

# Interleukin 10 Secretion and Impaired Effector Function of Major Histocompatibility Complex Class II-restricted T Cells Anergized In Vivo

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## Summary

Continuous antigenic stimulation in vivo can result in the generation of so-called “anergic” CD4<sup>+</sup> or CD8<sup>+</sup> T cells that fail to proliferate upon antigenic stimulation and fail to develop cytolytic effector functions. Here we show that class II major histocompatibility complex-restricted T cells specific for influenza hemagglutinin (HA) that become anergic in mice expressing HA under control of the immunoglobulin  $\kappa$  promoter exhibit an impaired effector function in causing diabetes in vivo, as compared to their naive counterparts, when transferred into immunodeficient recipients expressing HA under the control of the insulin promoter. Furthermore, HA-specific T cells anergized in vivo contain higher levels of interleukin (IL)-4 messenger RNA (mRNA) than naive and recently activated T cells with the same specificity and more than a 100-fold higher levels of IL-10 mRNA. The higher expression of the IL-10 gene is also evident at the protein level. These findings raise the interesting possibility that T cells rendered anergic in vivo have in fact become regulatory T cells that may influence neighboring immune responses through the release of IL-10.

Many T cells reactive to self peptides are deleted in the thymus (1). This is also true for T cells reactive to superantigens (2). Central deletion is not the only mechanism of immune tolerance, and additional mechanisms must exist to ensure tolerance to antigens that are not encountered in the thymus in sufficient quantity. In vivo tolerance experiments conducted with mature T cells have shown that peripheral tolerance may result from deletion as well as anergy induced by MHC-presented peptides (3–6) as well as superantigens (7–9) in mature CD4<sup>+</sup> (5–9) as well as CD8<sup>+</sup> T cells (3, 4). It has also been reported that some antigens expressed in certain tissues may be ignored either because T cells have no access to them or perhaps because cells of such tissue cannot stimulate T cells (10, 11). It appears unlikely that ignorance is the reason for tolerance to a large variety of antigens since, except for privileged sites where antigens released from certain tissues cannot be presented by hemopoietic APCs, fragments of tissue-specific proteins can be presented by neighboring APCs (12). In in vitro experiments, it was reported that already activated T cell clones could be rendered “anergic” by antigen pre-

sented on nonprofessional APCs that lack costimulatory molecules (13). Anergic cells were reported to have a block in p21<sup>ras</sup> activation, a decrease in the activities of MAP kinase pathways, a reduced induction of c-Fos and JunB proteins, and a failure to form and phosphorylate the activator protein 1 required for IL-2 gene transcription (14–18). More recently, negative regulation of TCR-mediated IL-2 gene transcription has been described as an additional mechanism of T cell anergy in vitro (19, 20). In all cases, anergy induced in vitro could be overcome by the addition of exogenous IL-2 (21, 22). It remains to be seen whether anergy induced in vivo rests on similar mechanisms. Although some experiments showed a similar type of anergy of in vivo tolerized CD8<sup>+</sup> T cells (23), others found that the anergic state could not be overcome by the addition of IL-2 (3, 6, 24). Moreover, several experiments strongly indicate that anergy in vivo can be preceded by an activation of T cells (3, 6, 9, 25) and it was recently shown that blocking the B7/CD28 interaction prevents in vivo priming, whereas blocking the B7/CTLA4 interaction prevents in vivo tolerance induction (25). The latter observation suggests that tolerance was induced by B7 expressing APCs. We have observed that in transgenic mice expressing both a class II-restricted TCR- $\alpha/\beta$  specific for influenza hemagglutinin and the hemagglutinin under the control of the Ig- $\kappa$  promoter,

