IMMUNE REGULATION

T_{fr} cells lack IL-2Rα but express decoy IL-1R2 and IL-1Ra and suppress the IL-1–dependent activation of T_{fh} cells

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Follicular regulatory T (T_{fr}) cells from lymph node germinal centers control follicular helper T (T_{fh}) cell–dependent B cell activation. These scarce cells, often described and purified as $CD25^+$ cells, are thought to be derived from thymic regulatory T (T_{reg}) cells. However, we observed that mouse T_{fr} cells do not respond to interleukin-2 (IL-2), unlike T_{reg} cells. Stringent immunophenotyping based on B cell lymphoma 6 (Bcl6), programmed cell death protein 1 (PD-1), and CXCR5 expression revealed that T_{fr} cells are actually $CD25^-$, in mice and humans. Moreover, T_{fr} cell characterization based only on CXCR5 and PD-1 high expression without excluding $CD25^+$ cells resulted in contamination with T_{reg} cells. Transcriptome studies of $CD4^+CXCR5^+PD\text{-}1^+Bcl6^+Foxp3^+CD25^-$ T_{fr} cells revealed that they express the IL-1 decoy receptor IL-1R2 and the IL-1 receptor antagonist IL-1Ra, whereas T_{fh} cells express the IL-1R1 agonist receptor. IL-1 treatment expanded T_{fh} cells in vivo and activated their production of IL-4 and IL-21 in vitro. T_{fr} cells suppressed the IL-1–induced activation of T_{fh} cells as efficiently as the IL-1 receptor antagonist Anakinra. Altogether, these results reveal an IL-1 axis in the T_{fh} cell control of B cell responses and an IL-2/IL-1 dichotomy for T_{reg} cell control of effector T cells versus T_{fr} cell control of T_{fh} cells.

INTRODUCTION

Germinal centers (GCs) form within peripheral lymphoid organs to foster T cell–dependent stimulation of B cells that results in the production of high-affinity antibodies (*1*, *2*). To produce such antibodies, GC B (GCB) cells interact with follicular helper T (T_{fh}) cells to enable their activation and differentiation into plasma cells (*3*–*5*). The T_{fh} cell lineage is imprinted by expression of B cell lymphoma 6 (Bcl6), a transcription factor that represses alternative fates and promotes T_{fh} cell differentiation and function (*6*). Bcl6 promotes the expression of CXCR5 (*7*–*9*), which controls the homing of T_{fh} cells to the B cell follicle and GCs (*10*, *11*). Interleukin-6 (IL-6) and IL-21 contribute to T_{fh} cell differentiation (*12*, *13*) when naïve T cells are stimulated through their T cell receptor (TCR) and costimulatory molecules, such as ICOS (inducible costimulator) and CD28 (*7*, *14*, *15*). T_{fh}-mediated CD40L/CD40 signaling in the presence of IL-21 and IL-4 induces proliferation, isotype switching, and differentiation of B cells (*16*–*20*).

The generation of autoantibodies and the development of autoimmune diseases can result not only from the activation of autoreactive T and B cells but also from a dysregulation of GC formation and maintenance by T_{fh} cells (*21*, *22*). Limiting the number of T_{fh} cells within GCs is critical to warding off the emergence of autoantibodies (*23*–*25*). Thus, regulation of T_{fh} cells is essential for limiting GC reactions against self-antigens and preventing autoimmunity (*26*, *27*).

Follicular regulatory T (T_{fr}) cells were found to limit the GC reaction and to reduce antibody production within B cell follicles in human tonsils (*28*) and in mice (*29*). These cells were initially reported to express Bcl6, programmed cell death protein 1 (PD-1), and CXCR5, the canonical markers of T_{fh} cells, as well as forkhead box P3 (Foxp3) and CD25, the canonical markers of regulatory T (T_{reg}) cells (*30*, *31*). T_{fr} cells were first described as $CD25^{hi}$, similar to T_{reg} cells (*29*, *32*), and T_{fr} cells are characterized/purified according to this marker in most studies. Except for mice expressing a reporter gene of Foxp3 expression, there is no other physiological surface marker that allows one to separate live T_{fr} cells from T_{fh} cells. T_{fr} cells were proposed to develop not only from thymus-derived T_{reg} cells but also from $Foxp3^-$ precursors in a PD ligand 1–dependent manner (*33*).

How T_{fr} cells control the GC reaction remains unclear. In vitro suppression assays showed that T_{fr} cells suppress B and T_{fh} cell responses, inhibiting B cell activation and class switch recombination and decreasing T_{fh} cell production of cytokines such as IL-4 (*34*, *35*). T_{fr} suppression of T_{fh} cells has been reported to be dependent on cytotoxic T lymphocyte–associated protein 4 (*36*, *37*). Depletion of T_{fr} cells in $Bcl6^{fl/fl} \times Foxp3^{CRE}$ mice had no impact on the size of the T_{fh} cell or GCB cell compartments (*38*). However, vaccinated T_{fr}-depleted mice have altered antigen-specific antibody responses, with significantly increased immunoglobulin A (IgA) levels and decreased avidity of IgGs against the immunogen, suggesting a qualitative role of T_{fr} cells in antibody production (*38*).

The antigen specificity of T_{fh} and T_{fr} cells is not well known, and diverging results have been reported regarding T_{fr} cells. Concordant results showed that T_{fh} cells are specific for the immunizing antigen (*33*, *39*, *40*). In contrast, a previous study showed that T_{fr} and T_{fh} cells share TCRs specific for the immunizing antigen (*33*), whereas a recent one reported oligoclonal expansions in T_{fh} cells and a broad TCR usage in T_{fr} cells from the same GCs (*41*).

In this work, we identified T_{fr} cells as a rare population of $CD4^+CXCR5^+PD\text{-}1^+Foxp3^+$ cells that do not express CD25 and likewise do not respond to IL-2. On the basis of a stringent characterization of T_{fr} cells, we analyzed their transcriptome and compared it with that of T_{fh} and T_{reg} cells. Transcriptome studies clustered T_{fr} cells with T_{fh} cells rather than T_{reg} cells and revealed a previously uncharacterized IL-1 axis in T_{fr} cell suppression of T_{fh} cells. Together, our work should prompt a reassessment of the biology of T_{fr} cells based on their better characterization and highlights an IL-2/IL-1 dichotomy for T_{reg} cell control of effector T (T_{eff}) cells versus T_{fr} cell control of T_{fh} cells.

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RESULTS

T_{fr} and T_{fh} cell response to IL-2

We investigated the in vivo response of T_{fh} and T_{fr} cells to IL-2. Mice expressing the green fluorescent protein (GFP) as a reporter gene of Foxp3 expression were injected with an adeno-associated virus coding for IL-2 (AAV–IL-2) that allows continuous production of IL-2 (*42*). Twenty-one days after AAV–IL-2 injection, mice were immunized or not immunized with ovalbumin (OVA), and their spleens were analyzed 10 days after the immunization. $CD19^+CD4^-$ B cells, which are known to be $CXCR5^{hi}$ and $PD\text{-}1^-$, were used to select the appropriate gating for defining $CD4^+CXCR5^{hi}PD\text{-}1^{hi}$ follicular T (T_{fol}) cells (fig. S1). GFP expression was then used to identify the subsets of $Foxp3^+$ T_{fr} cells and $Foxp3^-$ T_{fh} cells within T_{fol} cells.

IL-2 increased the proportion (Fig. 1A) and numbers (fig. S2) of splenic T_{reg} cells, as previously reported (*42*–*44*), irrespective of the immunization. The marked increase of T_{fh} cells induced by immunization was reduced by IL-2 (Fig. 1B). In contrast, whereas immunization had no impact on the proportion of T_{fr} cells, IL-2 induced a significant decrease of their proportion in $CD4^+$ cells (Fig. 1C). The combined effects of IL-2 on T_{fh} and T_{fr} cells led to a decreased T_{fr}/T_{fh} ratio for both immunization and IL-2 treatment, alone or in combination (Fig. 1D).

