γδ T Cells Affect IL-4 Production and B cell Tolerance

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γδ T cells can influence specific antibody responses. Here, we report that mice deficient in individual γδ T cell subsets have altered levels of serum antibodies including all major subclasses, sometimes regardless of the presence of αβ T cells. One strain with a partial γδ deficiency that increases IgE antibodies also displayed increases in IL-4-producing T cells (both residual γδ T cells and αβ T cells), and in systemic IL-4 levels. Its B cells expressed IL-4-regulated inhibitory receptors (CD5, CD22, CD32) at diminished levels while IL-4 inducible IL-4Rα and MHCII were increased. They also showed signs of activation and spontaneously formed germinal centers. These mice displayed IgE-dependent features found in hyper IgE syndrome, and developed anti-chromatin, anti-nuclear and anti- cytoplasmic autoantibodies. In contrast, mice deficient in all γδ T cells had nearly unchanged Ig levels, and did not develop autoantibodies. Removing IL-4 abrogated the increases in IgE, anti-chromatin antibodies and autoantibodies in the partially γδ-deficient mice. Our data suggest that γδ T cells, controlled by their own crosstalk, affect IL-4 production, B cell activation and B cell tolerance.

gammadelta T cell | Interleukin-4 | Autoimmunity | Immunoglobulin | Tolerance

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Introduction

The role of γδ T cells within the vertebrate immune system is not yet fully understood but it has become clear that they exert a strong influence on the immune responses. These cells represent a system of specialized subsets with different developmental kinetics, tissue distributions and functional roles (1). Moreover, at least some of the subsets appear to balance each other's influence on the immune system (2). Like αβ T cells and B cells, γδ T cells express antigen receptors encoded by rearranging genes (3, 4), which enable adaptive responses to antigenic challenge. Following such stimulation in the course of diseases, γδ T cell populations can undergo large changes in size and subset composition (5). The γδ T cell populations also change during ontogeny, and due to inter-individual genetic differences (6, 7). Conceivably, such changes might alter γδ T cell balance and with it γδ T cell influence on other immune cells.

In mice and humans, it was found that functional attributes of γδ T cells segregate with expressed γδ T cell receptors (TCR) (8, 9) although functional differentiation has also been observed within or across TCR-defined subsets, and correlated with other markers such as CD27 and CD8 (10, 11). The murine TCRγ locus contains seven Vγ genes, six of which are functional and expressed on the cell surface (3, 12). In the normal mouse spleen, the largest γδ T cell population expresses Vγ1, followed by Vγ4^{pos} cells and smaller populations expressing Vγ2 and Vγ7 (13). Vγ5^{pos} and Vγ6^{pos} cells are not present in substantial numbers. In earlier studies relying on cell transfer and targeted inactivation with antibodies, we and others found that splenic Vγ1^{pos} and Vγ4^{pos} cells exert opposite influences on host responses to infection, allergic sensitization and malignancy (8, 10, 14, 15). The data suggested that these two γδ T cell subsets balance each other in their influence on the immune responses (2).

In the current study, we further tested this idea by examining antibody levels and B cells in non-immunized mice genetically deficient either in individual γδ T cell subsets or in all γδ T cells. The focus on antibodies derives from our earlier observation that mutant mice selectively deficient in Vγ4 and Vγ6 (B6.TCR-Vγ4$^{-/-}$/6$^{-/-}$) produce substantially more IgE antibody than wt controls or mice deficient in all γδ T cells (B6.TCR-δ$^{-/-}$) (10). Here, we report that deficiency in individual γδ T cell subsets (16, 17) can change antibody production and B cell activation in non-immunized mice, to a degree that jeopardizes self-tolerance. However, the effect cannot simply be ascribed to an altered γδ T cell balance. Instead, it correlates with functional changes that occur within the remaining γδ T cells themselves, when they are no longer restrained by normal γδ cross talk. Our data show that this cross talk controls the amount of IL-4 produced by a subset of γδ T cells and other T cells, resulting in downstream effects on antibodies, B cells and self-tolerance.

Results

Genetic γδ T cell-deficiencies change systemic antibody levels in non-immunized mice

Having found that IgE antibody responses in OVA/alum-immunized and non-immunized mice are sensitive to the functional balance within the γδ T cell compartment (10), we wondered if this balance also affects other pre-immune antibodies. We therefore examined a panel of background-matched mouse strains with genetic deficiencies in TCR γ or δ genes for systemic antibody levels (Fig. 1). The panel includes C57BL/6 mice (wt control), B6.TCR-δ$^{-/-}$ mice (lacking all γδ T cells) (18), B6.TCR-Vγ1$^{-/-}$ mice (lacking Vγ1^{pos} γδ T cells) (17) and B6.TCR-

Significance

This study changes our understanding of the relationship between T cells and B cells. While it is known that T cells provide help for specific B cell responses it is unclear if and to what extent T cells also influence pre-immune B cell functions. We show here that γδ T cells modulate systemic antibody levels in non-immunized mice including all major subclasses, and especially IgE antibodies. One mouse strain deficient in certain γδ T cells developed various autoantibodies whereas mice deficient in all γδ T cells had relatively normal antibodies. Based on these and other findings we conclude that γδ T cells, influenced by their own crosstalk, affect IL-4 production, B cell activation and B cell tolerance.

Reserved for Publication Footnotes

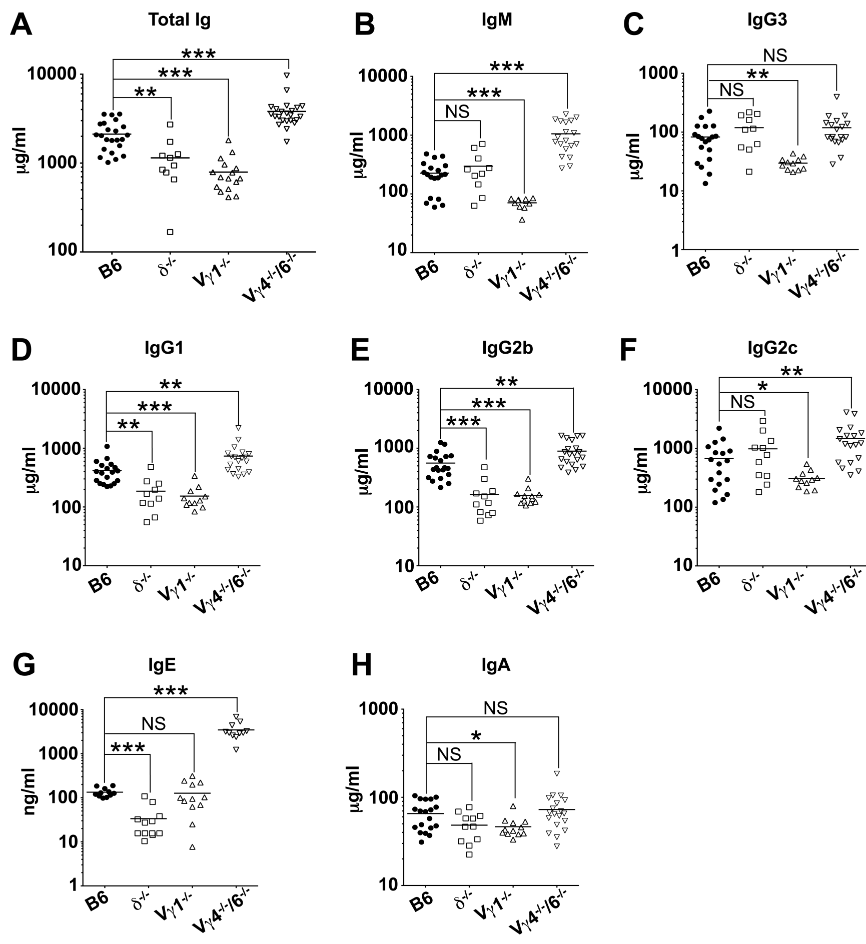

Fig. 1. Influence of γδ T cells on systemic antibody levels (*A-H*) Antibody ELISA in sera from 8-12 wks old female mice, including C57BL/6 (B6), B6.TCR-$\delta^{-/-}$ ($\delta^{-/-}$), B6.TCR-$V\gamma1^{-/-}$ ($V\gamma1^{-/-}$), and B6.TCR-$V\gamma4^{-/-}$/$V\gamma6^{-/-}$ ($V\gamma4^{-/-}/6^{-/-}$). (*A*) Total serum Ig, (*B-H*) ELISA of Ig subclasses as indicated, n = 10 - 23 mice per group, $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$, NS not significant

