Self-antigen presentation by mouse B cells results in regulatory T-cell induction rather than anergy or clonal deletion

Running title: B cells vs. DC in self-tolerance induction

Section: Immunobiology

Sara Morlacchi*, Cristiana Soldani*, Antonella Viola** and Adelaida Sarukhan*

*Istituto Clinico Humanitas, IRCCS, Milan, Italy, **Department of Translational Medicine, University of Milan, Italy

Corresponding Author:
Adelaida Sarukhan
telephone +39 0282245134
fax +39 0282245101
email: adelaida.sarukhan@humanitasresearch.it
Abstract

Multiple mechanisms operate to ensure T cell tolerance towards self-antigens. Three main processes have been described: clonal deletion, anergy and deviation to CD4+ regulatory T cells (Tr) that suppress autoreactive T cells that have escaped the first two mechanisms. While it is accepted that DCs and B cells contribute in maintaining T cell tolerance to self antigens, their relative contribution and the processes involved under physiological conditions remain only partially characterized. In this study, we used different transgenic mouse models to obtain chimeras where a neo self-antigen is expressed by thymic epithelium and/or by DC or B cells. We found that expression of cognate ligand in the thymus enhances antigen-specific FoxP3+ cells independently of whether the self-antigen is expressed on thymic epithelium or only on DC, but not on B cells. On the contrary, self-antigen expression by B cells was very efficient in inducing FoxP3+ cells in the periphery, while self-antigen expression by DC led mainly to deletion and anergy of antigen-specific FoxP3− cells. The results presented in this study underline the role of B cells in Tr induction and may have important implications in clinical protocols aimed at the peripheral expansion of Tr cells in patients.
Introduction

T cell tolerance towards self-antigens is achieved through a series of mechanisms that have evolved to keep autoimmunity in check. Three main processes, that start in the thymus but continue in the periphery, have been described: clonal deletion of cells reactive to proteins present in the thymus, including peripheral tissue antigens expressed by thymic epithelial cells and blood-borne antigens; anergy induction as a result of antigen recognition under non co-stimulatory or inhibitory conditions that remain only partially understood; and the generation of Tr cells that further limit peripheral reactivity towards self. The relative contribution of each of these mechanisms to the maintenance of T cell tolerance has been subject of some debate. However, it is clear that the breakdown of any of these mechanisms, that start in the thymus and continue in the periphery, can lead to the development of autoimmune disease, even though the kinetics, severity and target organs may be different. Thus, the generation and maintenance of FoxP3+ Tr has been shown to be critical in avoiding severe lymphoproliferative disease and extensive multiorgan infiltration throughout the entire lifespan of the organism. Similarly, mice deficient for Fas or Fas ligand and thus incapable of efficiently deleting T cells in the periphery develop lymphoproliferation and generalized autoimmunity, although apoptosis has been reported to be dispensable in T cell tolerance induction to a systemic self-antigen. Recently, E3 ubiquitin ligases such as Cbl-b, Itch, Grail and TRAF6 have been implicated in the development and maintenance of T cell anergy and mice deficient for these genes display resistance to anergy induction and enhanced susceptibility to spontaneous and induced autoimmune diseases. However, some genes typically expressed by anergic cells are also expressed by Tr and it has been difficult to dissociate anergy induction from Tr induction in vivo and determine their relative contribution in the establishment of self tolerance.

Tolerance induction to target antigens may represent a therapeutic strategy in the treatment of autoimmune diseases and allergy. Thus, it is important to understand the conditions by which these different processes occur and, in particular, the role of the different populations of antigen presenting cells (APCs) in tolerance induction (regulatory cell induction versus anergy versus deletion). The critical role of DCs not only in the induction of efficient immune responses but also in the maintenance of T cell tolerance, via the cross-presentation of tissue-restricted self antigens and their transport to the lymph nodes under steady-state conditions, has been well documented. A large body of recent experimental data suggest that antigen-presentation by DCs under non-inflammatory conditions leads to the generation or expansion of Tr, although the exact mechanisms involved remain only partially understood.

B cells are considered to be poorly immunogenic APCs and it was reported that animals can be rendered tolerant to antigens presented by naive B cells. More recent studies show that B cells
can positively or negatively regulate T cell-mediated responses through antibody-independent mechanisms and modulate the development, proliferation and survival of Tr \(^{17}\). In fact, B cells can efficiently generate Tr \textit{in vitro} \(^{18-20}\) but, on the other hand, they were reported to induce deletion of self-reactive T cells \textit{in vivo} \(^{21}\). Thus, the role of B cells in induction of T cell tolerance to self-antigens under physiological conditions, and in particular, in the induction of Tr, remains poorly defined.

