Stat3 Programs Th17-Specific Regulatory T Cells to Control GN

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ABSTRACT

A pathogenic role for Th17 cells in inflammatory renal disease is well established. The mechanisms underlying their counter-regulation are, however, largely unknown. Recently, Th17 lineage-specific regulatory T cells (Treg17) that depend on activation of the transcription factor Stat3 were identified. We studied the function of Treg17 in the nephrotoxic nephritis (NTN) model of crescentic GN. The absence of Treg17 cells in Foxp3Cre3Stat3fl/fl mice resulted in the aggravation of NTN and skewing of renal and systemic immune responses toward Th17. Detailed analysis of Stat3-deficient Tregs revealed that the survival, activation, proliferation, and suppressive function of these cells remained intact. However, Tregs from Foxp3Cre3Stat3fl/fl mice lacked surface expression of the chemokine receptor CCR6, which resulted in impaired renal trafficking. Furthermore, aggravation of NTN was reversible in the absence of Th17 responses, as shown in CD4Cre3Stat3fl/fl mice lacking both Treg17 and Th17 cells, suggesting that Th17 cells are indeed the major target of Treg17 cells. Notably, immunohistochemistry revealed CCR6-bearing Treg17 cells in kidney biopsy specimens of patients with GN. CCR6 expression on human Treg17 cells also appears dependent on STAT3, as shown by analysis of Tregs from patients with dominant-negative STAT3 mutations. Our data indicate the presence and involvement of Stat3/STAT3-dependent Treg17 cells that specifically target Th17 cells in murine and human crescentic GN, and suggest the kidney-specific action of these Treg17 cells is regulated by CCR6-directed migration into areas of Th17 inflammation.


Landmark studies by Mossman and Coffman1,2 more than 20 years ago have revolutionized our understanding of T cell biology by introducing the concept of functionally different T-helper (Th) cell subsets. Ever since, the Th cell family has grown substantially and currently includes Th1, Th2, Th17, and Tfh cells, as well as the latest additions, Th9 and Th22.3,4 Although Th cells are crucial for protection against pathogens, misdirected overactivation of these cells causes disease. In this context, Th2 cells mediate allergies, and Th1 and Th17 cells are central to the development of autoimmunity.5,6

To exert their functions, Th cells must be equipped with effective machinery optimized for their specific task. This includes subtype-specific effector cytokines and trafficking receptors. Expression of these characteristic receptors and cytokines is directed by lineage specific transcription factors. T-bet, Stat1, and Stat4 induce a Th1 phenotype,7,8
Gata3 and Stat6 induce Th2 cells,9,10 and Stat3 and RORγt are crucial for induction of Th17 cells.11–13

This effective armada of immune effectors needs close surveillance, especially in the light of potential development of autoimmunity. A central role for downregulation of Th-cell responses has been ascribed to a different T-cell lineage with a regulatory phenotype.14 According to their function, these cells were termed regulatory T cells (Treg), and studies showed that their development largely depends on the transcription factor Foxp3.15–17 Until recently, Foxp3+ Tregs were thought to be a singular population. However, given the diversity and high specialization of proinflammatory Th cells, it seems highly unlikely that a single type of anti-inflammatory T cell can effectively control immune homeostasis.18

Not surprisingly, well regarded studies from the last few years have proposed the concept of lineage-specific Tregs, which correspond to the respective proinflammatory Th cell counterpart. Campbell et al. have shown that Th1 immunity is under control of Th1-specialized Tregs.19 Interestingly, development of these Treg1 cells was shown to depend not only on the Treg characteristic transcription factor Foxp3 but also on T-bet, which was formerly identified as a crucial mediator of Th1 development. Thus, while differing in Foxp3 expression, Th1 and Treg1 cells share the same transcription factor T-bet for their programming. A concept that seems logical as for effective and specific suppression, a certain degree of similarity between the anti-inflammatory Treg subtype and its proinflammatory target appears to be necessary. Independent studies by another group have extended this concept of lineage-specific Tregs by showing that Th2 responses are under control of Treg2 cells, which share the transcription factor IRF4.20 Finally, in one study, Th17 immunity was specifically suppressed by Treg17 cells.21 Treg17 development depended on the transcription factor Stat3, which is also responsible for programming of Th17 effector cells.12–22 Mice with selective deletion of Stat3 in Tregs lack Treg17 cells and developed spontaneous severe colitis because of enhanced Th17 responses.21