T_{fr} cells do not express CD25

This negative impact of IL-2 on T_{fr} cells led us to carefully assess their expression of CD25 (IL-2Rα, a component of the high-affinity receptor for IL-2) by flow cytometry. We first stringently defined T_{fol} cells on the basis of high CXCR5 and PD-1 expression within $CD4^+$ cells. This gating defined T_{fol} cells as all $CXCR5^{hi}PD\text{-}1^{hi}$ cells expressing Bcl6 (*6*), whereas $CXCR5^{int/lo}PD\text{-}1^{int/lo}$ non-T_{fol} cells did not (Fig. 2A). Within each of these two cell populations, we could detect the presence of a subset of $Foxp3^+$ cells, thus defining $CD4^+CXCR5^{hi}PD\text{-}1^{hi}Bcl6^+Foxp3^+$ T_{fr} cells and $CD4^+CXCR5^{int/lo}PD\text{-}1^{int/lo}Bcl6^-Foxp3^+$ T_{reg} cells (Fig. 2B). T_{fr} cells did not express CD25, unlike T_{reg} cells that expressed it highly (Fig. 2C). Similar observations were made with cells obtained from C57BL/6, BALB/c, and NOD (nonobese diabetic) genetic backgrounds (fig. S3). Expression levels of Bcl6 and CD25 were comparable among $CXCR5^{hi}PD\text{-}1^{hi}$ T_{fh} and T_{fr} cells (fig. S4).

To confirm the bona fide nature of $CXCR5^{hi}PD\text{-}1^{hi}Foxp3^+CD25^-$ T_{fr} cells, we studied their suppressive activity in a classic in vitro assay investigating the inhibition of IL-4 and IL-21 production by T_{fh} cells from immunized mice cocultured with B cells (*34*). Adding T_{fr} cells to these cocultures significantly decreased IL-4 production (Fig. 2D) and almost completely suppressed IL-21 production (Fig. 2E).

Because T_{fr} cells were initially described as $CD25^+$ (*32*), we speculated that this could be due to contamination by $CXCR5^{int/lo}PD\text{-}1^{int/lo}$ T_{reg} cells. We thus analyzed CD25 and Bcl6 expression on a gradient of subsets defined by the intensity of CXCR5 and PD-1 expression among $CD4^+Foxp3^+$ cells (Fig. 2F, left). Whereas $CXCR5^{hi}PD\text{-}1^{hi}$ T_{fr} cells homogeneously express Bcl6 and are $CD25^-$, the progressive decrease in the intensity of CXCR5 and PD-1 expression was associated with a parallel loss of Bcl6 expression and an increase in CD25 expression (Fig. 2F, middle and right). These results indicate that a nonstringent characterization of T_{fr} cells leads to the inclusion of T_{reg} cells.

T_{fr} cells' transcriptomic profile distinguishes them from T_{reg} and T_{fh} cells

We analyzed the expression profile of a set of 545 immune-related genes in T_{fh}, T_{fr}, and T_{reg} cells from NOD and BALB/c mice using NanoString technology. Hierarchical clustering based on the entire set of genes clustered T_{reg} cells samples apart from T_{fol} cell samples (fig. S5). We further used the 81 genes that better separate the three cell subsets (see the Supplementary Materials) to perform an additional hierarchical clustering (Fig. 3A) that again identified two main clusters of T_{fol} and T_{reg} cells, regardless of the genetic background. Within the T_{fol} cluster, T_{fr} and T_{fh} cells were well separated. We evaluated the accuracy and statistical robustness of this clustering by multiscale bootstrap resampling using the pvclust R package (Fig. 3B) (*45*, *46*). This statistical process attributes accuracy to each branch of the clustering by calculating an approximately unbiased (AU) *P* value as a percentage (*45*). The higher the percentage, the higher the accuracy of a given branch of the clustering. In our data set, the AU *P* values for the separation of T_{reg} and T_{fol} cells, as well as for the separation of T_{fr} and T_{fh} cells within T_{fol} cells, were 100%, indicating the robustness of the cell subset identification.

Differential gene expression in T_{reg}, T_{fh}, and T_{fr} cells

We next analyzed the genes differentially expressed between T_{fh}, T_{fr}, and T_{reg} cells using Ingenuity Pathway Analysis (IPA). The IPA's Upstream Regulator analytic tool identified NFATc1, a subunit of the NFAT (nuclear factor of activated T cells) complex known to interact with Foxp3 (*47*, *48*), as a key component differentiating the transcriptome profiles of our three cell populations. NFATc1 is down-regulated (blue) in T_{reg} cells and up-regulated (orange) in T_{fh} and T_{fr} cells, in

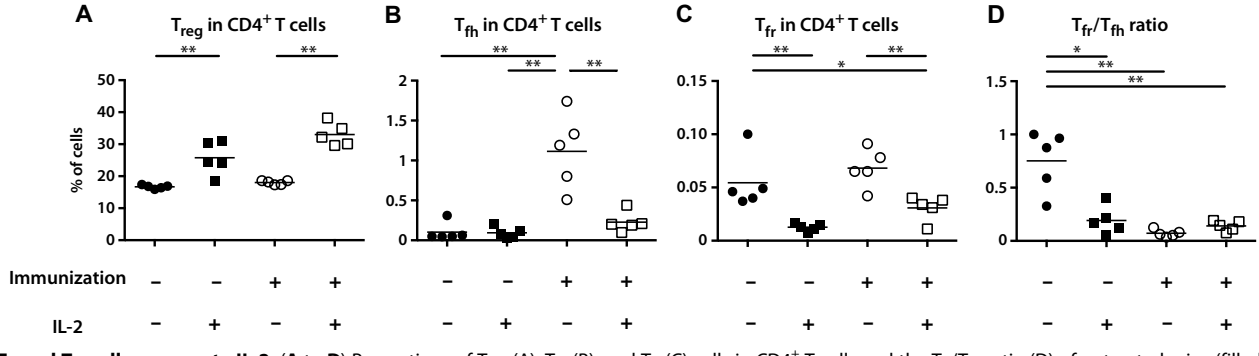

Fig. 1. T_{fr} and T_{fh} cell response to IL-2. (**A** to **D**) Proportions of T_{reg} (A), T_{fh} (B), and T_{fr} (C) cells in $CD4^+$ T cells and the T_{fr}/T_{fh} ratio (D) of untreated mice (filled circles), IL-2–treated mice (filled squares), immunized mice (empty circles), and immunized IL-2–treated mice (empty squares). $n = 5$; $^*P < 0.05$, $^{**}P < 0.01$, Mann-Whitney *U* test. Data are representative of three independent experiments.

accordance with (i) the observed expression levels of genes regulated by NFATc1 and (ii) their expected positive or negative regulation by NFATc1 (*49*), as modeled by IPA. Foxp3 expression aside, the pattern of NFATc1-dependent gene expression was identical between T_{fh} and T_{fr} cells. In contrast, each of the NFATc1 up-regulated genes in T_{reg} cells was down-regulated in T_{fr} and T_{fh} cells, and vice versa (Fig. 4A).

