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Highlights

FRONTLINE:

# Breakpoints in immunoregulation required for Th1 cells to induce diabetes

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We describe a novel TCR-transgenic mouse line, TCR7, where MHC class II-restricted, $CD4^+$ T cells are specific for the subdominant $H\text{-}2^b$ epitope ($HEL_{74–88}$) of hen egg lysozyme (HEL), and displayed an increased frequency in the thymus and in peripheral lymphoid compartments over that seen in non-transgenic littermate controls. $CD4^+$ T cells responded vigorously to HEL or $HEL_{74–88}$ epitope presented on APC and could develop into Th1 or Th2 cells under appropriate conditions. Adoptive transfer of TCR7 Ly5.1 T cells into Ly5.2 rat insulin promoter (RIP)-HEL transgenic recipient hosts did not lead to expansion of these cells or result in islet infiltration, although these TCR7 cells could expand upon transfer into mice expressing high levels of HEL in the serum. Islet cell infiltration only occurred when the TCR7 cells had been polarized to either a Th1 or Th2 phenotype prior to transfer, which led to insulitis. Progression from insulitis to autoimmune diabetes only occurred in these recipients when Th1 but not Th2 TCR7 cells were transferred and CTLA-4 signaling was simultaneously blocked. These findings show that regulatory pathways such as CTLA-4 can hold in check already differentiated autoreactive effector Th1 cells, to inhibit the transition from tolerance to autoimmune diabetes.

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## Introduction

Autoimmune diseases that target specific organs such as the pancreatic islets, thyroid and other organs of the endocrine or nervous systems have been shown to cluster together in individuals where there is a large

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**Abbreviations:** **HEL:** hen egg lysozyme · **RIP:** rat insulin promoter

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inherited component for susceptibility which has been attributed genetically to MHC and non-MHC chromosomal regions [1]. The nature of the defects which lead to such diseases is as yet unclear. Self-reactive T cells can be readily detected in the circulation and peripheral tissue of animals and humans and most do not develop any apparent autoimmune disease.

Induction of clonal deletion and clonal anergy are mechanisms for arresting self-reactive T cells in the thymus or in the peripheral immune system [2, 3]. These mechanisms require a critical threshold of antigen/MHC and TCR affinity to be triggered, which in some cases cannot be achieved since the low amounts of antigen in the thymus or the periphery are not sufficient to trigger autoreactive T cells of low TCR avidity [2–10]. Heterogeneity of autoreactive T cells has hampered the ability to relate this possible cause of autoimmunity to function. In addition to such mechanisms of deletion and anergy there have been many reports suggesting that organ-specific autoimmune diseases may occur either by excessive stimulation of autoreactive T cells [11–13], or inadequate dampening of their responsiveness by defects or decreases in Th2 cells [14], regulatory T cells [12, 15, 16] or NKT cells [17, 18].

A high self-reactive T cell frequency [19], appropriate costimulation by islet β cells [20], a Th1 cytokine profile [8, 21, 22] and high TCR-MHC affinity [9] have been observed to potentiate diabetes in otherwise resistant models, or to accelerate diabetes in neonatal diabetes-prone mice. Moreover, there is evidence that the onset of diabetes is controlled by a regulatory population [16, 23, 24]. Blockade of cytotoxic T lymphocyte-associated antigen 4 (CTLA-4) [25, 26] has also been shown to provoke rapid diabetes in certain model systems on a NOD background [27, 28] in addition to exacerbating other autoimmune disorders [29, 30]. In addition, it has also been suggested that blockade of CTLA-4 signaling may interfere with the function of naturally occurring regulatory T cells to suppress inflammation [31, 32]. More recently, cooperative roles of CTLA-4 and regulatory T cells have been proposed in tolerance for an islet antigen [33]. It is likely, that a combination of genetic defects in the threshold of reactivities of autoreactive T cells, their regulation and possibly environmental factors such as viral infections may ultimately result in such autoimmune pathologies.

Different models of TCR transgenics have been described that make use of islet-reactive T cell clones that could be isolated from autoimmune mice and therefore fell under the threshold of thymic negative selection, but thus far the islet antigens have been unidentified [21, 34, 35]. Alternatively, islet-reactive $CD4^+$ T cells with a well-defined TCR recognizing an antigen with known specificity and avidity may be used, if the antigen is expressed in the pancreatic islet β cells [2, 3, 10, 35].

To study the immunoregulatory checkpoints that inhibit the development of diabetes [36], we tracked the fate of $CD4^+$ T cells, at different stages of activation or differentiation, in a model where TCR specific for an islet-expressed neo-antigen and the islet antigen were kept constant. Transfer of these islet antigen-specific T cells into recipient mice expressing the neo-antigen thus enabled the study of the immunoregulatory circuits in place in the periphery to check first entry into the pancreatic islets and second destruction of the islet β cells, and subsequent diabetes.