In this study, we have sought to determine the role of DCs and B cells in the induction of T cell tolerance towards a neo-self antigen \textit{in vivo}. We show that antigen presentation by these two different cell types results in distinct types of tolerance. Presentation by DCs favors deletion and anergy, whilst presentation by B cells leads to peripheral Tr induction.
Methods

Mice
Balb/c (H-2\textsuperscript{d}) mice were from Charles River Laboratories (Italy). TCR-HA transgenic mice expressing a TCR\(\alpha\beta\) specific for peptide 111–119 from influenza virus hemagglutinin (HA) presented by I-E\textsuperscript{d} have been previously described\textsuperscript{22}, and are on the Balb/c background. These mice were crossed with mice expressing influenza HA under the control of the ubiquitous pgk promoter to generate TCR-HAxpgk-HA double-transgenic mice\textsuperscript{23} or to mice expressing influenza HA under the control of the Igk L chain promoter and enhancer to generate TCR-HAxIg-HA mice\textsuperscript{23}. FoxP3-GFP knock-in mice (C.Cg-Foxp3\textsuperscript{tm2Tch/J}) were purchased from the Jackson Laboratory and backcrossed onto the TCR-HAxIg-HA and TCR-HAxpgk-HA mice. Ig-HA mice were also backcrossed onto Rag\textsuperscript{-/-} Balb/c mice. All mice were used between 6 and 10 weeks of age.


Antibodies and reagents

The clonotypic 6.5 mAb, which recognizes the transgenic TCR-HA, was produced in our laboratory and was used coupled to biotin or PE. All other antibodies for flow cytometry were purchased from BD Pharmingen. The polyclonal Ki67 mAb was purchased from Abcam. Cells were analyzed on a flow cytometer (FACS Canto; Becton Dickinson). Cell sorting was performed using a FACS Aria (Becton Dickinson). Facs data were analysed using Diva software and FlowJo software. The HA peptide (SVSSFERFEIFPK) was purchased from Invitrogen.

Vybrant CFDA-SE cell tracer kit (Invitrogen) and CellVue Maroon (Molecular Targeting Technologies, Inc) were used according to manufacturer’s instructions.

In vitro proliferation and suppression assays

For in vitro experiments, total LN and spleen suspensions from the different transgenic or chimeric mice were stained with CD4 and 6.5 antibodies and sorted on a FACS Aria to obtain HA-specific 6.5\textsuperscript{+} Tc (CD4\textsuperscript{+} 6.5\textsuperscript{+} GFP\textsuperscript{-}) or 6.5\textsuperscript{+} Tr (CD4\textsuperscript{+} 6.5\textsuperscript{+} GFP\textsuperscript{+}) cells, respectively. Dendritic cells (DC) were obtained from the spleen of Balb/c mice by positive selection with anti-CD11c microbeads (Miltenyi Biotec). All assays were performed in complete DMEM (Lonza), supplemented with 2-mercaptoethanol (Invitrogen) and 10% FCS. 2.5x10\textsuperscript{4} Tc cells were incubated with DCs (1.5 x 10\textsuperscript{4}) in flat bottom 96 well plates in the absence or presence of Tr at a Tc:Tr ratio of 1:1 or 2:1. After 2
days of culture, supernatants were collected for the quantification of cytokines and 1μCi \(^3\)H-methylthymidine was added for an additional 16h. All conditions were performed in triplicates. Some experiments were performed staining T cells with CellVue Maroon, and the proliferation was analyzed by flow cytometry after 3 days of co-culture. For experiments shown in figure 3A, spleen cell suspensions were obtained by collagenase digestion, stained with CD11c and CD19 antibodies, and sorted electronically. They were then co-cultured in vitro with CD4\(^+\)6.5\(^+\) T cells from TCR-HA single transgenic mice.

**Bone marrow chimeras**

Pgk-HA or Balb/c recipient mice were irradiated 700 rads and reconstituted with bone marrow obtained from TCR-HA FoxP3-GFP mice (2x10\(^6\) cells), together with an equal amount of bone marrow from Rag\(^\gamma\), Rag\(^\gamma\) Ig-HA mice or 0.5-0.7x10\(^6\) CD19\(^+\) cells sorted from the bone marrow of an Ig-HA mouse. Mice were kept on antibiotics for two weeks after transfer. Reconstitution was confirmed after 7 weeks and mice were analysed between 8 and 12 weeks after transfer. In all chimeric mice, the percentage of B cells in the spleen was comparable at the time of analysis (71.8%; se 1.6).

