

The capacity of Th2 lymphocytes to deliver B-cell help requires expression of the transcription factor STAT3

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CD4⁺ T-cell help for B cells is crucial for effective Ab responses. Although follicular T helper (Tfh) cells have emerged as the main providers of T-cell help to B lymphocytes during the germinal center reaction, much less is known about the helper capacities of other effector CD4⁺ T cells. The purpose of the present study was to evaluate the acquisition of B-cell help capacity of canonically derived T helper 2 (Th2) cells, a Th-cell subset originally considered responsible for B-cell help in vivo. We demonstrate herein that developing Th2 cells in mice co-express activated forms of signal transducer and activator of transcription 6 (STAT6) and STAT3 and that STAT3 expression was required for the capacity of Th2 cells to provide B-cell help. Thus, Th2 lymphocytes share a common, STAT3-mediated activation program for the acquisition of optimal B-cell help capacity with Tfh cells. Moreover, the expression of STAT3 in Th2 cells enhanced the IgG1-to-IgE class switch ratio in vivo, a finding with important implications for understanding the molecular basis of allergic diseases.

Keywords: Humoral response · STAT3 · Th2 cells

Introduction

Upon activation, naïve T helper (Th) cells can differentiate into a diverse set of effector cell subsets regulating various immunological functions, such as clearing viruses (Th1 cells), extracellular parasites (Th2 cells), and fungi (Th17 cells), or inhibiting immune-mediated damage (Treg cells) [1]. Effector CD4⁺ Th-cell specification is greatly influenced by the cytokines present in the microenvironment during the Ag priming phase. A major means by which cytokines exert their effect is through the signal transducer and activator of transcription (STAT) family transcription factors. Thus, IL-12 promotes Th1-cell development through the activation of STAT4 that induces IFN- γ and enhances expression of the transcription factor T-bet. Similarly, IL-4 activates STAT6 and induces GATA3, a master driver of the Th2-cell differentiation program, while IL-6, a STAT3-signaling cytokine, cooperates with

TGF- β to guide ROR γ t expression and Th17 lineage development [1, 2].

Follicular helper T (Tfh) cells have recently been identified as a subset of CD4⁺ T cells with specialized function in enhancing germinal center (GC) formation and providing help to B cells [3]. Several cytokines, including the IL-6 and IL-21, have been shown to drive Tfh-cell differentiation by promoting the activation of STAT3 and the expression of the transcriptional repressor BCL6, considered as the critical regulator of Tfh-cell development [4–7]. The main cytokine-signature of Tfh cells is IL-21, which provides crucial signals to B cells leading to Ab production, memory, and plasma cell differentiation in the context of thymus-dependent humoral immune responses [8–11]. This particular helper subset has recently been shown to display substantial plasticity, as Tfh cells within the GC can express cytokines characteristic of other Th cells [12–15]. In support of these observations, both in

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vitro- and in vivo-derived Tfh cells have been found to produce Th1, Th2 or Th17 effector cytokines upon restimulation in appropriated polarizing conditions [16]. The diversity in the nature of Th cells that provide B-cell help is further emphasized by the work of Nakayama et al. [17] who recently demonstrated that IL-12-induced differentiation of Th1 cell was marked by a transitional Th1/Tfh cell-like phenotype expressing feature of both Tfh (IL-21, BCL6) and Th1 (IFN- γ , T-bet) cells.

Th2 cells have long been considered to play a pivotal role in helping B cells, based on the superior ability of Th2 clones to support in vitro Ab production, and the well-documented capacity of Th2-derived cytokines (such as IL-4) to sustain B-cell growth, differentiation, and isotype switch [18–21].

The discovery that Tfh cells secreted the Th2 signature cytokine IL-4 [12–15] further suggested a strong relationship between Th2 and Tfh cells. Similarly, IL-21, as well as the transcription factor STAT3, has been shown to be expressed and important for the development of both Tfh- [11, 22, 23] and Th2-like lymphocytes [24–27]. Tfh cells however differentiated normally in vivo in Th2-unfavorable settings [11] and IL-4 or IL-5 knockout (KO) mice, characterized by defective Th2-cell responses, displayed readily detectable Ab production and GC formation [28, 29].

The relation of Th2 cells to the Tfh-cell subset is therefore a subject of intense investigations as understanding the relative roles of Tfh and Th2 cells in providing help for B cells and the mechanisms they employ to achieve this effector function will be important for the rational design of vaccines and therapies for immune-mediated disorders.

In this work we have specifically analyzed the molecular pathways underlying the capacity of Th2 cells to provide help to B lymphocytes. Our results indicate that upon Th2-cell differentiation, accumulation of activated forms of STAT3 is responsible for the execution of a Tfh-like differentiation program in Th2 cells, leading to the delivery of B-cell help both in vitro and in vivo. Our observations strengthen the important and central role played by STAT3 in regulating the capacity of multiple helper subsets in promoting humoral responses, irrespectively of their Th1/Th2/Th17 initial differentiation program.

Results

Th2 cells express a set of genes associated with Tfh-cell function and promote B-cell help in vitro

To assess and compare the relative capacity of cells differentiated under Th2- and Tfh-promoting conditions to provide B-cell help, we used a well-described and widely adopted in vitro differentiation protocol to polarize naïve CD4⁺ T cells into distinct effector cell subpopulations [16, 23, 30]. In particular, minimal conditions employing single cytokines (exogenous IL-4 or IL-6) and/or blocking antibodies were used in an attempt to mimic in vivo conditions. The efficacy of our in vitro protocol was verified by intracellular cytokine staining (data not shown) and gene expression analysis. T lymphocytes activated under neutral (i.e. in the absence of exogenously added cytokines and/or blocking

