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STAT5 programs a distinct subset of GM-CSF-producing T helper cells that is essential for autoimmune neuroinflammation

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T helper (T_H)-cell subsets, such as T_H1 and T_H17, mediate inflammation in both peripheral tissues and central nervous system. Here we show that STAT5 is required for T helper-cell pathogenicity in autoimmune neuroinflammation but not in experimental colitis. Although STAT5 promotes regulatory T cell generation and immune suppression, loss of STAT5 in $CD4^+$ T cells resulted in diminished development of experimental autoimmune encephalomyelitis (EAE), a mouse model of multiple sclerosis. Our results showed that loss of encephalitogenic activity of STAT5-deficient autoreactive $CD4^+$ T cells was independent of IFN-γ or interleukin 17 (IL-17) production, but was due to the impaired expression of granulocyte-macrophage colony-stimulating factor (GM-CSF), a crucial mediator of T-cell pathogenicity. We further showed that IL-7-activated STAT5 promotes the generation of GM-CSF-producing $CD4^+$ T cells, which were preferentially able to induce more severe EAE than T_H17 or T_H1 cells. Consistent with GM-CSF-producing cells being a distinct subset of T_H cells, the differentiation program of these cells was distinct from that of T_H17 or T_H1 cells. We further found that IL-3 was secreted in a similar pattern as GM-CSF in this subset of T_H cells. In conclusion, the IL-7-STAT5 axis promotes the generation of GM-CSF/IL-3-producing T_H cells. These cells display a distinct transcriptional profile and may represent a novel subset of T helper cells which we designate as T_H-GM.

***Keywords*:** T helper cell; experimental autoimmune encephalomyelitis; GM-CSF; IL-7; STAT5

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Introduction

In response to antigenic assault, naïve $CD4^+$ T cells differentiate into various subsets of effector T helper (T_H) cells with differential cytokine production profiles and distinct functions [1-3]. Inflammatory cytokines direct the differentiation of antigen-specific $CD4^+$ T cells by inducing the expression of subset-specific transcription factors [1]. For instance, interleukin 12 (IL-12) activates STAT4 and induces T-bet expression, which promotes T_H1 differentiation and IFN-γ production [4-6]. IL-6 signaling through STAT3, in concert with TGF-β, induces RORγt expression and initiates the differentiation of T_H17 cells [7-9], which is further enhanced by TNF-α, IL-23, and IL-1β [10].

By coordinating both innate and adaptive effector cell activities, $CD4^+$ T cells including T_H1, T_H2, and T_H17 play critical roles in host defense against infectious agents and in the pathogenesis of various autoimmune

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diseases [11]. For example, both T_H1 and T_H17 cells are considered major mediators of autoimmune neuroinflammation in multiple sclerosis (MS) and experimental autoimmune encephalomyelitis (EAE) [2, 12, 13]. However, mice deficient in IFN-γ or IL-12 (p35) show exacerbated EAE development, whereas mice deficient in IL-23 (p19) are resistant to EAE [14-17]. Loss of RORγt or STAT3, the master regulators of T_H17 cells, attenuates the development of EAE [8, 18]. These studies suggest that T_H17, rather than T_H1 cells, are the main encephalitogenic population in autoimmune neuroinflammation. However, the role of T_H17 in MS and EAE is still in debate since none of the T_H17-hallmark cytokines, including IL-17, IL-17F, and IL-22, is mandatory for EAE development [12, 19, 20]. More recently, granulocyte-macrophage colony-stimulating factor (GM-CSF) secreted by autoreactive T cells was identified as a potential encephalitogenic factor to sustain neuroinflammation [21-23].

STAT5 transmits IL-2 signal and is crucial for regulatory T (T_{reg}) cell development [24], but also negatively regulates T_H17 differentiation [25, 26]. However, its function in T cell-mediated autoimmune diseases has not been well documented. In this study, surprisingly, we found that STAT5 was indispensible for the encephalitogenicity of autoreactive $CD4^+$ T cells in EAE. Further investigation showed that IL-7-STAT5 signaling axis induced optimal GM-CSF production in pathogenic $CD4^+$ T cells, which was important for inducing effective neuroinflammation. *In vitro* studies showed that GM-CSF-producing $CD4^+$ T cells regulated by IL-7-STAT5 signaling axis may represent a new T_H subset with a distinct differentiation program and cytokine production profile.

Results

Mice with Stat5 deletion in T cells are resistant to EAE

To examine the role of STAT5 in T cell-mediated pathogenesis, we induced EAE in *Cd4-Cre*; $Stat5^{f/f}$ ($Stat5^{-/-}$) mice [27], where *Stat5a/b* loci were specifically deleted in $CD4^+$ and $CD8^+$ T cells, and $Stat5^{f/f}$ ($Stat5^{+/+}$) mice. We found diminished incidence and severity of EAE disease in $Stat5^{-/-}$ mice compared with $Stat5^{+/+}$ mice (Figure 1A and Supplementary information, Figure S1A and S1B), which was opposite to our expectation based on an inhibitory role of STAT5 in T_H17 generation. Consistent with EAE resistance, we found a remarkable reduction of immune cell infiltration in the CNS of $Stat5^{-/-}$ mice (Figure 1B and Supplementary information, Figure S1C-S1F). However, the frequencies of IL-17^+ and IFN-γ^+ cells among $CD4^+$ T cells in the central nervous system (CNS) were comparable between $Stat5^{+/+}$ and $Stat5^{-/-}$ mice (Figure 1C), suggesting that the resistance to EAE in $Stat5^{-/-}$ mice is independent of T_H1 and T_H17 cells. Furthermore, we detected decreased $CD4^+CD25^+$ population and reduced Foxp3 expression in $Stat5^{-/-}$ mice (Figure 1D and Supplementary information, Figure S2A), indicating the resistance to EAE is unlikely due to altered T_{reg} cell development.

Intrinsic defect in encephalitogenicity of STAT5-deficient $CD4^+$ T cells

To examine whether T cell-specific deletion of *Stat5* resulted in peripheral lymphopenia, we analyzed T cell populations in spleens of MOG_{35-55}/CFA-immunized mice. Consistent with a previous report [28], we detected reduced $CD8^+$ T cell number but similar number of $CD4^+$ T cells in $Stat5^{-/-}$ mice compared with $Stat5^{+/+}$ mice (Supplementary information, Figure S2B and S2C). Furthermore, we detected increased frequencies of both IL-17^+ and IFN-γ^+ $CD4^+$ T cells in the spleens of $Stat5^{-/-}$ mice (Supplementary information, Figure S2D). To validate the function of STAT5 in T_H1 and T_H17 generation, we performed *in vitro* T-cell differentiation. As reported [25, 26], STAT5 mediated the suppressive effect of IL-2 on T_H17 differentiation (Supplementary information, Figure S3A and S3B). STAT5 deficiency led to slightly decreased T_H1-cell generation (Supplementary information, Figure S3C). Therefore, the resistance to EAE in $Stat5^{-/-}$ mice is unlikely due to impaired T_H1- and T_H17-cell generation *in vivo*.

To address whether the resistance to EAE in $Stat5^{-/-}$ mice is caused by STAT5 deficiency in $CD4^+$ T cells, we reconstituted $Rag2^{-/-}$ mice with $Stat5^{+/+}$ or $Stat5^{-/-}$ $CD4^+$ T cells followed by EAE induction. We found that $Rag2^{-/-}$ mice receiving $Stat5^{-/-}$ $CD4^+$ T cells were resistant to the disease compared with mice receiving $Stat5^{+/+}$ $CD4^+$ T cells (Supplementary information, Figure S4A and S4B), demonstrating that $Stat5^{-/-}$ $CD4^+$ T cells were impaired in mediating EAE development. The expression of chemokine receptors such as CCR6 and CXCR3, which are critical for T_H17 or T_H1 cell entry into the CNS [29, 30], was not impaired in $Stat5^{-/-}$ $CD4^+$ T cells (Supplementary information, Figure S5A), indicating that $CD4^+$ T cells with STAT5 deficiency are likely capable of infiltrating the CNS. Consistent with this, we observed comparable numbers of $CD4^+$ T cells in the CNS of $Stat5^{+/+}$ and $Stat5^{-/-}$ mice before disease onset (on days 7 and 9) (Supplementary information, Figure S5B). However, during the later phase, significantly more $CD4^+$ T cells accumulated in the CNS of $Stat5^{+/+}$ mice compared with $Stat5^{-/-}$ mice (Supplementary information, Figure S5C). These results indicate that $Stat5^{-/-}$ $CD4^+$ T cells can infiltrate the CNS but fail to induce