## Results and discussion

### Generation and characterization of hen egg lysozyme-specific TCR transgenics: TCR7 mice

A novel TCR-transgenic mouse line was produced, by making DNA constructs (Fig. 1A) carrying functionally rearranged anti-hen egg lysozyme (HEL) TCR Vα11 and Vβ3 chain genes obtained from a HEL-specific T cell hybridoma, which recognizes a subdominant but not cryptic peptide of HEL [36]. Cell surface expression of the transgenic TCR was confirmed by transfection of the α and β chain constructs into a TCR-negative T cell hybridoma ($58\alpha^-\beta^-$), [37] and flow cytometric analysis for Vβ3 (Fig. 1A), which requires the co-expression of Vα (in this case Vα11). Two transgenic founder mice carrying the anti-HEL TCR α and β chain genes were produced using fertilized eggs from inbred C57BL/6J mice. T cell development appeared the same in the two lines, and one line, TCR7, was maintained on this genetic background and used in subsequent studies.

Thymic $CD4^+$ T cell development in TCR7 transgenic mice displayed strong positive selection with a fivefold rise in the frequency of CD4 single-positive thymocytes over that seen in non-transgenic littermate controls (Fig. 1B). Moreover, the frequency of $CD4^+$ cells expressing Vβ3 was also elevated in the peripheral lymphoid compartments of TCR7 transgenic mice as compared with non-transgenic controls and >80% of the $CD4^+$ splenic or inguinal lymph node T cells expressed Vβ3 (Fig. 1B).

Spleen cells, or purified $CD4^+$ T cells, from TCR7 transgenic mice stimulated *in vitro* with various concentrations of HEL protein or the I-$A^b$-restricted $HEL_{74–88}$ epitope for which the transgenic TCR is specific [36], made a vigorous response to HEL, which was absent in the non-transgenic control cells (Fig. 1C). In addition, naive $CD4^+$ sorted TCR7 cells developed normally to either a Th1 phenotype secreting IFN-γ with little IL-4, or to a Th2 phenotype secreting IL-4 with little IFN-γ when stimulated with $HEL_{74–88}$ presented by irradiated splenic APC under the appropriate conditions

 

of IL-12 and/or anti-IL-4 for Th1, or IL-4 and/or anti-IL-12 for Th2 development (Fig. 1D).

## Fate of TCR7 cells after adoptive transfer to recipient mice expressing HEL

As reported with a distinct HEL-specific TCR-transgenic (H-$2^k$-restricted) mouse line [10], when TCR7 mice were crossed with rat insulin promoter (RIP)-HEL mice that express membrane HEL on pancreatic β cells (RIP-HEL), autoreactive HEL-specific TCR7 T cells were able to leave the thymus and some of the cells migrated to the pancreas and caused insulitis but not diabetes (data not shown). To study further the checkpoints required to prevent such autoreactive $CD4^+$ T cells from first migrating to the pancreas, and then inhibiting them from inducing diabetes, transfer experiments were performed using $Ly5.1^+$ HEL-specific TCR-transgenic cells and $Ly5.2^+$ recipients of the following types: RIP-HEL (express membrane HEL on pancreatic β cells, and