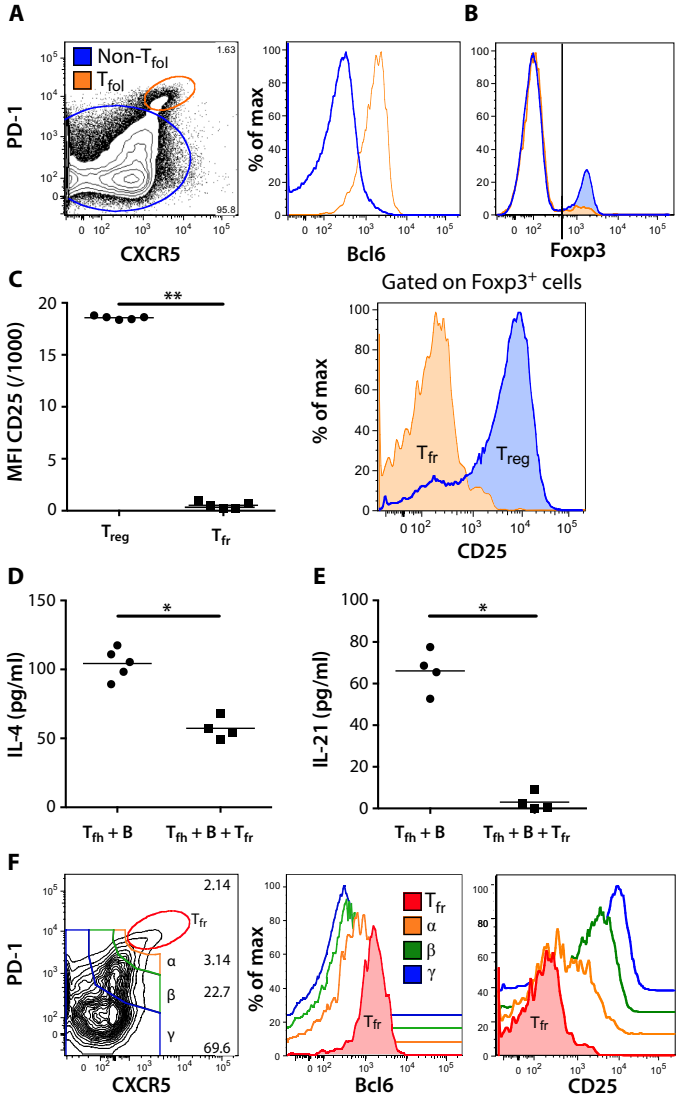

Fig. 2. T_{fr} cells do not express CD25. (**A**) Flow cytometry contour plots (left) showing CXCR5^{hi}PD-1^{hi} T_{fol} (orange) and CXCR5$^{int/lo}$PD-1$^{int/lo}$ non-T_{fol} (blue) cells within CD4$^+$ T cells and histograms showing their Bcl6 expression status (right). (**B**) Foxp3 expression of T_{fol} and non-T_{fol} cells defining T_{reg} cells and T_{fr} cells (both Foxp3$^+$). (**C**) Mean fluorescence intensity (MFI) of CD25 expression levels in T_{reg} cells and T_{fr} cells ($n = 5$). Data are representative of three independent experiments (left). Representative histogram of the expression of CD25 in T_{fr} and T_{reg} cells (right). (**D** and **E**) IL-4 (D) and IL-21 (E) production by T_{fh} cells from immunized mice, in the presence ($n = 4$) or absence ($n = 4$ to 5) of T_{fr} cells. Results are representative of two independent experiments. (**F**) Flow cytometry plot identifying different populations according to their PD-1 and CXCR5 expression within CD4$^+$Foxp3$^+$ T cells (left) and their expression of Bcl6 (middle) and CD25 (right). For (C) to (E), *$P < 0.05$, **$P < 0.01$, Mann-Whitney *U* test.

IPA then identified the pathways differentially modulated between the cell subsets. T_{fr} cells appeared as very active cells, with markedly up-regulated pathways related to movement, survival, viability, and proliferation. In contrast, pathways related to death and mortality were up-regulated in T_{fh} cells (Fig. 4B).

Supervised analyses then identified ILs and their receptors as markedly differentially expressed among the three cell subsets. IL-2Rα, IL-2Rβ, and IL-2Rγ are highly expressed by T_{reg} cells, whereas T_{fr} and T_{fh} cells only express IL-2Rβ and IL-2Rγ (Fig. 4C, left). IL-1Rs were not expressed by T_{reg} cells and had distinct expression profiles for T_{fol} subsets (Fig. 4C, right). T_{fh} cells only expressed the agonist IL-1 receptor IL-1R1, which transmits the inflammatory message of IL-1. In contrast, T_{fr} cells expressed lower levels of IL-1R1 than T_{fh} cells but high levels of IL-1R2, the IL-1 receptor decoy (*50*), and, to a lesser extent, IL-1Rn, the IL-1R antagonist (*51*).

IL-4 and IL-21, cytokines involved in T_{fh}-mediated B cell help (*16*, *20*, *52*), were mostly expressed by T_{fh} cells (fig. S6). IL-2 expression could not be detected in any of the subsets, whereas IL-1β expression was at the limit of detection (number of transcripts ≈ 100) and was similar in each subset (fig. S6). Costimulation molecules involved in B-T cell cooperation were also differentially expressed in T_{fol} cells. CD40L was mostly expressed on T_{fh} cells, whereas OX40 (fig. S6) was mostly expressed on T_{fr} cells. Thus, the IL-1 receptors CD40L and OX40 are the main molecules that discriminate T_{fh} and T_{fr} cells, as also confirmed by flow cytometry for IL-1R2 and OX40 (Fig. 4, D and E, and fig. S7).

IL-1–dependent T_{fh} cell activation

To assess the functional relevance of our IL-1–related gene expression profile, we investigated the role of IL-1β in T_{fh} cells. We treated immunized mice with recombinant IL-1Ra (Anakinra), which blocks the IL-1β response in humans (*53*) and mice (*54*–*56*), or with murine IL-1β.

Compared with untreated immunized mice (OVA + Alum), inhibition of IL-1β during immunization led to a significantly reduced proportion of T_{fh} cells (Fig. 5A), whereas injection of IL-1β induced a significant increase of T_{fh} cell proportion. This increase was observed even when immunization was performed without Alum, indicating the autonomous effect of IL-1β on T_{fh} cells. The increased T_{fh} cell response induced by IL-1β translated into an increased production of anti-OVA–specific IgG (Fig. 5B).

We then tested the contribution of IL-1β to the production of IL-4 by T_{fh} cells from immunized mice (Fig. 5C). B cells alone did not produce IL-4, whereas baseline T_{fh} cell IL-4 production was around 50 pg/ml. Coculture of B and T_{fh} cells led to a doubling of IL-4 production, whereas the addition of IL-1β rather than B cells led to an even more pronounced increase of IL-4 production by T_{fh} cells. Thus, T_{fh} cells are directly activated by IL-1β.

We next investigated the IL-1β dependence of the T_{fh} cell suppression by T_{fr} cells. When T_{fr} cells (at a T_{fh}/T_{fr} ratio of 2) were added to a coculture of T_{fh} and B cells, we observed a 50% reduction in IL-4 production (Fig. 5D), which corresponds to the value of basal IL-4 production by T_{fh} cells (Fig. 5C). This inhibition was equivalent to the inhibition observed when adding Anakinra (Fig. 5D). This also suggests that some cells in the culture produced IL-1β. We confirmed by flow cytometry that this production was mainly from B cells (fig. S8). Similar results were observed for the production of IL-21, leading to a sevenfold decrease of IL-21 production when T_{fr} cells are added to the culture (Fig. 5D).

T_{fr} cells could suppress the IL-1β–induced T_{fh} cell activation (Fig. 5E). Whereas IL-1β led to a 10-fold increase of IL-4 and IL-21