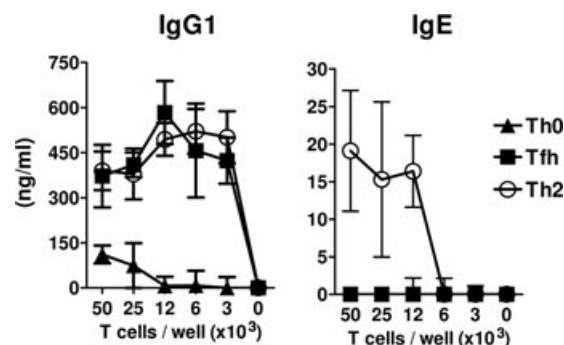


Figure 1. Th2 and Tfh cells promote B-cell help function in vitro. Naïve CD62L⁺CD4⁺ T cells purified from the spleens of C57BL/6 mice were stimulated with anti-CD3 and anti-CD28 mAbs coated on plastic plates for 48 h under neutral (Th0, triangles) or polarizing (Tfh squares, Th2 open circles) conditions and rested 24 h in fresh medium. Serial dilutions of irradiated recovered Th cells were incubated with freshly purified naïve B cells from C57BL/6 mice (5×10^5 cells/well) and anti-CD3 mAb (500 ng/mL). Supernatants of the T/B-cell coculture were tested on day 7 for (A) IgG1 or (B) IgE contents by ELISA. Results are expressed as mean $\pm$ SD of triplicates and are from one experiment representative of four independent experiments performed.

antibodies) conditions (Th0) were used as control. Both populations of CD4⁺ T cells stimulated in the presence of IL-4/anti-IFN- γ (referred to as Th2 cells thereof) and IL-6 (Tfh) promoted B-cell proliferation (data not shown) and immunoglobulin class switch and secretion (Fig. 1). Of interest and according to data from the literature [23, 31, 32], Th2 and Tfh cells induced a similar increase in IgG1 secretion (Fig. 1A), while only Th2 cells induced IgE class switch (Fig. 1B), revealing both similar and distinctive features in the B-cell help capacities of these Th-cell populations. Similar results were obtained when Th-cell subsets were derived in IMDM medium in the presence of T-cell depleted splenocytes as APCs, a protocol known to promote Tfh-cell activation [16] (data not shown).

As a step toward the identification of factors potentially responsible for the observed B-cell help function of Th2 cells, we next examined the time course expression of several genes coding for cytokines and transcription factors known to be associated with prototypical Th2- or Tfh-cell differentiation programs. As expected, expression of GATA3, IL-4, IL-5, and IL-13 was restricted to cells cultured under Th2-promoting conditions (Fig. 2A–D), further confirming the validity of our in vitro differentiation protocol. In addition, Th2 cells expressed equivalent amounts of the BCL6 mRNA as Tfh cells, associated with lower amount of Blimp-1, respectively, the master switch up- and downregulators of Tfh-cell differentiation (Fig. 2E and F). As previously reported [24, 33, 34], Th2 cells expressed c-Maf and IL-21, although with a 2 days delay when compared to Tfh (Fig. 2H and I). IL-6, a well-described Tfh-inducer cytokine, was transiently, and specifically expressed by cells cultured in Th2-promoting conditions (Fig. 2G). Both Th2 and Tfh cells expressed IL-10 after a 2 days culture but prolonged IL-10 expression was maintained in Th2 cells only (Fig. 2J). Further examination of the phenotype of in vitro-derived Th cells revealed that both Th2- and Tfh-cell populations included