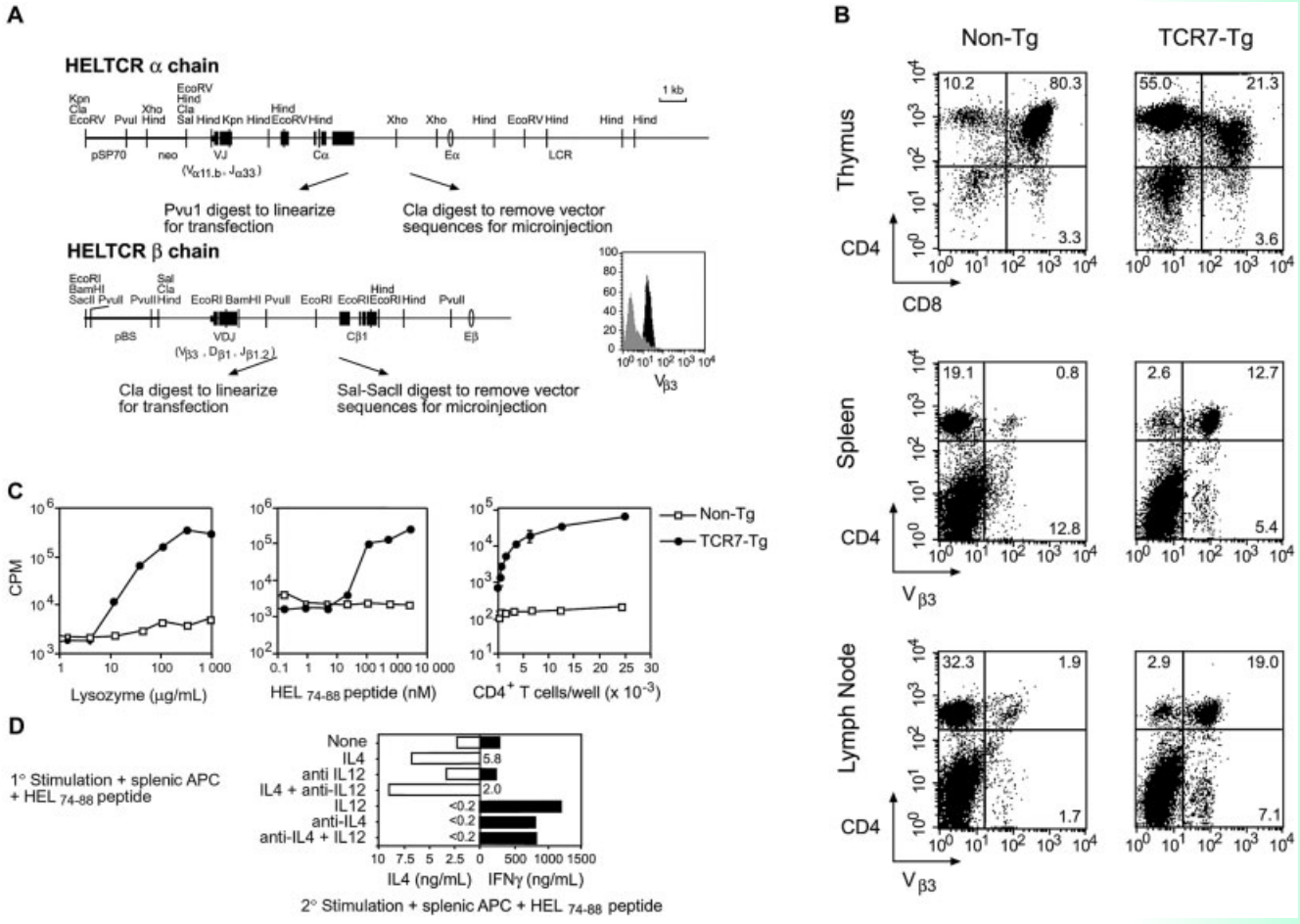


**Figure 1.** Generation and characterization of TCR7 mice. (A) The anti-HEL TCR α chain construct was made by insertion of the anti-HEL TCR α chain VJ region DNA fragment obtained from the BO4H9.1 T cell hybridoma [36] and *neo* gene into the pVCαK TCR α chain construct [45] as described in *Materials and methods*.The FACS histogram shows $58\alpha^-\beta^-$ cells [37] stained with an anti-Vβ3 antibody (the transgene β chain specificity) and only observed when co-transfected with the anti-HEL TCR α and β chain constructs. Equivalent staining was also obtained with an anti-CD3 antibody, indicating that both the α and β chain genes were expressed on the cell surface. (B) CD4/CD8 profiles of thymi from non-transgenic (Non-Tg) littermate (left) and TCR7 transgenic (TCR7-Tg) mice (right) are shown in the upper row of panels with the corresponding CD4/Vβ3 expression for the spleen and inguinal lymph node displayed below. The percentage of cells present in each quadrant is also indicated in the top right of the individual plots. (C) T cell proliferation was measured by [$^3$H]thymidine incorporation for either $1\times10^6$ whole spleen cells from non-transgenic (Non-Tg, open symbols) or TCR7 transgenic (TCR7-Tg, closed symbols) mice stimulated with the indicated doses of HEL protein (Lysozyme) or $HEL_{74–88}$ peptide, or for the indicated number of CD4 purified spleen cells from these mice stimulated with $1\times10^6$ irradiated splenic APC in the presence of 1.1 μM $HEL_{74–88}$ peptide (triplicate cultures). (D) Naive (Mel-$14^{bright}$) $CD4^+$ spleen cells sorted from TCR7 transgenic mice were set up in primary cultures with $HEL_{74–88}$ peptide and APC (irradiated spleen cells) for 7 days in the absence of polarizing cytokines (None) or in the presence of either Th2- or Th1-inducing conditions as described in *Materials and methods*. Cells were then restimulated with $HEL_{74–88}$ peptide + APC for 48 h, and IL-4 and IFN-γ content of the supernatants determined by ELISA (limit of sensitivity: IFN-γ, <125 pg.mL; IL-4, <100 pg/mL). Results are representative of three experiments and data are represented as the mean ± SD of triplicate cultures.

 

have 1.2 ng/mL HEL in the serum); M-HEL, (express HEL on most cell types under control of the class I promoter and have 150–200 ng/mL HEL in the serum); solHEL (express soluble HEL in the liver and have 10–20 ng/mL in the serum) and non-transgenic mice as controls.

TCR7 (Ly5.1) donor cell frequency in the spleen of non-transgenic, solHEL or RIP-HEL recipients dwindled rapidly over the 5 days after transfer, to almost undetectable levels due to insufficient activation (Fig. 2A). By contrast, donor TCR7 cells expanded and persisted and their frequency remained elevated in identical transfers to M-HEL recipient mice (Fig. 2A). Expression of HEL under the class I promoter has previously been demonstrated to result in enhanced processing and thus reactivity to HEL [10], suggesting that the cells are failing to be maintained in the periphery of non-transgenic, solHEL or RIP-HEL mice when they have to compete with a normal T cell compartment, whereas they get sufficient engagement to promote survival in M-HEL mice.