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$V\gamma4^{-/-}/6^{-/-}$ mice (lacking $V\gamma4^{pos}$ and $V\gamma6^{pos}$ γδ T cells) (16, 19). In the absence of all γδ T cells, total absolute serum Ig levels were somewhat decreased (∼ 2 fold relative to wt mice). However, when only $V\gamma1^{pos}$ cells were missing, total Ig levels were decreased more drastically (∼ 6 fold). In marked contrast, the absence of $V\gamma4^{pos}$ plus $V\gamma6^{pos}$ cells was associated with an increase in total Ig levels (∼ 4 fold relative to wt mice) (Fig. 1*A*). Similar patterns were seen within Ig subclasses although the extent of the changes varied. Thus, IgM, IgG3, IgG2c and IgA levels were not substantially affected by the absence of all γδ T cells (Fig. 1*B, C, F, H*) although at least one of the partial γδ deficiencies changed all of these antibodies. The largest serum antibody changes in the partially γδ T cell-deficient mice were seen with IgM, IgG1, IgG2b and IgE (Fig. 1*B, D, E, G*) and the smallest with IgG3 and IgA (Fig. 1*C, H*). Plotting frequencies of the Ig subclasses relative to total Ig showed a dramatic increase of IgE antibodies in B6.TCR-$V\gamma4^{-/-}/6^{-/-}$ mice whereas IgA antibodies were relatively decreased (Fig. S1). In B6.TCR-$V\gamma1^{-/-}$ mice, on the other hand, IgA was relatively increased whereas IgE was changed little. Overall, the partially γδ-deficient mice greatly differed from wt and totally γδ-deficient mice in the composition of their serum Ig, and synopsis of the data revealed that genetically induced changes in the mix of γδ T cells affect levels and composition of antibodies present in non-immunized mice.

Genetic γδ T cell-deficiency can affect antibodies independently of αβ T cells

To address a potential requirement of αβ T cells, we re-examined the effect of genetic γδ-deficiency in the context of a αβ T cell-deficient background (Fig. 2). Similar changes in the antibodies would indicate independence from αβ T cells, while absent or different changes would indicate a requirement for them. Deficiency in $V\gamma4^{pos}$ and $V\gamma6^{pos}$ cells on an αβ-deficient background (B6.TCR-$\beta^{-/-}$/$V\gamma4^{-/-}/6^{-/-}$ vs B6.TCR-$\beta^{-/-}$) still much increased total Ig, IgM, IgG3, IgG2b, IgG2c and, to a lesser degree, IgA (Fig. 2*A,B,C,E,F,H*), as had been found in the comparison of αβ T cell-sufficient mice (Fig. 1), suggesting that the γδ-effect on these antibodies is largely αβ T-independent. However, it no longer increased IgG1 and IgE (Fig. 2*D,G*), revealing that the γδ-effects on these antibody subclasses are αβ T-dependent. Despite much lower levels in the αβ T cell-deficient mice, anti-chromatin antibodies were still significantly increased by this partial γδ-deficiency (Fig. 2*I*, compare with Fig. 4*C,D*), indicating that the regulatory effect of γδ T cells on the generation of anti-chromatin antibodies involves both αβ T cell-dependent and -independent pathways. Additional evidence for a role of αβ T cells was seen after treating B6.TCR-$V\gamma4^{-/-}/6^{-/-}$ mice with an antibody specific for TCR-β, which transiently inactivates αβ T cells (20). This treatment failed to decrease absolute levels of anti- chromatin antibodies (although it decreased their frequency relative to total Ig, which increased), but it clearly reduced IgG1 and IgE levels, consistent with a role for αβ T cells (Fig. S2*A*). Taken together, the data shown in Fig. 1, Fig. 2 and Figs. S1 and S2 revealed that an unbalanced repertoire of γδ T cells affect antibody levels in ways both independent of and dependent on αβ T cells.

As with αβ-sufficient mice, the complete absence of γδ T cells in αβ-deficient mice (B6.TCR-$\beta^{-/-}$ vs B6.TCR-$\beta^{-/-}/\delta^{-/-}$) imparted smaller effects on antibody levels than did a partial γδ T cell

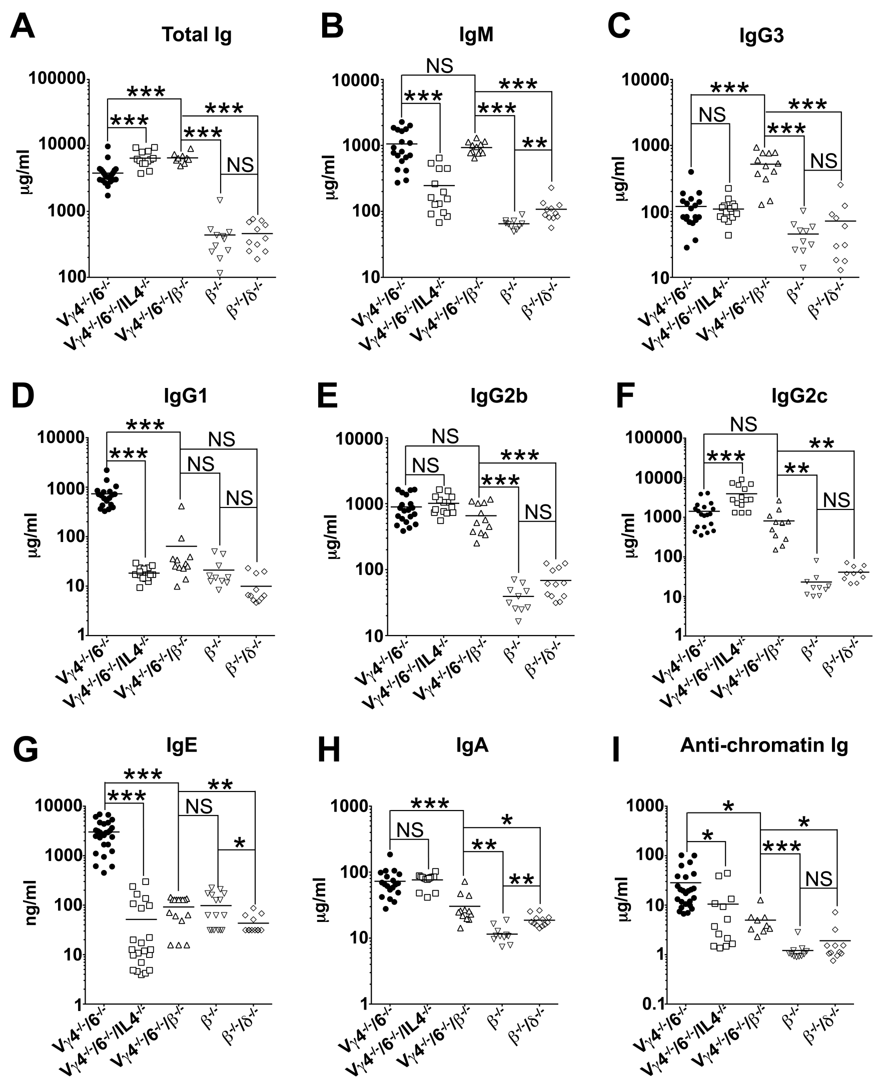

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Fig. 2. Influence ofγδ T cells on systemic antibody levels in the absence of αβ T cells or IL-4 (*A-I*) Antibody ELISA in sera from 8-12 wks old female mice, including B6.TCR-Vγ4$^{-/-}$/Vγ6$^{-/-}$ (Vγ4$^{-/-}$/6$^{-/-}$), B6.TCR-Vγ4$^{-/-}$/Vγ6$^{-/-}$/IL-4$^{-/-}$ (Vγ4$^{-/-}$/Vγ6$^{-/-}$/IL-4$^{-/-}$), B6.TCR-Vγ4$^{-/-}$/Vγ6$^{-/-}$/TCR-β$^{-/-}$ (Vγ4$^{-/-}$/Vγ6$^{-/-}$/β$^{-/-}$), B6.TCR-β$^{-/-}$ (β$^{-/-}$) and B6.TCR-β$^{-/-}$/δ$^{-/-}$ mice (β$^{-/-}$/δ$^{-/-}$). (*A*) Total serum Ig, (*B-H*) ELISA of Ig subclasses as indicated, (*I*) total anti chromatin Ig. n = 9 - 21 mice per group, *P<0.05, **P<0.01, ***P<0.001, NS not significant

deficiency, and such effects were further diminished due to the overall lower antibody production in the αβ-deficient mice.

Mice deficient in Vγ4^{pos} and Vγ6^{pos} γδ T cells display spontaneous germinal center formation and increases in activated B cells.