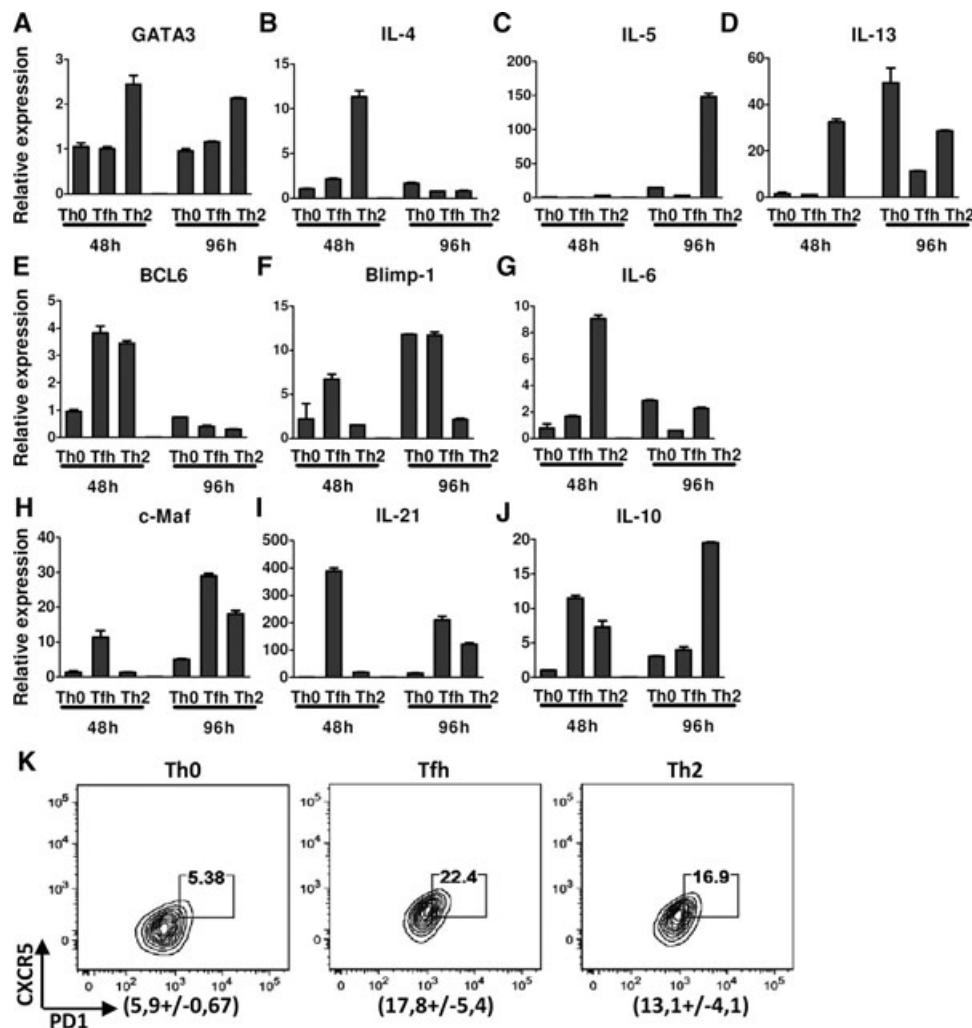


Figure 2. Th2 cells exhibit delayed expression of Tfh-cell attributes. (A–J) Naïve CD62L⁺CD4⁺ T cells purified from the spleens of C57BL/6 mice were stimulated with anti-CD3 and anti-CD28 mAbs coated on plastic plates under neutral (Th0), Tfh, or Th2-prone conditions for 48 or 96 h. The gene expression level of (A) GATA-3, (B) IL-4, (C) IL-5, (D) IL-13, (E) BCL-6, (F) Blimp-1 (G) IL-6, (H) c-MAF, (I) IL-21, and (J) IL-10 was assessed by quantitative RT-PCR. Data are shown as the mean $\pm$ SD of duplicates and are from one experiment representative of three independent experiments performed. (K) CD62L⁺CD4⁺ T cells were activated in IMDM media in the presence of irradiated T-depleted splenocytes for 3.5 days and analyzed for CXCR5 and PD1 expression. The mean % of CXCR5⁺PD1⁺ cells $\pm$ SD of three independent experiments is shown under each representative plot.

a greater percentage of CXCR5⁺PD1⁺ cells, compared to Th0 cells (Fig. 2K).

Taken together, our data demonstrate that the Th2-cell polarization pathway is accompanied by expression of genes previously shown to play a critical role in Tfh-cell function.

Th2 cells express activated form of STAT3 downstream of a STAT6-signaling pathway

We then compared the signaling events leading to Th2- and Tfh-cell differentiation in our culture model. As expected, Th2-cell differentiation led to the rapid and sustained accumulation of phospho-STAT6, as judged by flow cytometry analysis (Fig. 3A and B). Phospho-STAT3 was gradually induced in the course of Th2-cell differentiation, with detectable expression beginning on

day 2 and reaching expression levels similar to pSTAT6 on day 3. We next sought to test whether activation of STAT3 during Th2-cell polarization was subordinated to the IL-4/STAT6 axis. As shown in Figure 3C, STAT6-deficient Th2 cells displayed strongly reduced amounts of pSTAT3, indicating that STAT3 phosphorylation observed in Th2 cells was under the control of the IL-4/STAT6 signaling pathway.

IL-6 and IL-10, two cytokines known to activate STAT3, were expressed during Th2-cell differentiation in a STAT6-dependent fashion (Fig. 3D), thus representing likely candidates for the observed accumulation of phospho-STAT3 under Th2 culturing conditions. To explore this hypothesis, WT or IL-6-deficient T lymphocytes were activated under Th2 polarizing conditions in the presence or not of blocking antibodies directed against both IL-10 and its receptor (α -IL-10R). While phosphorylation of STAT6 was independent of IL-6 and IL-10 signaling under these culture