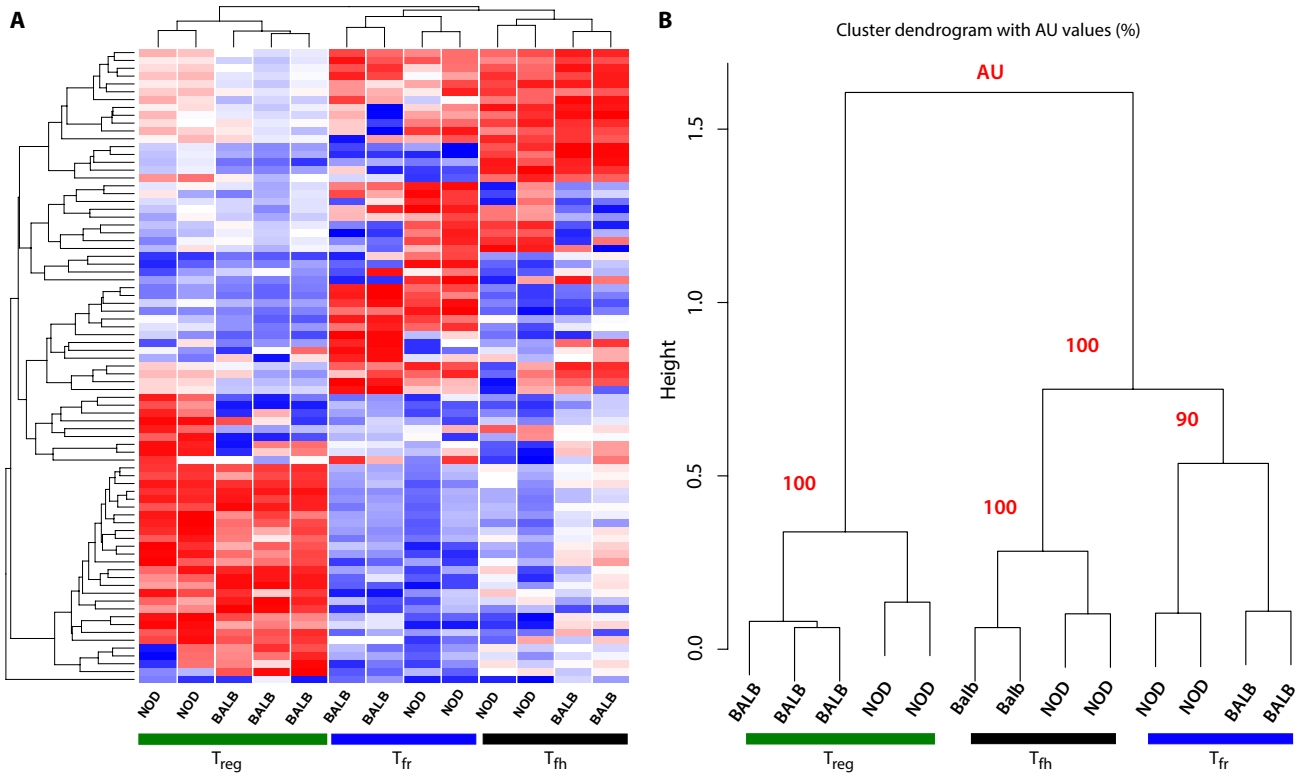

Fig. 3. T_{fr} cells' transcriptomic profile distinguishes them from T_{reg} and T_{fh} cells. (**A**) Heat map comparing the gene expression profiles of T_{reg} (green), T_{fr} (blue), and T_{fh} (black) cells from two genetic backgrounds after immunization. Red, high gene expression; blue, low gene expression. (**B**) Dendrogram of T_{reg}, T_{fr}, and T_{fh} cells representing the hierarchical cluster analysis performed with the pvclust R package.

production by T_{fh}, the addition of T_{fr} cells markedly inhibited this IL-1β–dependent IL-4 and IL-21 production (Fig. 5E). Together, these results indicate that there is an IL-1β-dependent activation of T_{fh} cells that can be suppressed by T_{fr} cells.

T_{fr} cell response to immunization with self-antigens or foreign antigens

We compared the response of T_{fr} cells to antigenic stimulation with two representative antigens. OVA immunization led to a higher increase in T_{fh} cells compared with insulin (INS) immunization (Fig. 6A), and vice versa, for the proportion of T_{fr} cells (Fig. 6B). The resulting T_{fr}/T_{fh} ratio was thus about fourfold higher after immunization with a self-antigen compared with a foreign antigen (Fig. 6C). This translated well in a higher proportion of $CD19^+Foxp3^-Bcl6^+GL7^+IgD^-$ GCB cells among $CD19^+$ B cells (fig. S9) after OVA immunization compared with INS immunization (Fig. 6D). There was an inverse correlation between the proportions of T_{fr} cells and GCB cells (Fig. 6E). When IL-1β was added during INS immunization, GCB cell values were in the range of those obtained after OVA immunization (Fig. 6F), indicating that the lower expansion of GCB cells after INS immunization was not intrinsically limited by a lower frequency of INS-specific B cells. There was again an inverse correlation between the proportions of T_{fr} cells and GCB cells (Fig. 6G)

Phenotype of T_{fr} cells in humans

We investigated the phenotype of T_{fh} and T_{fr} cells in humans by analyzing these cells in spleen or mesenteric lymph node cells obtained from healthy organ donors, or from tonsils. By using the same gating strategy that we used for mice (Fig. 7A), we could show the absence of CD25 expression on human T_{fr} cells (Fig. 7B). As for mice, we analyzed CD25 expression on a gradient of subsets defined by the intensity of CXCR5 and PD-1 expression among $CD4^+Foxp3^+$ cells (Fig. 7C, left). Although $CXCR5^{hi}PD\text{-}1^{hi}$ T_{fr} cells are homogeneously $CD25^-$, the progressive decrease in the intensity of CXCR5 and PD-1 expression was associated with a parallel increase in CD25 expression (Fig. 7C, right). As for mice, IL-1R2 was expressed only on T_{fr} cells (Fig. 7D).

DISCUSSION

IL-2 is now widely developed for stimulating T_{reg} cells in the context of inflammatory and autoimmune diseases (*57*). To better understand the consequences of an IL-2 treatment, and because T_{fr} cells are thought to derive from T_{reg} cells, we assessed T_{fr} cell response to IL-2. We found that T_{fr} cell proportion was reduced rather than augmented during IL-2 treatment. This intriguing observation led us to thoroughly assess their CD25 phenotype and to determine that T_{fr} cells do not express CD25, neither in mice nor in humans. Contrary to T_{reg} cells, which comprise both $CD25^+$ and $CD25^-$ subsets (*58*), careful investigations assessing expression of the canonical Bcl6 marker (Fig. 2F) led us to conclude that T_{fr} cells are homogeneously $CD25^-$ and $CXCR5^{hi}PD\text{-}1^{hi}$. These results initially obtained in C57BL/6 mice were further confirmed in BALB/c and NOD mice (fig. S3) and also observed for human T_{fr} cells (Fig. 7C). Although a recent study on T_{fr} cell conditional depletion in $Bcl6^{fl/fl}Foxp3^{CRE}$ mice suggested that

T_{fr} cells are $CD25^{lo}$ rather than $CD25^{+}$ (*38*), we believe that they are truly $CD25^{-}$, as also supported by their lack of expansion (Fig. 1C) after treatment with IL-2. IL-2 treatment not only expands $CD25^{+}$ T_{reg} cells but also induces an increase of CD25 expression by $CD25^{lo/-}$ lymph node T_{reg} cells (*59*, *60*). Our results now define T_{fr} cells as a very small population of $CD4^{+}CXCR5^{hi}PD\text{-}1^{hi}Foxp3^{+}Bcl6^{+}CD25^{-}$ cells. Therefore, when analyzing T_{fr} cells, which are scarce, if a very stringent gating on the highest CXCR5 and PD-1 expression and/or an exclusion of $CD25^{+}$ cells is not implemented, there will be significant contamination of the studied population by T_{reg} cells that we estimated at >50% with commonly used gating. Thus, many of the properties initially assigned to T_{fr} cells have likely been attributed to a mixed population of T_{reg} and T_{fr} cells. Our results suggest that there is a need to revisit these properties in more stringently selected T_{fr} cells. It should be realized that this will be difficult, because T_{fr} cells are reduced to a minute population.