Apart from these proof-of-concept studies, not much is known about the biology and function of lineage-specific Tregs. This is especially true for the field of nephrology, where no data exist so far. Multiple studies by us and others have cemented a central role for Th17 cells in the development and progression of GN.23–27 On the other hand, Tregs potently downregulate nephritogenic immunity and protect against renal tissue injury.28,29 This led us to the hypothesis that a Th17-specific Treg17 subpopulation exists and plays a central role for immune surveillance during GN. Our studies therefore aimed to (1) clarify the role of Stat3 for Treg17 generation, (2) define the role of Treg17 in experimental GN, (3) investigate Treg17 cell mechanisms of action, and (4) characterize Treg17 cells in human renal disease.

RESULTS

Mice Lacking Stat3 in Tregs Show Enhanced Th17 Responses but Do Not Develop Spontaneous Disease

To generate mice with selective deficiency of Treg17 cells, mice expressing cre recombinase under control of the Foxp3 promoter were intercrossed with mice harboring a loxP flanked Stat3 gene (Foxp3Cre×Stat3fl/fl). Efficiency and specificity of the targeted deletion were confirmed by PCR from FACS-sorted Tregs and effector T cells (Supplemental Figure 1A). Systemic leukocyte composition in spleen and blood of naive Foxp3Cre×Stat3fl/fl and Foxp3Cre control mice was similar in both strains (Supplemental Figure 1B). In addition, the activation status of Th cells and Tregs was similar (Supplemental Figure 1C). Nevertheless, frequencies of Th17 cells were increased in the peripheral circulation of Foxp3Cre×Stat3fl/fl mice (Figure 1A). In line, polyclonal T-cell receptor stimulation of naive splenocytes by anti-CD3 revealed increased levels of Th17 cytokines IL-6, TGF-β, and IL-17, whereas levels of Th1 (IFN-γ) or Th2 cytokines (IL-4), as well as IL-2 were similar (Figure 1B). Of note, no spontaneous histologic or functional renal alterations were observed (Figure 1C and Supplemental Figure 1, D–I). Hematoxylin and eosin–stained sections from liver and colon also showed no signs of spontaneous inflammatory disease (not shown).

NTN Is Aggravated in Treg17-Deficient Mice with Reduced Treg Recruitment and Enhanced Th17 Responses

To investigate the role of Treg17 cells for the course of crescentic GN, we induced NTN in Foxp3Cre and Foxp3Cre×Stat3fl/fl mice. Ten days after induction, analysis revealed increased glomerular and interstitial damage in Foxp3Cre×Stat3fl/fl mice. This finding was underscored by functional data, revealing a pronounced increase of BUN levels and albuminuria (Figure 1A). Infiltration by CD3+ T cells, macrophages, and neutrophils, the leukocyte subpopulations known to mediate tissue damage during NTN, was significantly increased (Figure 2, B–D). Strikingly, however, renal Treg recruitment was significantly reduced. This finding could be recapitated by quantitative RT-PCR from renal cortex showing reduced Foxp3 expression compared with wild-type animals (Figure 2E). Interestingly, flow cytometric cytokine analysis of renal leukocytes revealed a shift toward Th17 responses, whereas Th1 responses and percentages of IL-17–secreting γδ T cells remained unchanged (Figure 2F). These findings were underscored by FACS analysis of the Th17 transcription factor RORγt in renal CD4+ T cells (Figure 2G).