**Adoptive transfer experiments**

CFSE-labelled CD4\(^+\)6.5\(^+\) T cells from a Thy1.1 TCR-HA mouse (1x10\(^6\)) were injected into pgk-HA, Ig-HA or Balb/c recipients (Thy1.2). Some Balb/c recipients had been reconstituted 7-8 weeks earlier with pgk-HA bone marrow. Recipient mice were sacrificed 3 days after adoptive transfer, and cell suspensions from LN and spleen were analysed by flow cytometry.

**Immunohistochemistry**

Frozen spleen sections (10µm) from the different chimeric mice were fixed with 4% formalin, rehydrated with PBS and permeabilized with PBS/Tween 0.1%. Then the slides were incubated with the anti-FoxP3 Alexa647-conjugated (1:50, BD) and with the primary antibody anti-CD19 (1:50, e-Bioscience) that was revealed with the Alexa488 anti-rat antibody (1:500, Molecular Probes). After washes, nuclei were counterstained with Hoechst 33258 (1µg/ml) and mounted with ProLong (Molecular Probes). Negative controls included slides incubated with the secondary antibodies alone. Acquisition of images was made by confocal microscopy Fluoview FV1000 (Olympus, Tokio, Japan) with an oil immersion objective (60x 1.4 NA Plan-Apochromat; Olympus). The statistical analysis was performed using: Student’s unpaired two-tailed t test, ANOVA one-way test analysis with Tukey's Multiple Comparison Test. For each specimen, Foxp3\(^+\)
cells were counted in randomly selected CD19 positive follicles at 20x magnification (x400). Values are expressed as mean ± SE.

Statistical analysis
Results are expressed as means ± standard deviation. Student t test was performed where indicated and p values <0.05 (*), 0.01 (**) and 0.001 (***) were considered significant.

Results
The pattern of self-antigen expression determines the fate of antigen-specific T cells
Although it is well established that Tr selection is positively regulated by expression of the agonist ligand in the thymus \(^{24,25}\), Tr can also be generated in periphery \(^{26}\), through processes that are not fully understood. Here, we took advantage of two mouse models - pgk-HA and Ig-HA mice - expressing the neo self-antigen HA in the thymus. The pgk-HA mouse expresses HA on thymic epithelium, although expression on other cell types in periphery has not been explored. The Ig promoter in Ig-HA mice drives expression of HA in B cells \(^{27}\), but also in dendritic cells \(^{28}\) and thymic epithelium \(^{25}\). These two different transgenic mice were backcrossed onto mice expressing a transgenic TCR (recognizable by the clonotypic antibody 6.5) specific for the immunodominant peptide of HA in the context of IE\(^{id}\) molecules. In addition, in this study, all transgenic mice were on a FoxP3-EGFP background, in order to identify and isolate FoxP3 positive cells by GFP expression. Both TCR-HAxpkg-HA and TCR-HAxIg-HA mice, despite thymic deletion of HA-specific T cells due to negative selection, are known to generate HA-specific Tr \(^{29}\). Indeed, when compared to the TCR-HA single transgenic mouse, mice having thymic expression of HA showed enhanced selection of HA-specific FoxP3\(^+\) thymocytes (Figure 1A upper row). This was also true for the periphery, where, despite the strong deletion of CD4\(^+\)6.5\(^+\) cells observed for both types of double transgenic mice (Figure 1A lower row and Figure 1B left graph), the percentage of FoxP3\(^+\) cells among the 6.5\(^+\) population was significantly higher than in the single transgenic mice (Figure 1A lower row and Figure 1B right graph). It is interesting to note that in the periphery of single transgenic TCR-HA mice there were more CD4\(^+\)6.5\(^+\)FoxP3\(^+\) cells in periphery with respect to the thymus, suggesting either expansion of thymic-derived Tr cells in periphery or their de novo generation, even in the absence of the cognate antigen. This is true both for the percentage of FoxP3\(^+\) cells among CD4\(^+\)6.5\(^+\) cells, as observed in Figure 1 (thymus versus lymph nodes), and for the absolute numbers of CD4\(^+\)6.5\(^+\)FoxP3\(^+\) cells (8.37 + 1.3 \times 10^4 cells in the thymus versus 85.5 + 30.7 \times 10^4 cells in the lymph nodes plus spleen, reflecting an average 10-fold increase).
Interestingly, when comparing absolute numbers of CD4+6.5+ cells in secondary lymphoid organs (lymph nodes and spleen) of the three types of mice, we observed an age-dependent accumulation in the number of FoxP3+6.5+ cells in TCR-HAxIg-HA mice (Figure 1C, left graph). In fact, the number of total CD4+6.5+ cells accumulated with age (Figure 1C, right graph) and this increase was in great part due to a striking increase in total cellularity of secondary lymphoid organs of TCR-HAxIg-HA mice, clearly evident from the age of 12 weeks (Figure 1D). In contrast, this increase in total cellularity was not observed for TCR-HAxpgk-HA mice. In fact, the absolute number of FoxP3+6.5+ and total CD4+6.5+ cells in TCR-HA single transgenic mice or TCR-HAxpgk-HA mice tended to decrease with age, independently of FoxP3 expression (Figure 1C), since the T conventional (Tc)/Tr ratio remained relatively constant with age (data not shown).

