mice (test-Tg) after a standard glucose shock test (as described in Materials and Methods) compared to that of N non-Tg mice (test-N) (p < 0.01, paired t test). Unmanipulated N non-Tg (pre-imm N) and BI-Tg (pre-imm Tg) were used as the baseline controls. The mean basal level of BI secretion in Tg mice (pre-imm Tg) was 0.18 ± 0.05 ng/ml. The anti-human C-peptide antibody used for RIA does not cross-react with mouse C-peptide according to the manufacture and Selden et al. [27]. Figure 1 shows the representative data from four independent experiments. Bars denote S.D..

**Figure 2:** Tissue-specific BI expression in Tg mice. Autoradiograph (upper panel) from RT-PCR shows transcription of BI RNA in Tg pancreas (lane 2-3) and not in Tg thymus (lane 7-8,10-12). Other lanes showed the transcription of MI RNA in Tg and N non-Tg pancreas (lane 4-6) and new-born day 1 thymi (lane 13-14). Total RNA samples were prepared and BI or MI specific primers were added for PCR (in parenthesis as BI or MI, described in Materials & Methods) as follows: lane 1, HI cDNA control (BI); lane 2, E18 adult pancreas (BI); lane 3, E16 adult pancreas (BI); lane 4, E18 adult pancreas (MI); lane 5, E16 adult pancreas (MI); lane 6, N non-Tg pancreas (MI); lane 7, E18 adult thymus (BI); lane 8, E16 adult thymus (BI); lane 9, N non-
Tg thymus (Bl): lane 10, E16 fetal day 18 thymus (Bl); lane 11, E16 new-born day 1 thymus (Bl); lane 12, E16 neonate week one thymus (Bl); lane 13, E16 new-born day 1 thymus (MI); lane 14, N non-Tg new-borne day 1 thymus (MI). Results from various E18 thymi were the same as those of E16 (data not shown). β-actin primers were used as the internal control for RT-PCR as shown. Lower panel shows the sensitivity (at least $10^5$ fold) of the semi-quantitative PCR assay: (I) 200 ng of Tg pancreas cDNA samples with 10x serial dilutions amplified, (II) 200 ng of Tg pancreas cDNA was diluted into 200 ng N liver cDNA samples at the ratio indicated (10x dilution) for amplification and the last sample used in both I & II was 200 ng Tg thymic cDNA for comparison. Prolonged exposure (up to 6 days) did not result in any detectable expression above $10^5$ dilution level in (I) and (II).

**Figure 3:** Increased ratio of frequency of Bl-specific Th2/Th1 cells in Bl-Tg mice, compared with that of non-Tg mice. Details of the experiments were described in the Materials and Methods. Data were plotted as the number of cells per well v.s. the log of the fraction of negative wells. The dashed lines indicate the frequency of precursors by extrapolation from 0.37 negative wells. The estimated frequency (1/f) of Bl-specific, IFN-γ & IL-4 secreting cells in normal non-Tg and Bl-Tg mice were 81.6 ± 8.8 (S.E.M.) & 19.8 ± 2.6 v.s. 27.4 ± 4.6 & 78.2 ± 6.7 (per 10^6 cells), respectively. Values differed significantly between each group (p < 0.015; p < 0.004). $X^2$ and 95% confidence limits are not shown. Figure 3 shows the representative data from two independent experiments.
Figure 4: BI can be presented in the Tg thymus. Thymic APCs prepared from Tg mice can activate BI-specific T cell hybridoma B73 in the absence of BI in vitro. 3-5 (per group) N non-Tg and Tg mice were over-night fasted and glucose-shocked before thymi were freshly (20 min post glucose-shock) prepared for tissue culture in the presence of BI (N & Tg, respectively) or in the absence of BI (N-no Ag & Tg-no Ag, respectively) and IL-2 bioassay as described in Materials and Methods. Thymic suspensions prepared from BI immunized (day 3) N and Tg mice were also used for the activation of hybridoma B73 in the absence of BI in vitro (N-imm and Tg-imm, respectively). For both N-no Ag(6008±2576)/N-imm(14032±3424) and Tg-no Ag(47060±9650)/Tg-imm(49095±8764) groups, there was a significant (about 4-8 fold) difference (p < 0.01, paired t test). There was no difference in the results of thymic AP assays regarding the timing (3, 5 or 7 days) after BI immunization. The results obtained from another BI-specific hybridoma SI-38 were the same. Figure 3 shows the representative data from three independent experiments. Bars denote S.D..

Figure 5: Mature (single positive) thymocytes are hyporesponse to BI stimulation in Tg mice and not in N non-Tg mice. Mature thymocytes were prepared (as described in Materials and Methods) from 3-5, BI or OVA (100 ug) immunized (7 days prior) N non-Tg and Tg mice, then subjected to culture (10^6 cells/well) with BI (N-BI & Tg-BI, respectively) or OVA (N-Ova & Tg-Ova, respectively) and irradiated (2000 rads) syngeneic splenocytes (10^6 cells/well) for 48 hours. ^H-thymidine (1 uCi/well) was added for the last 12-14 hrs of culture. For BI stimulation,
there was a significant difference (p < 0.01, paired t test) between N non-Tg and Tg mice. Figure 4 shows the representative data from three independent experiments. Bars denote S.D.. The results obtained from day 10 post-immunization were the same.

**Figure 6:** High dose rIL-2 (150 U/ml) restored in vitro Tg T-cell proliferation to levels comparable with the non-Tg control. Bulk in vitro T cell proliferation of normal non-Tg (N) and BI-Tg (Tg) mice, specific to BI, was assessed as described in the Materials and Methods, in the presence (filled and opened squares) or absence (N & Tg) of rIL-2 (150 U/ml). The results of ³H-thymidine uptake after the addition of rIL-2 in culture were not significantly different from each other (p > 0.05). Figure 6 shows representative data from three independent experiments.
Chapter IV

Title:

The mechanism of active suppression for self tolerance in beef insulin transgenic mice: in vitro analyses and in vivo bystander suppression

Data presented in this chapter will be part of the manuscript submitted to the journal for future publication by

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**1 Introduction**

The phenomenon of active suppression has been documented [Review 1], especially in the field of transplantation and neonatal tolerance [2-4], although it is controversial concerning the phenotypes of suppressor cells, the Ag-specificity, MHC-restriction and its mechanisms. Ag-specific active suppression can be adoptively transferred to syngeneic, naive hosts with intact cells [1,2,5] or soluble factors [6-8], suggesting a mechanism that is distinct from clonal deletion and clonal anergy. Clonal deletion (or apoptosis) and clonal anergy have been invoked as the major mechanisms for central and peripheral tolerance [reviewed in 9-10]. Recent studies [11-16] showed that some regulatory T cells, in human and murine models, secret IL-4 and IL-10 (Th2 phenotype) or TGF-β, suggesting directly that soluble factors can downregulate the immune responses mediated by other Th cells in vivo or in vitro. A large bulk of data have demonstrated that IL-4 & IL-10 can direct the Th2 differentiation and inhibit the responses of Th1 cells [17-19].