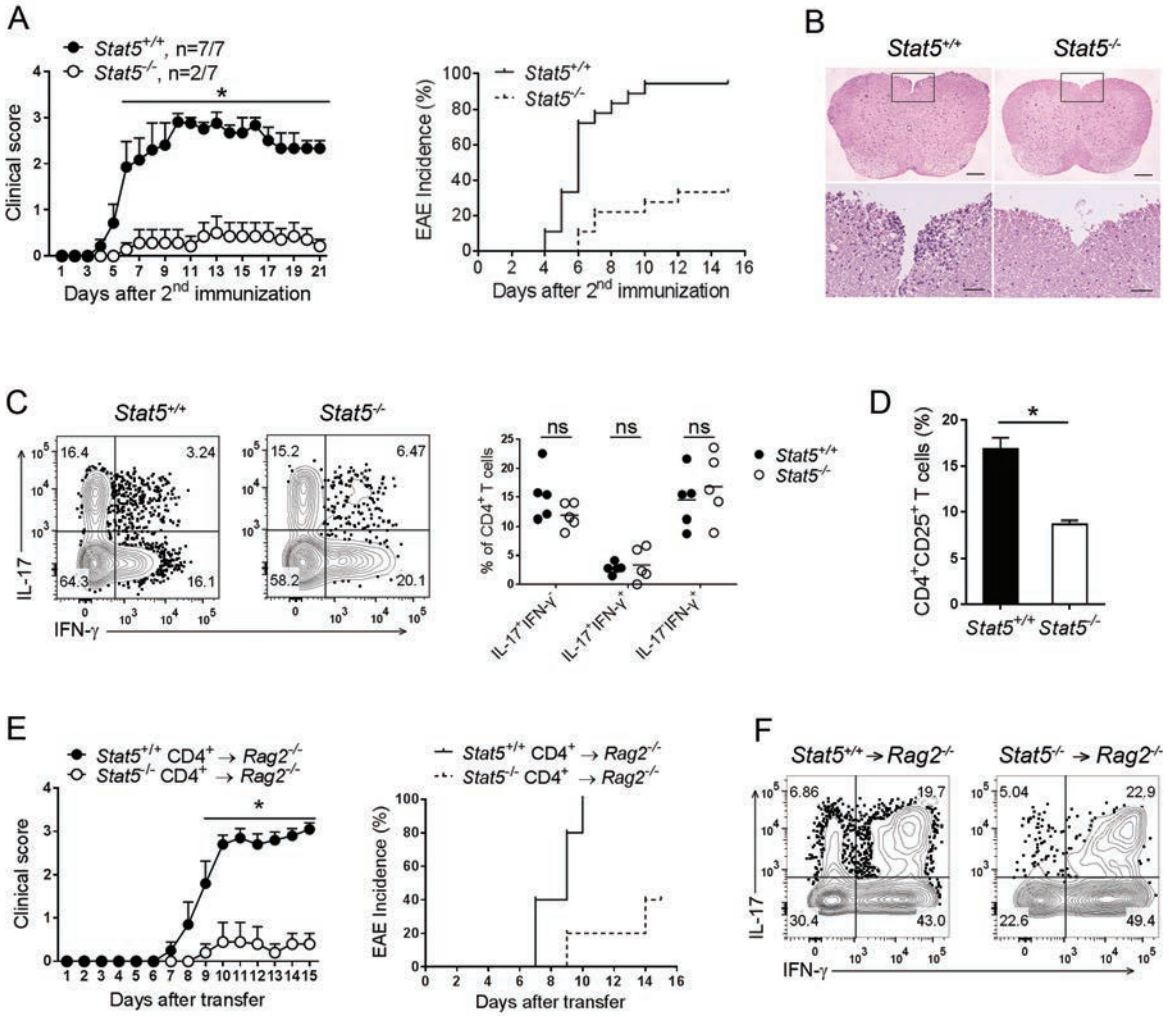


Figure 1 *Stat5*-conditional knockout mice are resistant to EAE. **(A)** Clinical EAE scores (left) and incidence (right, *n* = eighteen of three experiments pooled) of *Stat5*$^{+/+}$ and *Stat5*$^{-/-}$ mice immunized twice with MOG_{35-55}/CFA. **(B)** Histology of spinal cord sections obtained from EAE mice on day 9 after 2nd immunization. Scale bars, 200 μm (top), 50 μm (bottom). **(C)** Flow cytometric analysis of IL-17 and IFN-γ expression by CNS-infiltrating CD4$^+$ T cells at peak of disease. **(D)** Percentage of CD4$^+$CD25$^+$ T cells in the CNS at peak of disease (*n* = 3). **(E, F)** Clinical EAE scores (**E**, left) and incidence (**E**, right) of *Rag2*$^{-/-}$ mice (*n* = 5 per group) after adoptive transfer of 2 × 10^6 MOG_{35-55}-reactive *Stat5*$^{+/+}$ or *Stat5*$^{-/-}$ CD4$^+$ T cells, respectively. IL-17 and IFN-γ expression by CNS-infiltrating CD4$^+$ T cells was measured by intracellular cytokine staining at the peak of disease **(F)**. Data represent at least two independent experiments. **P* < 0.05; ns, not significant.

effective inflammatory responses. To further exclude the possibility that EAE resistance is due to reduction in *Stat5*$^{-/-}$ CD4$^+$ T cell number in the CNS, we transferred more *Stat5*$^{-/-}$ CD4$^+$ T cells than *Stat5*$^{+/+}$ cells into *Rag2*$^{-/-}$ mice followed by MOG_{35-55}/CFA immunization, so that comparable numbers of autoreactive CD4$^+$ T cells were present in the CNS after disease onset. We still observed reduced disease severity in mice receiving *Stat5*$^{-/-}$ CD4$^+$ T cells (Supplementary information, Figure S6A and S6B). Together, these results suggest that the resistance to EAE disease caused by STAT5 deficiency in CD4$^+$ T cells is unlikely due to impaired CD4$^+$ T cell infiltration or survival in the CNS.

To confirm that EAE resistance caused by STAT5 deficiency is due to intrinsic impairment of autoreactive CD4$^+$ T cells, we isolated CD4$^+$ T cells from MOG_{35-55}/CFA-immunized mice and transferred *ex vivo*-expanded MOG_{35-55}-reactive *Stat5*$^{+/+}$ and *Stat5*$^{-/-}$ CD4$^+$ T cells into *Rag2*$^{-/-}$ mice separately without additional immunization. Mice receiving *Stat5*$^{+/+}$ cells developed EAE 1 week after the transfer (Figure 1E). In contrast, mice receiving *Stat5*$^{-/-}$ CD4$^+$ T cells had significantly reduced disease

severity and incidence (Figure 1E). Of note, the frequencies of IL-17$^+$ and/or IFN-γ$^+$ cells among CD4$^+$ T cells in the CNS were comparable between the two groups (Figure 1F). CD4$^+$ and CD8$^+$ T cell co-transfer experiments demonstrate that the resistance to EAE observed in *Stat5*$^{-/-}$ mice was not due to CD8$^+$ T cells (Supplementary information, Figure S7A and S7B). Therefore, *Stat5*$^{-/-}$ CD4$^+$ T cells are intrinsically defective in encephalitogenicity, independent of T_H1- and T_H17-cell generation.

STAT5 deficiency in CD4$^+$ T cells causes impaired expression of GM-CSF

Communication between CNS-infiltrating CD4$^+$ T cells and myeloid cells is critical for inducing effective neuroinflammation [21, 22]. GM-CSF production by encephalitogenic CD4$^+$ T cells, not other types of cells, is essential for microglial cell activation, peripheral myeloid cell recruitment and EAE development [23]. To test whether GM-CSF production was impaired upon *Stat5* depletion, we examined GM-CSF expression in MOG_{35-55}-specific CD4$^+$ T cells. We found that GM-CSF production was robustly increased in a dose-dependent manner in *Stat5*$^{+/+}$, but not in *Stat5*$^{-/-}$ cells, upon antigen re-stimulation (Figure 2A). Antigen-specific CD4$^+$ T cells with STAT5 deficiency contained significantly reduced percentages of GM-CSF-producers in both IL-17$^+$ and IL-17$^-$ populations (Figure 2B). Of note, the frequency of IL-17-producing CD4$^+$ T cells was increased with STAT5 deficiency (Figure 2B). Together, these results suggest that STAT5 is required for GM-CSF expression by antigen-specific CD4$^+$ T cells.

Next, we examined GM-CSF expression in the CNS during EAE development. Although IL-17 and IFN-γ expression by CNS-infiltrating *Stat5*$^{-/-}$ CD4$^+$ T cells was not impaired (Figure 1C), we detected a significantly diminished frequency of CD4$^+$GM-CSF$^+$ cells in the CNS of *Stat5*$^{-/-}$ mice compared with control mice (Figure 2C). Similarly, in passive EAE induction, *Rag2*$^{-/-}$ mice transferred with STAT5-deficient MOG_{35-55}-reactive CD4$^+$ T cells also showed a reduced frequency of CD4$^+$GM-CSF$^+$ T cells in the CNS compared with mice transferred with wild-type (WT) cells (Figure 2D). Time-course analysis of cytokine induction in the whole CNS tissues showed that GM-CSF mRNA expression in *Stat5*$^{+/+}$ mice was markedly increased as early as day 8 after MOG_{35-55}/CFA immunization, whereas GM-CSF induction in *Stat5*$^{-/-}$ mice was significantly diminished (Figure 2E). Meanwhile, no significant difference in IL-17 or IFN-γ expression was detected between *Stat5*$^{-/-}$ and *Stat5*$^{+/+}$ mice on day 8 post-immunization (Figure 2E). The reduced IL-17 expression in the CNS of *Stat5*$^{-/-}$ mice at a later stage (day 14, Figure 2E) could be explained by the inability of *Stat5*$^{-/-}$ CD4$^+$ T cells to induce effective neuroinflammation with a result of decreased inflammatory cell infiltration (Supplementary information, Figures S1 and S5C). We also observed the expression level of IL-23, an important inflammatory cytokine mainly produced by dendritic cells (DCs) [16], was reduced in the CNS of *Stat5*$^{-/-}$ mice compared with *Stat5*$^{+/+}$ mice, paralleling decreased IL-17 expression level (Figure 2E). Interestingly, the expression level of IL-23 in the CNS of *Stat5*$^{+/+}$ mice was only significantly increased on day 14 after disease induction (Figure 2E), suggesting that IL-23 might not be required for GM-CSF expression and EAE induction at the early stage. Together, these results demonstrate that STAT5 deficiency in CD4$^+$ T cells results in impaired GM-CSF expression, which is associated with EAE resistance.