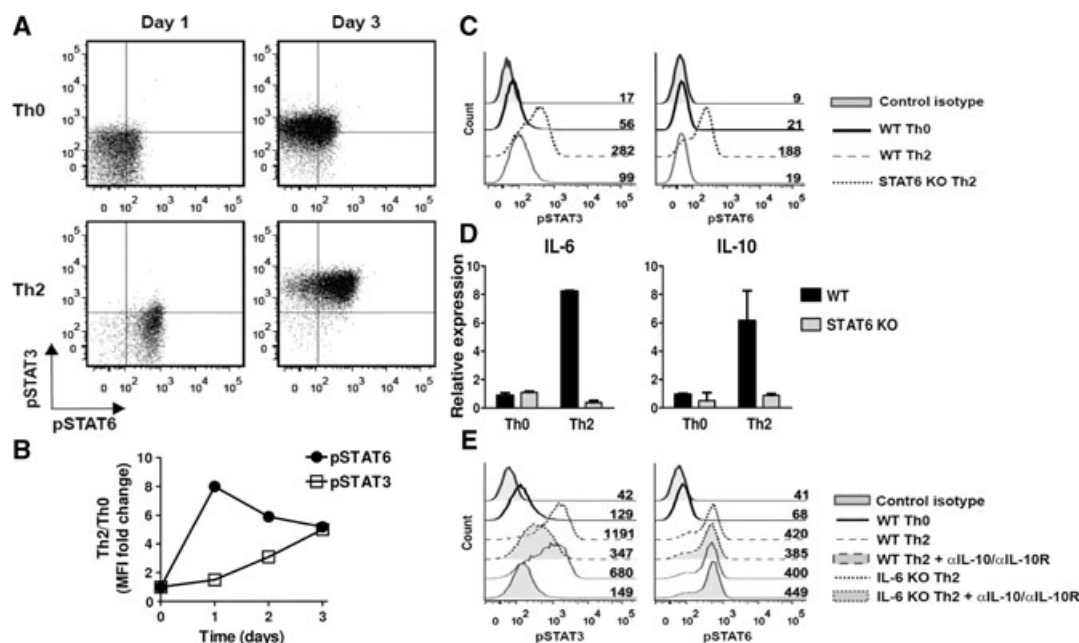


Figure 3. STAT3 is activated during Th2-cell differentiation. (A, B) Naïve CD4⁺ T cells were stimulated with anti-CD3 and anti-CD28 mAbs coated on plastic plates under neutral (Th0) or Th2 polarizing conditions. After 1, 2, or 3 days of polarization, cells were stained for intracellular phospho-STAT3 and phospho-STAT6. (A) Dot plots corresponding to days 1 and 3; (B) the fold change in the median fluorescence intensity (MFI) between Th2 and Th0 conditions over a 3-day activation course (value before activation (day 0) was set to 1) is shown. (C) Flow cytometry analysis of phospho-STAT3 and phospho-STAT6 expression in WT and STAT6-KO activated for 3 days under Th0 or Th2 condition. (D) RT-PCR analysis of IL-6 and IL-10 expression by WT and STAT6-deficient CD4⁺ T cells activated for 48 h in neutral or Th2 condition is shown as mean +SD of duplicates and is representative of three independent experiments performed. (E) Phospho-STAT3 and phospho-STAT6 expression in WT and IL-6-KO Th2 cells cultured for 3 days in the presence or absence of anti-IL-10/anti-IL-10R mAbs. (C, E) Numbers in histograms correspond to the median fluorescence intensity. The data shown are representative of three (A, B, D) or two (C, E) independent experiments performed.

conditions (Fig. 3E, right panel), accumulation of phospho-STAT3 was reduced when IL-10, and to a lesser extent, IL-6 signaling was inhibited. Interfering with both IL-6 and IL-10 signaling led to a near complete inhibition of phospho-STAT3, suggesting that IL-6 and IL-10 contribute to induce and maintain high phospho-STAT3 levels during in vitro Th2-cell differentiation (Fig. 3E, left panel).

STAT3 regulates the capacity of Th2 cells to provide B-cell help

Since phospho-STAT3 was generated in Th2 cells, we investigated the influence of this transcription factors on the expression of genes associated with a typical Th2 (GATA3, IL-4) or a common Th2/Tfh (c-Maf, IL-21) differentiation program. To address this question, we cultured naïve WT, STAT6-KO, or STAT3^{fl/fl}/CD4-CRE (referred to as STAT3^{CD4-/-}) CD4⁺ cells under neutral (Th0) or Th2-promoting conditions. As expected, STAT6 deficiency led to an impaired expression of all Th2-associated mRNAs tested, including GATA3, c-Maf, BCL6, IL-4, IL-5, IL-6, IL-10, and IL-21 (Fig. 4A and data not shown).

STAT3^{CD4-/-} T cells cultured under pro-Th2 conditions upregulated GATA3 and IL-4 gene expression, but failed to express significant levels of c-Maf and IL-21 (Fig. 4B and C). Accordingly, intracellular cytokine staining confirmed that STAT3 was required for optimal IL-21 secretion by both Th2- and Tfh-like cells, while being dispensable for IL-4 production by Th2 cells (Fig. 4D).

In agreement with RT-QPCR analysis, STAT6-deficient Th cells cultured in the presence of IL-4 and blocking anti-IFN- γ mAbs displayed reduced IL-4 and IL-21 intracellular protein levels (Fig. 4E). Collectively, data from Figure 3 and 4 indicate that STAT3 is activated upon in vitro Th2-cell differentiation and plays an important role in allowing Th2 cells to secrete IL-21.

We next sought to evaluate the ability of cells lacking expression of STAT6 or STAT3 to provide help to WT naïve B cells in vitro when cultured under pro-Th2 conditions. The capacity of Th2-like cells to promote B-cell differentiation into Ab secreting cells was completely inhibited in the absence of STAT6 (Fig. 5, upper panels) and severely impaired in the absence of STAT3 (Fig. 5, lower panels), suggesting that both transcription factors might contribute to the B-cell help function of Th2 cells.

The role of STAT3 in the generation of B helper T cells was further confirmed in vivo. CD3 ϵ ^{-/-} or RAG- γ ^{-/-} recipient mice were transferred with in vitro-derived Th2 cells from control or STAT3^{CD4-/-} mice, and subsequently immunized against NP-KLH (RAG- γ ^{-/-} recipient mice were also transferred with WT syngeneic B cells). Flow cytometric analysis performed 2 weeks after reconstitution confirmed that equivalent numbers of CD4⁺ T cells were detected in the spleen of recipient mice (data not shown), suggesting that homeostatic cell proliferation and in vivo T-cell survival were not affected by lack of STAT3 expression. As shown in Figure 6A and B, a significant proportion of both WT and STAT3-deficient Th2 cells acquired expression of CXCR5 and PD1 upon