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thymic deletion of hemmagglutinin (HA)<sup>1</sup>-specific T cells takes place but some self-reactive cells also accumulate in the periphery (6). These cells show signs of activation in vivo, but are anergic in terms of proliferation when restimulated in vitro. In contrast to in vitro anergized T cell clones, unresponsiveness could not be overcome by addition of exogenous IL-2. In this system, one can study T cells that have been anergized in vivo by mechanisms that are perhaps physiologically more relevant than those analyzed in vitro. Furthermore, the possibility of isolating the TCR transgenic cells by means of a clonotypic antibody, permits the analysis of the anergic cells from TCR-HA × IG-HA mice in greater detail. In the present study we have characterized the anergic HA-specific cells in terms of their effector function and cytokine production in vivo. We show that the anergic cells, in contrast to their naive precursors, fail to induce rapid and fulminant diabetes when injected into recipients that express HA under the control of the insulin promoter (INS-HA). Nevertheless, these cells do not appear to be entirely anergic, as they produce much higher levels of IL-10 messenger RNA (mRNA) and protein than their naive counterparts, suggesting that they may have a regulatory role.

## Materials and Methods

**Mice.** The TCR-HA transgenic mice expressing a TCR- $\alpha/\beta$  specific for peptides 111–119 from influenza HA presented by I-E<sup>d</sup> have been previously described (6, 26). Mice expressing HA (IG-HA) have the HA transgene under control of the Ig- $\kappa$  promoter and enhancer elements and are on BALB/c background (6). TCR-HA × IG-HA double transgenic mice were bred in our animal facilities. TCR-HA mice on recombination activating gene (RAG)-2<sup>-/-</sup> background have been described (25). INS-HA mice, already described (27), were backcrossed onto the RAG<sup>-/-</sup> background and were bred onto the H-2<sup>d</sup> haplotype. Transgene expression was determined by PCR. Animal care was in accordance with institutional guidelines.

**T Cell Proliferation Assays.** In vitro assays were performed in complete IMDM as previously described (6). Responder cells (2 × 10<sup>5</sup>/well) were isolated from the spleen of TCR-HA × IG-HA or TCR-HA mice and cultured in the presence of 5 × 10<sup>5</sup> total irradiated (2,200 rads) BALB/c splenocytes and different concentrations of the HA peptide. Responder cells were also stimulated with coated anti-CD3 at 10  $\mu$ g/ml or with coated 6.5 mAb at 100  $\mu$ g/ml in absence or presence of IL-2 (30 UI/ml). <sup>3</sup>H incorporation was measured over the last 18 h of a 66-h culture.

**Antibodies and Flow Cytometry.** The following mAbs were used for staining: F23.1-biotinylated (specific for the TCR- $\beta$  chain of HA-reactive TCR), 5(6)-carboxyfluorescein-*N*-hydroxy-succinimide ester (FLUOS)-labeled 6.5 clonotypic mAb, and SA-PE (Southern Biotechnology, Birmingham, AL). All stainings were done in 96-well plates (5 × 10<sup>5</sup> cells/well) in 20  $\mu$ l of mAb in PBS plus 2% FCS plus 0.1% sodium azide for 20 min on ice. Between first and second step reagents, cells were washed in PBS

plus 2% FCS plus 0.1% sodium azide, as was done after the last step. Data were analyzed on a FACScan<sup>®</sup> (Beckton Dickinson, Mountain View, CA), using Lysis II software.

For cell sorting experiments, splenocytes were depleted of surface Ig<sup>+</sup> cells using Dynabeads (Dyna, Oslo, Norway), and cells were subsequently stained with F23.1 and 6.5 mAbs. F23.1<sup>+</sup>6.5<sup>hi</sup> cells were sorted with a FACS<sup>®</sup> Vantage.