IL-7-STAT5 signaling induces GM-CSF expression in autoreactive CD4$^+$ T cells

We next investigated the possible cytokine(s) that signal through STAT5 to regulate GM-CSF expression. We stimulated CD4$^+$ T cells with IL-23 and IL-1β, two cytokines that drive GM-CSF expression in T_H17 cells [21, 22]. We found that neither IL-23 nor IL-1β was able to induce STAT5 activation (Figure 3A). Furthermore, IL-1R1 expression was not changed, whereas IL-23Rα expression was increased in *Stat5*$^{-/-}$ CD4$^+$ T cells (Figure 3B), indicating that STAT5-mediated GM-CSF expression is unlikely dependent on IL-23 and IL-1β signaling. In contrast, both IL-2 and IL-7 potently activated STAT5 (Figure 3A). Therefore, we further examined the roles of these two cytokines in GM-CSF induction in CD4$^+$ T cells. Splenocytes were isolated from MOG_{35-55}/CFA-immunized mice before disease onset and challenged with MOG_{35-55} alone versus in the presence of IL-2 or IL-7 *ex vivo*. We did not detect an obvious effect of IL-2 on the frequency of GM-CSF-producing cells in CD4$^+$CD44^{hi} population (Supplementary information, Figure S8). In contrast, IL-7 significantly increased the frequency of GM-CSF-producing cells in CD4$^+$CD44^{hi} population and GM-CSF secretion in a STAT5-dependent manner (Figure 3C and 3D).

IL-7Rα is expressed by naïve and effector CD4$^+$ T cells, suggesting that IL-7 may directly act on both populations to regulate GM-CSF expression. To address this, sorted CD62L^{hi}CD44^{lo} (naïve) and CD62L^{lo}CD44^{hi} (effector) CD4$^+$ T cells from *Stat5*$^{-/-}$ and *Stat5*$^{+/+}$ mice during EAE development were activated by anti-CD3 plus anti-CD28 in the presence or absence of IL-7, followed by GM-CSF expression examination. As shown in Figure 3E, CD62L^{lo}CD44^{hi} T cells expressed GM-CSF more robustly than CD62L^{hi}CD44^{lo} cells. IL-7 promoted

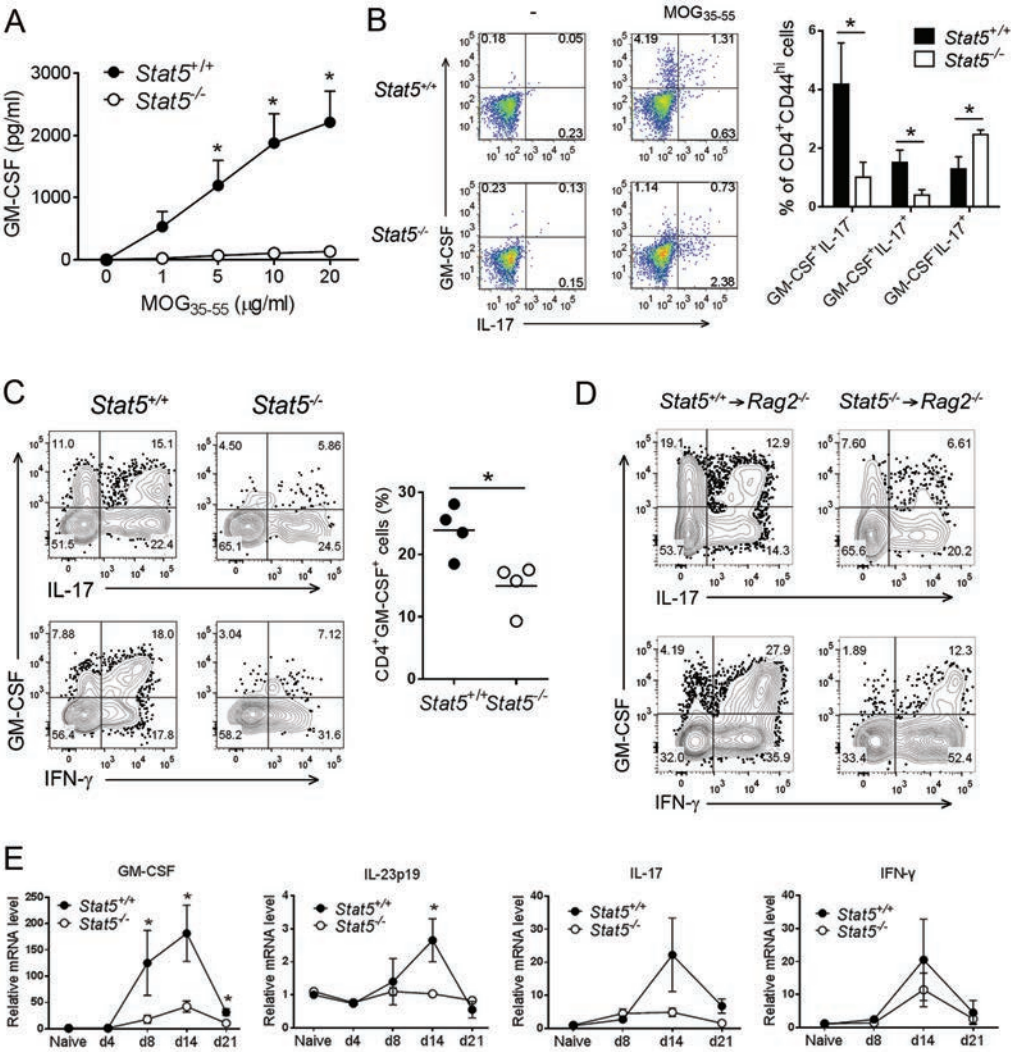


Figure 2 Diminished induction of GM-CSF in *Stat5*$^{-/-}$ CD4$^+$ T cells. **(A**, **B)** Splenocytes were obtained from MOG_{35-55}/CFA-immunized *Stat5*$^{+/+}$ and *Stat5*$^{-/-}$ mice (n = 3 per group) before disease onset and challenged with MOG_{35-55} at various concentrations for 24 h. GM-CSF secretion was measured by ELISA **(A)**. Golgiplug was added in the last 4 h of MOG_{35-55} (20 μg/ml) challenge and the frequencies of IL-17$^+$ and GM-CSF$^+$ cells among CD4$^+$CD44^{hi} T cells were measured **(B)**. **(C)** IL-17, IFN-γ and GM-CSF expression by CNS-infiltrating CD4$^+$ T cells of *Stat5*$^{+/+}$ and *Stat5*$^{-/-}$ mice was measured by intracellular cytokine staining at peak of disease. **(D)** IL-17, IFN-γ, and GM-CSF expression by CNS-infiltrating CD4$^+$ T cells of *Rag2*$^{-/-}$ recipient mice at peak of EAE induced by adoptive transfer of MOG_{35-55}-reactive CD4$^+$ T cells. **(E)** CNS tissues were collected from naïve or MOG_{35-55}/CFA-immunized mice for RNA extraction (n = 3 per group at each time point). Time-course analysis of cytokine mRNA expression was performed with RT-PCR. The RT-PCR data were normalized to Rn18S, and expression in naïve mice was set to 1. Data represent two independent experiments. *P < 0.05.

GM-CSF expression in both cell subsets, which was abrogated by STAT5 deficiency (Figure 3E).

Consistent with the facilitating effect of IL-7-dependent T-cell differentiation on EAE, mice treated with an IL-7Rα-specific antibody (clone SB/14) during EAE development showed a significant reduction of disease severity accompanied with reduced CNS inflammation, but without T cell depletion [31] (Supplementary information, Figure S9A-S9C). Notably, blocking IL-7 signaling resulted in decreased GM-CSF expression in CNS-infiltrating CD4$^+$ T cells (Supplementary information, Figure S9D-S9F). These findings demonstrate that IL-7 induces STAT5 activation to promote GM-CSF expression in autoreactive CD4$^+$ T cells, which is critical for the development of neuroinflammation.