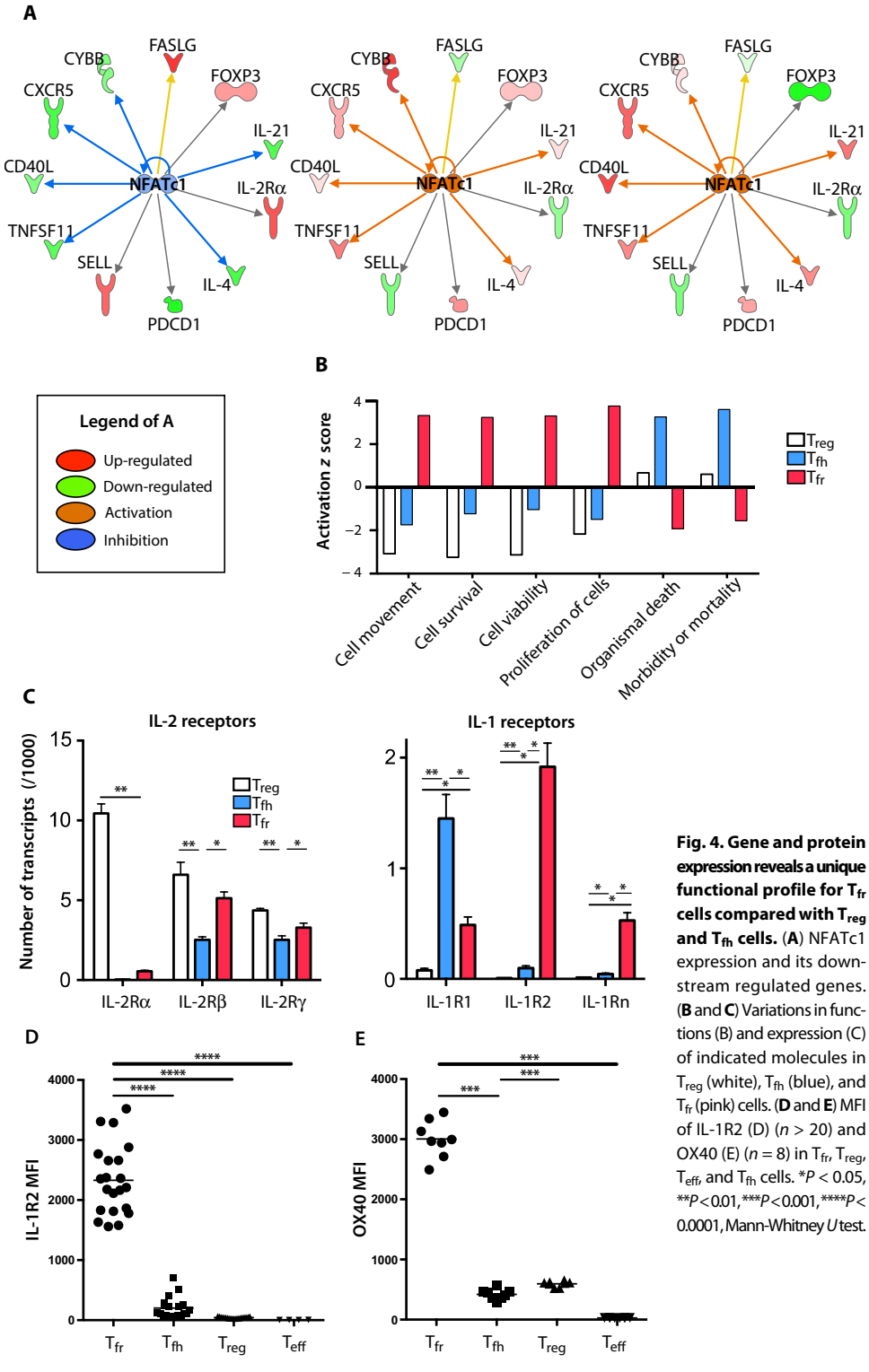

Fig. 4. Gene and protein expression reveals a unique functional profile for T_{fr} cells compared with T_{reg} and T_{fh} cells. (**A**) NFATc1 expression and its downstream regulated genes. (**B** and **C**) Variations in functions (B) and expression (C) of indicated molecules in T_{reg} (white), T_{fh} (blue), and T_{fr} (pink) cells. (**D** and **E**) MFI of IL-1R2 (D) ($n > 20$) and OX40 (E) ($n = 8$) in T_{fr}, T_{reg}, T_{eff}, and T_{fh} cells. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$, Mann-Whitney *U* test.

With this phenotypic characterization, we were able to sort only 2000 to 6000 T_{fr} cells per spleen of immunized mice. Nonetheless, we could confirm the functionality of T_{fr} cells. In vitro, they suppress T_{fh}-mediated production of IL-4 and IL-21 (Fig. 2, D and E), necessary cytokines for T_{fh}-mediated differentiation of antibody-producing B cells (*61*). Such a reduction of IL-21 and IL-4 production has been described as the main mechanism by which T_{fr} cells inhibit antibody responses (*34*). The in vivo suppressive activity of $CD25^{-}$ T_{fr} cells is also supported by the observation that the sole elimination of Bcl6/Foxp3 cells led to highly abnormal T_{fh} cell and GCB cell responses (*38*). As we show that our cells are the only $Bcl6^{+}Foxp3^{+}$ cells (Fig. 2F), this work strongly supports our conclusions. In vivo, the increase in T_{fr} and T_{fh} cells upon immunization was reciprocally dependent on the self/nonself nature of the antigen, suggesting different antigenic specificities, as recently reported (*41*).

To further revisit T_{fr} cell functionality on the basis of this previously unknown characterization, we studied the transcriptomic profile of T_{fr} cells and compared it with that of T_{fh} and T_{reg} cells. We used the NanoString technology, which is reported to be more quantitative for small cell populations (*62*). These analyses first showed that T_{fr} cells were clustered with T_{fh} and not with T_{reg} cells (Fig. 3A and fig. S5). This clustering was shown to be robust by a multiscale bootstrap sampling statistical validation (Fig. 3B). This suggests that the transcriptional program controlled by Bcl6 imprints the transcriptome landscape of T_{fr} cells more than the transcriptional program controlled by Foxp3. The first study describing T_{fr} cells (*29*) showed a clustering of T_{fr} cells with T_{reg}