Systemic Immunity in Treg17-Deficient Mice Is Skewed toward Th17

To analyze whether skewing of immune responses toward Th17 was an exclusively renal or rather a systemic phenomenon, systemic cellular and humoral immunity was assessed at day 10 of NTN. Splenocyte numbers (Figure 3A) and gross splenic
Figure 1. Foxp3<sup>Cre</sup>xStat3<sup>fl/fl</sup> mice showed enhanced Th17 responses without developing a spontaneous renal phenotype. (A) FACS analysis of IL-17<sup>+</sup> and ROR<sup>+</sup>T<sub>17</sub> cells in peripheral blood after stimulation with phorbol 12-myristate 13-acetate/ionomycin. (B) Cytokine ELISAs from culture supernatants of anti-CD3 mAb-stimulated Th17 cells in peripheral blood after stimulation with phorbol 12-myristate 13-acetate/ionomycin. (C) Renal histology, BUN, and albumin-to-creatinine ratio of naive 10-week-old Foxp3<sup>Cre</sup>xStat3<sup>fl/fl</sup> mice (representative photographs of periodic acid-Schiff–stained kidney sections; magnification, ×400). Circles represent individual mice, and horizontal lines indicate mean values. *P<0.05; **P<0.01.

T-cell composition, including Tregs, were not significantly altered in Foxp3<sup>Cre</sup>xStat3<sup>fl/fl</sup> mice (Figure 2A). However, in line with increased Th17 responses in the kidneys, we found significantly higher percentages of splenocytes expressing the Th17 defining transcription factor RORγt in Foxp3<sup>Cre</sup>xStat3<sup>fl/fl</sup> mice (Figure 2B). In contrast, the proportion of T-bet–positive Th1 cells was unaltered (Figure 3B), as was the activation status of splenic Th cells (Figure 3C). Importantly, the rate of Ki67<sup>+</sup> proliferating T helper cells and Tregs was also similar (Figure 3D). Analysis of antigen-specific splenic cytokine production revealed a significant increase of the Th17-associated cytokine IL-6 whereas IL-17 production and several Th1 and Th2 cytokines were similar (Figure 3E, Supplemental Figure 2B). Analysis of systemic humoral immune responses revealed significant increases in serum levels of total mouse antisheep globulin IgG (Supplemental Figure 3A), as well as sheep immunoglobulin-specific IgG2b and IgG2c (Supplemental Figure 3C).

Renal Recruitment of Stat3-Deficient Tregs Is Impaired by Lack of Trafficking Receptor CCR6

Because renal Treg numbers were reduced in Treg17-deficient mice, we suspected Treg trafficking abnormalities. Indeed, analysis of chemokine receptor CCR6 showed almost complete and selective absence on renal Tregs from Foxp3<sup>Cre</sup>xStat3<sup>fl/fl</sup> mice (Figure 4A). Expression of the Th1 characteristic trafficking receptor CXCRI3 was not impaired (not shown). Renal expression of the CCR6 ligand CCL20 did not differ between the groups, excluding deficient chemokine production as cause of reduced Treg recruitment (Figure 4B). Next, we performed T-cell trafficking assays by injecting splenocytes from CD45.1<sup>+</sup> wild-type or CD45.2<sup>+</sup> Foxp3<sup>Cre</sup>xStat3<sup>fl/fl</sup> mice in a 1:1 ratio into RAG1-deficient recipients 1 day before the induction of NTN (Figure 4C). FACS analysis 14 days after NTN induction revealed reduced frequencies of Stat3-deficient CD45.2<sup>+</sup>Tregs in the kidneys, whereas their frequencies in spleens were identical to CD45.1<sup>+</sup> wild-type Tregs (Figure 4D). Furthermore, analysis of kidney-to-spleen ratios revealed selective impairment of Treg trafficking because recruitment of effector T cells remained intact (Figure 4C). In line, renal effector T cells from both groups expressed similar levels of CCR6, while CCR6 expression was nearly absent on renal Tregs derived from Foxp3<sup>Cre</sup>xStat3<sup>fl/fl</sup> mice (Figure 4E).