TCR7 $CD4^+Ly5.1^+$ cells were detected in the spleen of M-HEL mice at an approximate frequency of 3–6% of live lymphocytes (Fig. 2A and data not shown) and exhibited evidence of activation, with modulation of $CD44^{hi}$, $CD45RB^{lo}$ and $Mel\text{-}14^{lo}$ seen for at least 150 days after transfer (data not shown). In addition, 10 days after transfer, the HEL-specific proliferative

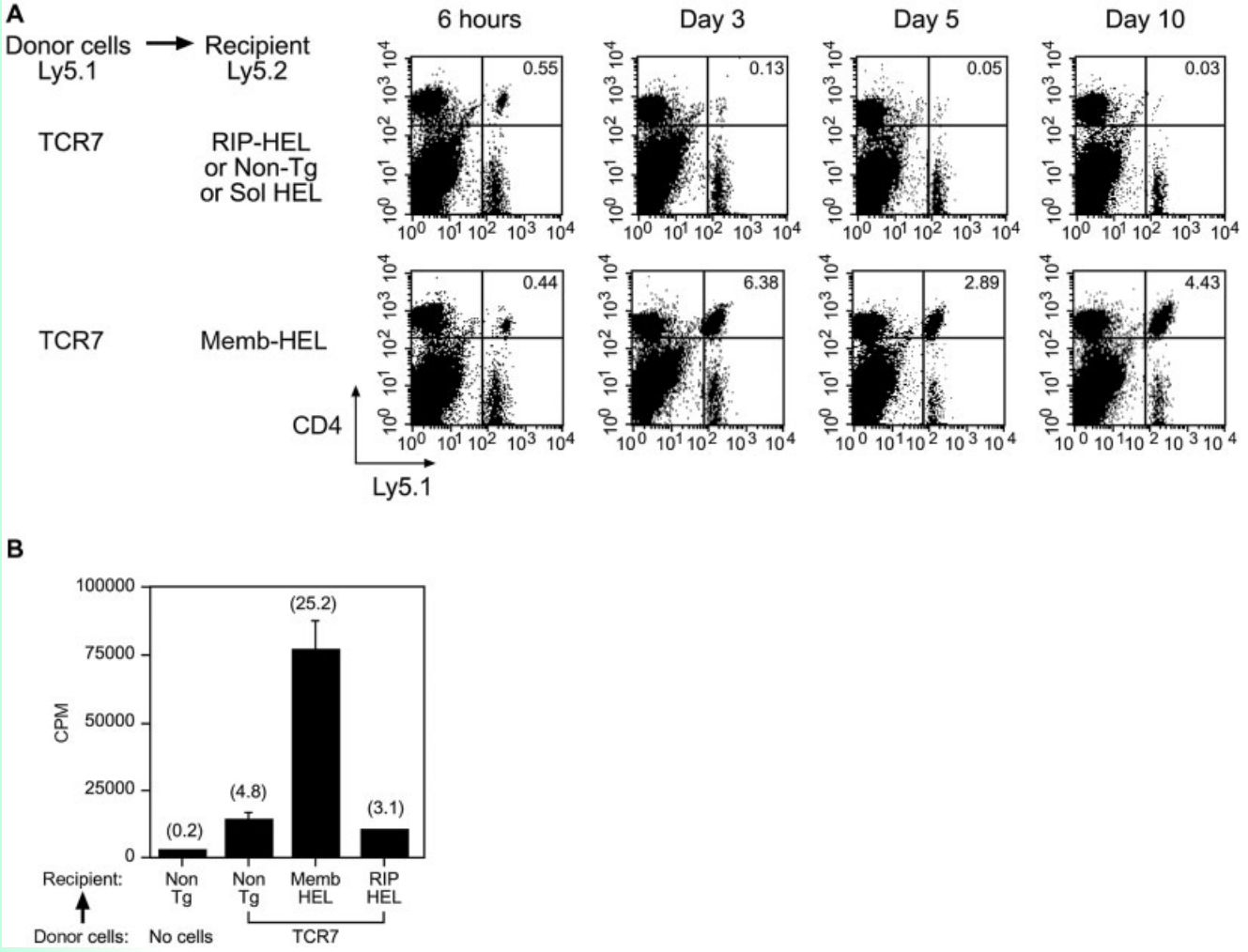


**Figure 2.** Long-term expansion of TCR7 T cells is seen only after adoptive transfer to M-HEL, but not to solHEL, RIP-HEL or non-transgenic recipients. (A) Recipient spleens were harvested from 6 h to 10 days after the indicated Ly5.2 recipient mice were adoptively transferred with $40\times10^6$ TCR7 Ly5.1 cells. Flow cytometric analysis for $CD4^+$ $Ly5.1^+$ cells was used to detect the presence of donor T cells within recipient spleens. Their frequency was calculated as the percentage of $CD4^+Ly5.1^+$ cells within a live lymphocyte gate of the recipient spleen and the numbers shown are representative of five mice per group and at least three identical experiments. (B) Proliferative responses are shown for spleen cells from the indicated recipient mice after cultures identical to those described in Fig. 1D. Also shown are the percentages of $CD4^+V\beta3^+$ T cells expressed as a fraction of the live lymphocytes present within each population used for the proliferation assays. Similar results were found in at least three identical experiments.

 

capacity of CD4-enriched spleen cells from M-HEL transgenic recipients was strongly enhanced over that of untransferred non-transgenic controls, while there was no augmented proliferation in non-transgenic, solHEL or RIP-HEL groups that had received TCR7 cells (Fig. 2B). The capacity of these transferred TCR7 $CD4^+$ T cells to respond to HEL *in vitro* paralleled the frequency of $CD4^+V\beta3^+$ TCR7 cells present in the cultures and demonstrated their ability to respond to HEL.