Because total Ig levels and most Ig subclasses were elevated in non-immunized B6.TCR-Vγ4$^{-/-}$/6$^{-/-}$ mice (except IgG3 and IgA), we examined B cells, the immediate precursors of antibody secreting cells, in these mice. First, we compared wt and B6.TCR-Vγ4$^{-/-}$6$^{-/-}$ mice in terms of their splenic anatomy. Despite their elevated antibodies, the γδ-deficient mice had smaller B cell follicles (Fig. 3*A*), consistent with their reduced numbers of mature splenic B cells (9.7+/-1.8 vs 36.3+/-6.7 mature B cells x10^6/spleen). However, in marked contrast to the wt mice, they developed germinal centers (GCs) as early as 4 wks of age, as indicated by the PNA^{pos} cells surrounded by B220^{pos} B cells, and numbers of GCs further increased with age (8 or 16 wks), whereas none were seen in the wt controls (Fig. 3*A*). In addition, comparative flow cytometric analysis of splenic B cells showed a larger relative frequency of germinal center B cells in B6.TCR-Vγ4$^{-/-}$6$^{-/-}$ mice (Fig. 3*B*).

Phenotypic comparison with wt B cells revealed that B cells of B6.TCR-Vγ4$^{-/-}$/6$^{-/-}$ mice expressed more CD69 (early activation antigen), and CD80 and CD86 (inducible ligands for CD28), both in terms of molecules/cell (MFI) and cellular frequencies (%), whereas CD25 and CD62L were not substantially changed (Fig. 3*C*). B6.TCR-Vγ4$^{-/-}$/6$^{-/-}$ B cells also expressed more MHCII molecules/cell (Figs. 3*D*, 7*A*), slightly more IL-4Rα and more CD23 than the wt B cells (Fig. 7*A*), but less of the inhibitory receptors CD5, CD22 and FcγRIIB (CD32) (Fig. 7*B,C*).

The same partial γδ T cell deficiency also induces a hyper-IgE phenotype and the development of autoantibodies

The activated B cells, increased absolute Ig levels, and altered Ig class composition in B6.TCR-Vγ4$^{-/-}$/6$^{-/-}$ mice suggested deficient regulatory control, with a potential for secondary pathologies and a break of tolerance. Serum IgE levels, already much elevated in 8-12 wks old B6.TCR-Vγ4$^{-/-}$/6$^{-/-}$ mice, were further increased at 8 months of age (Fig. 4*A*). In these mice, cell-bound IgE was readily detected on B cells (CD19^{pos}, IgE^{hi}; blood, lymph nodes and spleen, and a small population in bone marrow) and granulocytes (CD19^{neg}, IgE^{hi}; blood, bone marrow and a smaller population in spleen) (Fig. 4*B*). Increases in basophilic, neutrophilic and especially eosinophilic cells were also seen (Fig. S3), a phenotype reminiscent of hyper IgE syndrome (21-23).

To assess auto-reactivity, we examined B6.TCR-Vγ4$^{-/-}$/6$^{-/-}$ mice and the other genetically matched mutant mouse strains for serum levels of anti-chromatin Ig. By comparison

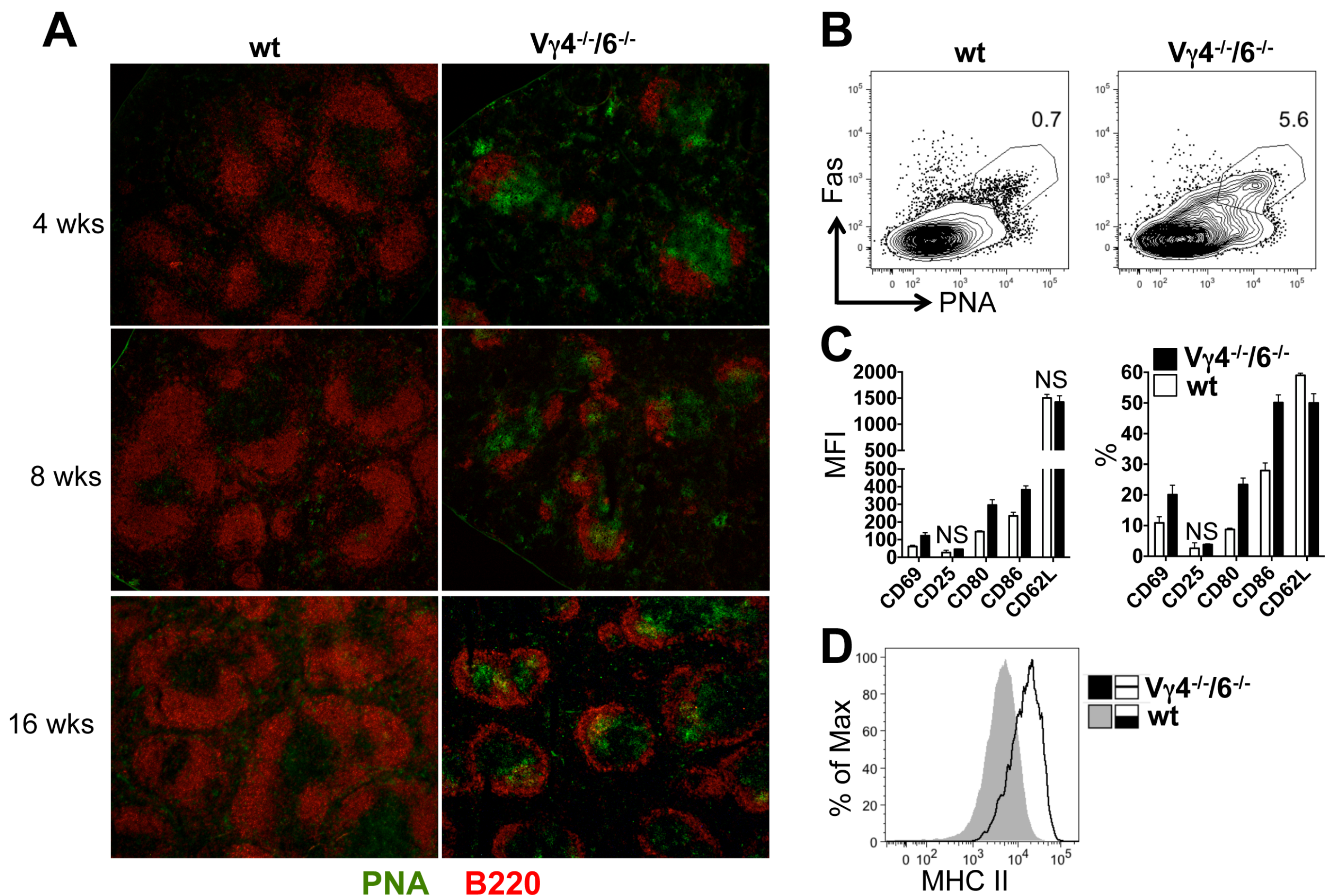

Fig. 3. Spontaneous germinal center formation, B cell activation and increased levels of BAFF in partially γδ T cell-deficient mice(*A*)Spontaneous germinal center formation and smaller B cell follicles in B6.TCR-Vγ4$^{-/-}$/Vγ6$^{-/-}$ mice. Spleens of wt B6 and B6.TCR-Vγ4$^{-/-}$/Vγ6$^{-/-}$ (Vγ4$^{-/-}$/6$^{-/-}$) mice were sectioned, fixed and stained with antibodies specific for B220 (red) to detect B cells and with peanut agglutinin (PNA, green) to detect germinal centers, at the indicated ages. The slides shown are representative for three experiments.(*B*) Increased relative frequency of germinal center B cells (compared to total B cells) in the spleen of B6.TCR-Vγ4$^{-/-}$/Vγ6$^{-/-}$ (Vγ4$^{-/-}$/6$^{-/-}$) mice. B cell populations in 8 wks old female C57BL/6 (wt) and B6.TCR-Vγ4$^{-/-}$/Vγ6$^{-/-}$ (Vγ4$^{-/-}$/6$^{-/-}$) mice were compared. Germinal center B cells (GCB) were identified using the indicated markers after first gating on lymphocytes and B220^{pos} cells. The staining profiles shown are representative of 5 mice in each group.(*C*) Comparison of total B cells in the spleens of 8 wks old female C57BL/6 (wt) and B6.TCR-Vγ4$^{-/-}$/Vγ6$^{-/-}$ (Vγ4$^{-/-}$/6$^{-/-}$) mice for expression of the cell surface molecules CD69, CD25, CD80, CD86 and CD62L. Splenic B cells were identified based on their cell surface expression of B220 and CD19, and stained in addition with antibodies specific for the listed cell surface molecules. Both mean fluorescence (MFI) and relative frequencies (%) are shown. Comparisons that did not reveal significant differences are identified (NS). n = 4 mice per group.(*D*) Comparison of total B cells in the spleens of 8 wks old female C57BL/6 (wt) and B6.TCR-Vγ4$^{-/-}$/Vγ6$^{-/-}$ (Vγ4$^{-/-}$/6$^{-/-}$) mice for cell surface-expressed MHCII molecules. Staining profiles shown are representative of 5 mice/group (see also Fig. 7*A*).