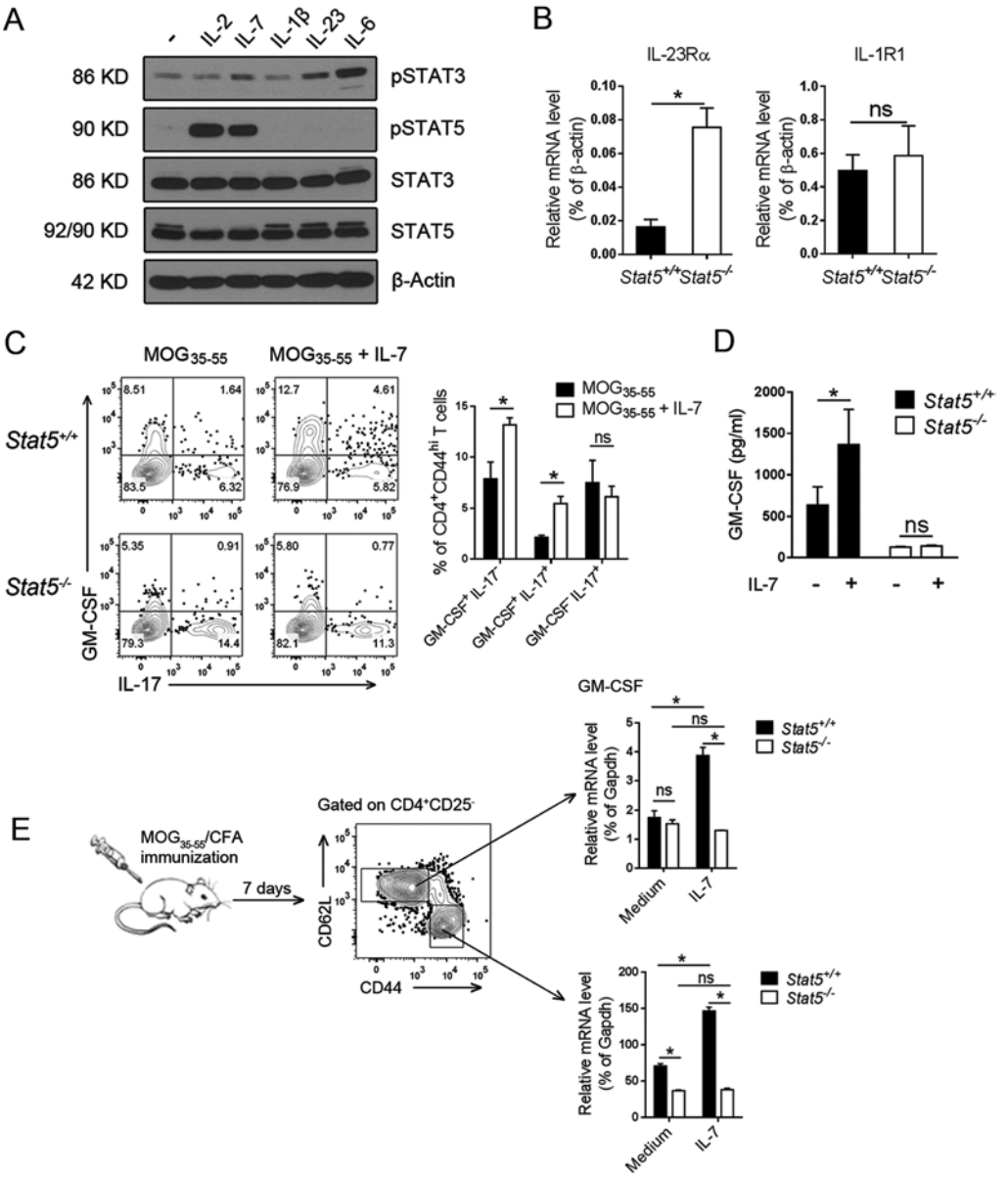


Figure 3 IL-7 promotes GM-CSF expression through STAT5 activation in autoreactive $CD4^+$ T cells. **(A)** Purified $CD4^+$ T cells were cultured with TGF-β and IL-6 for 3 days, followed by resting for 6 h. Then cells were treated with various cytokines for 30 min, and pSTAT3 and pSTAT5 levels were determined by immunoblotting. STAT3 and STAT5 were further detected after stripping. **(B)** The mRNA expression of IL-23Rα and IL-1R1 in splenic $CD4^+$ T cells of *Stat5*$^{+/+}$ and *Stat5*$^{-/-}$ EAE mice (n = 3). **(C**, **D)** Splenocytes were obtained from MOG_{35-55}/CFA-immunized *Stat5*$^{+/+}$ and *Stat5*$^{-/-}$ mice before disease onset and challenged with MOG_{35-55} (20μg/ml) in the absence or presence of IL-7 for 48 h. Frequencies of GM-CSF$^+$ and IL-17$^+$ cells among $CD4^+CD44^{hi}$ T cells were measured by intracellular cytokine staining and flow cytometry **(C)**. Right panel in **C** shows overall frequencies of GM-CSF$^+$ and IL-17$^+$ cells in *Stat5*$^{+/+}$ group (n = 3). GM-CSF secretion was measured by ELISA **(D)**. Data represent two independent experiments with three mice per group. **(E)** Splenic $CD62L^{hi}CD44^{lo}$ and $CD62L^{lo}CD44^{hi}$ T cells from MOG_{35-55}/CFA-immunized mice were sorted out. Cells were stimulated with anti-CD3 and anti-CD28 in the absence or presence of IL-7 for 4 h and then harvested for the analysis of GM-CSF expression by RT-PCR. *P < 0.05; ns, not significant.

T_H17 or T_H1 differentiation condition inhibits GM-CSF expression

To further understand IL-7/STAT5-mediated GM-CSF expression in $CD4^+$ T cells, we stimulated naïve $CD4^+$ T cells with various conditions. We found that anti-CD3 together with anti-CD28 induced the expression of both

GM-CSF and IFN-γ (Supplementary information, Figure S10A). Interestingly, both T_H1 (IL-12 + anti-IL-4) and T_H17 (blocking both IFN-γ and IL-4 in combination with TGF-β + IL-6 or IL-6 + IL-23 + IL-1β) differentiation conditions greatly suppressed the expression of GM-CSF (Figure 4A and 4B). Conversely, neutralization of both IL-12 and IFN-γ promoted the generation of GM-CSF-producing cells, consistent with a previous report [21], which was not affected by IL-23 and IL-1β (Figure 4A). In addition to TGF-β-mediated inhibition of GM-CSF expression [22], we found that IL-6, an essential cytokine for T_H17 differentiation, had a profound inhibitory effect on GM-CSF expression (Figure 4C), indicating that STAT3 could be a negative regulator of GM-CSF expression. We used STAT3-deficient $CD4^+$ T cells to test this hypothesis. As expected, naïve *Stat3*$^{-/-}$ $CD4^+$ T cells were impaired in T_H17 differentiation (Supplementary information, Figure S10B). In WT cells, T_H17 differentiation condition (anti-IFN-γ + anti-IL-4 + IL-6 + IL-23 + IL-1β) greatly inhibited GM-CSF expression (Figure 4A). However, deficiency of STAT3 abrogated the inhibitory effect of IL-6 on GM-CSF expression (Supplementary information, Figure S10B). Interestingly, even without exogenous IL-6, STAT3 exhibited a suppressive effect on GM-CSF expression as *Stat3*$^{-/-}$ cells showed increased GM-CSF expression compared to WT cells (Supplementary information, Figure S10B). In addition, GM-CSF expression in $CD4^+$ T cells is independent of RORγt and T-bet [22]. Thus, our data support that differentiation of GM-CSF-producing $CD4^+$ T-cell is distinct from T_H1 or T_H17.

IL-7-STAT5 promotes GM-CSF-producing T_H-cell differentiation

Our findings above suggest the possibility of a potential new T_H cell subset that is regulated by IL-7-STAT5 signaling. To further test this possibility, we investigated GM-CSF-producing T_H cell differentiation *in vitro* by activating naïve $CD4^+$ T cells with anti-CD3 and anti-CD28 in the presence of different concentrations of IL-7. We found that addition of 0.5 ng/ml IL-7 greatly increased the frequency of GM-CSF-producing cells and the secretion of GM-CSF, which were further increased upon increase in IL-7 concentration (1 ng/ml) (Figure 4D and 4E). Without STAT5, IL-7 was unable to promote the generation of GM-CSF-producing cells (Figure 4F and 4G). Chromatin immunoprecipitation (ChIP) analysis showed that IL-7 activated STAT5 directly bound to promoter regions of the *Csf2* gene (Supplementary information, Figure S11A and S11B). We noticed the presence of a small proportion of IFN-γ-producing cells in this condition (Figure 4D). Therefore, we included IFN-γ-blocking antibody in the culture and found that a combination of IL-7 and anti-IFN-γ induced the highest frequency of $GM\text{-}CSF^+$ cells, where few $IL\text{-}17^+$ or $IFN\text{-}\gamma^+$ cells were detected (Figure 4H). Therefore, the *in vitro* generation of GM-CSF-producing T_H cells requires the transcription factor STAT5, optimal concentration of IL-7, and IFN-γ neutralization in addition to TCR and CD28 signaling.