The autoreactive TCR7 cells transferred to M-HEL recipients induced enhanced levels of IgG autoantibodies to double-stranded DNA in their sera [38], which was not seen in either non-transgenic, solHEL or RIP-HEL transgenic recipients (data not shown). Although this is consistent with a systemic lupus erythematosus disorder [39], these mice did not develop raised proteinurea, and only a mild chronic ascending pyelonephritis was seen in their kidney (data not shown), rather than the glomerulonephritis characteristic of systemic lupus erythematosus. Analysis of the pancreas of RIP-HEL transgenic recipients of HEL-specific TCR7 cells showed no infiltration into the pancreas over a 4-month period after transfer and these mice were normoglycemic (data not shown). Given that a small frequency of $CD4^+V\beta3^+$ cells remained in these mice and could be expanded upon immunization without inducing diabetes (data not shown), we reasoned that the donor TCR7 cells were clonally ignorant of the pancreas-restricted neo-self antigen in RIP-HEL mice. Furthermore, transfer into M-HEL mice did not result in any organ-specific disease despite great expansion in the periphery of TCR7 $CD4^+$ T cells after transfer.

## Blockade of CTLA-4 signaling allows Th1 but not Th2 cells to cause diabetes

In other systems of diabetes, failure of activation of autoreactive T cells could be overcome either by peripheral immunization or infection [4, 40] or by transfer of differentiated Th1 cells [21, 22, 41] into neonatal diabetes-prone mice, suggesting that induction of pathology and escape from peripheral tolerance mechanisms required appropriate T cell activation/differentiation and possibly the appropriately susceptible genetic background [4, 40]. We attempted to provoke autoimmune infiltration of the islets by activating the TCR7 cells prior to transferring to the RIP-HEL mice. Only when TCR7 cells were stimulated with HEL and polarized to a Th1 phenotype in the presence of IL-12 and anti-IL-4, were the transferred cells capable of inducing a moderate islet infiltrate (Fig. 3A). This was reminiscent of the pathology typically seen in autoimmune diabetes, with a presence of lymphocytes, plasma cells and rare neutrophils. In contrast, transfer of Th2 TCR7 cells, induced by stimulating with $HEL_{74–88}$ and APC in the presence of IL-4 and anti-IL-12, induced a peri-islet infiltration, which was comprised primarily of eosinophils with fewer lymphocytes, plasma cells and rare neutrophils (Fig. 3B). No insulitis was observed in RIP-HEL recipients of TCR7 cells primed in non-polarizing conditions using cross-linked anti-CD3 plus anti-CD28 (data not shown).

Interestingly, there was never a prolonged elevation of blood glucose levels under any of these circumstances (Fig. 3C) and the insulitis observed after transfer of either Th1 or Th2 TCR7 cells appeared to be held in check throughout an 8-month monitoring period, in contrast to reports in other models where Th1 cells specific for islet antigens induced diabetes [8, 21]. This difference could be due to the genetic background of the TCR7 mouse (C57BL/6 *versus* those used in other studies) [8, 21]. Alternatively it is possible that since the TCR7 mice respond to a subdominant peptide of $HEL_{74–88}$ (Fig. 1C) the ability of islet-specific Th1 cells to further infiltrate the islets and cause diabetes can still be held in check by additional peripheral regulatory mechanisms [8, 21, 42].

We thus assessed whether or not treatment with anti-CTLA-4 mAb would provoke disease as it had in other systems of diabetes on a NOD background [28, 43]. Indeed RIP-HEL mice developed diabetes with 100% incidence by 50 days after transfer of Th1 TCR7 cells when co-administered with anti-CTLA-4 mAb at the time of transfer (Fig. 3C). In contrast, anti-CTLA-4 mAb treatment did not induce diabetes in RIP-HEL mice either untransferred (data not shown) or after their receipt of Th2 TCR7 cells (Fig. 3C). Interestingly, anti-CTLA-4 treatment made no noticeable difference to either the extent of infiltration or the cell types present in RIP-HEL recipients of either Th1 or Th2 TCR7 cells (Fig. 3D, E). Therefore, the mechanism by which anti-CTLA-4 mAb provokes diabetes in this system appears to segregate with the Th1 TCR7 cell subset and has no detectable effect on the autoreactive potential of Th2 TCR7 cells, probably as a result that Th1 cytokine production can provoke autoimmune pathologies, whereas in contrast Th2 cells have been suggested to inhibit them [42].