Gershon and Kondo [20] originally observed a lower Ab response to sheep RBCs in irradiated mice adoptively transferred with a mixture of bone marrow cells and "hyporesponsive" thymocytes than in those receiving bone marrow cells alone, suggesting T cell-mediated active suppression. Both CD4+ and CD8+ T cells have been reported to mediate and/or transfer active suppression [1,13-14,21-23,65], which could be either MHC class I or class II-restricted [24-27].

It has been demonstrated in human lepromatous vs tuberculoid leprosy that IL-4 produced by leprosy-specific CD4+ or CD8+ Th2 cells, may suppress the Ag-specific cell-mediated immunity required for protection [1,13-14]. More recently, some regulatory T cells involved in
oral tolerance model have been reported to produce TGF-β that mediated in vivo and in vitro active suppression [16,21-22,65]. A number of animal studies have suggested that, in transplantation biology, polarization of the cytokine production towards Th2 (IL-4 & IL-10) profile in the grafted recipient may be beneficial for the prolongation of graft survival [28-31]. In particular, donor Ag-specific IL-10-producing regulatory T cells in the local environment (such as gut-associated lymphoid tissues) may even prolong the "third party" allograft survival in a bystander fashion [32]. In the study of cancer immunobiology, TGF-β has been shown to shift the Th1/Th2 balance to Th2 development via IL-10-mediated inhibition of the Th1 responses in "tumour-bearing" mice [33]. A recent study by Scott et al. [34] also suggested the existence of such Ag-specific Th2 (or Th0) regulatory T cells which can suppress the spontaneous autoimmune diabetic insulitis in mice. However, the mechanism(s) that mediate the active suppression by regulatory Th2 cells, in the context of self tolerance, remain unclear.

We have previously demonstrated, using a BI-Tg mouse model, that i) Ag-specific hyporesponsiveness to the self-Ags can be induced and/or maintained in peripheral T cell pools. ii) CD4+ Th2-like regulatory T cells are involved in maintaining peripheral hyporesponsiveness (as measured by anti-BI Ab response), and iii) multiple levels of regulation such as thymic selection, peripheral anergy and active suppression are likely involved in hyporesponsiveness to physiological soluble self-Ag, BI, in the same host [35-36]. These Tg mice carry low levels of BI [10^{-10}-10^{-11} M] in the serum and there is a differential activation of BI-specific Th2 cells in Tg mice [35-36]. To further investigate the possible molecule(s) that mediate the active suppression for self tolerance, we used an in-vitro transwell system to coculture the BI-specific Th1 and regulatory Th2 cells and an in-vivo co-immunization strategy. The results described
here suggest that it is the soluble cytokine "TGF-β", not IL-4 or IL-10, that mediates the suppression of Ag-specific Th1 cells in vitro and such active suppression can be exerted in a bystander fashion in vivo, associated with self tolerance.

2 Materials and Methods

2.1 Mice

Normal Balb/c mice were purchased from the Charles River Laboratory (Montreal, Quebec) or the Jackson Laboratory (Bar Harbor, ME). The BI-Tg mice has been described elsewhere [35]. The homozygous BI-Tg mice used in this study were F5 to F7 generations which stably produced BI as described previously [36].

2.2 Reagents and Tissue culture

BI and ovalbumin (OVA) were purchased from Sigma Co., (St. Louis. MO). Complete Freund’s adjuvant (CFA) from Gibco BRL (ONT). rmTGF-β was from R & D systems (Minneapolis, MN). Neutralizing Mabs against mIL-4 (Rat IgG1), mIL-10 (Rat IgG1 or IgG2b) and TGF-β (Rat IgG1 or Mouse IgG1) were from Pharmingen (CA), Pharmingen and R & D systems, respectively. Murine anti-CD4 Mab was from Beckenson Dickinson Corp (CA). Isotypic control Abs (Rat IgG1 or IgG2b, & mouse IgG1) were from Sigma Chemical Co., (St. Louis. MO). All tissue culture was performed, otherwise indicated, in complete RPMI 1640 medium with 10% fetal calf serum.
2.3 Transwell co-culture experiments

To measure the interactive T-cell proliferation of Th1 and regulatory Th2 cells in vitro, a transwell coculture system (Costar, Cambridge, MA), a dual-chamber culture separated by a semi-permeable polycarbonate membrane with a pore size 0.4 um allowing no direct cell-to-cell contact [21], was used. Briefly, 1 x 10^5 resting BI-specific Th1 (TP9) and Th2 (P24X) clones were cultured with BI and 10^6 naive syngeneic T-depleted splenocytes prepared as described elsewhere [21], in 800 ul of 10% RPMI medium in the lower and upper wells, respectively. Each transwell was performed in triplicate. After 52-56 hrs of culture, each well was pulsed with 4 uCi of [3H]thymidine for another 16-18 hrs. Then, each well was split and reseeded to 6 wells in a plat-bottomed 96-well plate for harvesting into filter mats and counted in a β-counter. To investigate which molecule mediated the suppression of Th1 clonal proliferation in vitro, anti-cytokine (TGF-β, IL-4 & IL-10) Abs (up to 10 ug/ml) were added at the beginning of the transwell coculture experiments described above.

2.4 FACS analysis

To determine the percentage of the dead or dying Th1 cells in the transwells during the coculture period, total cells were harvested, at the 48 and 72 hrs, from the lower chamber of the transwell in which the BI-specific Th1 cells (TP9 or TP5; 8 x 10^5 cells) were cocultured with APCs (4 x 10^5 T-depleted 2000-rads splenocytes) in the absence or presence of regulatory Th2 cells (same numbers) in the upper chamber of the transwell. Then, they were subject to the FACS staining with 1% propidium iodide and FITC-anti-mCD4 Mab as described elsewhere.
The percentage of the live CD4+ T cells were plotted accordingly.

2.5 Cytokine TGF-β bioassay

The detection of cytokine TGF-β was performed by using a bioassay which inhibits the proliferation of a sensitive cell line as described [37-38]. Briefly, MvlLu (mink lung-derived epithelium) cells were co-cultured at 20,000 cells/96 well flat-bottomed plates containing 10 % FACS in α-MEM and 100 ul (frozen & thawed, and 70°C treated for 15 min before used) culture supernatants taken from stimulation and clonal proliferation of BI-specific Th1 or Th2 clones (at 72 hrs, as described above). After 14-16 hrs, the cells were pulsed with 1 uCi/well [3H]thymidine for another 6-8 hrs, trypsinized, harvested onto filter mats, and counted in a β-counter. All studies included a standard rmTGF-β titration curve so that the activity in the supernatants could be expressed as ng/ml.