GM-CSF-producing T_H cells represent a potential new subset distinct from T_H1 or T_H17

To further characterize GM-CSF-producing T_H cells, we differentiated T_H1, T_H17, and GM-CSF-producing T_H cells from naïve $CD4^+$ T cells *in vitro*. The expression of RORγt and T-bet was examined. We found that unlike T_H1 or T_H17 cells, the expression of T-bet or RORγt was minimal in GM-CSF-producing T_H cells (Figure 5A). Next, we performed microarray analysis to examine gene expression profiles of T_H1, T_H17, and GM-CSF-producing T_H cells. We identified a list of 202 genes preferentially expressed in T_H1 cells compared with naïve, T_H17 and GM-CSF-producing T_H cells, among which IFN-γ, Gzmb, and T-bet were on the top of the list (Figure 5B, left panel and Supplementary information, Table S1). Similarly, T_H17 feature genes, including IL-17, IL-17F, RORγt, and RORα, were identified in the list of 411 genes specific to T_H17 cells (Figure 5B, middle panel, and Supplementary information, Table S1). The GM-CSF-producing T_H cell-specific gene list contains 210 genes with genes encoding GM-CSF and IL-3 as the top genes in the list (Figure 5B, right panel and Supplementary information, Table S1).

Next, we further verified the microarray findings. Cytokine expression analysis showed that GM-CSF was predominately expressed in GM-CSF-producing T_H cells compared with T_H1 or T_H17 cells (Figure 5C and 5D). Interestingly, IL-3, a cytokine that is coregulated with GM-CSF [32], was also highly expressed in GM-CSF-producing T_H cells, not T_H1 or T_H17 cells (Figure 5D). Further examination showed that IL-3 expression was also regulated by IL-7-STAT5 signaling (Figure 5E and 5F).

IL-2, which also signals through STAT5, did not promote GM-CSF-producing T_H differentiation (Supplementary information, Figure S12A), possibly due to the lack of IL-2Rα expression on naïve $CD4^+$ T cells and thus the unresponsiveness of STAT5 to the IL-2 signal early in differentiation (Supplementary information, Figure S12B-S12D). To further confirm this possibility, we stimulated activated $CD4^+$ T cells with IL-2 or IL-7, and found both cytokines induced STAT5 activation, STAT5 binding to *Csf2* promoter, and increase in GM-CSF mRNA levels (Supplementary information, Figure S13A-S13C). Notably, IL-2 induced a prolonged STAT5

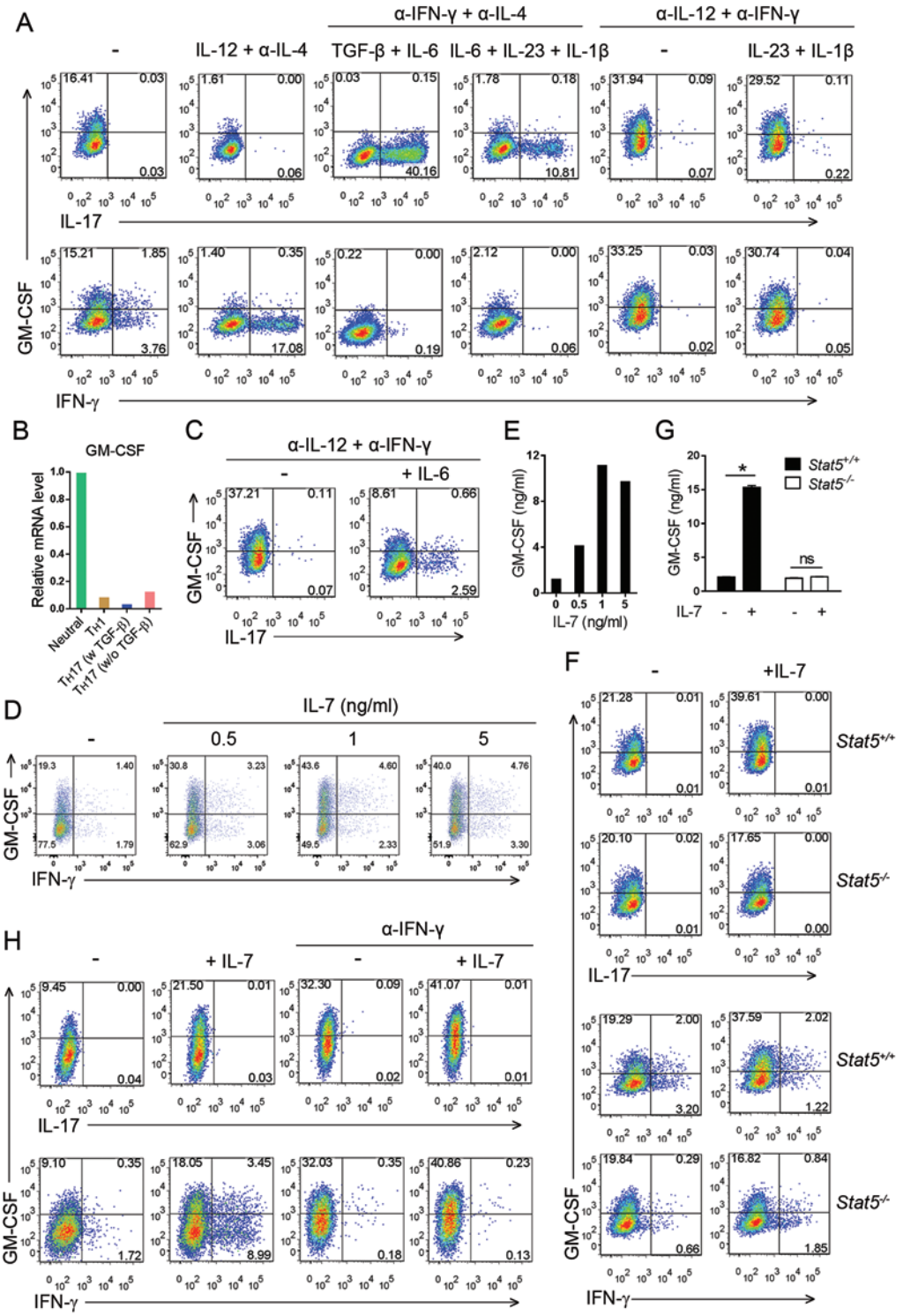
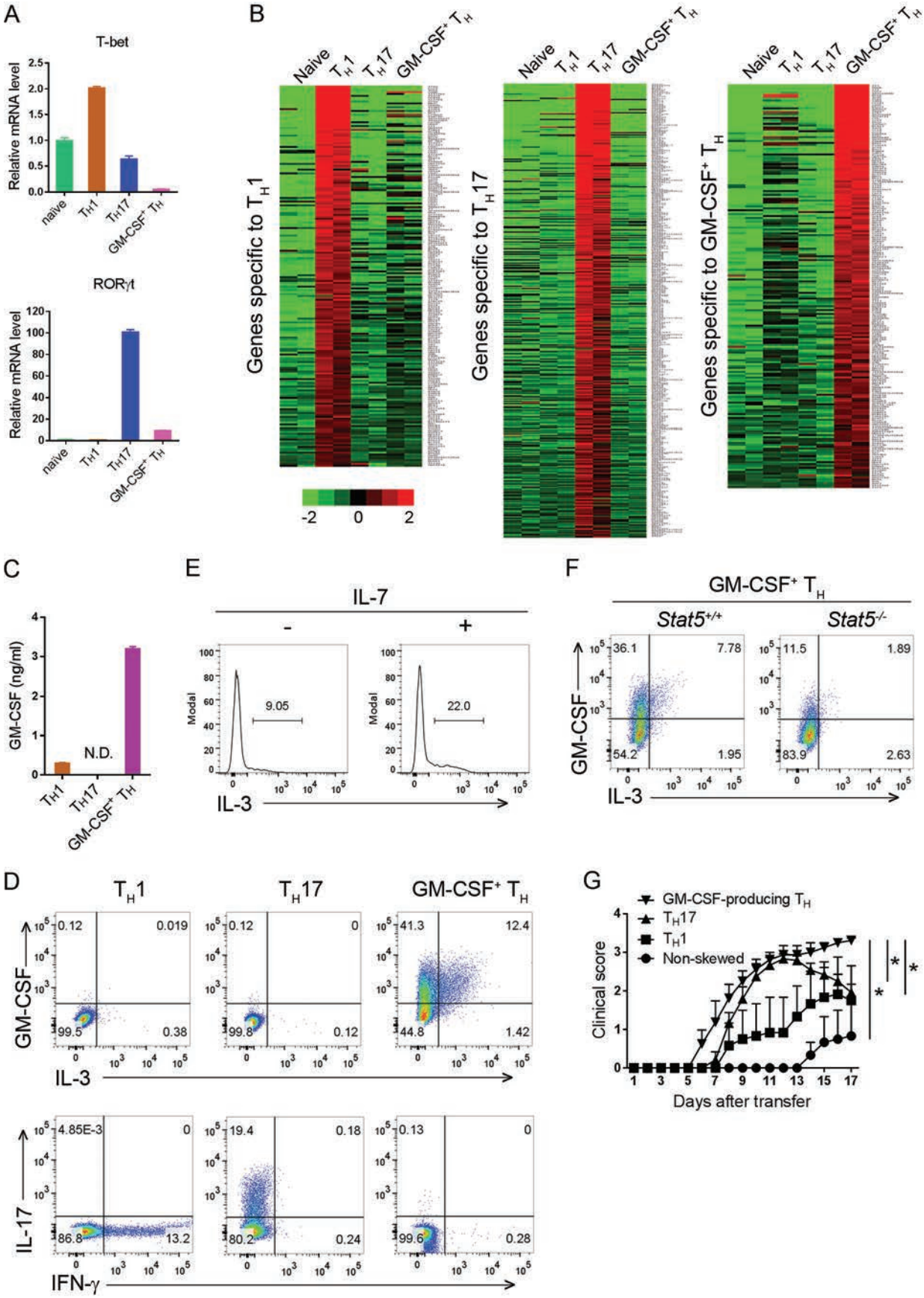