These studies suggest that the HEL-specific Th1 cells, although able to infiltrate the pancreas, cannot overcome the threshold of immune regulation to evoke disease. The inhibition of CTLA-4 signaling may interrupt the activation or function of regulatory T cells, as previously suggested [31, 32], or alternatively remove intrinsic negative signaling in the Th1 cell by CTLA-4 [26, 33] *per se*, thus lowering its threshold of activation. Our studies show that even fully differen-

 

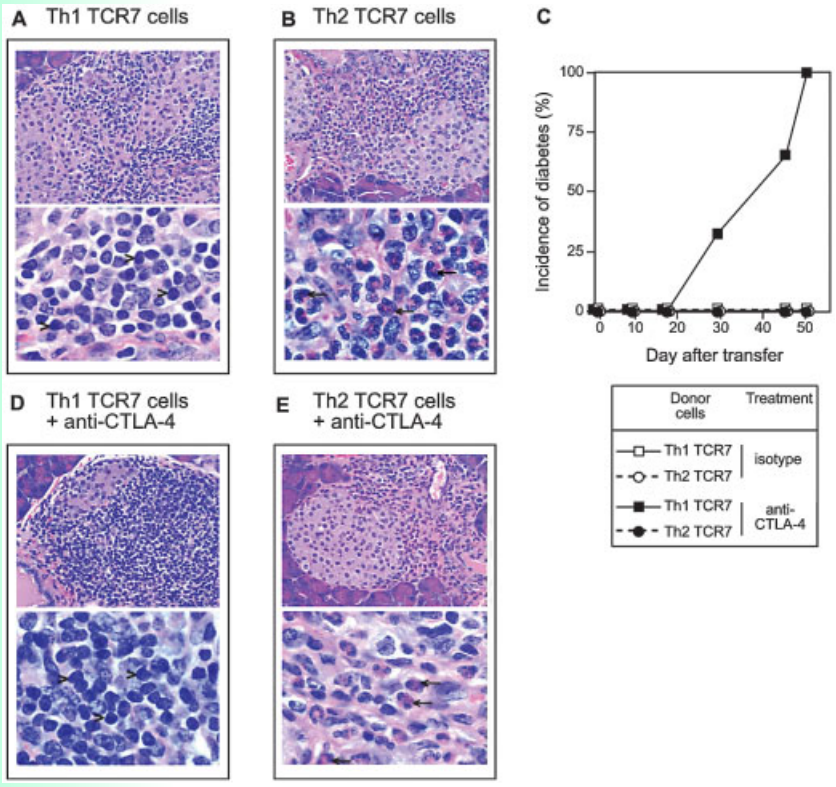


**Figure 3.** Requirements for diabetes in RIP-HEL mice: Transfer of Th1 TCR7 cells results in insulitis that can induce diabetes in the absence of CTLA-4 signaling. Th1- and Th2-polarized TCR7 spleen cells were prepared and $9\times10^6$ $CD4^+V\beta3^+$ cells were transferred to recipients. Representative H&E-stained sections are shown for pancreata removed from RIP-HEL recipients 50 days after transferring either (A) Th1- or (B) Th2-polarized TCR7 cells. (C) RIP-HEL mice were injected i.p. with 200 μg of either anti-CTLA-4 mAb or isotype-matched control mAb 1 day prior to and on days 3 and 5 after transfer of Th1 or Th2 TCR7 cells as described above. Diabetes development was monitored by regular blood glucose measurements and the incidence of disease is shown for five mice per group from two identical experiments. H&E-stained pancreata were also prepared where a representative section from anti-CTLA-4-treated RIP-HEL recipients of either (D) Th1- or (E) Th2-polarized TCR7 cells is shown. Pancreata of isotype control mAb-treated RIP-HEL recipients of Th1- or Th2-polarized TCR7 cells were identical to those shown in (A, B). For all sections, the upper panel shows ×40 original magnification and the lower panel shows ×160 original magnification. The presence of lymphocytes (>) predominates in (A, D), while eosinophils (←) comprise the majority of cells present in (B, E).

tiated Th1 cells that have migrated to the pancreas and can cause insulitis are still subject to immune regulatory mechanisms.

## Concluding remarks

We describe here a novel TCR-transgenic line, TCR7, where MHC class II-restricted, $CD4^+$ T cells are specific for the subdominant $H\text{-}2^b$ epitope ($HEL_{74–88}$) of HEL. Adoptive transfer of TCR7 (Ly5.1) T cells into RIP-HEL (Ly5.2) recipient hosts did neither lead to expansion of these cells nor result in islet infiltration, although these TCR7 cells could expand upon transfer into mice expressing high levels of HEL in the serum. Islet cell infiltration only occurred when the TCR7 cells had been polarized to either a Th1 or Th2 phenotype prior to transfer, which led to insulitis. However, progression from insulitis to autoimmune diabetes only occurred in these recipients when Th1 but not Th2 TCR7 cells were transferred and CTLA-4 signaling was simultaneously blocked. These findings show that even already differentiated autoreactive effector Th1 cells that have migrated into the pancreas fail to induce diabetes and are still held in check by powerful regulatory elements.

These regulatory mechanisms are revealed using blocking anti-CTLA-4 antibodies which may be operating *via* regulatory T cells or directly on effector cells, to inhibit the transition from tolerance to autoimmune diabetes. These findings are in keeping with previous reports that variations in the CTLA-4 molecule are associated with susceptibility to type I diabetes in humans and in the NOD mouse model for this disease [44], and provide a new model for further study of the molecular and cellular pathways involved in type I diabetes.