We then compared the functional properties of HA-specific FoxP3+ and FoxP3- cells obtained from these mice. By staining cells with a cell dye, we could determine by flow cytometry the division of CD4+6.5+FoxP3+ and FoxP3- cells in total lymph node cell preparations, when stimulated with different peptide doses in vitro. The 6.5+FoxP3+ cells generated in the three different mice were capable of proliferating at a comparable level, due to exogenous IL2 provided by the 6.5+FoxP3- cells present in the same well. In sharp contrast, 6.5+FoxP3- T cells from TCR-HAxIg-HA mice did not divide at any peptide dose (Figure 2A). Furthermore, very low IFN-γ levels were detected in the supernatant of TCR-HAxIg-HA cultures, as compared to those from TCR-HA and TCR-HAxpgk-HA mice (Figure 2B). These data strongly suggested that HA-specific conventional T cells were in a state of anergy in TCR-HAxIg-HA mice but they were functional in TCR-HAxpgk-HA mice. To understand if this different proliferative capacity of FoxP3- cells was due to differences in the suppressive capacity of their FoxP3+ counterparts, we sorted CD4+6.5+FoxP3+ and FoxP3+ cells from both mice and stimulated them in vitro with the HA peptide. As shown in Figure 2C, FoxP3+ cells from TCR-HAxpgk-HA mice proliferated similarly to control HA-specific T cells and, as expected, the FoxP3+ cells did not proliferate due to the absence of exogenous IL2. In contrast, FoxP3- cells from TCR-HAxIg-HA mice were unable of responding in vitro to peptide stimulation, similar to their FoxP3+ counterparts (Figure 2C).

Overall, these data suggested that self-antigen-specific cells undergo a different fate depending on the pattern of expression of the self-antigen.

Under homeostatic conditions, HA is expressed and presented by splenic B cells and DCs in Ig-HA mice. As a result, sorted CD19+ or CD11c+ cells from the spleen of Ig-HA mice induced proliferation of HA-specific T cells in vitro (Figure 3A). In contrast, neither DCs nor B cells from pgk-HA mice were capable of inducing HA-specific proliferation in vitro, unless peptide was added to the culture (Figure 3A). However, CFSE-labeled HA-specific cells transferred into pgk-HA recipients did show some in vivo proliferation, even though at levels much lower than those
observed when the same cells were transferred into Ig-Ha recipients (Figure 3B). This proliferation
was in great part due to antigen presentation by non-hematopoietic cells, such as liver sinusoidal
endothelial cells, because HA-specific T cell transfer into chimeric recipients, where only the
hematopoietic compartment was of pgk-HA origin, resulted in very poor T cell proliferation (Figure
3B, last row). However, we cannot completely discard that, under certain inflammatory conditions,
HA can be presented by DCs in pgk mice because bone marrow derived DCs from pgk mice were
able to induce HA-specific T cell proliferation when stimulated by LPS, albeit at levels lower than
those elicited by DCs from Ig-HA mice (supplementary Figure 1).

Self-antigen expression on B cells or DCs induces Tr development or T cell deletion/anergy,
respectively

Based on the differences observed between the two double transgenic mouse models described
above, we tried to further dissect the role of B cells versus DCs in tolerance induction towards self
antigens. For this, we generated bone marrow chimeras (Figure 4A) where HA-specific T cells
(bone marrow from TCR-HA, FoxP3-GFP mice) would develop in a context with no HA
expression (with Rag\(^{-}\) bone marrow, in a Balb/c recipient: ctrl chimera), HA expression on thymic
epithelium (with Rag\(^{-}\) bone marrow in a pgk-HA recipient: T chimera), on thymic epithelium plus
DC (with Rag\(^{-}\)Ig-HA bone marrow in a pgk-HA recipient: TD chimera), or on thymic epithelium
plus B cells (with CD19\(^{+}\) cells from Ig-HA bone marrow in a pgk-HA recipient: TB chimera).
Based on the results described above (Figure 3B), HA expression by non-hematopoietic cells in the
periphery of the pgk-HA recipients cannot be excluded but one would expect it to remain constant
between the T, TD and TB chimeras. In all chimeras, the TCR-HA FoxP3-GFP bone marrow
generated a population of TCR transgenic T cells together with non-HA expressing B cells and
DCs. HA-expressing DCs or B cells were generated by the co-administration of RAG/IgHA bone
marrow (TD chimeras) or CD19\(^{+}\) cells from Ig-HA bone marrow (TB chimeras), respectively.
The different chimeras were analyzed 8-12 weeks after reconstitution. As can be seen in Figure 4B
and 4C, the presence of HA in the thymus (chimeras T, TD and TB) resulted in a partial deletion of
6.5\(^{+}\) cells compared to the control (ctrl chimera) but enhanced selection of CD4\(^{+}\)6.5\(^{+}\)FoxP3\(^{+}\)
thymocytes. In periphery, the same tendency was seen, both in lymph nodes and in spleen.
However, chimeras expressing HA on both thymic epithelium and B cells (TB chimeras) displayed
the higher percentage and absolute numbers of FoxP3\(^{+}\) HA-specific T cells in secondary lymphoid
organs (Figure 4C).