Figure 4 Regulation of GM-CSF-producing T_H cells *in vitro*. **(A-E)** Naïve $CD4^+$ T cells were primed with plate-bound anti-CD3 and soluble anti-CD28 in the presence of a combination of various cytokines and neutralizing antibodies as indicated. GM-CSF, IL-17, and IFN-γ expression was analyzed by intracellular cytokine staining **(A**, **C**, **D)**, RT-PCR **(B)** or ELISA **(E)**. (**F**, **G**) *Stat5*$^{+/+}$ and *Stat5*$^{-/-}$ naïve $CD4^+$ T cells were activated with anti-CD3 and anti-CD28 in the presence IL-7 for 3 days. GM-CSF, IL-17, and IFN-γ expression was analyzed by intracellular cytokine staining **(F)**. GM-CSF secretion was measured by ELISA **(G)**. **(H)** Naïve $CD4^+$ T cells were activated with anti-CD3 and anti-CD28 in the presence of IL-7 or/and anti-IFN-γ as indicated. GM-CSF, IL-17, and IFN-γ expression was analyzed. Data represent more than two independent experiments. **P* < 0.05; ns, not significant.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-
+
Modal
9.05
22.0
IL-3
F
GM-CSF+ TH
Stat5+/+
Stat5-/-
GM-CSF
IL-3
G
GM-CSF-producing TH
TH17
TH1
Non-skewed
Clinical score
Days after transfer

activation compared with IL-7 (Supplementary information, Figure S13A).

To test the hypothesis that the GM-CSF-producing T_H subset is the primary encephalitogenic effector cells, we performed adoptive transfer of different subsets of MOG_{35-55}-reactive $CD4^+$ T cells into *Rag2*$^{-/-}$ mice for EAE induction. As shown in Figure 5G, GM-CSF-producing T_H cells were preferentially able to induce a more robust EAE compared with T_H17 and T_H1 subsets.

Together, these data demonstrate that IL-7-STAT5-signaling controls the differentiation of a new T helper cell subset that is distinct from T_H1 or T_H17 and predominantly expresses GM-CSF and IL-3.

STAT5-deficient CD4$^+$ T cells retain the capacity to induce colitis

The finding from the EAE model prompted us to test whether STAT5-deficient T cells also lacked pathogenicity in other T cell-mediated autoimmune diseases. We assessed the pathogenic potential of STAT5-deficient autoreactive $CD4^+$ T cells in colitis by reconstituting *Rag2*$^{-/-}$ mice with *Stat3*$^{-/-}$, *Stat5*$^{-/-}$ or WT $CD4^+CD25^-CD45RB^{hi}$ naïve T cells. Consistent with a previous report [33], mice reconstituted with *Stat3*$^{-/-}$ T cells continued to gain weight, whereas mice receiving *Stat5*$^{-/-}$ or WT T cells lost weight (Figure 6A), and showed enlarged spleens and mesenteric lymph nodes (MLNs) (Figure 6B). Marked colonic inflammation and inflammatory cell infiltration in the colon were observed in mice that received either WT or *Stat5*$^{-/-}$ T cells at 8 weeks after reconstitution (Figure 6C and 6D). Consistent with the resistance to colitis in mice receiving *Stat3*$^{-/-}$ cells, an obviously reduced frequency of $CD4^+$ T cells and IL-17-producing $CD4^+$ T cells in their lamina propria lymphocytes (LPLs) was observed compared with mice receiving *Stat5*$^{-/-}$ or WT cells (Figure 6D and 6E). There was no defect in either IL-17 or IFN-γ production by $CD4^+$ T cells in LPLs of mice reconstituted with *Stat5*$^{-/-}$ T cells (Figure 6E). However, we detected a significant reduction in GM-CSF-producing T cells in the absence of STAT5 when transferred into *Rag2*$^{-/-}$ mice (Figure 6E). Therefore, GM-CSF-producing $CD4^+$ T cells might not play a critical role in colitis. This view was further supported by the observation that although there were comparable GM-CSF$^+$ cells in *Stat3*$^{-/-}$ and WT-transferred T cells, *Stat3*$^{-/-}$ $CD4^+$ T cells failed to induce colitis.

Discussion

Effector T_H cells, differentiated from naïve T cells after TCR-mediated antigen recognition with the influence of costimulation and the instruction from specific cytokines, are classified by their specific cytokine expression and immune-modulatory functions [1, 3, 34]. Here we have demonstrated that IL-7, signaling through STAT5, induces the development of a potential new T_H subset that predominantly expresses GM-CSF (GM-CSF-producing T_H cells). In addition to GM-CSF, we identified that this T_H subset highly expresses IL-3, a cytokine important in regulating the function of myeloid-derived immune cells. The development of GM-CSF-producing T_H cells is independent of the mechanisms required for T_H1 or T_H17 development. In fact, conditions for T_H1 or T_H17 differentiation suppressed the development of GM-CSF-producing T_H cells. It is known that differentiated effector T_H cells could produce specific cytokines to create a cytokine environment that favors the differentiation of their own while suppressing the differentiation of other T_H subsets. For instance, IL-12 as well as IFN-γ induces T_H1 differentiation. The differentiated T_H1 cells produce large amounts of IFN-γ to amplify T_H1 differentiation, whereas they suppress T_H17 or GM-CSF-producing T_H differentiation. It is possible that the GM-CSF-producing T_H cells produce certain cytokines to promote the differentiation of their own, but suppress the differentiation of T_H1 and/or T_H17 cells. It is also possible that this new subset of T_H cells produce cytokines such as GM-CSF to enhance T_H1 or T_H17 responses via inducing the production of inflammatory cytokines from myeloid cells. Further studies are needed to explore such possibilities.

While this manuscript was under submission, a report was published, showing that IL-17 and GM-CSF expres-

Figure 5 Distinct features of GM-CSF-producing T_H cells. **(A)** The mRNA expression of T-bet and RORγt in naïve, T_H1 (IL-12 + anti-IL-4), T_H17 (TGF-β + IL-6 + anti-IFN-γ + anti-IL-4) and GM-CSF-producing T_H (IL-7 + anti-IFN-γ) cells. The RT-PCR data were normalized to Gapdh, and expression in naïve T cells was set to 1. **(B)** Naïve $CD4^+$ T cells were differentiated into T_H1, T_H17 and GM-CSF-producing (GM-CSF$^+$) T_H cells *in vitro*. Microarray analysis was performed to examine their gene expression profiles. Hierarchical clusters of preferentially expressed genes for T_H1, T_H17, or GM-CSF-producing T_H cells were shown (biological duplication). **(C)** GM-CSF secretion by three T_H subsets *in vitro*. **(D)** Flow cytometric analysis of cytokine expression (GM-CSF, IL-3, IL-17, and IFN-γ) by three T_H subsets *in vitro*. **(E)** Frequency of IL-3$^+$ cells generated with or without IL-7. **(F)** GM-CSF and IL-3 expression by WT or STAT5-deficient GM-CSF-producing T_H cells. **(G)** Clinical EAE scores of *Rag2*$^{-/-}$ mice (n = 3-6 mice per group) after adoptive transfer of 6×10^5 various MOG_{35-55}-reactive T_H subsets. Data represent two independent experiments. *P < 0.05.