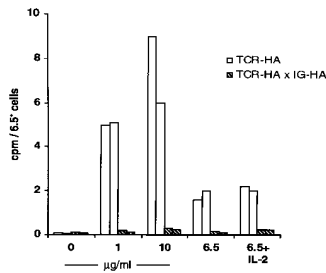
**Transfer Experiments.** Sorted F23.1<sup>+</sup>6.5<sup>hi</sup> spleen cells from TCR-HA × IG-HA mice, TCR-HA mice, or total lymph node and spleen cells from TCR-HA mice on RAG<sup>-/-</sup> background were washed in serum-free IMDM and were injected into the lateral tail vein of RAG<sup>-/-</sup> INS-HA<sup>+/+</sup> mice. Recipients were tested for urine glucose levels every day and diabetes was confirmed by blood glucose levels.

**Histology.** Pancreata were quick frozen in O.C.T. compound, and 5- $\mu$ m sections were obtained and fixed in acetone for 10 min. For immunohistochemistry, primary antibodies consisting of biotin-conjugated rat anti-mouse CD4 (clone GK1.5) and rat anti-mouse CD8 (clone 53-67.2) were applied on sections for 45 min at the appropriate dilution. After washing in PBS, slides were incubated with a biotinylated anti-rat  $\kappa$  antibody (Immunotech, Marseille, France) for 30 min, washed again, and finally incubated with streptavidin-peroxidase (Vector Laboratories, Inc., Burlingame, CA). Color reaction was revealed with 3-amino-9-ethyl-carbazole (AEC; Sigma Chemical Co., St. Louis, MO), and the slides were counterstained with hematoxylin. For the insulin staining, a guinea pig anti-porcine insulin antibody (Dako, Glostrup, Denmark) and a peroxidase-conjugated anti-guinea pig antibody (Dako) were used as primary and secondary antibodies, respectively.

**Competitive Reverse Transcriptase PCR for Cytokine mRNA Expression.** Total RNA was isolated from F23.1<sup>+</sup>6.5<sup>hi</sup> sorted splenocytes (2 × 10<sup>5</sup> cells) from TCR-HA single transgenic and TCR-HA × IG-HA double transgenic mice using RNeasy Mini Kit (Qiagen, Hilden, Germany). Reverse transcription was carried out using Superscript II reverse transcriptase (RT; GIBCO BRL, Gaithersburg, MD). The cytokine polycompetitor plasmid pQRS was used to quantitate amounts of transcripts for IL-4, IL-10, IFN- $\gamma$ , and the constitutively expressed hypoxanthine guanine phosphoribosyltransferase (HPRT), using the primers and PCR conditions as described previously (28). PCR products were analyzed by electrophoresis on 2% agarose gels and visualized by ethidium bromide staining under UV illumination, and then photographed (665 film; Polaroid, St. Albans, Hertfordshire, UK). Image densitometric analysis was performed using National Institutes of Health Image 1.61 software (SCR, Bethesda, MD) by integrating the volume in individual amplicons. After subtraction of background values, the density ratio of the competitor band to the target mRNA was determined and relative amounts of cytokine mRNA were calculated based on the starting amount of competitor.

**Enzyme-linked Immunospot Assay for IL-10.** The enzyme-linked immunospot (ELISPOT) assay was performed as previously described (29). In brief, F23.1<sup>+</sup>6.5<sup>hi</sup> sorted splenocytes from TCR-HA single transgenic and TCR-HA × IG-HA double transgenic mice were transferred by threefold dilutions (starting at 10<sup>4</sup> cells/well) in duplicate to 96-well microtiter plates (Millipore, Bedford, MA) that had been coated with the capturing mAb to IL-10 (JES5-2A5; PharMingen, San Diego, CA). After 20 h, cells were removed and spot-forming cells visualized with biotinylated detecting mAb (SXC-1; Pharmingen) and avidin peroxidase in conjunction with 3-amino-9-ethylcarbazole (Sigma Chemical Co.) substrate. Spots were counted under a dissecting microscope, and the frequency of antigen-specific cells was determined in both types of mice.

<sup>1</sup>Abbreviations used in this paper: AEC, 3-amino-9-ethyl-carbazole; ELISPOT, enzyme-linked immunospot; HA, hemmagglutinin; HPRT, hypoxanthine guanine phosphoribosyltransferase; mRNA, messenger RNA; RAG, recombination activating gene; RT, reverse transcriptase.