Treg Activation and In Vitro Suppressive Capacity Are Not Impaired by Lack of Stat3

FACS analysis showed slightly enhanced splenic Treg activation in Foxp3<sup>Cre</sup>xStat3<sup>fl/fl</sup> mice in response to immunization with sheep IgG (Figure 5A). Intracellular cytokine staining excluded Tregs as a direct source of proinflammatory cytokines, while IL-17 secretion by Stat3<sup>fl/fl</sup> Tregs was nearly absent on renal Tregs derived from Foxp3<sup>Cre</sup>xStat3<sup>fl/fl</sup> mice (Figure 4E). Reduced IL-6 receptor expression on Tregs from Foxp3<sup>Cre</sup>xStat3<sup>fl/fl</sup> mice as suggested by the initial report<sup>21</sup> was not reproducible by our experiments (Figure 5C). Suppression assays by coculturing splenic wild-type Foxp3<sup>Cre</sup> T cells alone or with FACS sorted Tregs from Foxp3<sup>Cre</sup>xStat3<sup>fl/fl</sup> mice showed similar ability to suppress IL-2 secretion. The ability to induce IL-10 production was even enhanced by Stat3-deficient Tregs, possibly reflecting their somewhat higher activation status (Figure 5D).

Aggravation of NTN Is Reversible in the Absence of Th17 cells

To assess whether aggravation of renal disease in Foxp3<sup>Cre</sup>xStat3<sup>fl/fl</sup> mice was mediated by overshooting Th17 responses, mice with targeted deletion of Stat3 in all CD4-bearing T cells were generated by crossbreeding Stat3<sup>fl/fl</sup> mice with CD4<sup>Cre</sup> mice. These mice lack Treg17 as well as Th17 cells,
with other T-cell populations left intact. In line with our hypothesis, analysis of histologic and functional renal damage did not reveal significant differences at day 10 after NTN induction (Figure 6, A and B). Renal leukocyte subpopulations showed similar numbers, with only a slight increase of interstitial macrophages in CD4\textsuperscript{Cre-3}Stat3\textsuperscript{fl/fl} mice (Supplemental Figure 4, A–D). Overall frequencies of renal Th cells were unchanged, whereas Th17 cells were completely absent with a compensatory increase in IFN-γ-producing Th1 cells (Figure 6C). In line, systemic immune responses showed absence of RORγt-expressing Th17 cells and splenocyte IL-17 production. T-bet–expressing Th1 cells as well as production of...
IFN-γ and various other cytokines remained intact (Figure 6D, Supplemental Figure 4E).

CCR6 expression was absent on both effector and Tregs in the peripheral blood of CD4CreStat3fl/fl animals CCR6 was specifically missing on Tregs. Expression of the Th1 characteristic receptor CXCR3 was similar in both strains of mice (Figure 6E). Analysis of humoral immune responses showed identical levels of anti-sheep globulin-specific IgG and most subclasses except for elevation of IgG2c (Supplemental Figure 4, F and G).

**DISCUSSION**

Despite intensive research during the past decades, the mechanisms underlying renal inflammatory diseases are still poorly understood. As a consequence, current therapeutic options are nonspecific and often highly toxic. However, cells of the Th17 response represent a promising novel target. We and others could demonstrate their central importance for initiation and progression of renal injury.23–27,30–32 Thus, understanding the mechanisms that lead to downregulation of highly nephritogenic Th17 responses is of great importance. Our study therefore evaluated the new concept of Th17-specific anti-inflammatory Treg17 cells by using mice with conditional deletion of Stat3 in Foxp3+ Tregs.21 No data regarding the role of Treg17 cells in nephritis exist so far.