2.6 Co-immunization, ELISA and adoptive transfer

Normal Balb/c and BI-Tg mice were immunized i.p. with BI and/or OVA (50ug) in CFA (1:1 ratio) concomitantly or individually. At the 12th day post-immunization, serum was collected from individual mice in each group for analysis of BI or OVA-specific Ab by ELISA as described [35]. Dilution of the serum samples were made from 1:10 to 1:2000 for the titration analysis of the Ab titres. For adoptive transfer, after 10 to 12 days following restimulation of Th2 clones [CDC35, P24X, P6X, ref 35] in vitro, cells from the clones were centrifuged over Ficoll gradients (1500rpm, 15 min, Sorvall RT6000), washed twice in PBS, and 2 x 10^6 cells in 500 ul PBS were injected i.v. into 2-3 syngeneic normal non-Tg Balb/c mice per group. A
control group (N) of mice received PBS only. All recipients were immunized i.p. immediately afterwards, with BI and/or OVA (50 μg) in CFA (1:1 ratio). Serum was collected 12 days after immunization for Ag-specific ELISA as described.

3 Results:

3.1 Cytokine TGF-β is involved in the decreased proliferation of BI-specific Th1 clones cocultured with regulatory Th2 clones.

To examine the potential suppression of Ag-specific Th1 cells by regulatory T cells in vitro, a transwell system was used to co-culture the BI-specific Th1 (TP9) and Th2 (P24X) clones. The results showed that there was a significantly lower (about 50-75%; p < 0.01, paired t test) proliferation of Th1 clone (TP9) when Th2 clone (P24X) was cocultured (Fig 1A). The control Th2 clone (CDC35, I-A^d restricted rabbit Ig γ globulin-specific) did not induce a lower proliferation of Th1 cells (see figure legends). Similar results were obtained from other BI-specific Th1 (TP5) and Th2 (P6X) clones [ref 35, and data not shown]. When neutralizing Ab against TGF-β (10 μg/ml) was added during the coculture (with P24X), there was a significant reversal of the proliferation of the suppressed Th1 (TP9) clone which was only 20-25% lower than its proliferation in single culture (Fig 1B). An isotypic Ab control did not have any effects at all (see figure legends). The data suggested that TGF-β is involved in the suppressive effect of BI-specific regulatory Th2 cells in vitro.

To examine whether the in-vitro suppression introduced any apoptotic effects on BI-
specific Th1 cells. TP9 cells were recollected for a FACS analysis at the end of 48 and 72 hrs. in single culture or co-culture experiments. The results (Fig 2) showed that there was no significant difference in the percentage of cell death obtained from the singly cultured and cocultured (with P24X) Th1 cells, suggesting that active suppression mediated by regulatory Th2 cells in vitro was not associated with apoptosis.

3.2 Significantly higher amounts of TGF-β are produced by BI-specific regulatory Th2 cells.

Two BI-specific Th1 (TP9 & TP5) clones and three regulatory Th2 (P24X, P6X, P4X) clones were used to compare the amounts of TGF-β secretion detected by sensitive mink lung epithelium assay (up to pg/ml) in which the TGF-β inhibits the proliferation of the epithelial cell line. Limits of detection of this assay were 10 pg/ml. The results showed that there was significantly (P < 0.05; paired t test) higher amounts (3-4 fold) of cytokine TGF-β produced by regulatory Th2 cells than the Th1 cells (Fig 3) and this correlated with the in vitro suppressive effect of BI-specific regulatory Th2 cells observed in transwell cocultures (Fig 1A).

3.3 IL-4 and IL-10 are not associated with the suppressed proliferation of BI-specific Th1 cells cocultured with regulatory Th2 cells

To test the possibilities of other type II cytokines mediating in vitro immunosuppression, neutralizing Ab against IL-4 or IL-10 (upto 10 ug/ml) was added to the Th1/Th2 transwell cocultures described above. The results showed that there was no significant reversal of the proliferation of the suppressed Th1 (TP9) clones for both Abs (Figure 4A). Interestingly, the
proliferative response of the Th2 clones (P24X) in co-culture was reduced significantly (about 50%) or marginally (less than 15%) when anti-IL-4 Ab or anti-IL-10 Ab was added, respectively (Figure 4B). The results suggested that IL-4 and IL-10 were not involved in in-vitro active suppression mediated by regulatory Th2 cells and that IL-4 was an important growth factor for regulatory Th2 cells (50% reduction of the proliferation in the absence of cytokine IL-4 production; Fig 4B).

3.4 Ag-specific suppression in BI-Tg mice can be exerted in a bystander fashion against the third party OVA Ag after co-immunization with BI and OVA.

In order to examine the potential in-vivo bystander suppression (via a OVA-specific Ab response using ELISA) in the context of self tolerance, we coimmunized the BI-Tg mice with BI and a third party control Ag (OVA) [50ug, 1:1 ratio in CFA]. In this case, normal naive Balb/c mice served as the control for both Ags. The results showed that by 12 days post-immunization, there was a 40-50% reduction of the humoral Ab (IgG) response against the third party Ag-OVA in the BI-Tg mice when the hosts were co-immunized i.p. with both Ags, while tolerance to BI immunization was maintained (Fig 5). The control BALB/c mice developed significant Ab response against both Ags in vivo. The data suggested that the active suppression observed in BI-Tg mice [35] can be exerted in a bystander manner.

3.5 Normal syngeneic Balb/c mice adoptively transferred with BI-specific regulatory Th2 clones and coimmunized with BI and OVA can also exert a bystander suppression as seen in BI-Tg mice.
To test whether the bystander suppression observed in Figure 5 could be transferred by regulatory Th2 cells in vivo, BI-specific Th2 clones [P24X] were adoptively transferred (i.v.) to syngeneic naive Balb/c mice before the co-immunization performed (described above). The results (Figure 6) showed that there was a similar degree of bystander suppression of the humoral (IgG) response against the third party Ag-OVA in vivo by the 12th day post-coimmunization. The results obtained from another regulatory Th2 clone [P6X] was the same (data not shown). The data suggested that the bystander suppression can be transferred via Ag-specific regulatory Th2 cells which are involved in the maintenance of peripheral tolerance in BI-Tg mice.