**Figure 1.** Proliferation of splenocytes from TCR-HA or from TCR-HA  $\times$  IG-HA mice upon antigenic stimulation in vitro.  $2 \times 10^5$  spleen cells were stimulated with  $5 \times 10^5$  irradiated BALB/c splenocytes in the presence of the indicated amounts of peptide or with coated 6.5 antibody at 100  $\mu$ g/ml. IL-2 (30 UI/ml) was added to cells stimulated with the 6.5 antibody. Total cpm values were divided by the number of 6.5<sup>+</sup> cells/well as determined by flow cytometry. Each bar represents mean values of triplicate determinations for each mouse. Response of splenocytes from each mouse to stimulation with immobilized CD3 antibody was similar (not shown).

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## Results and Discussion

**HA-specific T Cells from TCR-HA  $\times$  IG-HA Mice Cannot Respond to Further Antigenic Stimulation in Vitro.** As described previously (6), mice that express a transgenic TCR specific for peptides 111–119 from influenza HA (TCR-HA), as well as influenza HA under the control of the Ig- $\kappa$  promoter (IG-HA) contained CD4<sup>+</sup> T cells in periphery that expressed high levels of the transgenic TCR (detected by the idiotypic 6.5 antibody). The proportion of these cells, which increased with the age of these mice, exhibited activation markers (like CD69), but did not proliferate when stimulated with different concentrations of antigen in vitro (Fig. 1). Such cells did not respond when stimulated with the clonotypic antibody, even after addition of exogenous IL-2. In the following, we have further characterized these cells that appear to behave differently from in vitro anergized cells.

**T cells from TCR-HA Single but Not from TCR-HA  $\times$  IG-HA Double Transgenic Mice Cause Rapid and Fulminant Diabetes.** To determine whether the lack of proliferation in vitro was reflected by a lack of effector function in vivo, it was tested whether anergic 6.5<sup>hi</sup> cells could cause diabetes after transfer into rearrangement-deficient RAG<sup>-/-</sup> mice expressing HA under the control of the rat insulin promoter (INS-HA). In this system, transfer of  $10^4$  or  $10^5$  6.5<sup>+</sup> T cells from TCR-HA single transgenic mice on the RAG<sup>-/-</sup> background or of  $10^5$  6.5<sup>hi</sup> cells sorted from TCR-HA single transgenic mice induced diabetes at days 13, 9, and 10, respectively, after transfer (Table 1). The injected mice died within 12 to 16 d after transfer. In contrast,  $10^5$  6.5<sup>hi</sup> sorted cells from TCR-HA  $\times$  IG-HA double transgenic mice did not induce diabetes with the same kinetics or the same severity, since recipients only became diabetic 16 d after transfer of cells (Table 1) and survived for >40 d after transfer. The significant delay in the onset of diabetes in the RAG<sup>-/-</sup> INS-HA mice that received anergic cells was not due to the fact that these cells could not repopulate the host or could not home to the pancreas, as T cells were detected both in the lymph nodes and in the pancreas 11 d after transfer (Fig. 2). Histological examination of the pancreas of recipients of naive versus anergic 6.5<sup>hi</sup> cells revealed striking differences; in the former, infiltrating cells were de-

**Table 1.** Transfer of Diabetes into RAG<sup>-/-</sup> INS-HA Recipients by Cells from TCR-HA and TCR-HA  $\times$  IG-HA Mice

Donors*	No. of recipients with diabetes	Day of diabetes onset	Median survival after transfer
			<i>mean <math>\pm</math> SD</i>
TCR-HA $\times$ RAG <sup>-/-</sup>			
$10^5$ 6.5 <sup>+</sup> cells	10/10	9 $\pm$ 0.2	12 (12–16) <sup>‡</sup>
$10^4$ 6.5 <sup>+</sup> cells	5/5	13 $\pm$ 2	ND
TCR-HA			
$10^5$ 6.5 <sup>hi</sup> spleen cells	2/2 <sup>§</sup>	10 $\pm$ 0.2	18
TCR-HA $\times$ IG-HA			
$10^5$ 6.5 <sup>hi</sup> spleen cells	5/8 <sup>  </sup>	16 $\pm$ 1.7	>40

\*Genotype of mice from which donor cells were obtained.