We next tried to determine whether Tr generated in the different types of chimeras were functional.
The intensity of expression of FoxP3 is considered to be a marker for suppressive capacity, and
decreased Foxp3 expression results in the development of an aggressive autoimmune syndrome,
although anergic properties of the cells are maintained \(^3\). Interestingly, Tr cells from the TB chimeras were those with the highest FoxP3 expression (Figure 4D). CD4\(^+\)6.5\(^+\)FoxP3\(^+\) cells obtained from the different chimeras were all capable of suppressing HA-specific T cell proliferation \textit{in vitro} with comparable efficiency (Figure 4E). Remarkably, FoxP3\(^-\) T cells sorted from the different chimeras behaved very differently; while HA-specific T cells from T and TB chimeras were capable of proliferating \textit{in vitro} upon antigenic stimulation, those from TD chimeras were not (Figure 4E).

These results indicate that self-antigen expression by B cells is very efficient in promoting FoxP3\(^+\) T cells whereas self-antigen expression by DCs results in T cell anergy.

\textit{Self-antigen expression on thymic epithelium is not necessary for the enhancement of Treg generation by B cells}

In order to determine whether antigen expression by B cells alone (i.e. no HA expression on thymic epithelium) would also be efficient in promoting FoxP3\(^+\) T cells, we performed more experiments with bone marrow chimeras (Figure 5A). This time, all recipients were Balb/c - and thus did not express HA on thymic epithelium - but we reconstituted them with TCR-HA Foxp3EGFP bone marrow together with either DCs (bone marrow from Rag\(^{\text{2/-}}\)IgHA mice, D chimera) or B cells (CD19\(^+\) cells from IgHA bone marrow, B chimera) expressing HA. Figures 5B and 5C show that HA expression by DCs induced very efficient thymic deletion of CD4\(^+\)6.5\(^+\) cells, as previously reported \(^2\), whereas HA expression by B cells did not induce significant thymic deletion. These data are in agreement with the fact that B cells represent a very small population in the thymus and do not delete CD4\(^+\) T cells efficiently \(^3\). In accordance, HA mRNA expression was only detected in the D chimera (Supplementary Figure 2). Furthermore, these results indicate that very little B-cell derived HA antigen, if at all, is being cross-presented by DCs, at least in the thymus. As a result, the percentage of FoxP3\(^+\) cells among CD4\(^+\)6.5\(^+\) thymocytes was higher in the D chimera than in the other mice (Figure 5C). However, results were different in periphery, where the percentage of FoxP3\(^+\) cells among CD4\(^+\)6.5\(^+\) cells increased significantly in the B chimera (Figure 5B and 5C) and their absolute numbers were significantly higher than those in the D chimera (Figure 5C). Indeed, the absolute numbers of CD4\(^+\)6.5\(^+\)FoxP3\(^+\) cells in the D chimera were very low due to a significant deletion of HA-specific cells, both in thymus and periphery. \textit{In vitro} proliferation experiments performed with DCs sorted from the spleens of B and D chimeras confirmed that DCs from D mice expressed and presented HA antigen, as expected, whereas DCs from B mice were not
capable of inducing T cell proliferation, at least at detectable levels, thus excluding cross-presentation of B-cell derived HA antigen also in periphery (Supplementary Figure 3).

Immunohistochemical analysis revealed different distribution of Tr in spleens (Figure 5). In control mice the great majority of CD3 (not shown) and FoxP3 cells (Figure 5D) were outside the B cell zones. In D chimeras, some FoxP3+ cells were inside the B cell zones but basically remained at the margins, which are rich in follicular DCs 33. In contrast, B chimeras showed accumulation of FoxP3+ cells within the B cell zones. These data were confirmed by a quantitative analysis of FoxP3+ cell distribution within splenic B cell zones.