4 Discussion:

There are several possibilities to induce the immunological hyporesponsiveness such as specific (i.v.) immunization routes [40-41] without adjuvants, chemical modification of Ags [42], low dose of soluble Ag [43-44], blockade of the co-stimulatory signals [45-46], injection of SAgs [SEB; ref 47] and oral feeding regimen [16,21,22]. Our BI-Tg mice may represent an unique physiological model of "low zone tolerance" [35-36](serum BI: 10^{-10}-10^{-11} M) associated with an Ag-specific differential activation of Th2 cells as shown by limiting dilution analysis and T-cell cloning [35-36]. Differential activation of Th2 cells has been shown in other cases like hen egg lysozyme (HEL)-Tg mice [48], continuous administration of low dose soluble (HEL) proteins [49], and low dose oral feeding [50-51]. Interestingly, some BI-specific Th2-like clones can suppress the BI-Ab response after adoptive transfer to syngeneic naive host and BI immunization.
The Balb/c mouse strain is a BI high responder [52-53] and insulin A chain (1-13) is the dominant immunogenic peptide in H-2d mice [54]. Further, the mechanisms of Ag-specific hyporeponsiveness observed in BI-Tg mice, are likely to involve other process(es) such as thymic selection and peripheral anergy [36], besides active suppression described above. These findings significantly extend observations in other studies using soluble Ag systems such as F liver protein [55], circulating complement C5 protein [56], human insulin-Tg [57-58] and HEL-Tg models [48,59-61].

In our current study, using a co-immunization strategy, we were able to observe a concurrently incomplete (about 40-50%) suppression of the third party Ab (OVA) response in the BI-Tg mice (Fig 5) and in normal Balb/c mice adoptively transferred with BI-specific regulatory Th2 clones (Fig 6) suggesting that the active suppression involved in maintaining peripheral tolerance can be exerted in a bystander manner. These data are consistent with previous findings by Weiner et al [16,21-22,50], in which Ag-driven bystander suppression of the clinical scores of the experimental autoimmune encephalomyelitis (EAE) can be induced by "oral tolerance" in animal models. Low doses of fed Ag may favour active suppression, whereas high doses may favour clonal anergy and/or deletion [50-51].

The results obtained from the transwell coculture experiments suggested that 1) there is an Ag-specific suppression of Th1 cells (50-75%) by regulatory Th2 cells, 2) the cytokine TGF-β can significantly reverse the immunosuppression (about 70%) in vitro, and 3) IL-4 & IL-10 are not associated with the in-vitro suppression observed in current model (Fig 3A & 3B). It is not clear why there is a dissociation of the TGF-β and the type 2 cytokines for regulatory Th2 cells; often they are found together with Th2-like cells. This may suggest that effector functions
and activation of regulatory Th2 cells require different control mechanisms [62]. The cytokine TGF-β has been shown to play a vital role in regulating lymphocyte proliferation and activation (MHC class II and ICAM-I expression) of a multifocal inflammatory disorder using a TGF-β null mouse model [74], suggesting that it plays a contributing role in the maintenance of self tolerance. However, the results shown here are consistent with findings of the "low-dose-feeding" induced and (CD4+ or CD8+) TGF-β-mediated "bystander" suppression of EAE mouse model [21-22,50]. The dosage of the Ag appears to have significant impacts on the different mechanism(s) of hyporesponsiveness induced in vivo [43-44,50,59,63]. We have shown that there are at least two subpopulations of Ag-specific Th2 cells [35] and it is possible that regulatory Th2 cells may require other factor(s) to maintain their differentiation and proliferation, or an unique determinant on protein Ag may be recognized [23,64] by them for the active suppression described here. Further investigations are needed to understand this phenomenon. Clearly, cytokines IL-4 & IL-10 have also been shown to have immunosuppressive effects in an autoimmune non-obese diabetic (NOD) mouse model [12], a peptide analogue-induced EAE model [11], the prolongation of graft survival in transplantation hyporesponsiveness [28-31] and in human leprosy [13-14]. Recent study by Kapp et al. [66] suggested that CD8+ regulatory T cells may be involved in hyporesponsiveness induced by feeding with soluble Ag in vivo. Whether BI-reactive CD8+ T cells are associated with active or/and bystander suppression observed in the current model would require further analysis.

FACS analysis (Fig 2) suggests that regulatory Th2 cells do not kill or induce apoptosis of Ag-specific Th1 cells in vitro which is consistent with the results from human leprosy model [67]. Further studies are required to examine whether these less proliferating (or suppressed) Th1
cells are functionally inactivated or not, and this can be done by continuous culture and restimulation of these Th1 cells with APCs and Ag or adoptive transfer in vivo. However, it is not clear whether regulatory Th2 cells can directly inhibit the Ab production by Ag-specific B cells. Further, whether there is any effect on the functions of Ag presentation and/or processing by other types of APCs (dendritic cells or macrophages) remains to be explored. It has been documented that cytokine IL-10 can downregulate or inhibit the co-stimulatory functions of macrophages [68-69] required for optimal T cell activation and proliferation.

The bystander suppression observed so far [16,21,32,39 and current paper] is not fully understood biologically, especially in the context of "specificity" for self tolerance. Although, cytokine TGF-β and IL-10 may exert their functions as Ag non-specific suppressive factors [32-33,65]. Our recent experiments suggest that there is a specific window period (day 6 to 13) for bystander suppression of the third party Ab response to be exerted after the co-immunization in vivo and it is a rather "local" than "systemic" effect for regulatory T cells (Teng. et al. manuscript in preparation). TGF-β has been shown to have a wide range of biological effects in vitro and in vivo [review 70] and its local effect is believed to be tightly regulated. In specific situations (such as infectious diseases or autoimmunity), bystander suppression may regulate "cross-reacting" autoreactive T cells. Hence, hypothetically, there may be multiple check-points such as anergy [36,71] and bystander suppression [33] to maintain peripheral tolerance. It has been shown that some anergic Th cells may exert a suppressive function in certain in vitro and in vivo conditions [72,73].

Taken together, we have shown that active suppression of B1-specific Th1 cells by CD4+ regulatory Th2 cells involves cytokine TGF-β, and not IL-4 or IL-10, in vitro. Further, T cell-
mediated active suppression of the humoral immune response can be exerted in an Ag-specific and effector-nonspecific (bystander) manner when a self-Ag (BI) and a third party Ag are co-administered. Active suppression may play a distinct role for maintaining peripheral self tolerance.
5 References


8. Onda, T., Brunner, T., Meissier, H., Biossonnette, R., Cheverri, F., Baier, G., Fotedar, A., Green, D. R., Immunoregulatory activity of a T-cell receptor α chain demonstrated...