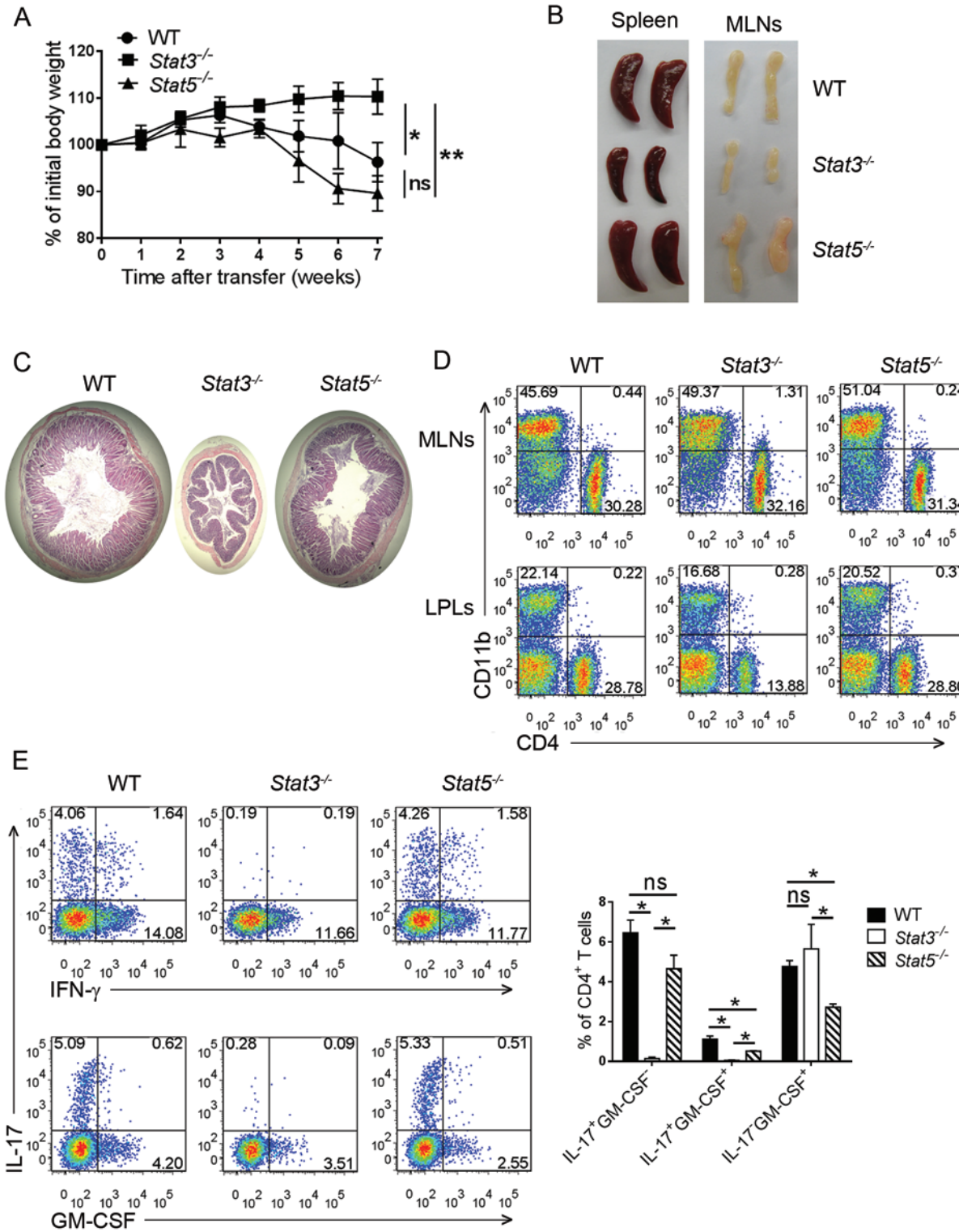

Figure 6 STAT5 is not required in T cell-dependent colitis. **(A)** *Rag2*$^{-/-}$ mice were reconstituted with CD4^{+}CD25^{-}CD45RB^{hi} naïve T cells derived from WT, *Stat3*$^{-/-}$ or *Stat5*$^{-/-}$ mice (3-5 mice per group). Body weight loss was monitored and calculated over 7 weeks. Data are representative of two independent experiments. **(B)** Systemic inflammation was assessed by comparing size of spleen and MLNs 8 weeks after transfer. **(C)** Colonic inflammation was assessed histologically. Images shown are representative of three mice per group. **(D)** Percentages of CD4^{+} T cells and CD11b^{+} cells in MLNs and LPLs were analyzed by flow cytometry. Data are representative of at least three mice per group. **(E)** IL-17, IFN-γ, and GM-CSF production by CD4^{+} T cells in LPLs was measured. **P* < 0.05, ***P* < 0.005.

sion in human T_H cells are antagonistically regulated [35]. Similar to our finding in mice, Noster *et al*. [35] found that GM-CSF-producing T_H cells represent a substantial population in the cerebrospinal fluid of MS patients, suggesting a pathogenic role of these cells in neuroinflammation. Our work utilizing the mouse model for a thorough functional study has provided clear genetic evidence showing the essential role of STAT5 in GM-CSF and IL-3 production in this novel T_H cell subset. This subset is essential, even in the context of normal T_H1 or T_H17 activity, for the pathogenesis of EAE. We propose to tentatively refer to this T helper cell subset as T_H-GM, which is critically regulated by STAT5 and predominantly produces GM-CSF and IL-3.

GM-CSF governs the activities of myeloid-derived cell populations and is implicated in various inflammatory and autoimmune diseases such as rheumatoid arthritis (RA) and MS. Therapeutic interventions targeting GM-CSF, such as Mavrilimumab (human anti-GM-CSFRα Ab) and MOR103 (human anti-GM-CSF mAb), are under phase 2 clinical trial in RA and RA/MS, respectively (*clinicaltrials.gov*). IL-3 is a cytokine also involved in several autoimmune diseases [36-39]. Therefore, directly targeting this new subset of T_H cells, the dominant source of such pathogenic mediators, in various autoimmune diseases could lead to better outcomes than targeting a single factor, such as GM-CSF or IL-3.

STAT5 transmits IL-2 signals. A previous widely accepted model is that T-cell survival and proliferation require IL-2, based on studies using *in vitro* systems. There is now much evidence arguing that IL-2 is dispensable for the induction of T cell-dependent immunity *in vivo* [40]. In our EAE model, we indeed observed *Stat5*-conditional knockout mice had fewer $CD4^+$ T cells in the CNS after disease onset as STAT5-deficient $CD4^+$ T cells had inability to induce effective inflammatory responses. However, in EAE induced by $CD4^+$ T cell transfer, STAT5-deficient $CD4^+$ T cells with a number comparable to that of WT cells in the CNS still failed to induce the disease (Supplementary information, Figure S6A and S6B). These results suggest that the role of STAT5 in $CD4^+$ T cell survival and proliferation is unlikely the major causal factor of EAE resistance in *Stat5*-conditional knockout mice.

IL-7, signaling through the common γc and IL-7Rα, is required for T cell homeostasis [41]. A previous report showed that IL-7 could acutely stimulate GM-CSF production from memory T cells [42]. Here, our work demonstrates that IL-7 through STAT5 activation induces the generation of pathogenic T_H-GM cells for GM-CSF production in mediating neuroinflammation. Our finding is in line with the association of IL-7Rα variants with MS in patient GWAS and the beneficial effect of IL-7Rα-neutralizing antibody in EAE treatment [43-45]. A recent report indicates IL-7 promotes IFN-γ production by T_H1 cells and contributes to a T_H1-driven subtype of MS [45]. However, mice deficient in IFN-γ show exacerbated EAE development [15]. Thus, the requirement for IL-7 in EAE development is unlikely due to its role in promoting IFN-γ production.

The function of IL-23, a cytokine required for T_H17 terminal differentiation [46], in EAE suggests the involvement of T_H17 cells in this disease [16]. However, the major cytokines produced by T_H17 cells, IL-17A, IL-17F, and IL-22, were found to be dispensable for the development of EAE [19, 20]. However, IL-23 was found to induce the expression of GM-CSF in T_H17 cells, a factor essential for encephalitogenicity of T cells [21, 22], which seems to strengthen the link between T_H17 cells and EAE. Paradoxically, a recent study reported IL-23/RORγt axis-suppressed GM-CSF expression in human T_H cells [35]. Moreover, anti-IL-23 receptor antibodies that worked well for treating peripheral inflammation such as psoriasis failed in treating MS [47], suggesting that the function of IL-23 in MS could be compensated by other factors. In this study, we found that T_H-GM cells produced greater amounts of GM-CSF than T_H1 or T_H17 cells (Figure 5), suggesting that T_H-GM cells are the major source of GM-CSF in T cell-mediated neuronal inflammation. Interestingly, we found that the expression of IL-23p19 in CNS during EAE development only occurred after the onset of the disease and *Stat5* gene deletion in T cells abolished its expression (Figure 2E), suggesting that IL-23 is not required for the initiation of EAE and signaling transduced by STAT5 is required for IL-23 expression in CNS in neuronal inflammation. It is possible that GM-CSF-producing T_H-GM cells provide GM-CSF to induce the expression of IL-23 from DCs, macrophages, and other CNS-residential cells to sustain the inflammation. Therefore, our study does not exclude a scenario where GM-CSF-producing T_H-GM cells cooperate with T_H1 and/or T_H17 to mediate the development of EAE. Further study on T_H-GM cells including their physiological functions is warranted for targeting these cells for the development of therapeutic interventions for human inflammatory diseases such as MS.

It is interesting to see that STAT5-regulated T_H-GM cells are not required in a T-cell transfer model of colitis (Figure 6). EAE represents sterile CNS inflammation, in which autoreactive $CD4^+$ T cells are the major producers of GM-CSF, a factor crucial for EAE pathogenesis [23]. However, numerous inflammatory cytokines, such as T_H1-related IFN-γ and T_H17-related IL-17F, are implicated in the pathogenesis of colitis [48]. STAT5-deficient

$CD4^+$ T cells are not impaired in T_H1 or T_H17 generation. In addition, GM-CSF could be induced in other types of cells in the intestine, such as Paneth cells [49]. Thus, STAT5-mediated GM-CSF production in T cells may not be mandatory for colitis.

In summary, we found that a distinct subset of T helper cells (T_H-GM), which is regulated by IL-7-STAT5 signaling axis and predominantly produces GM-CSF and IL-3, is critical for autoimmune neuroinflammation.