## Materials and methods

### Generation of TCRαβ-transgenic mice

HEL-specific TCR-transgenic mice were made using gene constructs encoding functionally rearranged α and β chain TCR genes obtained from the T cell hybridoma BO4H9.1, which recognizes $HEL_{74–88}$ peptide in the context of $I\text{-}A^b$ [36]. The HEL TCR α chain gene construct was made by insertion into the plasmid pBS a 4-kb *Eco*RI-*Eco*RV genomic fragment containing the functionally rearranged VJ region of the TCR α chain gene cloned from BO4H9.1 cells (a kind gift from N. Shastri). A 2.9-kb fragment containing the anti-HEL VJ region was then removed by digestion with *Bam*HI and subcloned into *Bgl*I-*Bam*HI-digested pUG-1 into which the 1.6-kb neomycin (*neo*) gene had been inserted (a gift from C. Somoza). From this, the *neo* and anti-HEL VJ genes (4.5 kb) were removed by digestion with *Xho*I and *Bam*HI and inserted into a TCR gene construct (pVCαK; a kind gift from A. Winoto [45]) containing the α chain constant region, enhancer, and locus control region. To do this, the plasmid pVCaK was first digested with *Sal*I and partially digested with *Bam*HI to remove the existing VJ region, leaving a 21.8-kb linearized vector and control regions. The *Xho*I-*Bam*HI fragment containing the anti-HEL VJ region and *neo* gene was then inserted, giving the complete anti-HEL TCR α chain gene construct.

The anti-HEL TCR β chain gene construct was made by inserting into pBS a 4.9-kb *Eco*RI-*Bam*HI DNA fragment containing the TCR β chain enhancer (obtained from a modified β shuttle vector (a kind gift from M. Davis [46]). This was done by treating the *Eco*RI-*Bam*HI fragment with Klenow and *Eco*RI linkers added and cloning into *Eco*RI-digested pBS. The resulting plasmid was linearized by partial digestion with *Hind*III and into this was inserted a 9-kb *Hind*III genomic DNA fragment encoding the functionally rearranged anti-HEL TCR β chain VDJ region and downstream constant region which had been cloned from the BO4H9.1 hybridoma (a gift from N. Shastri). Positioning the VDJ region upstream of the enhancer region gave the complete anti-HEL TCR β chain gene construct.

Stable co-transfection of the anti-HEL TCR α and β chain constructs into $58\alpha^-\beta^-$ cells (a T cell hybridoma lacking TCR expression; a gift of B. Malissen) followed linearization of the α chain by *Pvu*I digestion, and of the β chain by *Cla*I digestion, electroporation and G418 (Gibco BRL/Life Technologies, Gaithersburg, MD) selection (using 500 μg/mL G418) as described for Sp2/0 cells [47]. Representative transfectants were stained with fluorescinated anti-Vβ3 antibody (the transgene specificity; obtained from Pharmingen, San Diego, CA) and analyzed on the FACScan (Becton Dickinson, San Jose, CA).

For oocyte microinjection the α chain gene construct was removed from vector sequences by digestion with *Cla*I, and the β chain removed by digestion with *Sal*I-*Sac*II. The DNA was purified by cesium chloride centrifugation prior to microinjection into C57BL/6J eggs (Jackson Research Laboratories, Bar Harbor, ME). In this way, two founder mice were obtained, TCR7 and TCR9. TCR7 was used in the experiments described here, and maintained by crossing to C57BL/6J mice (Jackson Laboratories).

### Mice

Female C57BL/6 and C57BL/6.Ly5.1 mice were purchased from Jackson Research Laboratories. The generation of M-HEL (KLK-4), solHEL (ML-5) and RIP-HEL (ILK-3) transgenic mice (all on a C57BL/6 background) has been previously described [2, 3, 10, 35, 48, 49] and colonies of each line were maintained by breeding with C57BL/6 mice. These mice were a kind gift from Dr. C. C. Goodnow. In order to track HEL-specific TCR-transgenic T cells (TCR7), TCR7.Ly5.1 were obtained by breeding TCR7 mice with C57BL/6.Ly5.1 mice, while $TCR7.Ly5.1RAG2^{-/-}$ were obtained by crossing TCR7.Ly5.1 mice with $RAG2^{-/-}$Ly.5.1 mice (Jackson Research Laboratories) and then intercrossing $TCR7.Ly5.1RAG^{+/-}$.

All transgenic animals were genotyped by polymerase chain reaction on ear punch DNA [50] using primer sequences kindly provided by Dr. C. C. Goodnow for each HEL-transgenic line [10, 35], or forward endogenous 5′-GTCCTCCGTGTCCATATT-3′, forward transgenic 5′-ATCTGGTGAAAGGGCAAGGA-3′ and reverse 5′-CAAGGAGGAACCATGAGCA-3′ primer sequences for TCR7 mice. In all cases, 35 cycles of 94°C for 15 s, 60°C for 30 s and 72°C for 1 min were performed before polymerase chain reactions were separated on 2% agarose gels and visualized by ethidium bromide staining under UV illumination. $TCR7.Ly5.1RAG2^{-/-}$ mice were additionally screened by FACS analysis to confirm that they lacked lymphocytes other than those of a $CD4^+V\beta3^+$ profile. All mice were housed under specific pathogen-free conditions and were used at the age of 6–8 wk.