To understand whether in the B chimera B cells induced expansion of thymic-derived FoxP3+ cells or their de novo generation, we analyzed T cell proliferation by staining for Ki67, a cell proliferation marker 34. Independently of FoxP3 expression, CD4+6.5+ cells from the B chimeras were similar to those obtained from control mice in terms of in vivo proliferation, suggesting that the increase in the CD4+6.5+FoxP3+ cell compartment observed in B chimeras is due to de novo generation of Tr in periphery (Figure 5E) rather than to their expansion. We also observed that in D chimeras HA specific T cells were proliferating more in vivo than their “naive” counterparts, independently of their FoxP3 expression, suggesting that antigen presentation by DCs induces constitutive proliferation even under homeostatic conditions.

Finally, the suppressive capacity of FoxP3+T cells generated in B and D chimeras was confirmed in in vitro experiments performed after sorting of FoxP3+ and FoxP3- cells (Figure 5F). This experiment also confirmed that HA-specific T cells obtained from mice that express the antigen on DCs (D chimera) are unable to proliferate (Figure 5F).

Although we cannot formally exclude that the levels of HA expression by DCs and B cells in this transgenic system may be different and may also play a role, HA mRNA expression by these cells, determined by real-time PCR, was similar (Supplementary Figure 4).

Altogether, data obtained in B chimeras indicate that, even in the absence of antigen expression in the thymus, regulatory T cells can be efficiently generated in periphery by B cells expressing a self-antigen, and that this seems to be due to a de novo generation. In contrast, self-antigen expression by DCs is very efficient in deleting antigen-specific T cells, and in inducing anergy among the remaining FoxP3- cells.
Discussion

Tr induction in the thymus can be achieved by epithelial thymic cells but also by dendritic cells and thus seems to be more dependent on the maturation state of the thymocyte than on the cell presenting the antigen. We confirm here that expression of agonist ligand in the thymus, either on thymic epithelial cells or on thymic DCs, leads to both negative selection of antigen-specific T cells and induction of antigen-specific Tr. The stronger thymic deletion observed in the TCR-HA*Ig-HA mice as compared to the TCR-HA*pgk-HA mice (Figure 1A) is due to the fact that, in the former, HA is expressed by thymic epithelium and dendritic cells and, as expected, total mRNA HA expression levels are much higher in Ig-HA mice than in pgk-HA mice (Supplementary Figure 2). It has been recently reported that thymic expression of cognate antigen can induce IL17 producing, antigen-specific T cells in addition to FoxP3+ cells. Interestingly, we did indeed find more IL17 producing cells among CD4 cells in the thymus of both strains of mice expressing HA antigen in the thymus (supplementary Figure 5), however, they were not positive for the HA-specific TCR, indicating that Tr may favor IL17-producing T cell generation, but independently of their antigen specificity.

We also show here that in single transgenic TCR-HA mice, the percentage and absolute number of HA-specific Tr increases in the periphery as compared to the thymus as has already been reported, strongly suggesting conversion and/or expansion of Tr cells even in the absence of cognate ligand. When comparing both types of HA-expressing mice (TCR-HA*pgk-HA and TCR-HA*Ig-HA), we found that the percentage and absolute numbers of HA-specific cells increased with age in the TCR-HA*Ig-HA mice, while it decreased in the TCR and in the TCR-HA*pgk-HA mice. This increase in absolute numbers with age was in great part due to a striking increase in the size of secondary lymphoid organs in the TCR-HA*Ig-HA mice. The reasons for this are not clear, and no signs of autoimmunity could be observed in these mice as has been described for TCR-HA transgenic mice expressing HA antigen under the MHC class II promoter, although we do have some evidence for systemic B cell activation (AS, unpublished data).

Another striking difference between both types of mice was the fate of the HA-specific FoxP3- cells: GFP- cells from the TCR-HA*pgk-HA mice were not anergic upon further antigenic stimulation in vitro, in contrast to the GFP- cells from TCR-HA*Ig-HA mice. The fact that GFP- cells from TCR-HA*pgk-HA mice express slightly lower levels of the transgenic TCR compared to their counterparts from TCR-HA*Ig-HA mice (see Figure 1A) may contribute to explain this phenomenon, since it has been reported that downregulation of TCR levels may contribute to escape anergy induction.
The bone marrow chimera experiments performed in this study demonstrate that different types of APC can induce tolerance to self-antigens via different mechanisms. HA expression on B cells resulted in expansion of HA-specific Tr, without the considerable deletion and anergy induction of antigen-specific FoxP3- cells observed upon expression of HA by DCs. The experiments shown in Figure 5 permit us to conclude that the anergy of 6.5+FoxP3- cells from TCR-HAxIg-HA mice and from the TD chimeras is the result of antigen encounter on DC in the periphery and not of antigen encounter in the thymus or on non-hematopoietic cells. Furthermore, they confirm that HA expression by B cells alone was sufficient to induce HA-specific Tr, and suggest that this was the result of B-T cell interactions in secondary lymphoid structures. The reason we did not observe higher numbers of 6.5+FoxP3+ cells in the periphery of TCR-HAxIg-HA mice despite expression of HA by B cells, is that HA is also expressed by thymic epithelium and dendritic cells, inducing a strong deletion of these cells.