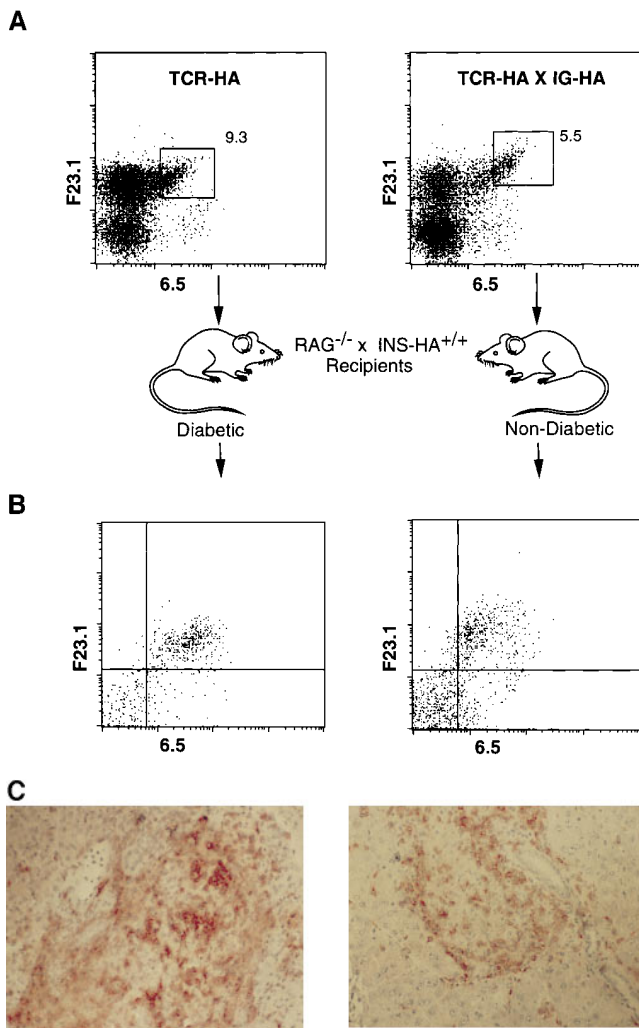
<sup>‡</sup>Range.

<sup>§</sup>One mouse was killed for analysis at day 11.

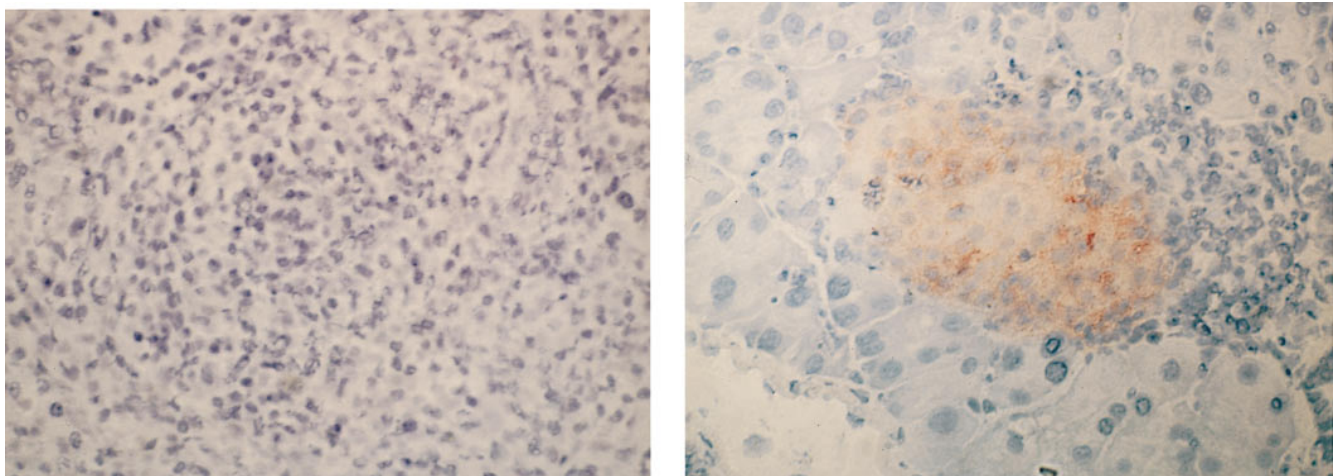
<sup>||</sup>Three mice were killed for analysis at days 11, 13, or 16, and were not diabetic at that time. The remaining mice did not die for the period of observation ranging from 40 to 105 d.