with wt mice, B6.TCR-Vγ4$^{-/-}$/6$^{-/-}$ mice had much increased (10-15 fold), whereas B6.TCR-Vγ1$^{-/-}$ mice had decreased, anti-chromatin antibodies (Fig. 4*C*). In the former, anti-chromatin antibodies were increased also relative to total Ig in these mice (Fig. 4*D*), suggesting impaired self-tolerance. Because deficiency in all γδ T cells failed to change anti- chromatin Ig levels, the increased levels seen in B6.TCR-Vγ4$^{-/-}$/6$^{-/-}$ mice likely reflect hyperactivity of the remaining γδ T cells, which are mostly Vγ1^{pos}. This interpretation is supported by the partially activated phenotype of these cells (see Fig. 6). To directly assess self-tolerance in B6.TCR-Vγ4$^{-/-}$/6$^{-/-}$ mice, we tested for anti-nuclear antibodies by immunofluorescence on fixed HEp-2 cells (24), (Fig. 4*E*). In contrast to the wt mice, serum of individual B6.TCR-Vγ4$^{-/-}$6$^{-/-}$ mice indeed tested positive for anti-nuclear antibodies (Fig. 4*E*, second to last row) as well as for a second type of antibody staining undefined cytoplasmic antigens (Fig. 4*E*, last row). Such antibodies – albeit at varying titers - were detectable in all mice tested (3 and 10 months of age). Comparing wt and B6.TCR-Vγ4$^{-/-}$/6$^{-/-}$ mice at 10 months of age, average autoantibody levels detected by the HEp-2 assay (based on mean fluorescence of stained cells) were > 15 fold higher in the mutant mice (Fig. 4*F* and Fig. 2*B*). Finally, we examined these mice for the presence of anti-dsDNA/histone and anti ssDNA antibodies in serum. B6.TCR-Vγ4$^{-/-}$/6$^{-/-}$ mice had substantially increased anti-dsDNA/histone and slightly increased anti ssDNA antibodies compared to wt mice (Fig. 4*G, H*). Together, these data indicate a loss of self-tolerance in B6.TCR-Vγ4$^{-/-}$/6$^{-/-}$ mice.

γδ T cells themselves control pre-immune antibody levels

We previously reported that adoptively transferred Vγ4^{pos} γδ T cells regulate specific IgE (10, 25). Because B6.TCR-Vγ4$^{-/-}$/6$^{-/-}$ mice display increased levels of pre-immune IgE, we suspected that Vγ4^{pos} γδ T cells also inhibit pre-immune IgE, and that this inhibition is released in B6.TCR-Vγ4$^{-/-}$/6$^{-/-}$ mice. We therefore treated wt C57BL/6 mice with an antibody against anti TCR-Vγ4, which specifically inactivates Vγ4^{pos} γδ T cells (19), and measured serum levels of IgE in treated mice and non-treated controls (Fig. 5*A*). Serum IgE levels in the antibody-treated mice increased substantially, confirming the inhibitory role of Vγ4^{pos} γδ T cells. On the other hand, remaining γδ T cells in the spleen of B6.TCR-Vγ4$^{-/-}$/6$^{-/-}$ mice were mostly Vγ1^{pos} (see below) and some

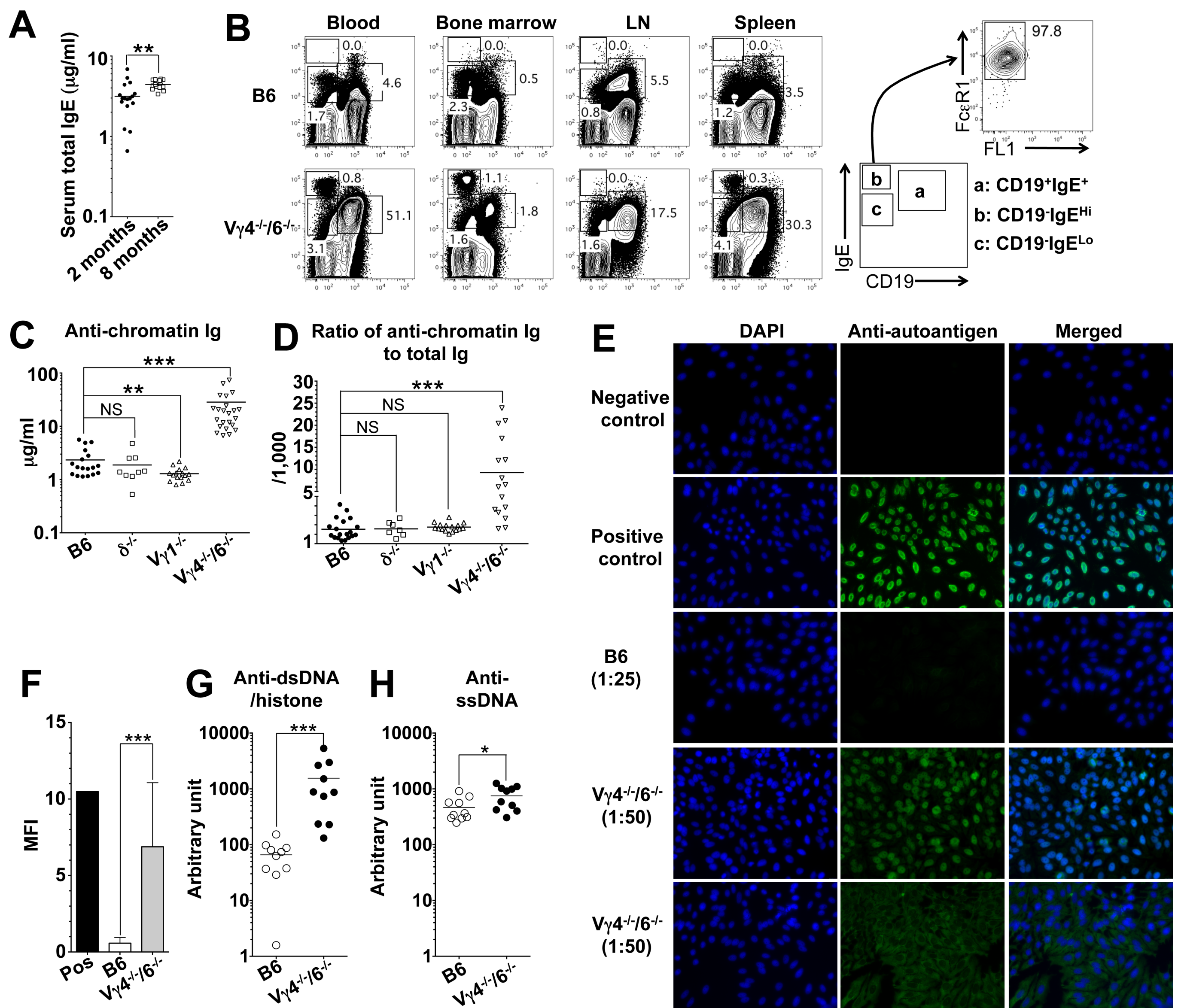

Fig. 4. IgE antibodies and autoantibodies in B6.TCR-Vγ4$^{-/-}$/Vγ6$^{-/-}$ mice (*A*)Comparison of 2 and 8 months old female B6.TCR-Vγ4$^{-/-}$/Vγ6$^{-/-}$ mice for IgE levels in serum using antibody ELISA. N = 15 mice/group, **P<0.01. (*B*) IgE antibody-"decorated" cells in blood, bone marrow, lymph nodes and spleen of a representative 8 wks old female B6.TCR-Vγ4$^{-/-}$/Vγ6$^{-/-}$ mouse. Cells were stained for IgE and CD19, and IgE^{pos} CD19^{neg} non-B cells were further tested for their expression of FcεR1. The staining profile shown is representative for 5 similar experiments. (*C,D*) Anti chromatin Ig ELISA in sera of the same mice as in Fig.1, n = 7-23 mice per group, **P<0.01, ***P<0.001, NS not significant. (*E*) Stains of fixed HEp-2 cells with DAPI alone for the detection of nuclei (negative control), DAPI plus anti nuclear Ab J5.8 (24) (positive control), or sera of 8-12 wks old female mice including C57BL/6 (B6) and B6.TCR-Vγ4$^{-/-}$/Vγ6$^{-/-}$ (Vγ4$^{-/-}$/6$^{-/-}$), at the indicated dilutions. Whereas the wt mice tested negative for autoantibodies, all γδ-deficient mice examined contained autoantibodies, some with anti nuclear (second to last row) and others with anti cytoplasmic (last row) specificity. (*F*) Quantitative comparison of mean fluorescence intensity (MFI) of HEp-2 cells stained with positive control antibody, or sera from wt or B6.TCR-Vγ4$^{-/-}$/Vγ6$^{-/-}$ mice, at a dilution of 1:50. n = 8, ***P<0.001.(*G, H*) Anti dsDNA/histone and ssDNA Ig ELISA, respectively, in sera of 12 wks old wt and B6.TCR-Vγ4$^{-/-}$/Vγ6$^{-/-}$ mice, n = 9 -10 mice per group, ***P<0.001.