Materials and Methods

Mice

Stat5$^{f/f}$ mice were provided by L Hennighausen (National Institute of Diabetes and Digestive and Kidney Diseases) [27]. *Stat3*$^{f/f}$ mice were generated as described [50]. *Cd4-Cre* transgenic mice were purchased from Taconic Farms. *Rag2*$^{-/-}$ mice were obtained from Jean-Pierre Abastado (Singapore Immunology Network). All mice are on a C57BL/6 genetic background and housed under specific-pathogen-free conditions at the National University of Singapore. All experiments were performed with 6-8-week-old mice and approved by the Institutional Animal Care and Use Committee of NUS.

In vitro T-cell differentiation

$CD4^+$ T cells were obtained from spleens and lymph nodes by positive selection and magnetic separation (Miltenyi Biotec), followed by purification of naïve $CD4^+$ T cell population ($CD4^+CD25^-CD62L^{hi}CD44^{lo}$) sorted with FACS Aria. Naïve $CD4^+$ T cells were stimulated with plate-bound anti-CD3 (3 μg/ml; BD Pharmingen) and anti-CD28 (1 μg/ml; BD Pharmingen) in the presence of different combinations of neutralizing antibodies and cytokines for 3-4 days: for neutral conditions, no addition of any cytokine or neutralizing antibody; for T_H1 conditions, IL-12 (10 ng/ml), and anti-IL-4 (10 μg/ml, BD Pharmingen); for T_H17 conditions, hTGF-β (3 ng/ml), IL-6 (20 ng/ml), anti-IFN-γ (10 μg/ml, eBioscience), and anti-IL-4 (10 μg/ml); for an alternative T_H17 conditions, IL-6 (20 ng/ml), IL-23 (10 ng/ml), IL-1β (10 ng/ml), anti-IFN-γ (10 μg/ml), and anti-IL-4 (10 μg/ml). For GM-CSF-producing cell differentiation, naïve $CD4^+$ T cells were stimulated with plate-bound anti-CD3 (3 μg/ml) and soluble anti-CD28 (1 μg/ml) with the addition of IL-7 (2 ng/ml) and/or anti-IFN-γ (10 μg/ml) as indicated. All cytokines were obtained from R&D Systems. All cells were cultured in RPMI 1640 supplemented with 10% FBS, 100 units/ml penicillin, 0.1 mg/ml streptomycin, 1 mM sodium pyruvate, 0.1 mM nonessential amino acid and 5 μM beta-mercaptoethanol. After polarization for 3-4 days, cells were washed and restimulated with phorbol 12-myristate 13-acetate (PMA) and ionomycin in presence of Golgiplug for 4-5 h, followed by fixation and intracellular staining with a Cytofix/Cytoperm kit from BD Pharmingen. Foxp3 staining was done with a kit from eBioscience. Cells were acquired on the LSR II (BD Biosciences) and analyzed with FlowJo software (Tree Star).

EAE induction

EAE induction procedures were modified from a previous report [51]. For active EAE induction, mice were immunized in two sites on the hind flanks with 300 μg MOG_{35-55} in 100 μl CFA containing 5 mg/ml heat-killed *M. tuberculosis* strain H37Ra (Difco) on day 0 and day 7. Pertussis toxin (List Bio Lab) was administrated intraperitoneally at the dosage of 500 ng per mouse on day 1 and day 8. For single MOG_{35-55}/CFA immunization, the similar procedure was performed on day 0 and day 1 only. In an alternative active EAE induction, LPS (600 μg/ml in IFA, O111:B4 from Sigma) was used as adjuvant. For active EAE induction in *Rag2*$^{-/-}$ mice, $CD4^+$ T cells derived from *Stat5*$^{f/f}$ or *Cd4-Cre; Stat5*$^{f/f}$ mice were transferred, followed by MOG_{35-55}/CFA immunization as described above. Clinical symptoms were scored as follows: 0, no clinical sign; 1, loss of tail tone; 2, wobbly gait; 3, hind limb paralysis; 4, hind and fore limb paralysis; 5, death. IL-7Rα neutralizing antibody (SB/14, BD Pharmingen) and isotype control was administrated intraperitoneally at 200 μg per mouse every other day. For analysis of CNS-infiltrating cells, both spinal cord and brain were collected and minced from perfused mice, and mononuclear cells were isolated by gradient centrifuge with Percoll (GE Healthcare).

For passive EAE induction with *Stat5*$^{+/+}$ or *Stat5*$^{-/-}$ $CD4^+$ T cells, splenocytes and LNs were harvested 10-14 days post-immunization and passed through a 70 μm cell strainer (BD Falcon). Cells were cultured *in vitro* for 3 days with MOG_{35-55} (20 μg/ml) in the presence of IL-23 (5 ng/ml) and IL-1β (2 ng/ml). After harvesting, $CD4^+$ T cells were purified by positive selection to a purity > 90%. $CD4^+$ T cells (2×10^6 in sterile PBS) were injected intraperitoneally into *Rag2*$^{-/-}$ mice, followed by Pertussis toxin administration on the following day. Mice were observed daily for the signs of EAE as described above. For EAE induction by transferring various T_H subsets, similar procedures were performed as described above. Different subsets skewing conditions were as follows: Non-skewed, MOG_{35-55} only; T_H1: MOG_{35-55} plus IL-12 (10 ng/ml) and anti-IL-4 (5 μg/ml); T_H17: MOG_{35-55} plus TGF-β (3 ng/ml), IL-6 (10 ng/ml), anti-IFN-γ (5 μg/ml) and anti-IL-4 (5 μg/ml); GM-CSF-producing T_H: MOG_{35-55} plus IL-7 (5ng/ml), and anti-IFN-γ (5 μg/ml). 6×10^5 $CD4^+$ T cells were transferred per recipient mouse.

T-cell transfer model of colitis

$CD4^+$ T cells were isolated from spleens and lymph nodes of WT, *Cd4-Cre; Stat3*$^{f/f}$ and *Cd4-Cre; Stat5*$^{f/f}$ mice by positive selection and magnetic separation (Miltenyi Biotech). Naïve $CD4^+$ T ($CD4^+CD25^-CD45RB^{hi}$) cells were sorted with BD FACS Aria (purify > 98%). *Rag2*$^{-/-}$ mice were reconstituted with 4×10^5 naïve $CD4^+$ T cells via intraperitoneal injection. Intestine inflammation was monitored up to 8 weeks. Cell suspensions were prepared from spleen, MLNs, and colon lamina propria by methods modified from previous report [52].

Histological analysis

For paraffin-embedded tissues, spinal cords, or colons were fixed in 4% PFA. Sections (5 μm) were stained with hematoxylin and eosin (H&E) to assess immune cell infiltration and inflammation. For frozen tissues, spinal cords were embedded in OCT (Tissue-Tek) and snap frozen on dry ice. Sections (10 μm) were fixed in ice-cold acetone and stained with primary anti-CD4 (Biolegend) and anti-CD11b (eBioscience), followed by incubation with fluorescence-conjugated secondary antibodies (Invitrogen).

Real-time PCR

Total RNA was extracted from cells with RNeasy kit (Qiagen) according to the manufacturer's instruction. Complementary DNA

(cDNA) was synthesized with Superscript reverse transcriptase (Invitrogen). Gene expressions were measured by 7500 real-time PCR system (Applied Biosystems) with SYBR qPCR kit (KAPA). Actinb, Gapdh, or Rn18S was used as internal control. The primer sequences are available upon request.

Microarray assay

For microarray analysis, RNA from naïve T cells, T_H1, T_H17 and GM-CSF-producing T_H cells was purified with RNeasy kit (Qiagen). Hybridization targets were amplified and labeled using Applause WT-Amp ST System according to the manufacturer's protocol (NuGEN). Labeled cDNA was hybridized to Affymetrix GeneChip Mouse Gene 1.0 ST according to the manufacturer's instructions. All microarray raw data (CEL files) were analyzed together using the Robust Multichip Average method to obtain the gene expression intensities. Normalization was then performed across all samples based on the cross correlation method [53]. Normalized data were further log2-tranformed and were used for identification of differentially expressed or T_H-cell specific genes. The cutoff fold change threshold of 1.5 was used for differential expression.

ELISA

GM-CSF level was assayed by Ready-SET-Go ELISA kit (eBioscience) according to the manufactures' instructions.

Chromatin immunoprecipitation assays

$CD4^+$ T cells isolated from *Stat5*$^{f/f}$ or *Cd4-Cre; Stat5*$^{f/f}$ mice were activated with plate-bound anti-CD3 and anti-CD28 for 3 days. Cells were stimulated with IL-7 (20 ng/ml) or IL-2 (25 ng/ml) for 45 min. Crosslink was performed by addition of formaldehyde at final concentration of 1% for 10 min followed by quenching with glycine. Cell lysates were fragmented by sonication and precleared with protein G Dynabeads, and subsequently precipitated with anti-STAT5 antibody (Santa Cruz) or normal rabbit IgG (Santa Cruz) overnight at 4 °C. After washing and elution, crosslink reversal was done by incubating at 65 °C for 8 h. The eluted DNA was purified and analyzed by RT-PCR with primers specific to *Csf2* promoter as described previously [54].

Statistics

Statistical significance was determined by Student's *t*-test using GraphPad Prism 6.01. $P < 0.05$ was considered significant. The *P* values of clinical scores were determined by one-way multiple-range analysis of variance (ANOVA) for multiple comparisons. Unless otherwise specified, data were presented as mean ± SEM.

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