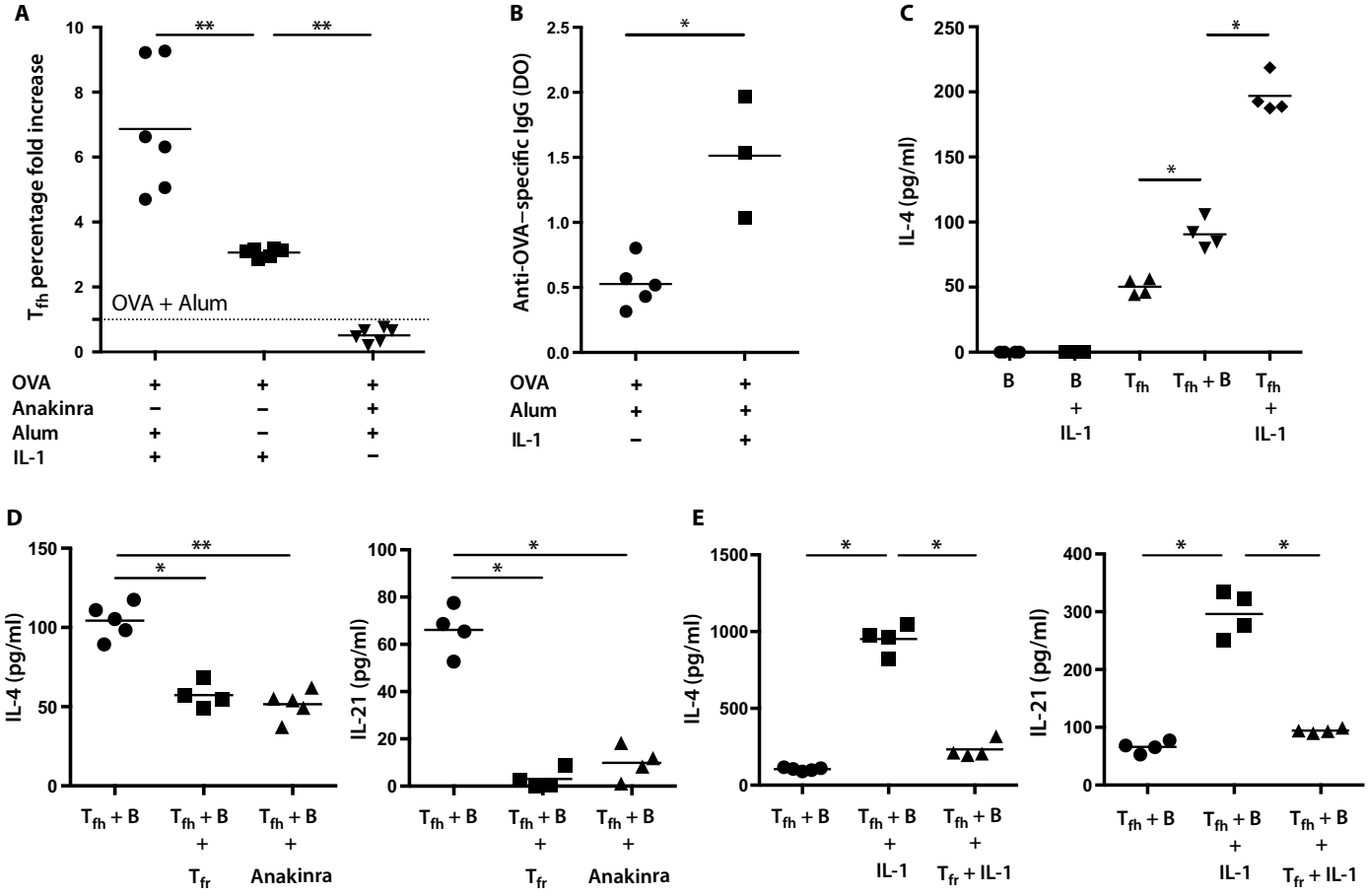

Fig. 5. IL-1β induces T_{fh} cell activation that is inhibited by T_{fr} cells. (**A**) Fold change in T_{fh} cell proportion among $CD4^+$ cells from indicated conditions in comparison with OVA + Alum control condition (dotted line) ($n = 6$ to 7). Data shown are from one experiment and representative of two independent experiments. (**B**) Serum anti-OVA–specific IgG production by mice immunized with OVA, treated ($n = 3$) or not treated ($n = 5$) with IL-1β. (**C**) IL-4 production by B cells and T_{fh} cells from immunized mice, in the presence or absence of B cells or IL-1β. (**D** and **E**) IL-4 and IL-21 production by T_{fh} cells from immunized mice cocultured with B cells, with or without T_{fr} cells or Anakinra (D) and with or without IL-1β and T_{fr} cells (E). (C to E) $n = 4$ to 5; $^*P < 0.05$, $^{**}P < 0.01$, Mann-Whitney *U* test.

cells; we believe that this could be due to contamination of the T_{fr} cells by T_{reg} cells.

Our observation does not give clues as to the origin of T_{fr} cells as cells either derived from thymic T_{reg} cells or induced from T_{fh} cells. The Bcl6-imprinted program could be secondarily imprinted over an initial imprinting by Foxp3, or vice versa. Lineage study and possibly TCR repertoire studies should help address this important issue.

We found that murine T_{fr} cells highly express the decoy receptor IL-1R2 (Fig. 4C). IL-1R2, an IL-1β and IL-1β negative regulator, has been observed on neutrophils, macrophages, monocytes, B cells, and $CD4^+$ T cells (*63*–*66*). T_{fr} cells also express IL-1Ra, the IL-1 receptor antagonist. We confirmed IL-1R2 expression on most, if not all, mouse and human T_{fr} cells by flow cytometry (Figs. 4D and 7D). IL-1R2 has also been described in some T_{reg} cells after in vitro activation (*65*, *66*) and in pancreatic (*67*) and infiltrating T_{reg} cells from colorectal tumors, but not from lung tumors or normal tissues (*68*). We could not detect significant expression of IL-1R2 in T_{reg} cells from immunized mouse splenocytes or human lymph nodes. In contrast, T_{fh} cells express the IL-1R1 agonist receptor but do not express IL-1R2 or IL-1Ra. These notable observations suggest that there could be an IL-1 axis in the regulation of T_{fh} cells by T_{fr} cells.

This IL-1 axis in the control of T_{fh} cell activation is directly supported by in vitro and in vivo results. The following observations collectively highlight the importance of IL-1β in T_{fh} cell activation and argue for a direct action of IL-1β on T_{fh} cells: (i) T_{fh} cells express IL-1R1 agonist receptor; (ii) IL-1β alone activates T_{fh} cell–dependent production of IL-4 and IL-21 in vitro; (iii) IL-1β alone (without adjuvant) triggers the expansion of T_{fh} cells in vivo in response to immunization; and (iv) Anakinra blocks the expansion of T_{fh} cells in vivo in response to immunization. Furthermore, the following observations demonstrate the biological relevance of antagonist IL-1Ra expression on T_{fr} cells: (i) T_{fr} cells suppress the production of both IL-4 and IL-21 by T_{fh} cells cocultured with B cells to the same extent as Anakinra, and (ii) T_{fr} cells suppress the IL-1β–triggered production of both IL-4 and IL-21 by T_{fh} cells.

Last, in the literature, the IL-1 axis in T_{fh} cell control of B cell response is indirectly supported by numerous observations: (i) administering IL-1 during immunization leads to enhanced antibody production (*69*–*71*); (ii) antibody production is significantly reduced in IL-1–deficient mice (*72*, *73*) but enhanced in mice lacking the expression of the IL-1 receptor antagonist IL-1Ra (*72*, *73*); (iii) the effect of IL-1 on antibody production works through induction of CD40L

(*73*), which is highly expressed on T_{fh} cells (fig. S6); and (iv) many adjuvants used for immunization are IL-1 inducers (*74*, *75*). These effects have been ascribed to IL-1β, rather than to IL-1α (*72*). These results, together with the observation that IL-1β alone activates T_{fh} cells in vitro and in vivo, support the idea that our results should be relevant to all immunization procedures increasing IL-1β production.

T_{fh} and T_{fr} cells are important for the regulation of humoral responses, in health and disease. Our results support that (auto)antibody production depends on IL-1β availability for T_{fh} cells, regulated by IL-1 antagonists expressed by T_{fr} cells. However, how general the contribution of the IL-1 axis is in the T_{fh}/B cell response to various antigenic stimulations (such as against other foreign antigens or self-antigens and during infection or autoimmune diseases) remains to be investigated, because this was not explored in this study. The mechanisms at work for its regulation also remain to be dissected. Selective knockout of IL-1R1 and IL-1R2/IL-1Ra in T_{fh} and T_{fr} should help address these questions. Last, the lineage of T_{fr} cells remains to be elucidated. Together, our results indicate a dual regulation of T cells in lymph nodes, one