In line with a putative deficiency of Treg17 cells, Foxp3Cre×Stat3fl/fl mice showed selectively enhanced systemic Th17 immunity with an otherwise morphologically and functionally normal immune system. Fortunately for our studies, their survival was normal; in contrast to the study by Chaudhry et al.,21 our mice did not develop signs of colitis. This is most likely due to the different microbial flora in our animal facility, which also fails to induce spontaneous inflammation in IL-10 deficient mice.29

To study the role of Treg17 cells under inflammatory conditions, we induced the Th17-dependent NTN model of acute crescentic GN.33 As hypothesized, the clinical course of disease was significantly aggravated in Treg17-deficient Foxp3Cre×Stat3fl/fl mice. In line with the concept, both renal and systemic immune responses were skewed toward Th17. Interestingly, despite generally enhanced renal leukocyte infiltration, renal Treg numbers and percentages were selectively reduced. In line with data by Chaudhry et al.,21 we found highly diminished expression of the chemokine receptor CCR6 on the surface of Tregs in Foxp3Cre×Stat3fl/fl mice. This is of special interest because a recent publication by our group showed the importance of CCR6 for trafficking of both Th17 cells and Tregs into inflamed kidneys.34 Mice constitutively lacking CCR6 showed severe aggravation of NTN with hyper-IgE syndrome (Job syndrome) due to dominant negative STAT3 mutations. Analysis revealed that, indeed, individuals who carry heterozygous STAT3 mutations hardly expressed any CCR6 on their regulatory and effector T cells compared with healthy controls. In contrast, CXCR3 expression was increased on both cell types. Expression of CD25 on Foxp3+ Tregs was unaltered (Figure 8, A and B).
reduced renal Treg numbers. We therefore hypothesized that one mechanism by which Treg17 cells control Th17 responses is expression of CCR6, which directs them into areas of Th17 inflammation. Competitive in vivo trafficking assays supported this hypothesis as recruitment of Tregs from Foxp3Cre×Stat3flo/flo mice into nephritic kidneys was greatly impaired. We next wanted to evaluate whether nontrafficking related properties of Tregs were affected by lack of Stat3. Detailed analysis, however, showed enhanced rather than reduced activation. In line, we documented unchanged in vivo proliferation and suppressive in vitro capacity. In contrast to the publication by Chaudhry et al., our analysis showed intact IL-6R expression on Tregs from Foxp3Cre×Stat3flo/flo mice. This discrepancy might be due to the fact that knockout mice in the study by Chaudhry et al. developed spontaneous systemic inflammation, which might lead to shedding of the IL-6 receptor, while the mice bred in our facility remained healthy.

More important, and in line with data from Chaudhry, we found that enhanced Th17 immunity in Foxp3Cre×Stat3flo/flo mice was not attributable to proinflammatory cytokine secretion by Tregs themselves because hardly any Treg-derived IL-17 or IFN-γ production was detected. Our results therefore pointed toward increased Th17 immune responses as an indirect effect mediated by insufficient Th17 suppression due to lack of Treg17 cells. To prove this hypothesis, we generated mice lacking Stat3 in all CD4+ cells, which include regulatory and also Th effector cells. These mice therefore lack both Treg17 and also Th17 cells. Analysis of renal and systemic immunity indeed confirmed complete absence of Th17 responses. In addition, in these mice, CCR6 expression was greatly reduced not only on Tregs but also on effector Th cells. In line with our concept, the observed aggravation of NTN in Treg17-deficient Foxp3Cre×Stat3flo/flo mice was completely reversed in the absence of Th17 responses. This finding shows that cells of the Th17 response are the specific target of Stat3-dependent Treg17 cells.

In a next step we wanted to investigate whether CCR6 bearing Treg17 cells are also present in human renal disease. Our analysis of renal tissue from patients with cANCA-associated vasculitis indeed showed infiltration of CCR6+ Treg cells which were recruited into areas of CCR6+ effector T cells. We thus hypothesize that in humans, Th17 responses are also under control of Treg17 cells. To evaluate whether human Treg17 cells also depend on STAT3 activation, we analyzed blood leukocytes from patients with hyper-IgE syndrome.