The role of B cells in inducing Tr has been strongly suggested by other reports. Thus, μMT KO B cell deficient mice express lower levels of Tr cells, are less efficient in Tr induction via oral feeding of antigen and do not recover normally after acute EAE induction. Furthermore, mouse and human B cells promote expansion allogeneic Tr ex vivo. The mechanisms involved in such induction as well as the activation state of the B cell required for such phenomena remain poorly defined. While rituximab-mediated depletion of B cells in pathogenic conditions has been reported to ameliorate autoimmune diseases, repopulation with normal B cells led to amelioration of autoimmune diabetes and adoptive transfer of B cells suppressed inflammatory responses in a mouse model of primary biliary cirrhosis. Naive B cells have been shown to generate Tr cells in the presence of a mature immunological synapse. However, in our TCR-HAxIg-HA mice, there is evidence of B cell activation (AS, unpublished results) and it has been shown that CD40L-activated human B cells can efficiently induce expansion of regulatory T cells so it is not clear whether the activation state of the B cell plays a role. Cytokines such as TGFβ3, TGFβ1 and IL10 produced by follicular B cells have also been reported to be involved in Tr induction by B cells. The precise mechanisms and molecules involved in our system are currently under investigation.

In conclusion, while we do not put in doubt that DC can generate Tr, we show that they actually may be more efficient in inducing tolerance via deletion and anergy of antigen-specific FoxP3- cells. Contrary to DC, B cells do not traffic from tissues to lymph nodes and thus their capacity to induce Tr specific for the non-hematopoietic self may be quite restricted under physiological conditions. However, B cells can be made to present antigens via the administration of chimeric antibodies that target Fc receptors, an approach that was described to efficiently induce antigen-specific T cell tolerance in mice. Furthermore, gene therapy approaches in mice with retroviral
vectors that introduce peptide-IgG constructs in B cells have been shown to successfully reduce the incidence or onset of different autoimmune diseases 50. Thus, the data presented in this study may have important implications in clinical protocols aimed at the peripheral expansion of regulatory T cells in patients.

Acknowledgements

We are grateful to Harald von Boehmer and Marinos Kallikourdis for critical reading of the manuscript and valuable suggestions. We thank Ludger Klein for the pgk-HA mice, Chiara Buracchi and Achille Anselmo for cell sorting and Marta Lezama for excellent animal care.

SM was supported by Fondazione Cassa di Risparmio delle Provincie Lombarde (CARIPLO) grant number 5808/2007. AV was supported by EU-FP7 SYBILLA no. 201106 and Associazione Italiana Ricerca sul Cancro. AS was supported by the Institut National de Sante et Recherche Medicale (INSERM, France) and by Fondazione CARIPLO no. 5808/2007.

Authorship and conflict of interest

SM, CS and AS performed experiments and analyzed data. SM and AS designed experiments and AV and AS wrote the manuscript.

The authors declare no conflict of interest
References


Figure Legends

Figure 1. HA expression in thymus induces negative selection of HA-specific T cells and an increase in HA-specific FoxP3+ cells, but with a different age-dependent kinetics depending on expression of the HA transgene. (A) Primary and secondary lymphoid organs from 12 week-old TCR-HA, TCR-HAxpkg-HA and TCR-HAxIg-HA mice were analyzed for FoxP3 and 6.5 expression among gated CD4+ cells. Shown are representative dot plots, with the percentage of total 6.5+ cells among CD4+ cells (value outside the parenthesis) and the percentage of FoxP3+ cells among CD4+6.5+ cells (value in parenthesis). (B) The same data are presented for a pool of 12 week-old mice, with an average of three mice per time-point. (C) Absolute numbers of 6.5+FoxP3+ cells and of total 6.5+ cells in periphery (lymph nodes and spleen) of the three different types of mice varies with the age. Each time-point represents two to five mice. (D) A representative image of the mesenteric lymph nodes (mLN) and spleen of a 16 week-old TCR-HA, TCR-HAxpkg-HA and TCR-HAxIg-HA mouse.