### Media, antigens, antibodies, cytokines and reagents

Cell culture medium was RPMI 1640 medium (Bio-Whittaker, Walkersville, MA) supplemented with 10% FCS (Hyclone Laboratories, Logan, UT), 1 mM L-glutamine, 10 mM Hepes, 100 U/mL penicillin, 100 μg/mL streptomycin, 1 mM sodium pyruvate and 0.05 mM β-2-mercaptoethanol (all Gibco BRL, Grand Island, NY). Unsupplemented Hanks' medium (Gibco BRL) was used for all adoptive cell transfers. HEL protein was obtained from Sigma, while the antigenic peptide ($HEL_{74–88}$) from HEL was synthesized on an Applied Biosystems model 430 peptide synthesizer (Foster City, CA). Recombinant mouse cytokines used were IL-2, IL-4 (both DNAX) and IL-12 (Pharmingen, San Diego, CA). Anti-IL-12 (C17.8.20 [51]), anti-IL-4 (11B11 [52]) and anti-CTLA-4 (UC10–9H10 [53]) were used with an isotype-matched control mAb when appropriate.

### $CD4^+$ T cell purification and $CD4^+$, Th1 and Th2 cell culture

$CD4^+$ T cells were enriched from spleen cell preparations by negative selection and purified further using a FACStar Plus flow cytometer (Becton Dickinson, Mountain View, CA) to achieve >99% $CD4^+Mel\text{-}14^+$ T cells as previously described [54]. Whole or CD4-enriched spleen cells were incubated for 48 h at 37°C in 96-well plates ($10^6$ or $5\times10^5$ cells per 200 μL per well, respectively), with either titrated amounts of HEL protein or $HEL_{74–88}$ peptide and red blood cell (RBC)-lysed C57BL/6 spleen cells ($1\times10^6$/well, 3000 rad) as APC. Super-

 

natants were harvested after 48 h of culture and stored at –80°C until analyzed for cytokine content, or cell cultures were pulsed for a further 20 h with 1 μCi [$^3$H]thymidine (Amersham) to measure proliferative responses.

Th1 and Th2 cell differentiation was achieved in culture using 1.1 μM $HEL_{74–88}$ and RBC-lysed C57BL/6 spleen cells ($1\times10^6$/well, 3000 rad) as APC with either IL-12 (10 ng/mL) ± anti-IL-4 (10 μg/mL) for Th1 conditions, or IL-4 (10 ng/mL) ± anti-IL-12 (10 μg/mL) for Th2 conditions as previously described [54]. Supernatants were collected after secondary stimulation (as in [54]) at 48 h and stored at –80°C until analyzed for IL-4 (Th2) and IFN-γ (Th1), which were detected using a two-site sandwich ELISA as previously described [54].

### Adoptive transfer of mice

Approximately $40\times10^6$ RBC-lysed spleen cells (containing approximately $4\times10^6$–$9\times10^6$ $CD4^+V\beta3^+$ T cells) from TCR7.Ly5.1 mice were transferred intravenously in 0.2 mL Hanks' medium to recipient mice (Ly5.2). Where indicated, the donor population was first polarized to a Th1 or Th2 phenotype as described above and previously [54]. Approximately $9\times10^6$ $CD4^+V\beta3^+$ T cells per recipient were transferred into C57BL/6 recipient mice. When indicated, the recipients were administered intraperitoneally (i.p.) several times (weekly) with either 200 μg per injection of anti-CTLA-4 or isotype-matched irrelevant hamster control antibody prepared in PBS.

### Flow cytometry

Single-cell suspensions of thymus, spleen or lymph node cells were depleted of contaminating RBC and stained as previously described [54] with various combinations of the following rat anti-mouse conjugated antibodies: FITC-conjugated Ly5.1; PE-conjugated Vβ3, Mel-14, CD45RB or CD69; Cy-Chrome-conjugated CD4 or CD8; allophycocyanin-conjugated CD4; or biotin-conjugated Vβ3 followed by streptavidin-allophycocyanin at pre-determined optimal doses (most antibodies from Pharmingen; secondary antibody from Caltag, Burlingame, CA). Viable cells (50 000 per sample), gated for lymphocytes by a combination of forward and side scatter, were analyzed on a FACSCaliber™ (Becton Dickinson) using CellQuest™ software.

### Histology

Hematoxylin and eosin (H&E) staining of 4-μm sections from buffered formalin-fixed paraffin-embedded pancreata was performed as described [55]. Multiple sections (at least 30) of pancreas from each animal were analyzed for insulitis as described [55]. Islets were judged to be free of infiltrates, suffering from peri-insulitis (if the infiltrates were limited to the surrounding ducts and vasculature and islet architecture appeared normal), suffering from moderate insulitis (if less than 50% of the islet was infiltrated), or suffering from severe insulitis (if 50% or greater of the islet was infiltrated and islet architecture was severely disrupted).

### Assessment of diabetes

Diabetes was assessed every 10 days by measurement of blood glucose using Accu-Chek Advantage® test strips and blood glucose monitor (Roche Diagnostics Corporation). Animals were considered diabetic after two sequential measurements above 250 mg/dL and this was confirmed by urine glucose measurements using dip sticks (Bayer Corporation, Elkhart, IN). Onset of diabetes was dated from the first of the sequential diabetic measurements.

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