Figure 4. Renal Treg recruitment is impaired because of a lack of CCR6. (A) FACS analysis of renal leukocytes from nephritic Foxp3Cre (n=4), Foxp3Cre×Stat3flo/flo (n=4), and CCR6−/− (n=1) mice. CCR6 expression of CD45+CD3+CD4+Foxp3+ Tregs (upper plots) and CD45+CD3+CD4+Foxp3− Th cells (lower plots). Quantification is shown in the right columns. (B) Renal expression of CCL20 mRNA from nephritic Foxp3Cre×Stat3flo/flo mice and Foxp3Cre controls. (C) In vivo T-cell trafficking and renal recruitment were explored by transferring CD45.1+ wild-type splenocytes and CD45.2+ Foxp3Cre×Stat3flo/flo splenocytes in a 1:1 ratio into RAG1−/− recipients (n=7). (D) Distribution of CD45.1+ wild-type and CD45.2+ Foxp3Cre×Stat3flo/flo Treg and Th cells. Representative FACS plots from renal tissue are shown on the left; quantification is shown in the right columns. (E) FACS analysis of CCR6 expression on CD45.1+ wild-type and CD45.2+ Foxp3Cre×Stat3flo/flo Treg and Th cells. Error bars represent SDs. Numbers in FACS plots represent percentages of gated events. **P<0.01; ***P<0.001.

patients experience recurrent infections due to reduced Th17 responses as a cause of dominant negative STAT3 mutations. In accordance with our animal data, we found greatly and selectively reduced CCR6 levels on Th effector and Tregs in these patients. These findings confirm trans-species conservation of Stat3/STAT3 dependency of CCR6 expression.

Although presence of CCR6 on Treg17 cells explains their potency to downregulate Th17 responses in inflamed target organs, the mechanisms leading to enhanced systemic Th17 immunity in the absence of Treg17 remain less clear. Similar to the data by Chaudhry et al., we found enhanced systemic production of Th17-inducing cytokines IL-6 and TGF-β1 in Foxp3Cre×Stat3fl/fl mice. Apart from providing TGF-β1, Tregs are known to promote Th17 cells by consumption of IL-2. However, we found no alterations of IL-2 secretion in Foxp3Cre×Stat3fl/fl mice; this finding is in line with the study by Chaudhry et al., who also reported identical IL-2 receptor expression by Tregs. Thus, the exact mechanisms leading to Th17-associated systemic cytokine overproduction remain unknown and require further studies. The same is true for the augmented antigen-specific IgG production in the absence of Treg17 cells. Because injury in the NTN model of GN develops independently of antibodies, elevated IgG levels are unlikely to contribute to the observed aggravation of disease, even though it cannot be ruled out completely. However, elevated levels of IgG2c were also observed in CD4Cre×Stat3fl/fl mice, which do not develop more severe nephritis than CD4Cre controls. One hypothesis is that increased antibody production might reflect enhanced Th cell activation. Alternatively, Treg/B-cell crosstalk might be disturbed in Foxp3Cre×Stat3fl/fl mice because activated B cells are also known to carry the chemokine receptor CCR6. Future studies will have to address these open questions. A further aspect that needs clarification is the cascade leading to induction of Stat3 expression and, thus, programming of Treg17 cells. In this respect again, Chaudhry et al. have published pioneering work that suggests dependence on signaling via the IL-10 receptor on Tregs. Finally, future studies are warranted to address the potential roles of Th1- and Th2-specific Treg1 and Treg2 cells in GN because no data exist to date.