of these cells are known to produce large quantities of IL-4 (26), a cytokine that is critical in the generation and maintenance of IgE antibodies (27). Furthermore, unrelated genetic mutations causing a selective increase of Vγ1^{pos} γδ T cells were also found to be associated with increased levels of IgE (28, 29). We therefore targeted Vγ1^{pos} cells in these mice with an antibody against TCR-Vγ1 that specifically inactivates Vγ1^{pos} γδ T cells (14). This treatment significantly diminished serum IgE as well as anti-chromatin Ig, and also serum titers of anti-nuclear autoantibodies (Fig. 5*B*). In contrast, total Ig remained unchanged, and the IL-4-independent IgG2c antibodies were increased.

In a complementary approach, we transferred the unbalanced γδ T cells from the spleen of non-immunized B6.TCR-Vγ4$^{-/-}$6$^{-/-}$ mice, which are mainly Vγ1^{pos} cells, to mice lacking all γδ T cells (B6.TCR-δ$^{-/-}$). As predicted, this significantly increased anti chromatin antibodies in the cell transfer recipients, both in absolute levels and relative to total Ig (Fig. 5*C*). In sum, these experiments support the notion that individual γδ subsets, or mixed but imbalanced γδ T cell populations, can alter antibody levels including IgE, anti-chromatin Ig and other autoantibodies.

Partial γδ T cell-deficiency changes size, composition and functional potential of the remaining γδ T cell population

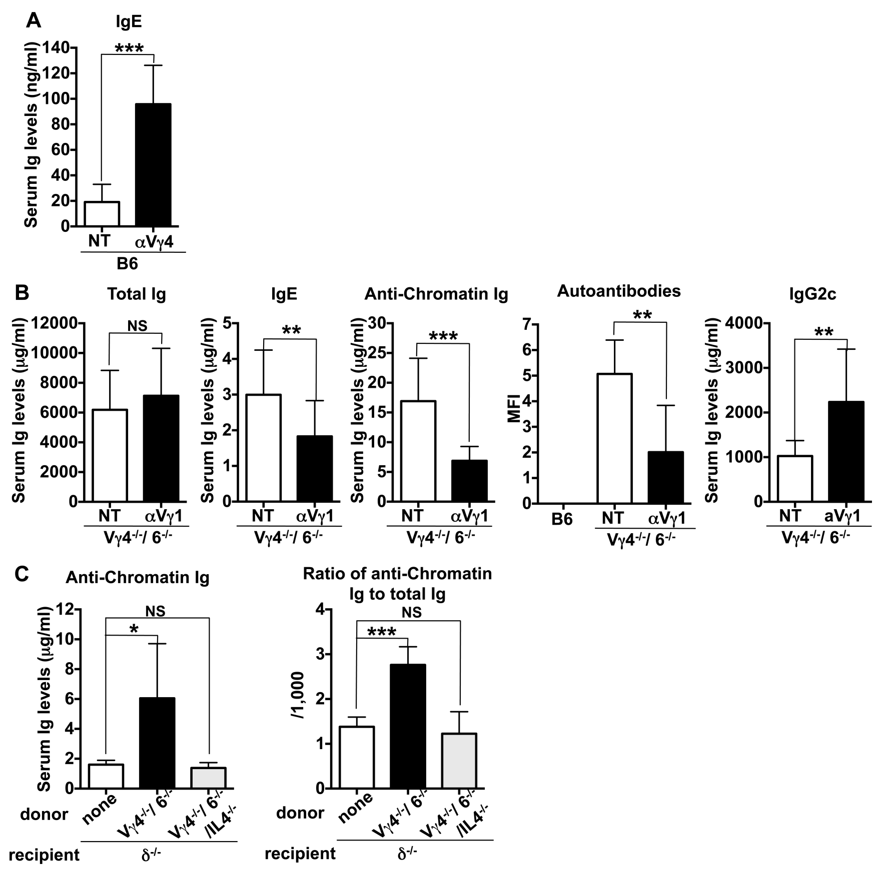

Fig. 5. Transient inactivation or adoptive transfer of γδ T cells alter antibody levels and B cells in the spleen (*A*) 8-12 wks old female C57BL/6 mice (B6) were injected i.v. with antibodies specific for Vγ4, and serum IgE levels examined by ELISA 10 days after the antibody treatment. n = 7 mice per group. (*B*) 8-12 wks old female B6.TCR-Vγ4$^{-/-}$/Vγ6$^{-/-}$ (Vγ4$^{-/-}$/6$^{-/-}$) mice were injected i.v. with antibodies specific for Vγ1, and serum levels of total Ig, IgE, anti chromatin Ig and IgG2c □ere□measured by ELISA 14 days after the antibody treatment (n = 8 mice). In addition, autoantibodies were measured using the Hep-2 staining assay (MFI) as described in Fig. 4. n = 4 mice per group. (*C*) 4 wks old female B6.TCR-δ$^{-/-}$ mice were injected with purified γδ T cells from B6.TCR-Vγ4$^{-/-}$/Vγ6$^{-/-}$ (Vγ4$^{-/-}$/6$^{-/-}$) or B6.TCR-Vγ4$^{-/-}$/Vγ6$^{-/-}$/□L-4$^{-/-}$ (Vγ4$^{-/-}$/6$^{-/-}$/IL-4$^{-/-}$), and anti chromatin Ig levels in sera measured 10 days after the cell transfer. n = 4 mice per group. *P<0.05, **P<0.01, ***P<0.001

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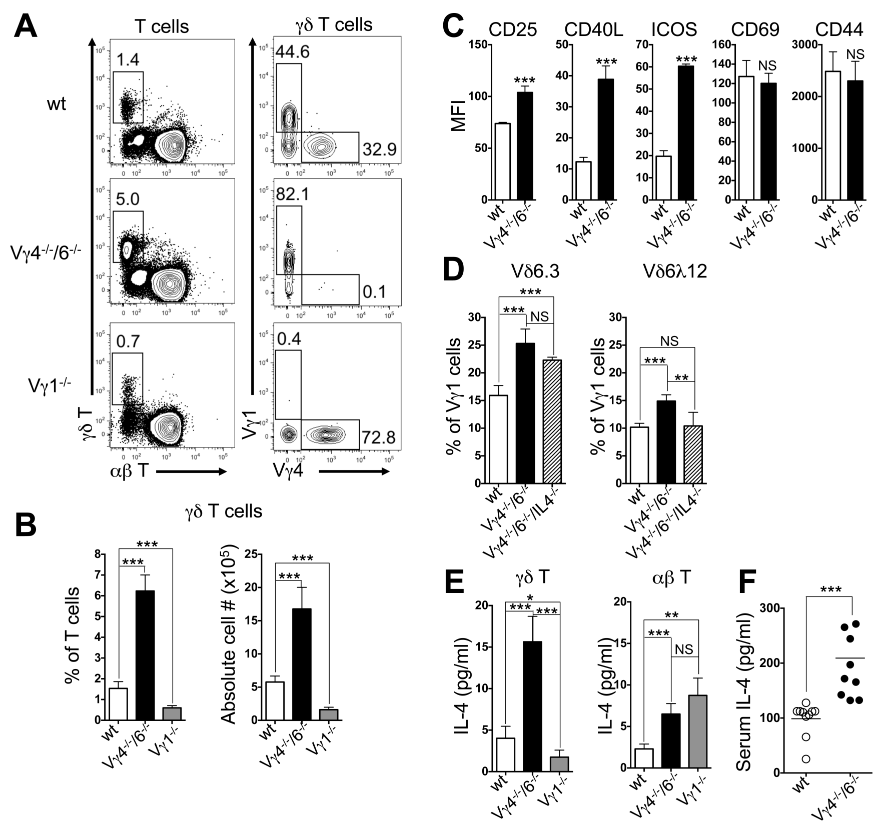