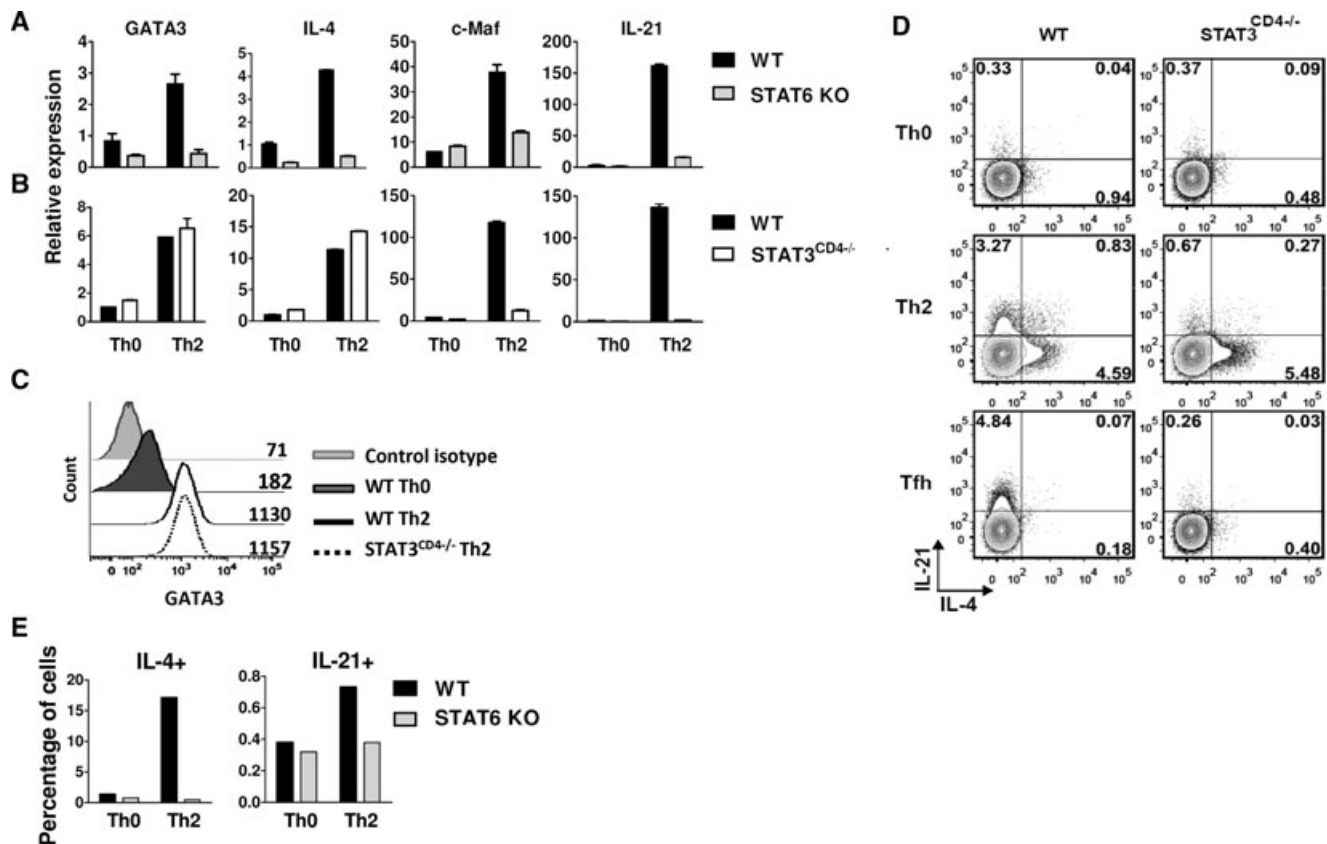


Figure 4. STAT3 is required for the expression of c-Maf and IL-21 upon Th2-cell differentiation. (A, B) RT-PCR analysis of the indicated gene expression by WT, (A) STAT6-KO and (B) STAT3^{CD4}^{-/-} Th cells activated for 48 h (GATA3, IL-4) or 96 h (c-Maf, IL-21) in neutral or Th2 conditions. Data are shown as mean + SD of duplicates and are from one experiment representative of (A) two or (B) three experiments performed. (C) Intracellular GATA3 staining of 48-h polarized WT and STAT3^{CD4}^{-/-} cells. (D) Flow cytometry analysis of IL-4 and IL-21 production. WT and STAT3^{CD4}^{-/-} Th0, Th2, and Tfh cells were activated for 48 h and further expanded for 5 days with IL-2 before restimulation with ionomycin and phorbol 12-myristate 13-acetate. Numbers indicate the percentages of cells in each quadrant. (E) WT and STAT6-KO Th cells were activated as in (D). Data are expressed as the percentage of IL-4⁺ and IL-21⁺ for each Th-cell population. Data shown are representative of two (E) to four (C, D) independent experiments performed.

in vivo transfer and immunization. GATA3 and BCL6 expression levels were further evaluated among CD4⁺CXCR5⁺PD1⁺ cells and found to segregate into GATA3^{int}BCL6^{hi}, GATA3^{hi}BCL6^{int}, and GATA3^{int}BCL6^{hi} subsets (Fig. 6C, right panels), the later subset being absent in CXCR5⁺PD1⁺ cells (data not shown). Notably, lack of STAT3 led to a specific loss of “Tfh-like,” GATA3^{int}BCL6^{hi} lymphocytes, with a concomitant increase in GATA3^{hi}BCL6^{int} cells. Analysis of two independent experiments (Fig. 6D) confirmed the role of STAT3 in regulating the relative expression of BCL6 and GATA3 upon in vivo transfer of cells previously cultured under Th2-polarizing conditions. In keeping with these data, mice reconstituted with STAT3-deficient Th2 cells displayed a severely impaired NP-specific IgG1 response, when compared to control mice reconstituted with STAT3-sufficient Th2 cells (Fig. 6E). Th2 cells lacking STAT3 expression were able to promote the secretion of adequate levels of NP-specific IgE antibodies, suggesting that these cells had not completely lost the capacity to interact with B cells in vivo (Fig. 6F). In agreement with the isotype secretion profile observed, splenocytes from mice reconstituted with STAT3-

deficient Th2 cells displayed reduced levels of IL-21 mRNA while retaining the capacity to express IL-4 when restimulated in vitro in an Ag-dependent fashion (Fig. 6G).

Discussion

Despite a wealth of data associating Th2-cell differentiation to enhanced humoral responses in vivo, the capacity of Th2 cells to deliver B-cell help and the mechanism governing this functional property are still a matter of debate.