Figure 1B
Figure 2

![Graph showing survival cells (1x10^6)](image)

- TP9
- TP9+P24X
Figure 3

TGF-beta (ng/ml)

TP5
TP9
P4X
P6X
P24X

0  50  100  150  200
Figure 4A

Graph showing CPM (counts per minute) as a function of BL- [uM] for different treatments:
- TP9
- TP9 (+P24X)
- TP9** (+P24X & anti-IL-10)
- TP9** (+P24X & anti-IL-4)
Figure 5

Ag-coated

N N* Tg Tg*
Figure 6

Ag-coated

O.D. value

bi

OVA
Figure 1: There is a decreased proliferation of the Bl-specific Th1 (TP9) clone in transwells cocultured with regulatory Th2 (P24X) clone and this phenomenon is significantly abolished by anti-TGF-β Mab. Resting TP9 cells (1 x 10^5) were cultured with 10^6 naive T-depleted 2000-rads splenocytes (Balb/c) and various (0.1, 1, 10, 100 uM) concentration of BI in the lower chamber in the absence (filled diamonds) or presence (shaded circles) of the Th2 (P24X) clones (1 x 10^5) in the upper chamber for 52-56 hrs and subsequent [³H]thymidine pulse for another 16-18 hrs. There was a significantly (P < 0.006; paired t test) reduced (range 50-75%) proliferation of TP9 clones (filled diamonds) when P24X clones were added in the upper transwells (shaded circles; Fig 1A). A control Th2 clone (CDC35, I-A^d restricted and rabbit γ-globulin specific) did not result in any significantly reduced proliferation of Th1 (TP9) clones (data not shown). When anti-TGF-β Ab (10 ug/ml) was added to the coculture (in the upper chamber), there was a significant (P < 0.01; paired t test) reversal of the proliferation of the Th1 (TP9: shaded triangles in Fig 1B) clones when compared to the TP9 and P24X cocultures (shaded squares: Figure 1-B). However, there was no significant difference (20-25% lower in CPM; P > 0.05; paired t test) when compared to the singly cultured Th1-(TP9) clones (filled diamonds: Fig 1B). An isotypic (Rat IgG 1) Ab did not have any effect at all, as shown in the Figure 1B (the crosses). Figure 1A & 1B each shows the representative data from three independent experiments. Note: Culture supernatants were collected to confirm the depletion of the cytokine TGF-β during the experimental period by using a cytokine bioassay described in Fig 3.
Figure 2: The observed suppression of BI-specific Th1 cells is not associated with killing or apoptosis in vitro. Total cells (1.2 x 10^6) were recollected at 48 and 72 hrs from the lower chambers (Th1-TP9 and APCs) for the FACS analysis by propidium iodide and FITC-anti-mCD4 Mab staining, when they were cultured individually (filled diamonds) or co-cultured with Th2 (P24X; crossed squares) clones. The results showed that there was no significant change in the total live cell population (78 ± 8% & 73 ± 9%; respectively) for Th1 clones at 48 and 72 hrs by FACS analysis. The concentrations of BI used (0.1, 1, 10, 100 μM) had similar results, although there was a higher % of cell death in both groups (survived: 61 ± 7% & 58 ± 6%; respectively) when 100 μM was used. Figure 2 shows the representative data from four independent experiments.

Figure 3: BI-specific regulatory Th 2 clones produce higher amount of cytokine TGF-β compared to that of Th1 clones. Supernatants were collected at the 72 hrs of the clonal stimulation of the 2 x 10^5 BI-specific Th1 and Th2 cells (TP5, TP9, P24X, P6X, P4X) with 10^6 naive Balb/c 3000-rads splenocytes in BI and were subject to immediate freezing at -70°C for later use. Mink lung epithelial cell line (MvLu) was used for the analysis of TGF-β secretion in the supernatants as described in the Material and Methods. The results showed that there was a higher amounts (3-4 fold) of TGF-β produced by the regulatory Th2 (P24X, P6X, P4X) clones, compared to the Th1 (TP5, TP9) clones. Standard curve was established using commercial rTGF-β in the bioassay. Figure 3 shows the representative data from three independent experiments.
Figure 4: The observed suppression of BI-specific Th1 cells is not associated with IL-4 or IL-10 in vitro, and cytokine IL-4 is an important growth factor for BI-specific regulatory Th2 cells. Anti-IL-4 or IL-10 Mab (10 ug/ml) was added to the upper chamber of the transwell at the beginning of the coculture as described in the Materials and Methods. The results showed that there was no significant change (P > 0.05) of the reduced proliferation of the Th1 clones for the two neutralizing Abs used (crossed squares for anti-IL-4 and anti-IL-10; Fig 4A) when P24X were cocultured in the transwells (filled circles; Fig 4A). The two isotypic control Abs had no effect on the proliferation of Th1 cells at all (data not shown). The results obtained from using lower amount (1 ug/ml) of neutralizing Abs were the same to the ones shown in the above Figure (data not shown). Further, there was an about 50% reduction of the proliferation of Th2 clones (P24X; Fig 4B) in coculture induced by anti-IL-4 neutralizing Ab (crossed squares), and not by anti-IL-10 (filled triangles) and the two isotypic control Abs (open circles: Fig 4B). Figure 4A & 4B each shows the representative data from three independent experiments. Note: Culture supernatants were collected to confirm the depletion of cytokine IL-4 & IL-10 during the experimental period by using the cytokine bioassays.

Figure 5: A bystander suppression of the OVA-specific Ab response observed in BI-Tg mice coimmunized with BI and OVA. Both groups (3-5 mice/group) of normal Balb/c and BI-Tg mice (N & Tg) were immunized i.p with BI (or OVA; 50 ug in CFA) independently as the controls, or both groups were co-immunized i.p. with BI and OVA (N* & Tg*) at the same time. Serum samples were collected at day 12 and subject to serial dilutions (500 to 2000 fold with PBS) for ELISA test as described in Material and Methods. The results showed that there was a significant
(40-50%) reduction of the Ab response against OVA when the Tg mice were co-immunized with both Ags, compared to the much higher anti-OVA Ab response in OVA-immunized Tg mice (P < 0.015, paired t test). Different dilutions (500 to 2000 folds) of the serum samples did not change the significance of the results, except that the relative O.D values became lower when higher dilutions were used. Figure 5 shows the representative data from 6 independent experiments.

Figure 6: A bystander suppression of the OVA-specific Ab response observed in naive Balb/c mice adoptively transferred with Bl-specific regulatory Th2 clones and co-immunized with Bl and OVA. Normal naive Balb/c mice (2-3 per group) were adoptively transfer i.v. with 2 x 10^6 regulatory Th2 clones (P24X or P6X) before the individual Bl (or OVA) immunization or Bl & OVA coimmunization (labelled AT & AT^*) strategy as described in Figure 5. The control mice did not receive adoptive transfer of regulatory Th2 cells (labelled N & N^*) before immunization. Serum samples were collected at day 12 and subject to serial dilutions (500 to 2000 fold with PBS) for ELISA test as described in Material and Methods. The results showed that there was a significant (about 40-50%) reduction of the Ab response against OVA when Bl and OVA were co-immunized in P24X-transferred Balb/c mice, compared to the higher anti-OVA Ab response in OVA-immunized, P24X-transferred Balb/c mice (p < 0.012; pair t test). The results obtained from adoptive transfer of another Th2 (P6X) clones were similar to the results described above. A control Th2 clone (CDC35) for adoptive transfer did not show any difference in Ab response to OVA immunization (data not shown). Figure 6 shows the representative data from three independent experiments.
Chapter V