Fig. 6. Changes in the residual γδ T cells of partially γδ T cell-deficient mice(*A*) Cytofluorimetric comparison of splenic γδ T cell populations in 10 wks old C57BL/6 (wt), B6.TCR-Vγ4$^{-/-}$/Vγ6$^{-/-}$ (Vγ4$^{-/-}$/6$^{-/-}$) and B6.TCR-Vγ□$^{-/-}$ (Vγ1$^{-/-}$) mice, profiles shown are representative of at least three independent experiments. (*B*) Absolute numbers and frequency (relative to total T cells) of splenic γδ T cells in the same mice as in (a). (*C*) Expression levels (MFI) of CD25, CD40L, ICOS, CD69 and CD44 by splenic γδ T cells in wt and Vγ4$^{-/-}$/6$^{-/-}$ mice, at 10 wks of age. (*D*) Relative frequencies of Vδ6^{pos} γδ T cells within the splenic Vγ1^{pos} subset of C57BL/6 (wt), B6.TCR-Vγ4$^{-/-}$/Vγ6$^{-/-}$ (Vγ4$^{-/-}$/6$^{-/-}$) or B6.TCR-Vγ4$^{-/-}$/Vγ6$^{-/-}$/□L-4$^{-/-}$ (Vγ4$^{-/-}$/6$^{-/-}$/IL-4$^{-/-}$) mice. (*E*) *In vitro* induced secretion of IL-4 by splenic γδ and αβ T cells in C57BL/6 (wt), B6.TCR-Vγ4$^{-/-}$/6$^{-/-}$ (Vγ4$^{-/-}$/6$^{-/-}$) and B6.TCR-Vγ1$^{-/-}$ (Vγ1$^{-/-}$) mice. n = 4 mice per group, *P<0.05, **P<0.01, ***P<0.001(*F*) *In vivo* production of IL-4 in C57BL/6 (wt) and B6.TCR-Vγ4$^{-/-}$/6$^{-/-}$ (Vγ4$^{-/-}$/6$^{-/-}$) mice, measured by serum ELISA 8.5 hrs after i.v. injection of the capture antibody. n = 9 -11 mice/group, ***P<0.001

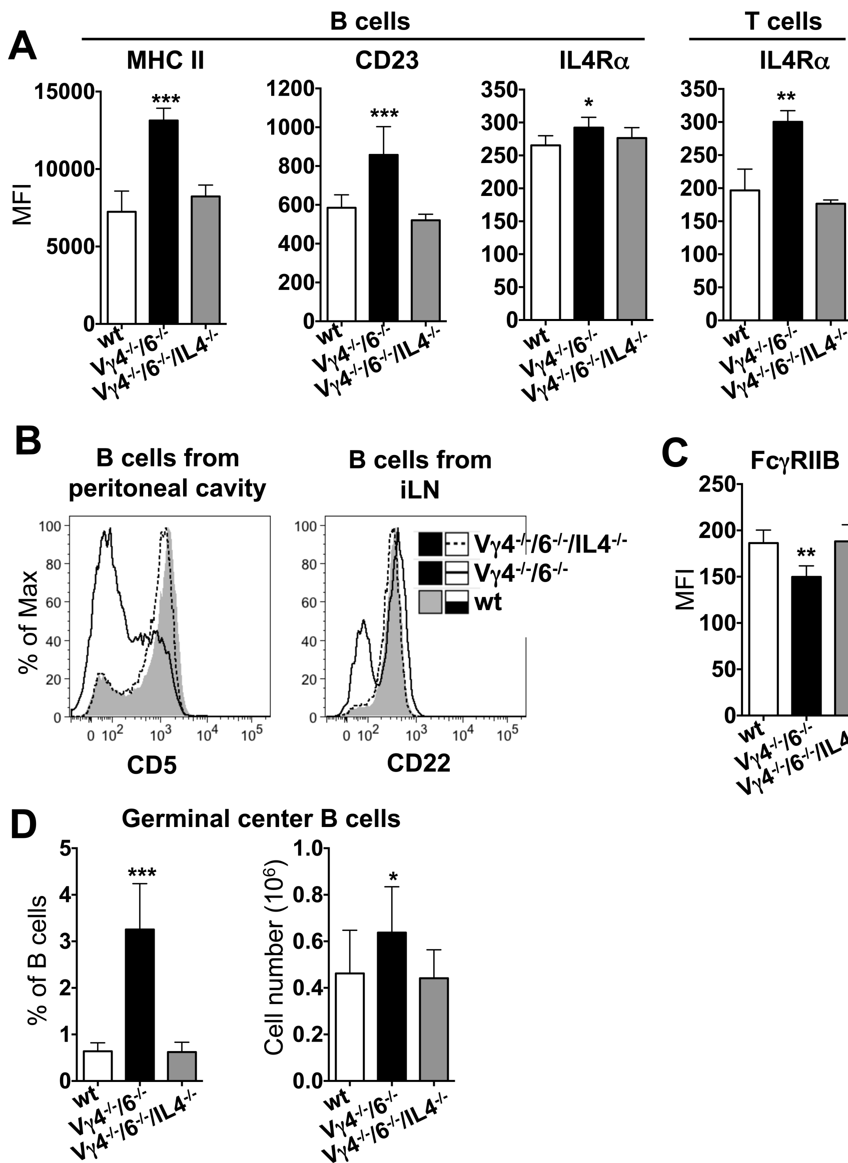

Fig. 7. Requirement for IL-4 in the altered B cell phenotype of B6.TCR-Vγ$4^{-/-}/6^{-/-}$ mice(*A*) Cytofluorimetric comparison of the expression levels (MFI) of MHCII and CD23 in total splenic B cells of C57BL/6 (wt), B6.TCR-Vγ$4^{-/-}/6^{-/-}$ (Vγ$4^{-/-}/6^{-/-}$) and B6.TCR-Vγ$4^{-/-}$/Vγ$6^{-/-}$/□L-$4^{-/-}$ (Vγ$4^{-/-}/6^{-/-}$/IL-$4^{-/-}$) mice, and of IL-4Ra in total splenic B cells and $CD4^{pos}$ T cells of the same mouse panel. n = 4 mice per group, $*P<0.05$, $**P<0.01$, $***P<0.001$ (*B*) Cytofluorimetric comparison of the expression profiles (MFI) of CD5 in peritoneal cavity B cells, and of CD22 in B cells of the inguinal lymph nodes, in the same panel of mouse strains as in (*A*). Profiles are representative of 4 mice in each group.

We examined the remaining γδ T cells in the spleen of partially γδ T cell-deficient mice for possible changes compared to γδ T cells in wt mice. Surprisingly, residual γδ T cells in the spleen of B6.TCR-Vγ$4^{-/-}6^{-/-}$ mice were increased in relative frequency and absolute numbers, whereas γδ T cells in B6.TCR-Vγ$1^{-/-}$ mice were decreased (Fig. 6*A,B*). Most splenic γδ T cells in B6.TCR-Vγ$4^{-/-}6^{-/-}$ mice were Vγ1^{pos} (Fig. 6*A*). They had a mixed phenotype suggesting altered composition and partial activation (increased expression of CD25, CD40L and ICOS but not CD69 and CD44) (Fig. 6*C* and Fig. S3*G*). Vγ1^{pos} cells in B6.TCR-Vγ$4^{-/-}/6^{-/-}$ mice expressed Vδ6 at higher frequencies than did wt Vγ1^{pos} cells (Fig. 6*D*), a TCR-phenotype associated with a propensity for IL-4 production (26). Indeed, splenic γδ T cells from B6.TCR-Vγ$4^{-/-}6^{-/-}$ mice produced far more IL-4 upon stimulation *in vitro* than wt splenic γδ T cells or those from B6.TCR-Vγ$1^{-/-}$ mice (Fig. 6*E*). Thus, a partial γδ T cell deficiency altered the composition, activation state and cytokine production of the residual γδ T cells, indicative of cross-regulation among γδ T cells. Of note, *in vitro* stimulated splenic αβ T cells from B6.TCR-Vγ$4^{-/-}6^{-/-}$ and B6.TCR-Vγ$1^{-/-}$ mice also produced more IL-4 than their wt counterparts (Fig. 6*E*), albeit less on a per cell basis than the γδ T cells in B6.TCR-Vγ$4^{-/-}6^{-/-}$ mice.