A major conclusion from this study is that naïve T cells developing under Th2-prone conditions acquire some features of Tfh cells, including BCL6 and c-Maf expression, IL-21 secretion, and the capacity to promote B-cell responses. The key molecular event underlying this dual Th2/Tfh developmental program appears to be the capacity of T cells stimulated in the presence of exogenous IL-4 to express phosphorylated forms of STAT3. Endogenously produced IL-6 and IL-10 appear as important effectors

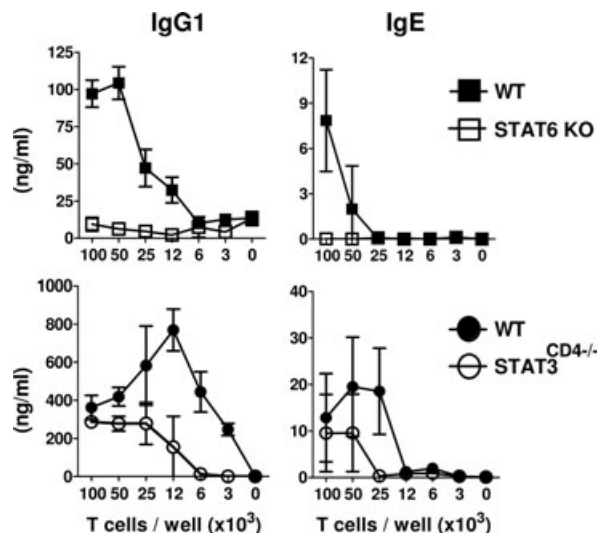


Figure 5. STAT3 and STAT6 contribute to the B-cell help capacity of Th2 cells. Naïve CD62L⁺CD4⁺ T cells from WT, STAT6-KO, or STAT3-KO mice were stimulated under Th2 conditions as in Fig. 1 and incubated with freshly purified naïve B cells (5×10^5 cells/well) and anti-CD3 mAb (500 ng/mL). Supernatants of the T/B-cell cocultures were tested by ELISA on day 7 for IgG1 and IgE. Results are expressed as mean $\pm$ SD of triplicates and are representative of two (top) to four (bottom) independent experiments performed.

linking IL-4/STAT6-regulated responses to STAT3 activation, as illustrated in Fig. 3. IL-6 and IL-10 represent well-described STAT3-signaling cytokines that are produced early during Th2-cell activation [35, 36] and the combined neutralization of IL-6 and IL-10 led to a significant reduction in phospho-STAT3 accumulation in Th2-like cells (Fig. 3E). Of note, loss of IL-6 or IL-10 activity only led to limited effects on STAT3 activation, suggesting functional redundancy between these two cytokines in promoting STAT3 phosphorylation during Th2-cell differentiation. Although IL-21 also represents a Th2-associated, STAT3-signaling cytokine, its neutralization did not affect phospho-STAT3 accumulation in Th2 developing cells (our own unpublished observations). Note also that phosphorylated forms of STAT3 could be detected in our experiment settings before IL-21 production (data not shown). However, once produced, IL-21 might act as a positive loop, to maintain STAT3 signaling, as suggested in a study with human intestinal lamina propria lymphocytes [37].

Despite sharing a common, STAT3-based, signaling pathway, Th2 and “bona fide” Tfh-like cells (i.e. Th cells activated in the presence of IL-6) expressed distinct cytokine profiles, leading to the secretion of selected immunoglobulin isotypes. While both Th2 and Tfh cells promoted the secretion of IgG1 immunoglobulins in vitro, only in vitro differentiated-Th2 were able to promote IgE isotype production, in agreement with their capacity to produce IL-4 and the well-established role of IL-4 in promoting IgE-switched B-cell immunity [38].

Although in vitro-derived Tfh cells did not produce IL-4 (this study and [16]), it should be noticed that Tfh cells are the major producers of IL-4 in secondary lymphoid organs

[12, 14, 15] and recent data elegantly showed that deletion of the *Il4* enhancer hypersensitivity site V resulted in a reduction in IL-4 expression in Tfh that is associated with defective IgG1 and IgE response [39, 40]. Therefore both Tfh and Th2 cells might contribute to IgG1/IgE secretion in vivo, probably acting with B cells at different location (i.e. GC and extra-follicular sites, respectively).

Upon in vivo transfer and immunization, some Th2 cells acquired expression of CXCR5 and PD1 in a STAT3-independent fashion (Fig. 6A and B). Of interest, only STAT3-competent Th2 cells were able to gain expression of the Tfh-cell master transcription factor BCL6, as well as capacity to provide B-cell help for IgG1 secretion in vivo (Fig. 6C–E). These observations are compatible with the concept of substantial plasticity for conversion of Th2 cells into Tfh in vivo, as previously suggested [15, 16] and highlight a major role of STAT3 in the acquisition of Tfh functional features in Th2 cells [16].

It is noteworthy that T-bet expression by Th1 cells opposes Tfh-cell development, suggesting that B-cell help capacity may represent a transitional, and thus unstable, state of developing Th1 cells [17]. In contrast to the transitional nature of the Th1/Tfh state, Th2 cells appear to express a common Th2/Tfh developmental program, compatible with a more stable Tfh-like function. The observation that BCL6 expression correlated with down-modulation of GATA3 expression upon in vivo transfer of Th2 cells (Fig. 6C) is reminiscent of data showing that BCL6 repressed GATA3 expression and transcriptional activity [41, 42]. Further work will be required to investigate whether BCL6 expression (and/or GATA3 down-modulation) during Th2-cell activation is responsible for the acquisition of B helper cell function observed in vivo.

Whether STAT3 plays a functional role in determining Th2 cell fate is still a matter of debate. Our observations are in agreement with previous studies demonstrating the full capacity of mouse and human CD4⁺ T cells lacking normal STAT3 expression to differentiate into IL-4 secreting cells when cultured under Th2 polarizing conditions [43–45] but appear at variance with a recent study from Stritesky et al. [27] describing an obligatory role for STAT3 in Th2-cell development. The reason for this discrepancy remains unresolved at this time, but may be related to the negative influence of BCL6 on GATA3 expression and Th2-cell development [41, 42]. In other words, STAT3 may promote Th2-cell development [27] while concomitantly inducing the expression of the GATA3-antagonistic transcriptional repressor BCL6. The overall influence of STAT3 on Th2-cell development may therefore depend on the relative expression of BCL6, which may vary according to experimental setup and/or timing of assay.