In conclusion, we show here for the first time a role of Stat3-dependent Treg17 cells in a model of crescentic GN and thus in a model of induced organ inflammation in general. Treg17 cells were shown to be crucial for downregulation of nephritogenic Th17 immune responses. One key mechanism of Treg17 cell action is expression of chemokine receptor CCR6, which facilitates trafficking into areas of Th17 inflammation. Importantly, we could also show presence of CCR6+ Tregs in biopsy specimens from humans with organ-related disease.
Figure 6. Aggravation of NTN is reversible in the absence of Th17 cells. (A) Representative periodic acid-Schiff-stained kidney sections (left: magnification ×400) and quantification of glomerular crescent formation and interstitial damage (right) at day 10 after NTN induction in CD4Cre or CD4Cre×Stat3fl/fl mice. (B) Quantification of BUN levels and albumin-to-creatinine ratio. (C) FACS analysis of renal T-cell subtype composition. (D) Assessment of RORγt+ Th17 and T-bet+ Th1 cells by FACS in splenic leukocytes from nephritic mice (left). Quantification of antigen-specific splenic cytokine production by ELISA (right). (E) Analysis of CCR6 and CXCR3 expression on regulatory and Th cells in peripheral blood from naive CD4Cre, CD4Cre×STAT3fl/fl, or Foxp3Cre×Stat3fl/fl mice (n=3 per group). Circles represent individual mice, and horizontal lines indicate mean values. Error bars represent SDs. *P<0.05; **P<0.01; ***P<0.001.

GN. Furthermore, analyzing blood from patients with STAT3-inactivating mutations, we confirmed STAT3 dependency of CCR6 expression. Our data therefore suggest the presence of Treg17 cells in the human system as well. This conservation across species indicates high functional importance of Treg17 and favors further research to better characterize Treg17 cell biology.

CONCISE METHODS

Animals
The loxP-site flanked Stat3fl/fl mice were a generous gift from Professor Shizuo Akira, Osaka University, Japan.42 Treg-specific deletion of Stat3 was achieved by crossbreeding with mice expressing a yellow fluorescent protein (YFP)-Cre recombinase fusion protein under the control of the Foxp3 locus,13 which were a kind gift from Professor Alexander Y. Rudensky, Memorial Sloan-Kettering Cancer Center, New York, New York. Efficiency and specificity of the Stat3 deletion were assessed by PCR from FACS-sorted CD45+CD4+YFP+ and YFP+ splenocytes. For detection of the Foxp3Cre, Foxp3wt, Stat3fl, or Stat33 alleles, primers were used as described elsewhere.21,42 CD4+ T cell–specific deletion of Stat3 was induced by intercrossing Stat3fl/fl mice with mice expressing Cre-recombinase under the control of a transgenic CD4 enhancer-promotor-silencer sequence (B6.Cg-Tg[Cd4-cre]1Cwi/BBu1), commercially available from The Jackson Laboratory. RAG1−/−, CD45.1, RORC−/−,23 and CCR6−/− mice were also purchased from The Jackson Laboratory. All mice were bred in our facility. All animals used in this study were on a C57BL/6 background and were raised under specific pathogen-free conditions.

Animal Experiments and Functional Studies
Nephrotoxic nephritis was induced in 8- to 10-week-old male Foxp3Cre×Stat3fl/fl and Foxp3Cre×Stat3wt/wt (referred to as Foxp3Cre) littermate controls or CD4Cre×Stat3fl/fl and CD4Cre×Stat3wt/wt (referred to as CD4Cre) littermate controls mice by intraperitoneal injection of 2.5 mg of nephrotoxic sheep serum per gram body weight.44,45 Organs were harvested at day 10 after injection. To assess in vivo T-cell trafficking and renal T-cell recruitment, splenic leukocytes were isolated from either Foxp3Cre×Stat3fl/fl (CD45.2+) or wild-type C57BL/6 (CD45.1+) mice. Viable cells were counted using trypan blue staining and intravenously injected into RA Gordon-deficient mice in a 1:1 ratio 1 day before induction of NTN. Renal and splenic leukocytes were isolated 14 days after induction as described below and analyzed for the distribution of CD45.2+ and CD45.1+ cells among the regulatory and effector T cells. For immunization studies, mice were injected with normal sheep IgG without adjuvant, and spleens were harvested at day 6. Animal experiments were performed according to national and institutional animal care and ethical guidelines and were approved by local committees (approval code G37/11). Urine samples were collected after housing of the mice in metabolic cages. Albuminuria was determined by standard ELISA (Bethyl Laboratories). BUN and urinary creatinine were quantified using standard laboratory methods.