Evidence that IL-4 mediates part of the dysregulated antibody phenotype of B6.TCR-Vγ$4^{-/-}/6^{-/-}$ mice, as well as the underlying γδ T cell crosstalk

Because of the increase in IgE and IgG1 antibodies in B6.TCR-Vγ$4^{-/-}/6^{-/-}$ mice (Fig. 1), both of which are known to be IL-4-dependent (30), as well as the heightened potential of their T cells to produce IL-4 (Fig. 6), we speculated that locally or systemically increased IL-4 might be largely responsible for the phenotype of these mice. Compared to other cytokines, serum levels of IL-4 are low, even in mice with increased IL-4 production (31), but we were able to detect substantial increases in the serum of B6.TCR-Vγ$4^{-/-}/6^{-/-}$ mice (Fig. 6*F*). We also examined known IL-4-sensitive molecules as surrogate indicators of IL-4 levels, including MHCII and CD23 on B cells, as well as IL-4Rα (especially on T cells), all of which are positively regulated by IL-4 (30, 32), and the B cell-inhibitory receptors CD5, CD22 and CD32, which are negatively regulated by IL-4 (33, 34). A comparison of wt and B6.TCR-Vγ$4^{-/-}/6^{-/-}$ mice (Figs. 3*D* and 7)

showed changes in all of these indicators consistent with increased IL-4 activity in B6.TCR-Vγ4$^{-/-}$/6$^{-/-}$ mice. Secondly, we generated B6.TCR-Vγ4$^{-/-}$/6$^{-/-}$ /IL-4$^{-/-}$ double mutant mice in order to test if this cytokine is a required mediator in the dysregulated antibody phenotype of B6.TCR-Vγ4$^{-/-}$/6$^{-/-}$ mice. Indeed, IgM, IgG1, IgE and anti chromatin Ig were all much diminished in the double mutants, IgG1 and IgE even below wt levels (compare Figs. 1 and 2), and anti-nuclear autoantibodies were no longer detectable (Fig. S2*B*). Likewise, germinal center B cells were no longer increased (Fig. 7*D*). In combination, these changes emphasize the importance of IL-4 for the antibody phenotype of B6.TCR-Vγ4$^{-/-}$/6$^{-/-}$ mice.

Finally, γδ T cells in B6.TCR-Vγ4$^{-/-}$/6$^{-/-}$ mice also were affected by IL-4. The comparison with B6.TCR-Vγ4$^{-/-}$/6$^{-/-}$ /IL-4$^{-/-}$ mice (Fig. 6*D*) showed that IL-4 further shifts the altered composition of Vγ1^{pos} cells in these mice towards the IL-4 producing type (26).

Discussion

The study described here started with a previously noted connection between partial genetic deficiency in γδ T cells and changed IgE levels (10). Experiments described within broaden this finding to include all major Ig subclasses as well as antibodies with specificity for auto-antigens, involving αβ T cell-dependent and -independent pathways. Further experiments provide additional evidence that γδ T cells per se, but not unrelated secondary consequences of the gene KO mutations, mediate the change in these antibodies.

A central finding of the current study is that partial genetic deficiency in γδ T cells, i.e. the loss of individual TCR-defined subsets, has a greater effect on antibody levels than total γδ T cell deficiency. Because the Vγ1^{pos} and Vγ4^{pos} subsets investigated here were previously associated with different and opposed functional roles (8, 9, 14), this observation fits with the idea that imbalanced γδ T cells rather than their complete absence, affect antibody levels (2). However, because the remaining γδ T cells in the partially γδ-deficient mice were changed in number, composition and inducible cytokine production, the data further indicate that crosstalk among γδ T cells (15) regulates the functional activity of individual subsets, and their potential influence on antibody levels. These findings with γδ T cell-deficient mice have implications for humans because humans vary greatly with regard to γδ T cell numbers and composition, due to genetic differences, during ontogeny, and as a consequence of diseases (5, 6). As with mice, such variation might affect residual γδ T cell function in humans, with consequences for antibody levels and other immune responses, and perhaps even host-microbial homeostasis (35).

In one mouse line with a partial γδ T cell deficiency (B6.TCR-Vγ4$^{-/-}$/6$^{-/-}$ mice), we found large increases of IgE and anti-chromatin antibodies as well as autoantibodies with anti-nuclear and anti-cytoplasmic specificities. This finding suggests that a γδ T cell functional imbalance can precipitate a breakdown of B cell tolerance. Consistently, B cells in these mice exhibited signs of activation in the absence of immunization, along with early age development of germinal centers in the spleen. This activated phenotype is reminiscent of certain spontaneously autoimmune mouse strains such as NZB/NZW mice (36-39). We have not yet determined if older B6.TCR-Vγ4$^{-/-}$/6$^{-/-}$ mice develop nephropathy and other autoantibody-related pathologies characteristic of SLE-prone strains, but we already found features typically associated with high levels of IgE antibodies such as increased IgE receptor expression, eosinophilia and activated mast cells. Hence, B6.TCR-Vγ4$^{-/-}$/6$^{-/-}$ mice represent a model for γδ-dependent antibody dysregulation leading to autoimmunity and hyper IgE syndrome (21-23).

The same B6.TCR-Vγ4$^{-/-}$/6$^{-/-}$ mice also displayed dysregulated IL-4 production. Thus, their *in vitro* induced T cells (both γδ and αβ T cells) were capable of producing this cytokine in larger quantity than wt counterparts, and at least the IL-4-producing γδ T cells were more frequent. Among γδ T cells, mainly Vγ1^{pos} cells are associated with IL-4 production, especially a Vγ1^{pos} NKT-like subset distinguished by co-expression of Vδ6 (26, 40, 41), and such cells were relatively and absolutely increased in B6.TCR-Vγ4$^{-/-}$/6$^{-/-}$ mice. Moreover, B6.TCR-Vγ4$^{-/-}$/6$^{-/-}$ mice showed increased *in vivo* production of IL-4, measured using the Cincinnati cytokine capture assay (42), and corresponding elevated expression of the IL-4-inducible MHCII and CD23 molecules on B cells and IL-4Rα (mainly) on T cells, together with increased systemic levels of the IL-4-dependent IgG1 and IgE antibodies, as well as decreased expression of the IL-4-regulated B cell inhibitory cell surface receptors CD5, CD22 and CD32 (33, 34). Inversely, the "complementary" B6.TCR-Vγ1$^{-/-}$ mice were deficient of IL-4 producing γδ T cells. Taken together, these findings indicate that γδ T cells and their crosstalk control IL-4 levels in non-immunized mice.

Several studies strongly implicate IL-4 in the breakdown of B cell tolerance. Thus, it was shown that IL-4 promotes Stat6-dependent survival of auto-reactive B cells *in vivo* (43). Further, as already mentioned, IL-4 reduces expression of the inhibitory receptors CD5 (34) as well as CD22, FcγammaRII (CD32), CD72 and paired immunoglobulin-like receptor (PIR)-B on B cells, also mediated through Stat6, and IL-4 abrogates the inhibitory effects that ensues when FcγRII or CD22 and BCR are co-ligated (33). More recently, it was found that IL-4 produces Fas-resistance in B cells, and a breakdown of B cell tolerance *in vivo* with autoantibody formation, proteinuria and tissue damage (44). Moreover, it was shown that IL-4 regulates Bim expression, promotes B cell maturation in synergy with BAFF, and confers resistance to B cell death at negative selection checkpoints (45). In IL-4 transgenic mice, constitutive expression of this cytokine causes autoimmune-type disorders (32). All of these observations indicate that IL-4 plays a critical role in B cell tolerance, and suggest that dysregulated IL-4 production in B6.TCR-Vγ4$^{-/-}$/6$^{-/-}$ mice might be responsible for their autoimmune phenotype (elevated anti-chromatin, anti-nuclear, anti-dsDNA/histone, anti-ssDNA and anti-cytoplasmic antibodies). The phenotype of IL-4-deficient B6.TCR-Vγ4$^{-/-}$/6$^{-/-}$ mice supports this idea. This deficiency abrogated elevated antibody levels including IgE, anti-chromatin and autoantibodies, and normalized nearly all of the examined immune features in B6.TCR-Vγ4$^{-/-}$/6$^{-/-}$ mice. Consistently, purified transferred γδ T cells from B6.TCR-Vγ4$^{-/-}$/6$^{-/-}$ /IL-4$^{-/-}$ mice no longer induced anti-chromatin Ig.