Finally, although optimal B-cell responses seem to be under the control of STAT3-activated helper cells irrespectively of their initial differentiation program (Th1-, Th2-, Th17-, or Tfh-like cells), the isotypic profile appears to be under the influence of the cytokine cocktail produced by the Th cell. Based on the well-known capacity of IL-21 to oppose IgE class switch [8, 46], we would speculate that STAT3-signaling pathways play an important role in limiting secretion of IgE by Ag-stimulated B cells. The

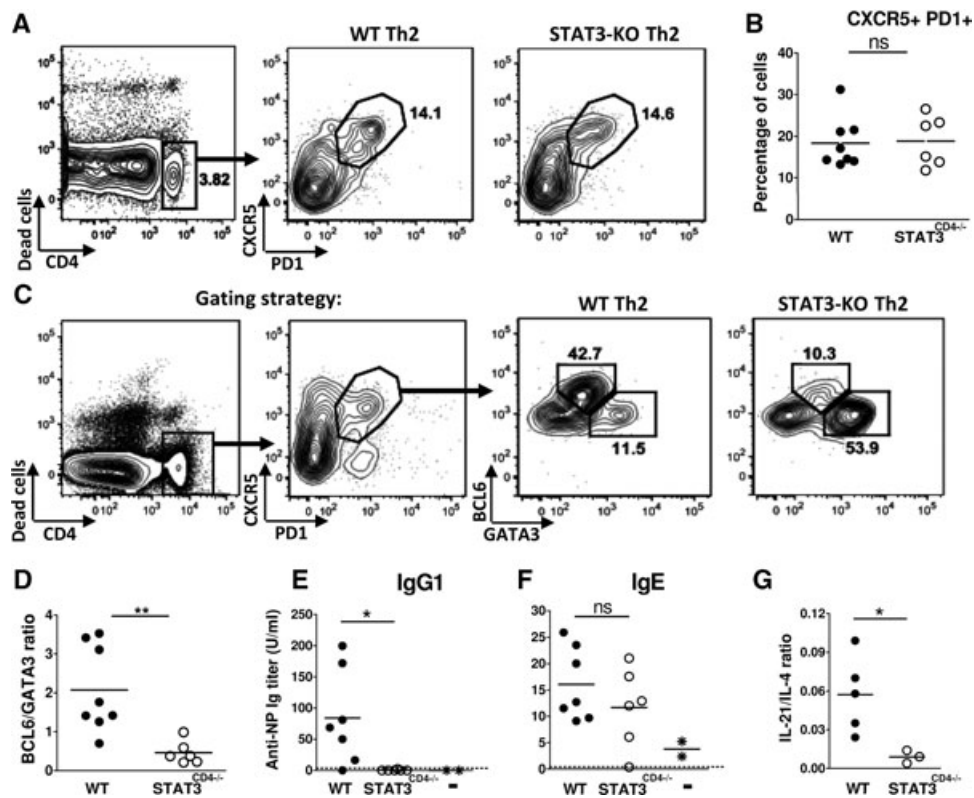


Figure 6. STAT3 differentially regulates IgG1 and IgE isotype secretion in vivo. (A–D) WT and STAT3^{CD4} naive T cells were activated for 72 h under Th2 polarizing conditions before transfer into CD3 ϵ ^{−/−} recipient mice. Recipient mice were immunized 1 day later with NP-KLH in Alum. Draining lymph nodes were analyzed for (A, B) CXCR5/PD1 (nonpermeabilized cells) and (C, D) GATA3/BCL6 (permeabilized cells) expression. (D) The ratio of GATA3^{int}BCL6^{hi}/GATA3^{hi}BCL6^{neg} cells among the Tfh-cell gate for each individual mouse is shown. Each symbol represents an individual mouse and data are pooled from two independent experiments. (E–G) RAG- γ ^{−/−} recipient mice were reconstituted with WT or STAT3^{CD4} Th2 cells together with CD4-depleted splenocytes and immunized as in (A). Individual sera were tested on day 14 for NP-specific (E) IgG1 and (F) IgE. Dashed lines correspond to nonimmune mice. Some recipient mice were sacrificed and their splenocytes were restimulated for 24 h in the presence of KLH (30 μ g/mL). (G) Relative expression of IL-21 to IL-4 was assessed by RT-PCR. These cytokines were not detected in the “CD4-depleted only” recipient mice (not shown). (B, D–G) Statistical differences between groups were analyzed with the Mann–Whitney test for two-tailed data. * $p < 0.05$.

observation that hyper-IgE syndrome (HIES) patients express high levels of IgE is compatible with this assumption.

In conclusion, the present study has established that (i) Th2 cells provide help to naïve B cells both in vitro and in vivo; (ii) similarly to classical Tfh cells, the B-cell helper capacity requires STAT3 expression, and (iii) B-cell helper function is autonomously acquired due to the capacity of developing Th2 cells to secrete STAT3-activating cytokines. We believe that the capacity to modulate STAT3 signaling in vivo may provide novel clues to develop long-term humoral immunity in response to vaccines and pave the way to innovative treatments of allergic diseases.

Materials and methods

Media and reagents

The medium used throughout this study was RPMI 1640 or IMDM supplemented with 5% fetal calf serum (FCS), penicillin,

streptomycin, glutamine, nonessential aa, 1 mM sodium pyruvate, and 0.05 mM 2-mercaptoethanol. Anti-CD3, anti-CD28 were produced in house. Anti-IFN- γ mAb (XMG1.2), anti-IL-10 mAb (JES5–2A5), recombinant hIL-2, mIL-4, and mIL-6 were purchased from eBioscience (San Diego, CA, USA). Anti-IL-10R mAb (1B1.3a) was purchased from BD Pharmingen (Erembodegem, Belgium).