tected in both the exocrine and endocrine (islets) tissue, the islets were completely disrupted, and at the day of diabetes onset, insulin-producing cells were no longer detectable. On the contrary, the distribution of anergic 6.5<sup>hi</sup> cells was restricted to the islets and insulin-producing cells were still detectable after diabetes onset (Fig. 3). Thus, the impaired in vitro response of the anergic HA-specific cells was reflected by an impaired effector function in vivo that may be caused by a defect in in vivo proliferation and/or effector function in the pancreas. The precise reason for the late development of diabetes is unclear. We cannot exclude the possibility that among the 6.5<sup>hi</sup> cells from TCR-HA  $\times$  IG-HA double transgenic mice, there are recent thymic emigrants that have not yet been rendered anergic and that are causing the slow and mild diabetes observed in the recipients.

**Anergic T Cells Contain High Levels of IL-10 mRNA.** In a first attempt to analyze the lack of effector function of the 6.5<sup>hi</sup> T cells from the double transgenic mice in some greater detail, the mRNA level of various cytokines was determined by competitive PCR. To this end, 6.5<sup>hi</sup> expressing cells from either TCR-HA single or TCR-HA  $\times$  IG-HA double transgenic mice were sorted, and mRNA levels were compared (Table 2). Although IFN- $\gamma$  mRNA was not detectable in T cells from both mice, only cells from the double transgenic mice contained low levels of IL-4 mRNA. By far, the most significant difference was observed with regard to IL-10; very high levels of this cytokine mRNA were found in 6.5<sup>hi</sup> cells from TCR-HA  $\times$  IG-HA double transgenic, but not from TCR-HA single transgenic mice (Fig. 4). These results were confirmed with sorted CD4<sup>+</sup>6.5<sup>+</sup> cells from the single and double transgenic mice. The lack of IL-10 transcripts in 6.5<sup>+</sup> cells from single TCR transgenic mice was not due to the lack of activation since 6.5<sup>+</sup> cells transferred to INS-HA recipi-



**Figure 2.** Detection of transferred cells in the lymph nodes and pancreas of  $RAG^{-/-}$  INS-HA recipients 11 d after transfer. (A) Splenocytes from TCR-HA or from TCR-HA  $\times$  IG-HA mice were stained with



**Figure 3.** Detection of insulin-producing cells in the pancreas of  $RAG^{-/-}$  INS-HA recipients. Mice transferred with 6.5<sup>hi</sup> cells from a TCR-HA (left) or from a TCR-HA  $\times$  IG-HA mouse (right) were killed at the day of diabetes onset or 7 d after diabetes onset, respectively. Pancreata were embedded in OCT and frozen; 5- $\mu$ m sections were stained with insulin antibodies and revealed with AEC. Insulin (red staining) was clearly detected in recipients of 6.5<sup>hi</sup> anergic cells and not in recipients of 6.5<sup>hi</sup> naive cells (original magnification: 400).