Discussion
Discussion

(I) Brief review of immunological tolerance

The mammalian immune system recognizes and responds to a vast array of foreign molecules. TCR/BCR diversity is generated by random V-(D)-J gene recombination. At the same time, a state of tolerance towards self constituents that are potentially recognized by auto-reactive lymphocytes must be monitored. During early development, T cells specific for self-Ags presented in the thymus are physically deleted by apoptosis [1]. Evidence from both in vivo and in vitro studies [2-4] suggest that DP thymocytes expressing TCRs with intermediate/low avidity for self-peptide/MHC complexes are positively selected in the thymus and differentiate into mature SP thymocytes. Thymocytes expressing TCRs with low/no avidity for self-peptide/MHC complexes do not survive and die by neglect. Thymocytes expressing high avidity for self-peptide/MHC complexes are deleted by apoptotic mechanism(s) at an early DP stage [5-6] in the cortex, or late SP stage in the medulla [7-9]. Determining factors involved in such interactions include: (i) the availability, nature and concentration of the Ag-peptides, (ii) the maturational stage of the thymocytes as defined by the ligand density of MHC molecules, TCRs, coreceptors and accessory molecules and their overall avidity, and (iv) the cell types (TE or BM-derived APCs) involved in Ag presentation. Examples supporting this model of elimination of auto-reactive T cells in the thymus include models for self SAgs [10-12], MHC-encoded allo-Ags [13-15], cellular auto-Ags [5] and serum components [6,9]. However, central tolerance can also result from clonal anergy. [16] when there is a tissue-specific Tg expression of Ags in cortical TE.
Peripheral lymphoid pools are tightly regulated and peripheral tolerance may serve as a fail-safe mechanism for preventing autoimmunity. It has been shown that the threshold for activation of mature T cells is higher than that for deletion [19-20] which in itself provides a margin of safety for self tolerance mechanisms. Clonal deletion of mature T cells occurs by apoptosis of diverse Vβ6+ and Vβ14+ TCRs reacting to SAgS [21-22]. The T cells remaining are difficult to activate (an anergic state) after the interaction with SAgS [23-25] or allo-AgS [14,26-27]. To activate T cells, two signals are needed. When naive T cells are triggered via TCR:CD3 complex without costimulation provided by competent APCs, anergy occurs [28-29].

This is characterized by a block in IL-2 production upon subsequent challenge with Ag and competent APCs. CD80 (B7.1), CD86 (B7.2), CD28 and CTLA4 are key regulating ligands:receptor pairs for triggering IL-2 production [30-32]. The CD40-ligand (gp39)/CD40 molecules are important in cognate T:B immune interactions [33-34]. Further fine tuning of the T cell activation may involve downregulation of the TCR or coreceptor levels to varying degrees on the cell surface in order to maintain peripheral hyporesponsiveness [14,27,35-36]. Thus, rare tissue-specific self-AgS presented on costimulator-deficient tissue APCs may preferentially result in clonal anergy of T cells. Another pathway mediated by the Fas/Fas-L molecules [37-39] regulates T cell apoptosis following stimulation by competent APCs. The regulation of activation-induced cell death by this Fas/Fas-L pathway is also involved in maintaining self tolerance in immune-privileged vital organs such as CNS, testes and eyes. It is clear that other factors contribute to the establishment and/or maintenance of peripheral tolerance. Among these factors are the local cytokine milieu; the timing, nature and dose of Ag expressed; differential Th cell activation; route of Ag administration; the APC involved; and the presence of local inflammatory
mediators and/or environments.

Active suppression of reactive DTH responses by regulatory T cells has been suggested to occur in murine models of neonatal tolerance [40] as well as autoimmune diseases such as IDDM [41] and EAE [42], oral tolerance models [43], and some adult allograft transplantation models [44,45]. The role of active suppression in self-nonself discrimination for immunological self-tolerance is controversial. Recent studies suggest that some Ag-specific type-2 Th cells secreting higher levels of IL-4/10 or TGF-β (anti-inflammatory or anti-proliferative; ref 46) are involved in mediating suppression of inflammatory type-1 Th cells in vitro and/or in vivo [45-51]. However, the exact mechanism(s) behind the activation of these cells, their effector functions and their Ag-specificity are unknown. Active suppression by regulatory T cells for self tolerance has not been shown to date in any Tg mouse models.

(II) Review of the data generated from the current thesis

The major goal of the current project was to elucidate the mechanism(s) involved in self tolerance towards a soluble self-Ag. Several Tg animal models have been established with the goal to address the mechanisms involved in establishment and maintenance of self tolerance to soluble self-Ags. Different mechanisms have been suggested based on the results of these studies. Our lab has previously characterized the TCR repertoire specific for insulins from different species (BI, SI, EI, PI) in Balb/c mice and identified a minimal immunogenic peptide A(1-13) [52,53]. These results prompted us to establish a BI-Tg model in an attempt to understand the mechanism of self tolerance towards a soluble Ag under physiological condition in vivo. In this model, the BI transgene is regulated by the host's glucose/insulin homeostasis in vivo with
tissue-specific expression of BI detected in the pancreatic β-islets and not in the thymus. These Tg mice are hyporesponsive to BI immunization at the level of both humoral and cell-mediated immune responses. Despite the low level of BI secretion into the serum (10^{-10}-10^{-11}M), it is evident that the BI-specific hyporesponsiveness is mediated by T cells and not by B cells (Fig 2, Chapter II) consistent with other self-tolerance models like HI-Tg, HEL-Tg mice where low levels of self-Ags [54,55] are also expressed. We believe that the BI-Tg mice may thus mimic the physiological situation in vivo and allow us to investigate the mechanisms of self tolerance.

A number of serum growth factors and hormones are tightly regulated and expressed at low levels such as human insulin [56] and thyroglobulin [57]. Our results using the BI-Tg mice model may be generally applicable to other soluble self-Ags.

The results of the ATX-BMR-AT experiment, in which adoptive transfer of splenic T cells from BI-Tg mice produced Ag-specific hyporesponsiveness despite no detectable thymic expression of the BI-Tg products (Fig 1 & 2, Chapter 2 and Fig 2, Chapter 3) suggested that: 1) the thymus is not necessarily required for tolerance to BI in Tg mice, and 2) we are dealing with mechanism(s) which are responsible for peripheral T cell tolerance in BI-Tg mice. However, BI can be presented in the thymus of the Tg mice and this is correlated with BI-specific hyporesponsiveness in mature thymocytes detected in Tg mice (Fig 4 & 5, Chapter 3). This latter result suggests that thymic selection to a peripheral soluble Ag, BI, may still occur in Tg mice. Consistent with this hypothesis, we have also found that 1) the dominantly expressed TCR gene pairs analyzed in normal Balb/c mice are absent in the BI-Tg mice repertoire, and 2) the majority of BI-specific T cell hybrids from Tg mice recognize the same dominant BI A(1-13) peptide as the hybrids from normal mice [58]. A recent study by Zal et al. [9] also suggests that
clonal deletion can occur at the SP thymocyte stage in the presence of circulating C5 protein (10^{-7} M).