Figure 2. Different behaviour of HA-specific FoxP3− cells in TCR-HAxpkg-HA and TCR-HAxIg-HA mice. (A) Total LN suspensions from TCR-HA, TCR-HAxpkg-HA, or TCR-HAxIg-HA mice were stained with cell-vue Maroon and incubated in vitro with different peptide doses. Proliferation of CD4+6.5+FoxP3− and FoxP3+ cells was determined by flow cytometry. (B) IFNγ present in the supernatant from the same cultures was determined by ELISA. Results correspond to the pool of triplicate wells, with cells obtained from one mouse of each genotype. Two independent experiments were performed with similar results. (C) CD4+6.5+FoxP3+ and FoxP3− cells from TCR-HAxpkg-HA (left graph) or TCR-HAxIg-HA (right graph) mice were electronically sorted and incubated in vitro with splenic derived DCs and different peptide doses. FoxP3− cells from a TCR-HA single transgenic mouse were used as control of proliferation. Thymidine was added after 48h of culture and left for an additional 16h. Experiments were performed in triplicate and one representative experiment out of three is shown.

Figure 3. HA presentation by hematopoietic cells in the periphery of TCR-HAxIg-HA but not in TCR-HAxpkg-HA mice. (A) CD11c+ and CD19+ cells were electronically sorted from Ig-HA and pgk-HA mice and different amounts of cells were tested for their capacity to induce HA-specific proliferation in vitro. Peptide (p, 0.1µg/ml) was added in one condition as positive control. (B) CD4+6.5+ cells from a Thy1.1 TCR-HA mouse were CFSE-labelled and transferred (1x10^6) into Balb/c, Ig-HA or pgk-HA recipients. Balb/c mice reconstituted 7 weeks before with pgk-HA bone marrow (last column) were also used as recipients. Three days after transfer, the proliferation of
CD4⁺Thy1.1⁺ cells in the lymph nodes was determined by flow cytometry. Representative dot plots are shown and the percentage of Thy1.1 positive cells within the CD4⁺ gate is indicated. Two mice per group and two independent experiments were performed with similar results.

**Figure 4.** Development and function of HA-specific FoxP3⁺ and FoxP3⁻ cells in the different chimeras expressing HA in the thymus. Thymi, lymph nodes and spleen cell suspensions from the different chimeras illustrated in (A) were analyzed for 6.5 and GFP expression within the CD4⁺ gate. Shown are representative dot plots (B) as well as the percentage of 6.5 cells in the CD4 gate, of FoxP3⁺ cells in the CD4⁺6.5⁺ gate and the absolute numbers of CD4⁺6.5⁺FoxP3⁺ cells in lymph nodes and spleen (C). Three mice per group were analyzed. (D) The mean fluorescence intensity of GFP within the CD4⁺6.5⁺GFP⁺ gate is shown. (E) CD4⁺6.5⁺GFP⁺ and GFP⁻ cells from lymph node suspensions of each type of chimera were electronically sorted and tested *in vitro* for their suppressive and proliferative capacity, respectively. For the suppression assay, naïve 6.5⁺ cells were incubated with Balb/c splenic DCs in absence or presence of peptide (0.1µg/ml), and in absence or presence of GFP⁺ cells from the different chimeras. For the proliferation assay, GFP⁻ cells from the different chimeras were co-incubated with DCs and peptide. The experiment was performed in triplicate and two independent experiments gave similar results.

**Figure 5.** Development and function of HA-specific FoxP3⁺ and FoxP3⁻ cells in the absence of HA expression in the thymus. Thymi, lymph nodes and spleen cells suspensions from the different chimeras illustrated in (A) were analyzed for 6.5 and GFP expression within the CD4⁺ gate. Shown are representative dot plots (B) as well as the percentage of 6.5 cells in the CD4 gate, of FoxP3⁺ cells in the CD4⁺6.5⁺ gate, and the absolute numbers of CD4⁺6.5⁺FoxP3⁺ cells (C). A total of three mice per group were analysed. (D) Immunofluorescence staining for CD19 (green), Foxp3 (purple) and nuclei (blue) in spleen sections of the different chimeras. The graphs represent the quantification of Foxp3-positive cells in CD19-positive follicules. Scale bar 50 µm. (E) The percentage of intracellular Ki67 positive cells within the FoxP3⁺ and FoxP3⁻ CD4⁺6.5⁺ cells was determined by flow cytometry. (F) CD4⁺6.5⁺GFP⁺ and GFP⁻ cells from lymph node and spleen suspensions of each type of chimera were electronically sorted and tested *in vitro* for their suppressive and proliferative capacity, respectively, as described in Figure 4.
Figure 1
Figure 2
Figure 3
Figure 5
Self-antigen presentation by mouse B cells results in regulatory T-cell induction rather than anergy or clonal deletion

Sara Morlacchi, Cristiana Soldani, Antonella Viola and Adelaida Sarukhan