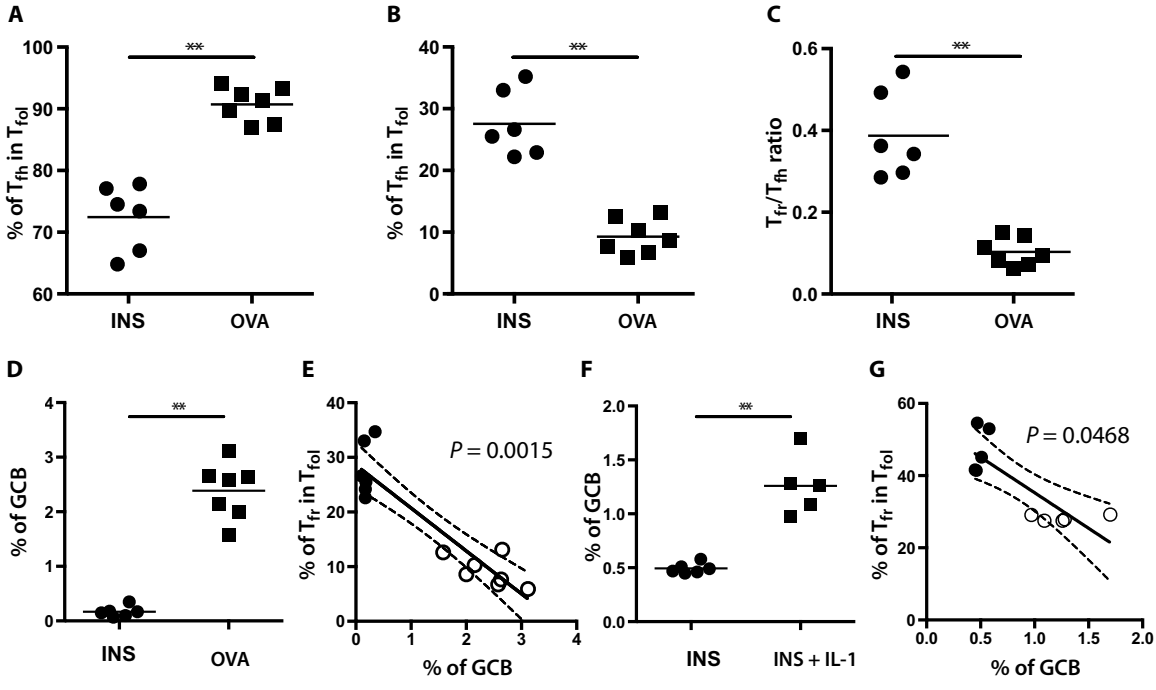

Fig. 6. T_{fr} cells respond better to immunization with self-antigens than with foreign antigens. (**A** to **C**) T_{fh} (A) and T_{fr} (B) cell proportions within T_{fol} cells and T_{fr}/T_{fh} ratio (C) after INS ($n = 6$) or OVA ($n = 7$) immunization. (**D** and **E**) GCB cell proportions within $CD19^+$ B cells (D) and correlation between the percentage of T_{fr} and GCB cells from INS-immunized (filled circles) or OVA-immunized (empty circles) mice (E). (**F** and **G**) GCB cell proportions within $CD19^+$ B cells (F) and correlation between the percentage of T_{fr} and GCB cells from INS-immunized mice receiving IL-1β (empty circles) or not (filled circles) (G). (A to C and F) $^{**}P < 0.01$, Mann-Whitney *U* test. (E and G) Each symbol represents one mouse, and the Spearman rank correlation value is shown.

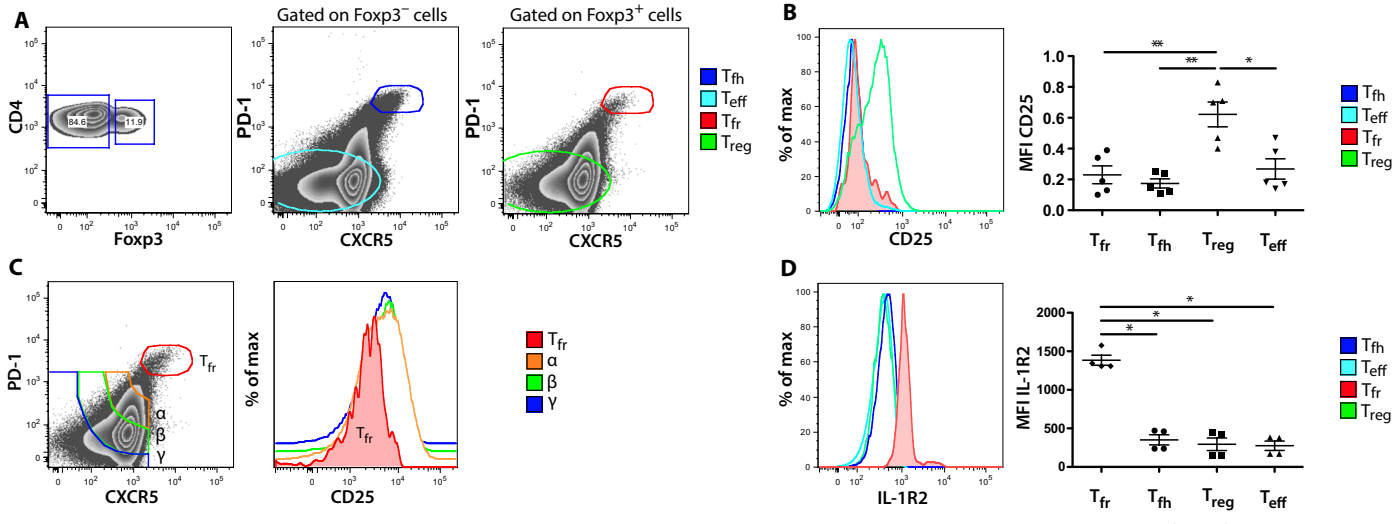

Fig. 7. Human T_{fr} cells do not express CD25. (**A**) Flow cytometry contour plots showing $CD4^+Foxp3^-$ and $CD4^+Foxp3^+$ T cells (left); $CXCR5^{hi}PD\text{-}1^{hi}$ T_{fh} cells (blue) and $CXCR5^{int/lo}PD\text{-}1^{int/lo}$ T_{eff} cells (cyan) within $CD4^+Foxp3^-$ T cells (middle); and $CXCR5^{hi}PD\text{-}1^{hi}$ T_{fr} cells (red) and $CXCR5^{int/lo}PD\text{-}1^{int/lo}$ T_{reg} cells (green) within $CD4^+Foxp3^+$ T cells (right). (**B**) Histograms showing CD25 expression level (left) and MFI (right) on gated subsets from (A). (**C**) Flow cytometry contour plot identifying different subsets according to PD-1 and CXCR5 expression within $CD4^+Foxp3^+$ T cells (left) and the corresponding expression of CD25 (right). (**D**) Histograms showing the IL-1R2 expression level of the subsets mentioned above (left) and their MFI (right). (B and D) $n = 5$; $^*P < 0.05$, $^{**}P < 0.01$, Mann-Whitney *U* test.

between T_{reg} and T_{eff} cells regulated by IL-2 outside GCs and the other between T_{fh} and T_{fr} cells regulated by IL-1β inside GCs.

MATERIALS AND METHODS

Study design

For human and mice flow cytometry assays, the sample sizes were of at least five individuals per experiment. For enzyme-linked immunosorbent assay (ELISA) and NanoString, sample sizes were of at least four per condition for which cells were sorted from pooled splenocytes from nine mice to ensure a sufficient number of T_{fr} cells. Sample size was determined on the basis of experimental feasibility and for statistical significance. The experiments were not randomized. The investigators were not blinded to the allocation during experiments and analyses.

Mice

Male and female NOD Foxp3-GFP and BALB/c Foxp3-GFP (C.129X1-Foxp3tm3Tch/J) mice (8 to 14 weeks old) expressing GFP under the control of the promoter of *Foxp3* gene were provided by V. Kuchroo of Brigham and Women's Hospital (Boston, MA). C57BL/6 Foxp3-GFP mice expressing GFP under the control of the promoter of *Foxp3* gene were provided by B. Malissen of the Centre d'Immunologie de Marseille-Luminy (France). All animals were maintained at the University Pierre and Marie Curie (UPMC) Centre d'Expérimentation Fonctionnelle animal facility (Paris, France) under specific pathogen–free conditions in agreement with current European legislation on animal care, housing, and scientific experimentation (agreement number A751315). All procedures were approved by the local animal ethics committee.

IL-2 treatment in mice

AAV8-CAG-IL2 recombinant vectors (rAAVs) were generated by triple transfection of human embryonic kidney 293T cells, as described previously (*42*). Mice were injected intraperitoneally once before immunization at day −21 (D−21) with 10^{10} viral genomes of rAAVs diluted in 100 μl of 1× phosphate-buffered saline (PBS1×).

Immunization

Mice were either immunized once (D0) and sacrificed at D10 for T_{fr}/T_{fh} phenotypic and transcriptomic studies or immunized twice (D0 and D14) and then euthanized at D28 for the analysis of GCB cells. Intraperitoneal injection was performed with 100 μg of OVA (OVA A5503, Sigma-Aldrich) mixed with 500 μg of aluminum hydroxide (Alum) gel (AlH303, Sigma) or with 4.5 IU of human INS (Umuline Rapide, Lilly) mixed with 500 μg of Alum.