In addition to these data implicating thymic selection events to BI in our Tg mice, anergy as an alternative mechanism for self tolerance can be inferred from the fact that in vitro BI-specific T cell proliferation in Tg mice can be significantly restored by exogenous IL-2 (Fig 6, Chapter III). Addition of IL-2 may allow expansion of some, but not all, anergic T cells that proliferate poorly in the absence of IL-2, a hypothesis which is consistent with the lower frequency of BI-specific Th1 cells detected in Tg mice (Fig 3, Chapter 3).

The best overall hypothesis to explain these data is that, at least for a soluble Ag expressed peripherally, self tolerance is a function of both intra- and/or extrathymic events. Both clonal deletion and anergy have been implicated as possible mechanisms for central vs peripheral tolerance, in different model systems (see Chapter I). It is likely that there are other factors involved in modulating the mechanism(s) of selection for tolerance induction and/or maintenance. Nevertheless, not all auto-reactive lymphocytes are deleted and anergy may serve to prevent autocrine proliferation in vivo. Although anergic T cells can respond to exogenous IL-2 in vitro, it has proven difficult to activate these T cells by IL-2 treatment in vivo in most cases [59]. Breaking self tolerance has been shown only in a few cases such as local tissue-specific Tg expression of IL-2 or, the costimulatory molecule B7-1 [60,61] and in NOD mice [62,63]. Local expression of IL-2 may enhance the development of autoimmune disease triggered by anti-self immune response [85]. All of these instances might be considered non-physiological. The pathological conditions which might activate lower affinity and/or anergized clones in vivo are unknown.
The results of limiting dilution analysis and T cell cloning experiments suggest that there is a preferential activation of Th2 cells to the soluble low dose BI, in Tg mice (Table 1, Chapter 2 & Fig 3, Chapter 3) consistent with other models [64-66]. It has been suggested that Ag dose is important in modulating the induction of low vs high zone tolerance [64-69].

Recent progress in the field of animal models for allograft transplantation has suggested that polarization to Th2 cytokines [for example by portal vein (p.v.) pre-transfusion with donor Ag, ref 70] may prolong graft survival in vivo [49,50]. This phenomenon may, in turn, be regulated by a set of mucosal γδ T cells produced after p.v. Ag challenge, which release a number of cytokines including IL-10, TGF-β and IL-4, leading to further polarization of T cell development to the Th2 pathway [71]. These data suggest an important role for regulatory γδ T cells in controlling allograft rejection. Functional changes in γδ T cell subsets could be implicated in dysregulation of some autoimmune conditions [72,73]. Unique γδ TCR subsets have also been implicated in regulation of local immune responses, including tolerance induction following inhalation of soluble Ags (ovalbumin) at the mucosal surfaces [74] and interactive responses with αβ T cells [75]. It is possible that in BI-Tg mice preferential triggering of a γδ TCR+ subset favours the activation of αβ TCR+ Th2 cells in the local environment.

Only some regulatory Th2 clones are able to suppress the BI-specific Ab responses after adoptive transfer into syngeneic naive Balb/c mice (Fig 4, Chapter II). Thus, the Th2 cell population existing after tolerance induction is still heterogenous (Fig 3, Chapter 2). When 100-fold less of these Th2 (2 x 10³) cells were transferred, no suppression of the BI Ab response was detected (Fig 4, Chapter 2), suggesting that suppression is dependent on the number of regulatory T cells transferred. These data are consistent with findings of Davies et al. [45] where
the degree of active suppression observed as a function of the number of the "infectious" CD4+ suppressors transferred in a murine skin allograft model. Quantitative measurement of active suppression in vivo in clinical settings may represent a means to monitor the outcome of organ transplantation and autoimmune processes.

We presented data suggesting that TGF-β may be implicated in the "in-vitro suppression" of Th1 cells observed in this model (Chapter V). However, it is not clear whether TGF-β is involved in active suppression in vivo. To answer this question, anti-TGF-β MAb could be administered in vivo after adoptive transfer of the regulatory Th2 clones into naive Balb/c mice. Ag-specific regulatory Th2 cells do not appear to suppress Th1 cells by inducing apoptosis or killing (Fig 2, Chapter IV). To follow the fate of the co-cultured BI-specific Th1 cells, subsequent restimulation of these Th1 cells with APCs can be performed to assess their phenotype and effector functions.

Alternative explanations for self tolerance in this BI-Tg mice model exists. One suggests that a unique epitope may be recognized by these regulatory Th2 cells which mediate suppression in vitro or in vivo. Thus, Sercarz and Krzych have suggested that suppressor (CD8+) and Th cells may recognize different determinants on protein Ags [76]. Further peptide-specificity analysis of the BI-specific Th1 and Th2 clones will decipher this puzzle by using defined BI peptides. It has been suggested in some situations that both CD4+ and CD8+ regulatory T cells can be induced to mediate active suppression in vivo after oral Ag feeding or exogenous soluble Ag immunization [48,51,77,78]. We do not know whether cooperative interactions with CD8+ T cells are involved in the phenomenon of CD4+ regulatory Th2 -mediated adoptively transferred hyporesponsiveness and/or bystander suppression in vivo.
In the models described, including allogenic transplant models, a murine EAE model, the NOD mouse DM model, and in human lepromatous leprosy, it has been shown that IL-4 and IL-10 are key molecules in the regulation of Th1-cell-mediated functional activity. It is not clear, based on the current BI-Tg model and the literature, what role another molecule (TGF-β), rather than the conventional type-2 cytokines (IL-4, IL-10, IL-13) plays in mediating Ag-specific suppression in vitro. Interestingly, TGF-β secretion is often found together with other Th2 cytokine production [79]. For example, Th3 cells co-expressing IL-4 & IL-10 with dominant TGF-β expression are found in MBP-specific TCR-Tg mice using an oral tolerance model of EAE [86]. The potential role of other type-2 cytokines such as IL-6 and IL-13 in the current BI-Tg model has not been examined.