**Table 2.** Quantification of Cytokine Transcripts in 6.5<sup>hi</sup> Sorted Splenocytes from TCR-HA and TCR-HA  $\times$  IG-HA Mice

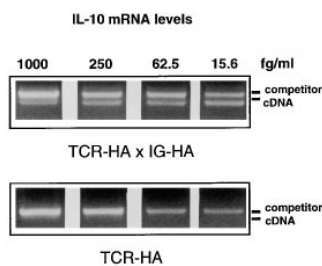
Cytokines*	TCR-HA $\times$ IG-HA	TCR-HA
IL-10	1.13 $\pm$ 0.13	<0.01
IL-4	0.10 $\pm$ 0.04	<0.01
IFN- $\gamma$	<0.01	<0.01

\*Cytokine transcript levels were calculated using competitive RT-PCR as described in Materials and Methods. Values represent the ratio (mean  $\pm$  SEM) of target gene to HPRT gene expression of two mice tested per group. The lower detection limit was 0.01.

ents did not produce IL-10 mRNA up to a point when the recipient mice became diabetic (not shown).

**High Levels of IL-10 mRNA Are Reflected in Protein Secretion.** To determine whether the increased levels of IL-10 mRNA reflected an increased secretion of the protein, we performed ELISPOT assays on 6.5<sup>hi</sup> sorted cells from single

F23.1 and 6.5 mAbs, and F23.1<sup>+</sup>6.5<sup>hi</sup> cells were gated as shown and were sorted accordingly. The purity of the cell populations after sorting was 97 and 95%, respectively.  $RAG^{-/-}$   $\times$  INS-HA recipients were injected with  $10^5$  6.5<sup>hi</sup> cells from TCR-HA or TCR-HA  $\times$  IG-HA mice, and were analyzed 11 d after transfer; the recipient of T cells from TCR-HA shown was diabetic, whereas the recipient of cells from the TCR-HA  $\times$  IG-HA donor was not. For B, two-color staining with the F23.1 and the 6.5 mAbs was performed on lymph node cell suspensions from each recipient. For C, the pancreas of the same mice (the left and right sections correspond to the recipient of 6.5 cells from the TCR-HA or the TCR-HA  $\times$  IG-HA mouse, respectively) was frozen and sections were stained with a mixture of CD4 and CD8 antibodies and revealed with AEC as described in Materials and Methods. The 6.5 mAb was not used because of its poor performance on histology sections, but isolation and staining of infiltrating lymphocytes in the pancreas of other transferred mice confirmed that they were all 6.5 positive. Note the total disruption of the anatomical structure of the pancreas having received the naive cells (left). Original magnification was 160 for both sections.



**Figure 4.** Competitive RT-PCR for IL-10 mRNA expression in  $6.5^{\text{hi}}$  sorted splenocytes from TCR-HA and TCR-HA  $\times$  IG-HA mice. IL-10 mRNA transcripts were compared between mice after standardization for the expression of the constitutively expressed HPRT gene (not shown). Constant amounts of cDNA samples were amplified