Mice, adoptive transfer, and immunization

Six- to eight-week-old C57BL/6 and Balb/c mice were purchased from Harlan (Horst, The Netherlands). IL-6-KO (C57BL/6 background) and STAT6-KO (Balb/c background) mice were from The Jackson Laboratory (Bar Harbor, ME, USA). CD3 ϵ -KO mice were from EMMA (CDTA, Orléans, France). STAT3^{flox/flox} mice (on a C57BL/6 background) were kindly provided by Dr. Shizuo Akira (Osaka University, Osaka, Japan), CD4-CRE (C57BL/6 background) mice by Dr. Geert Van Loo (University of Gent, Gent, Belgium) and RAG2^{−/−} γ ^{−/−} mice by Dr. James Di Santo

(Necker Hospital, Paris, France) [47]. STAT3^{flax/flax} and CD4-CRE mice were bred to generate T-cell compartment-specific STAT3-deficient mice (referred as STAT3^{CD4-/-}). All experiments using animals were done with the approval of the institute review board.

For adoptive transfers, CD3ε^{-/-} or RAG2^{-/-}γc^{-/-} (cotransferred with 10⁷ CD4-depleted WT splenocytes to provide a source of naïve B cells) mice were given 10⁷ in vitro-derived Th2 cells (from WT or STAT3^{CD4-/-} mice) intravenously, 24 h before immunization. Mice were immunized by injecting intraperitoneally 75 μg nitrophenyl-keyhole limpet hemocyanin (NP₂₅-KLH, Biosearch Technologies, Novato, CA, USA) with 3 mg of Imject Alum (Thermo Fisher Scientific, Rockford, IL, USA). Serum levels of NP-specific antibodies were determined by enzyme-linked immunosorbent assay (ELISA) according to standard procedures using mouse isotype-specific rat monoclonal antibodies (IMEX, Université Catholique de Louvain, Brussels, Belgium).

Cell purification

CD62L^{hi}CD4⁺ T cells were purified from naïve animals by magnetic separation, as previously described [48]. B cells were isolated by negative selection with anti-CD43-coupled magnetic beads (Miltenyi Biotech, Bergisch-Gladbach, Germany). The percentage of purified cell fractions in all experiments ranged between 90 and 98%, as estimated by flow cytometry (data not shown).

Priming of naïve T cells

Naïve CD62L^{hi} CD4⁺ T cells (10⁶ cells/well in 24-well plates) were preactivated for 48 h with plastic-coated anti-CD3 mAb (5 μg/mL) and soluble anti-CD28 mAb (1 μg/mL). Tfh-like cells were differentiated by the addition of IL-6 (20 ng/mL) and Th2 polarization was initiated by the addition of IL-4 (10 ng/mL) and anti-IFN-γ mAbs (10 μg/mL). T cells were recovered, extensively washed and rested 24 h in medium before being tested for cytokine secretion and B-cell help capacity. In some experiments, Th cells were further expanded for 3–5 days in the presence of IL-2 (10 ng/mL). Efficacy of polarizations was verified by intracellular cytokine staining or ELISA upon secondary stimulations (data not shown).

For QPCR experiments, Th cells were activated for 48–96 h upon polarizing condition and directly subjected to RNA extraction.

B-cell help assay

Serial dilutions of CD4⁺ T-cell populations were cultured for 7 days with purified syngeneic B cells (5 × 10⁵ cells/well) in the presence of anti-CD3 mAb (500 ng/mL). T cells were irradiated (2000 cGy) before the beginning of the coculture with B cells to prevent their outgrowth during the 7-day culture, as previously described [23]. IgG1 and IgE antibodies in the supernatants were

determined by ELISA, using rat monoclonal anti-mouse isotype antibodies as described [48].

Flow cytometry

Specific cell-surface staining was performed using a standard procedure with anti-CD4, anti-PD1 (eBioscience), and anti-CXCR5 mAbs (BD Pharmingen).

For intracellular cytokine staining, primed cells were restimulated for 4 h with phorbol 12-myristate 13-acetate (50 ng/mL) and ionomycin (250 ng/mL) (Sigma-Aldrich) in the presence of monensin (1/1000) (eBioscience). Cells were fixed and permeabilized with BD Cytotfix/Cytoperm kit (BD Pharmingen) and stained in a two-step procedure using APC-conjugated anti-mouse IL-4 (BD Pharmingen) and recombinant mouse IL-21R Subunit – Human Fc Chimera (R&D Systems, Minneapolis, MN, USA) followed by phycoerythrin (PE)-conjugated anti-human IgG (Jackson ImmunoResearch, West Baltimore Pike, PA, USA).

For intracellular phospho-STAT staining, differentiated T cells were collected and fixed with 4% formaldehyde for 10 min at 37°C. After fixation, cells were permeabilized for 30 min on ice with 90% methanol. Cells were then stained for phospho-STAT3 or phospho-STAT6 (BD Pharmingen) for 30 min at room temperature. Intracellular GATA3 and BCL6 (Ab from BD Pharmingen)-staining was performed according to the FoxP3 staining set protocol (eBioscience), according to the manufacturer's protocol. Cells were analyzed by flow cytometry with an FACS Canto II (BD Biosciences) and analyzed with the FlowJo Software.

RT-PCR

Total cellular RNA was extracted from cell lysates by the use of TRIzol reagent, and RT of mRNA was carried out using Superscript II reverse transcriptase (Invitrogen) according to manufacturer's instructions. Quantitative PCR was performed on StepOne Plus device (Applied Biosystems, Foster City, CA, USA) with Maxima SYBR Green/ROX qPCR Master Mix (Thermo Fisher Scientific, Waltham, MA, USA). Quantification (with RPL32 as endogenous housekeeping gene) was calculated using standard curves. Primer sequences are available on request.

Statistical analyses

Differences between groups were analyzed with the Mann-Whitney test for two-tailed data. A *p*-value less than 0.05 was considered as significant.

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Abbreviations: Tfh: follicular T helper · STAT: signal transducer and activator of transcription

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