The functional significance of the bystander suppression observed to date [80,81, and Chapter IV] is not understood, particularly in the context of specificity for self tolerance. Our data imply that bystander suppression (of the Ab response) to an unrelated Ag is possible only if the Ag is administered at the same time as the primary Ag to which the host is tolerant. TGF-β production is highly regulated and has a wide range of biological effects in vitro and in vivo. Our BI-Tg mice model may not offer the best approach to answer these questions, due to the lack of a marker for BI-reactive T cells in vivo. Our data are, however, consistent with previous findings by Weiner et al., in which Ag-driven bystander suppression of the clinical scores of the EAE can be induced by oral tolerance in mice. Low doses of fed Ag may favour active suppression, whereas high doses may favour clonal anergy or deletion [48,69,81,82]. The involvement of active suppression in self tolerance and its mechanism(s) largely remain unknown.
Our BI-Tg mouse model does provide direct evidence that CD4\(^+\) regulatory Th2 cells are important in the maintenance of tolerance towards a physiological soluble self-Ag, in peripheral lymphoid organs. Our transwell coculture data suggest furthermore, that the cytokine TGF-\(\beta\) may be involved in this complex regulation of Ag-specific Th1/Th2 interactions. Active suppression may modulate the activity of cross-reacting autoreactive T cells in maintaining peripheral tolerance.

(III) Some unanswered questions from the current thesis

Our data present the first time in which, in a soluble self-Ag Tg model, multiple mechanisms (deletion, anergy, suppression) have been shown to be involved in the induction and/or maintenance of self tolerance in central and peripheral lymphoid tissues (Chapter II, III). These, different mechanisms may be involved in the induction and/or maintenance of self tolerance for these BI-Tg mice at different stages, namely central and peripheral tolerance. To determine in more detail the origin and fate of the BI-specific T cells and the tissue location of selection events, a marker (such as TCR-Tg) would be required in this particular soluble-Ag Tg mice model.

Further studies are also needed to analyze how different Ag-peptide doses affect the qualitative endogenous cytokine production profile. It is possible that there is a preferential survival of a given Th2 cell phenotype among the initial heterogenous T cell population, capable of IL-4 production, after encounter with a low dose Ag. To test this hypothesis, analysis of the TCR gene usage in Th1 and Th2 clones obtained from normal and Tg mice can be pursued. It has also been suggested that Th1 and Th2 cells are triggered differently by soluble Ags. One
hypothesis suggests that cytokine(s) produced in the local environment might dictate responsiveness or hyporesponsiveness [48,64-66,69,81,82]. Thus, the outcome of activation of BI-specific CD4+ Th cells may be regulated by both Ag dose and by the cytokine present at the site of triggering. Whether these hypothesis are correct or not must await further study.

The clonal deletion mechanism operating in this system is clearly not complete. It is possible that some BI-specific hyporesponsive T cells may have a lower TCR avidity [58], requiring higher Ag doses for stimulation and thus apparently escaping the selection process [52,55-56]. Direct measurement of the binding affinity/kinetics between TCRs (normal and BI-Tg Th cells) and the BI (A1-13)/MHC class II (I-Aq) complex is currently ongoing in our lab. This uses soluble TCRs and MHC class II (I-Aq) molecules along with the BIAcore instrument. Data obtained from these studies will provide some important insights into this hypothesis.

Overall, several hypotheses including Ag dosage; existence of a distinct Th2 repertoire; peptide (or epitope)-specific recognition of regulatory Th2 cells, the TCR/peptide/I-Aq complex binding avidity required for T cell activation and/or selection; and the local cytokine milieu may all independently or cooperatively modulate the preferential activation of BI-specific Th2 cells in Tg mice. It is also possible that some γδ T cells are associated with the early activation of self-Ag specific Th cells, as suggested in other cases [71-74]. A future research goal would be to examine the influence of γδ T cells on the development of αβ TCR+ Th1/Th2 cells in this Tg mouse model using depletion of γδ T cells (with an anti-γδ TCR MAb in vivo) before Ag immunization.

Our recent experiments suggest that the bystander suppression observed in vivo using the co-immunization strategy described in Fig 5 & 6 of Chapter IV, may be a local phenomenon.
rather than a global systemic change in the immune response to control Ags (data not shown here). We observed no continuous suppression of third party immune responses despite continuous exposure to self-Ag in vivo. Thus, the specificity of self tolerance may be at least partially maintained by the activation of Ag-specific regulatory T cells for self-Ags. It has been suggested that chronic stimulation of T cells under the inflammatory environment (such as IL-2, TNF-α; ref 16,60,61) may facilitate the breakdown of self tolerance producing autoimmunity. Further studies are needed to address whether there is any biological significance for the bystander suppression observed in vivo. It remains unknown whether the function of regulatory Th2 cells we described also involves the regulation of APC functions or B cells. Further studies in the BI-Tg model are required to address the issue of whether TGF-β, mediates the bystander suppression seen in vivo (about 50% of the control Ab response) when a third party Ag (OVA) is used for co-immunization with BI in CFA (Fig 5 & 6, Chapter IV).

(IV) Future direction and conclusion

In summary, there are a number of outstanding questions which still need to be resolved in the BI-Tg model. These include: 1) what factor(s) alone, or in concert are involved in Ag-specific active suppression? 2) what is the peptide-specificity and the corresponding TCR gene usage of the BI-specific regulatory Th2 cells and Th1 cells? 3) is there a functional interaction of regulatory Th2 cells with B cells and/or other APCs? 4) what is the biological significance of bystander suppression in vivo? 5) how relevant is this bystander suppression of nominal Ab responses, given that self-Ag exists simultaneously in vivo?. The BI-Tg mice model may be developed further, using a marker (such as TCR-Tg) system for BI-specific T cells in vivo. With
such technology, we could further investigate: (i) the origin, tissue location of the selective events and the fate of Ag-specific T cells after activation; (ii) the differential activation of Th cells by soluble Ag; (iii) the affinity of different (Th1/Th2) TCRs for BI in the context of MHC class II I-A^d molecule, and (iv) the influence of insulins from different species in modulating the selection of the TCR repertoire and/or cytokine production from different Th subsets.

The work presented in the current thesis significantly extends the earlier findings in other soluble-Ag Tg model systems in which one dominant mechanism has been reported to explain the tolerance obtained [9,17,18,54,55,64,84]. Our data suggest that there are multiple levels of regulation for self tolerance in vivo, all operating in an Ag-specific fashion. These include thymic selection (probably deletion; ref 58), peripheral anergy and active suppression. The induction and/or maintenance of Ag-specific hyporesponsiveness in potentially harmful (autoreactive) T cells must be coordinated in the host. Whether various levels of regulation are the result of qualitatively or/and quantitatively different signals remains to be explored. The results from the BI-Tg model described in the current thesis provide clear and important new information analyzing the basic mechanism(s) for self-nonself discrimination in biological tolerance.
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Appendix: Figure 1

1.2 × 10^4 different BI-specific Th clones (Th1: TP9, TP5; regulatory Th2: P24X, P6X) were cultured with BI (10 uM) and 3000-rad irradiated naive Balb/c splenocytes in the presence or absence of different concentration mrIL-2 for 56-60 hrs with the addition of [3H]thymidine-(1μCi/well) for the last 16-18 hrs before harvesting for counting. The above experiments were repeated twice.