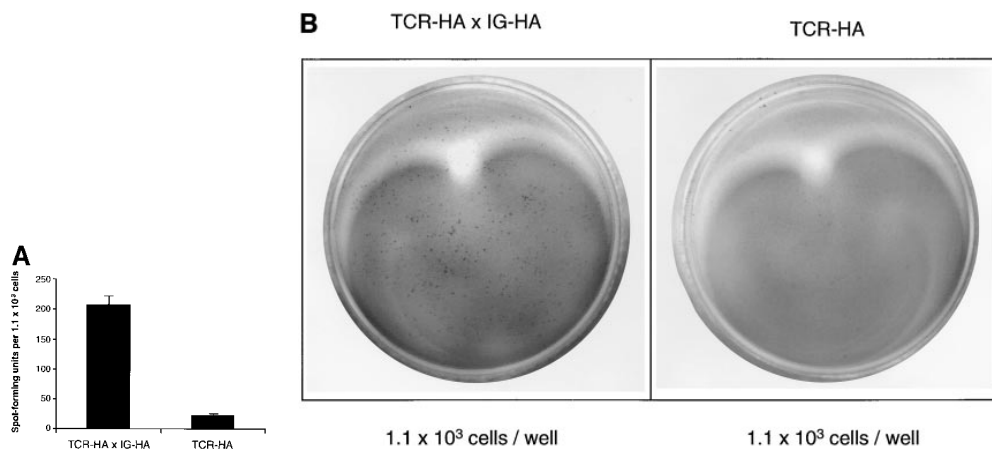
in the presence of serial fourfold dilutions of a multispecific internal plasmid control (pQRS). PCR products were separated on 2% agarose gels and visualized by ethidium bromide staining under UV illumination, and photographed. Image densitometric analysis was performed using National Institutes of Health Image 1.61 software. The concentrations of competitor used are indicated on the top. In all cases, the upper band is due to amplification of the competitor construct and the lower band is due to amplification of the cDNA. The point of equivalence between the competitor and the cDNA indicates the relative concentration of mRNA (15.6 fg/ml). Similar results were obtained in two separate experiments for each group.

and double transgenic mice. Fig. 5 clearly confirms that the anergic  $6.5^{\text{hi}}$  cells produced and secreted significantly higher levels of IL-10 than T cells from single transgenic mice.

These data raise the interesting possibility that so called anergic cells, as obtained *in vivo* through continuous stimulation with antigen, are in fact not really anergic but have assumed the phenotype of regulatory cells. IL-10 has been reported to downregulate costimulation by APCs (30), dendritic cell-driven IFN- $\gamma$  production by T cells (31), and T cell responses to antigen through inhibition of IL-2 pro-

duction and IL-2R  $\alpha$  chain expression (32, 33). Furthermore, adoptive transfer of autoreactive T cells genetically designed to secrete IL-10 were able to delay the onset of experimental autoimmune encephalomyelitis in mice (34). Finally, a recent study by Groux et al. (35) showed that OVA-specific T cell clones derived from *in vitro* cultures in the presence of IL-10 and chronic antigenic exposure, were able to prevent T cell-mediated inflammatory bowel disease when cotransferred with CD4 $^{+}$ CD45RB $^{\text{hi}}$  cells into SCID mice. These cells exerted their influence only upon stimulation with antigen, i.e., when the recipients were fed with OVA.

We show here that IL-10-producing T cells cannot only result from unphysiological maneuvers like genetic alteration or growth in IL-10-containing media, but also develop as a result of chronic antigenic stimulation *in vivo*. The IL-10 production by anergic T cells is in line with previous observations that repeated superantigen stimulation results in anergy as well as in IL-10 production (36) and with other data showing IL-10 production in SCID patients transplanted with HLA-mismatched hemopoietic stem cells (37). The exact source of IL-10 was not obvious from these experiments and it was not clear whether the unresponsive state of T cells in terms of proliferation and effector function correlated with IL-10 production. The results presented here directly show that T cells unable to proliferate in response to antigen *in vitro* and with an impaired specific effector function *in vivo*, can nevertheless produce high levels of IL-10 and therefore may regulate immune response through the release of IL-10.



**Figure 5.** ELISPOT for IL-10. The frequency of  $6.5^{\text{hi}}$  sorted splenocytes from TCR-HA single transgenic and TCR-HA  $\times$  IG-HA double transgenic mice that secreted IL-10 was determined. (A) Results are expressed as mean  $\pm$  standard error spot-forming units per  $1.1 \times 10^3$  cells of duplicate determinations. (B) Representative alkaline phosphatase-based ELISPOT demonstrating IL-10 secretion by  $6.5^{\text{hi}}$  sorted splenocytes from TCR-HA  $\times$  IG-HA sorted splenocytes. Similar results were obtained in two separate experiments.

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