It is not clear which *in vivo* cellular sources of IL-4 are critical. Some of our data indicating that αβ T cells are not required in the antibody phenotype of partially γδ-deficient mice would suggest that IL-4 from αβ T cells is not critical. Instead, γδ T cells could be a critical source of IL-4, at least initially. Consistently, whereas adoptive transfer of splenic γδ T cells from B6.TCR-Vγ4$^{-/-}$/6$^{-/-}$ mice to γδ T cell-deficient recipients (B6.TCR-δ$^{-/-}$) raised serum levels of αβ T cell-dependent anti-chromatin Ig, adoptively transferred γδ T cells from B6.TCR-Vγ4$^{-/-}$/6$^{-/-}$ /IL-4$^{-/-}$ mice failed to do so.

In conclusion, it appears that IL-4 is a critical mediator of the γδ-dependent humoral immune changes seen in B6.TCR-Vγ4$^{-/-}$/□$^{-/-}$ mice and in the "complementary" B6.TCR-Vγ1$^{-/-}$ mice. With experimental evidence at hand indicating that crosstalk between γδ T cells affects IL-4 production and protects B cell tolerance in non-immunized mice, the focus shifts to the questions of the nature, timing and place of this crosstalk between γδ T cells, and of the γδ T cell-B cell interaction that is shaped by it.

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Methods

Mice. C57BL/6 mice and several mutant strains of the same genetic background (B6.TCR-$\beta^{-/-}$, B6.TCR-$\delta^{-/-}$, B6.TCR-$\delta^{-/-}$/TCR-$\beta^{-/-}$) were originally obtained from The Jackson Laboratory and bred at NJH. TCR-V$\gamma4^{-/-}$/V$\gamma6^{-/-}$ mice were a gift from Dr. K. Ikuta (Kyoto University, Kyoto, Japan), were then backcrossed onto the C57BL/6 genetic background, and used after 11 backcross generations. B6.TCR-V$\gamma1^{-/-}$ mice were a gift from Dr. Simon Carding and distributed by Dr. C. Wayne Smith. Double knockout (KO) mice were generated by crossing the corresponding mutant strains and selecting double KO mice in the F2 generation. These mice (TCR-V$\gamma4^{-/-}$/V$\gamma6^{-/-}$/TCR-$\beta^{-/-}$, TCR-V$\gamma4^{-/-}$/V$\gamma6^{-/-}$/IL-$4^{-/-}$) were then bred as new homozygous strains. All mice were cared for at National Jewish Health (NJH) (Denver, CO), following guidelines for normal and immune deficient animals, and all experiments were conducted under a protocol approved by the Institutional Animal Care and Use Committee.

Serum antibody levels. ELISAs for detecting total Ig and subclasses were performed by coating Immulon 2 HB plates (Fisher Scientific Inc, MA) with polyclonal goat anti-mouse Ig κ (Bethyl Laboratories, TX). Sera were added at starting dilutions from 1:1,000 to 1:50,000, followed by 2- fold serial dilutions. Subsequently, serum Ig was detected with polyclonal HRP conjugated goat anti-mouse Ig(H+L), anti-IgM, anti-IgG1, anti-IgG2b, anti-IgG3, and anti-IgA (Southern Biotechnology, AL). Serum IgG2c was detected using cross-reactive HRP conjugated goat anti-mouse IgG2a (Southern Biotechnology, AL) (46). An anti-chromatin Ab ELISA was established using purified chromatin (47) to capture chromatin-specific antibodies in the serially diluted serum. Captured antibodies were then detected with polyclonal HRP conjugated goat anti-mouse Ig(H+L). Total IgE levels were measured by a sandwich ELISA using rat anti-mouse IgE at 2 µg/ml (clone R35-72; BD Biosciences) as a capture Ab, and biotinylated rat anti-mouse IgE H chain mAb (clone R35-118; BD Biosciences) at 2 µg/ml as detecting Ab, followed by streptavidin-conjugated HRP. All plate-bound HRP-conjugated antibodies were detected by tetramethylbenzidine substrate solution (Life Technologies, MD), read using a VERSAmax tunable microplate reader, and processed using SoftMax Pro 4.7.1 software. Serum levels of anti dsDNA/histone and anti ssDNA antibodies were measured as previously described (47). Measurement of antibodies in the presence of histone alone (Fig. S2C) confirmed that most of the anti dsDNA/histone antibodies were specific for dsDNA or the dsDNA/histone complex but not histone alone.

***In vivo* production of IL-4.** *In vivo* production of IL-4 was measured using the Mouse IL-4 *In Vivo* Capture Assay Set (BD Pharmingen), following the method of Finkelman & Morris (42).

Fluorescent test for autoantibodies. Sera were tested for autoreactivity against fixed HEp-2 liver cells (Bio-Rad Laboratories) as previously described (24). Briefly, slides with attached HEp-2 cells were incubated with diluted serum samples for 30 min at RT, washed with PBS for 5 min, and incubated with FITC-labeled rat anti-mouse Ig κ antibody (1□1000; clone 187.1,□Southern Biotech) for 30 min. After washing, slides were mounted with Fluoro-Gel II containing DAPI (Electron Microscopy Sciences, PA). Pictures were taken with an inverted microscope (Axiovert 200M; Carl Zeiss, Inc.) at 20x magnification. A montage of images was assembled using Slidebook 4.1 software (Intelligent Innovations Inc.).

Histological analysis 6-µm sections of frozen spleen embedded in Tissue-Tek OCT compound (Sakura Finetek USA, CA) were fixed in acetone, dried, and kept at −80°C. Sections were rehydrated in PBS and incubated in blocking buffer (PBS, 10% normal rat serum) at room temperature (20 min) in a humidified chamber. Slides were then stained simultaneously with FITC conjugated peanut agglutinin (PNA; Vector Laboratories, CA) and PE conjugated anti-mouse B220 (clone RA3-6B2; Biolegend) in staining buffer (PBS, 2% FCS, 0.1% sodium azide) and washed 2 times by PBS immersion (5 min). Stained slides were mounted with Biomeda Gel/Mount (Fisher Scientific, Pittsburgh, PA) and viewed with an inverted Zeiss 200M confocal microscope at 25°C. Images were collected with Slidebook software (Intelligent Imaging Innovations, CO).

Flow cytometric analysis. Cells obtained from single cell suspensions (2×10^5/well) were stained in 96 well plates (Falcon; BD Biosciences, Franklin Lakes, NJ) for the cell surface markers shown in the figures/tables, using the specific mAbs and □erivatized □eagents listed in Table S3.□Of□note,□CD93□os cells □ere □etec□□d using mAb□AA4.1.

Live cells were always gated based on forward and side scatter (lymphocyte gate), and unless indicated otherwise, forward scatter height and amplitude, and side scatter width and amplitude (to exclude or specifically include cellular conjugates), as well as expression of various B-□or T cell markers, or markers for granulocytes. All samples were analyzed on a LSRII flow cytometer, counting a minimum of 25,000 events per gated region, and the data were processed using FlowJo 9.5.2 software (Tree Star).

T cell purification and adoptive transfer. □uspensions of splenocytes were prepared by mechanical dispersion, treated with Gey's red cell lysis solution and passed through nylon wool columns to obtain T lymphocyte-enriched cell preparations, as previously described (14). Enriched cells were then incubated with biotinylated anti TCR antibodies (mAb GL3, anti TCR-δ) for 15 min at 4°C, washed and incubated with streptavidin-conjugated magnetic beads (Streptavidin Microbeads; Miltenyi Biotec, Bergisch Gladbach, Germany) for 15 min at 4°C, and passed through magnetic columns to purify total γδ T cells, as previously described in detail (48). This produced cell populations containing >85% viable γδ T cells as determined by dye exclusion and staining with specific anti TCR mAbs. The purified cells were then washed in PBS and re-suspended to a concentration of 2.5×10^7 cells/ml in PBS, and 5×10^6 cells/mouse were injected in 200 µl PBS via the tail vein of the transfer recipient.

Throughout this article, we use the nomenclature for murine TCR-Vγ genes introduced by Heilig and Tonegawa (49).

Treatment with antibodies against the TCR. Mice were injected with antibodies against the TCR as previously described (14, 20). Briefly, antibodies purified from hybridoma culture supernatants using a protein G-Sepharose affinity column (Pharmacia Biotech, Upsala, Sweden) were injected via the tail vein at 200 µg/mouse in 200 µl PBS, and effects of the treatment were analyzed 14 days later, as indicated in the Figures. We used mAb H57.597.2 specific for TCR-β (50) for the targeting of αβ T cells,□and□mAbs UC3 (anti mouse Vγ4) (51) and 2.11 (anti mouse Vγ1) (52) for targeting the respective subsets of γδ T cells.

Statistical analysis. Data are presented as means +/- SEM. The unpaired *t* test was used for two group comparisons, and ANOVA was used for analysis of differences in three or more groups. Statistically significant levels are indicated as follows: $p < 0.05$, $p < 0.01$, $p < 0.001$.

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