In vivo treatment

Mice treated with recombinant human IL-1Ra received Anakinra (1 mg per mouse; Amgen) every 24 hours from D−1 before immunization to D4 after immunization. Mice treated with IL-1β received recombinant mouse IL-1β (0.5 μg per mouse; BioLegend) at D1 and D2 after immunization.

Flow cytometry analysis of mouse cells

Fresh total cells from lymph nodes and spleens were isolated in PBS1×–3% fetal bovine serum (FBS) and stained for 20 min at 4°C with the following monoclonal antibodies at predetermined optimal dilutions: CD121b-BV421, CD19-PeCF594, CD4-V500, CD8a-AF700, Bcl6-APC, CD278-BV421, CXCR5-Biotin (BD Biosciences), GL7-e450, Foxp3-AF488, PD-1–PE (PD-1–phycoerythrin), CD134-APC (CD134-allophycocyanin) (eBioscience), streptavidin-APC (eBioscience) or streptavidin–APC-Cy7 (BD Biosciences), CD25-PC7 (eBioscience), or CD25–eFluor 660 (BD Biosciences). CXCR5 staining was performed using biotinylated anti-CXCR5 for 30 min at 20°C followed by APC- or APC-Cy7–labeled streptavidin at 4°C. Intracellular detection of Foxp3 was performed on fixed and permeabilized cells using appropriate buffer (eBioscience), following the manufacturer's recommendations. Cells were acquired on an LSR II flow cytometer (Becton Dickinson) and analyzed using FlowJo software (TreeStar Inc.). Dead cells were excluded by forward/side scatter gating.

Human tissues

Human tissues were obtained from prospective organ donors through an approved research protocol (no. 2014-108) authorized by the French Biomedicine Agency and the Ministry of Education and Research. Relatives of the donors provided informed consent for the collection of samples. Tissues were collected after the organs were flushed with cold preservation solution.

Tissue samples were maintained in cold PBS and brought to the laboratory within 2 to 4 hours of organ procurement where they were rapidly processed using mechanical digestion, resulting in high yields of living lymphocytes. For the spleen, an additional step was carried out to isolate mononuclear cells by density gradient centrifugation with Lymphoprep (STEMCELL Technologies).

Flow cytometry analysis of human cells

Cell pellets were resuspended in a 10^6 cells/50 μl of FBS concentration and then treated using the PerFix-nc kit (Beckman Coulter), following the manufacturer's recommendations. Cells were stained using a panel of fluorescence-conjugated fluorescein isothiocyanate (FITC), PE, PE–Texas Red (PE-TR), AF647, PE-Cy7, AF405, Pacific Blue, or BV510 monoclonal antibodies to detect the following cell proteins: CD4, CD25, CD127, Foxp3 (Beckman Coulter), CD3 (BD Biosciences), PD-1, CXCR5 (Ozyme), and IL-1R2 (R&D Systems). Flow cytometry data acquisition was performed on BD FACSAria II (BD Biosciences). Control samples included unstained and single fluorochrome-stained compensation beads (UltraComp eBeads, eBioscience). Flow cytometry data were analyzed using FlowJo software (TreeStar Inc.).

Cell sorting

Splenocytes from immunized mice were stained with Ter-119–biotin and B220-biotin antibodies for 20 min at 4°C and labeled with anti-biotin magnetic beads (Miltenyi Biotec) for 15 min at 4°C. B cells and erythrocytes were depleted on an autoMACS separator (Miltenyi Biotec), following the manufacturer's procedure. Enriched T cells were stained as described in the "Flow cytometry analysis of human cells" section, and the following subsets were sorted on BD FACSAria II (BD Biosciences), with a purity of >98%: $CD4^+CD8^-CXCR5^{hi}PD\text{-}1^{hi}Foxp3^-$ T_{fh} cells, $CD4^+CD8^-CXCR5^{hi}PD\text{-}1^{hi}Foxp3^+$ T_{fr} cells, and $CD4^+CD8^-CXCR5^{int/lo}PD\text{-}1^{int/lo}Foxp3^+$ T_{reg} cells.

Suppression assays

T_{fh}, T_{fr}, and $CD19^+$ B cells were sorted from D−10 OVA-immunized mice, following a previously described "Immunization" protocol. For all the conditions, 2×10^4 T_{fh} cells and 5×10^4 B cells were cultured for 96 hours in 96-well plates (Nunc) in complete RPMI 1640 (Thermo

Scientific) with anti-IgM (5 μg/ml; clone eB121-15F9, eBioscience) and three CD3/CD28 beads for one T cell (Dynabeads Mouse T-Activator, Thermo Scientific). We then added either (i) 1×10^4 T_{fr} cells with ("T_{fh} + B + T_{fr} + IL-1") or without ("T_{fh} + B + T_{fr}") 1 μg of recombinant mouse IL-1β (BioLegend), (ii) only 1 μg of recombinant mouse IL-1β ("T_{fh} + B + IL-1"), or (iii) Anakinra (500 ng/ml; Amgen) ("T_{fh} + B + Anakinra"). IL-4 and IL-21 secretion levels were measured by ELISA (eBioscience) in supernatants of cultured cells, according to the manufacturer's recommendations.

Gene expression analysis based on a NanoString immunology panel

Sorted cells were washed in PBS1× and stored in RNAqueous kit lysis buffer (Ambion Inc./Life Technologies) at −80°C. Total RNA was extracted according to the manufacturer's instructions, and quality was assessed on a bioanalyzer using the Pico RNA Reagent Kit (Agilent Technologies). Gene expression was analyzed using a NanoString mouse immunology panel, following the manufacturer's recommendation (see Supplementary Materials and Methods).

Statistical analysis

Flow cytometry, cytokine production, and gene expression data were analyzed using nonparametric Mann-Whitney *U* test on GraphPad Prism v5 [*P* values, such as $P > 0.05$ (not significant), $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, and $^{****}P < 0.0001$, are indicated in the figures]. Exact *P* values are provided in table S1. Nonparametric correlation analyses were performed by Spearman rank correlation coefficient calculation. NanoString data were analyzed using IPA (QIAGEN). Clustering analysis and multiscale bootstrap resampling were performed using heatmap.2 and pvclust R packages on R version 3.1.3 (cluster method, average; distance, correlation).

See the Supplementary Materials for additional Materials and Methods information.

SUPPLEMENTARY MATERIALS

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Materials and Methods
Fig. S1. Representative flow cytometry gating for T_{fol} cells.
Fig. S2. IL-2 increases the number of splenic T_{reg} cells.
Fig. S3. Expression of CD25 on T_{fr} and T_{fh} cells in three different genetic backgrounds.
Fig. S4. T_{fh} and T_{fr} cells have similar expressions of Bcl6 and CD25.
Fig. S5. Clustering of T_{reg}, T_{fr}, and T_{fh} cells based on the expression of the entire 545-gene set from the NanoString mouse immunology panel.
Fig. S6. Cytokines, CD40L, and OX40 expression of T_{fr}, T_{reg}, and T_{fh} cells.
Fig. S7. IL-1R2 and OX40 expression of T_{fr}, T_{reg}, T_{eff}, and T_{fh} cells.
Fig. S8. IL-1β production in coculture of B and T_{fh} cells.
Fig. S9. GCB representative gating after INS or OVA immunization.
Table S1. Exact *P* values of the asterisk symbols shown in figures.
Source data (Excel file)

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Immune regulation in the germinal center

Unregulated production of antibodies may contribute to the development of autoimmunity. Follicular regulatory T (T_{fr}) cells are thought to limit the germinal center (GC) reaction and to reduce antibody production within B cell follicles in both humans and mice, yet how T_{fr} cells control the GC reaction remains unclear. Ritvo *et al*. closely characterize T_{fr} cells and identify these cells as a rare population of $CD4^+CXCR5^+PD\text{-}1^+Foxp3^+$ cells that do not express CD25 and do not respond to interleukin-2. When compared with follicular helper T (T_{fh}) cells and regulatory T cells, T_{fr} cells clustered with T_{fh} cells. Moreover, they expressed decoy molecules for the interleukin-1 signaling pathway, suggesting a mechanism for the suppression of T_{fh} cells.