Morphologic Studies
Crescent formation and glomerular necrosis were determined in a minimum of 50 glomeruli per mouse in 2-μm-thick periodic acid-
4°C. The upper leukocyte fraction was aspirated and filtered through another 40-μm nylon mesh. Cells were washed, counted, and resuspended in PBS for staining and FACS analysis. Peripheral blood was drawn into EDTA-coated tubes, and red blood cell lysis was performed after staining for surface markers.

**Antigen-Specific Systemic Cellular and Humoral Immune Responses**

Splenocytes (4×10^6 cells/ml) were cultured under standard conditions in the presence of normal sheep IgG (10 μg/ml; Sigma-Aldrich, Taufkirchen, Germany) and supernatants were harvested after 72 hours. Commercially available ELISAs were used for detection of IFN-γ, IL-4, IL-5, IL-6, TNFa, IL-10, and IL-17A (Biolegend, San Diego, CA), IL-13 (Bioscience, San Diego, CA), TGF-β, IL-2 (BD Systems, Minneapolis, MN). Circulating sheep globulin–specific IgG titers were analyzed by ELISA (for total IgG; Biozol, Eching, Germany; for IgG1, IgG2b, IgG2c, and IgG3; Invitrogen, Frederick, MD).

**Flow Cytometry**

Cells were surface-stained for 30 minutes at 4°C with fluorochrome-labeled antibodies as previously described. Antibodies against CD45, CD3, CD4, CD19, CD44, CD69, CD62L, CCR6, CXCR3, γδ TCR, ICOS, PD-1, CD103, IL-6 receptor (Bioscience) were utilized. For intracellular and intranuclear staining, samples were processed using a commercial intranuclear staining Kit (Foxp3-Kit; Ebioscience). Fluorochrome-labeled antibodies against IL-17, IFN-γ, Foxp3, RORγt, Ki67, and T-Bet (Bioscience) were used as recently published. For intracellular cytokine staining, cells were activated with phorbol 12-myristate 13-acetate (50 ng/ml; Sigma-Aldrich) and ionomycin (1 μg/ml; Calbiochem-Merck) for 5 hours. After 30 minutes of incubation, brefeldin A (10 μg/ml; Sigma-Aldrich) was added. LIVE/DEAD staining (Invitrogen/Molecular Probes, Eugene, OR) was used to exclude dead cells during flow cytometry and to ensure viability of the cells after the stimulation procedure. Experiments were performed on a BD LSRII Cytometer (Becton Dickinson, Germany).

**Isolation of Leukocytes from Various Tissues**

Spleens were harvested in HBSS and passed through 70-μm nylon meshes. After lysis of erythrocytes with ammonium chloride, cells were washed and passed over 40-μm meshes. Cells were then washed again, counted, and resuspended in PBS for culture or FACS analysis.

**Quantitative Real-Time PCR Analysis**

Total RNA of renal cortex was isolated according to a standard Trizol protocol and purified by using a Nucleospin Kit (Macherey & Nagel, Düren, Germany). Real-time PCR was performed as described previously47 (all primer sequences available upon request).

**Treg Suppression Assay**

CD4+ splenocytes were enriched by using magnetic-activated cell sorting according to the manufacturer’s protocol (MACS CD4+ T cell Kit II; Miltenyi Biotec, Germany). To separate regulatory from effector T cells, cells derived from Foxp3Cre or Foxp3Cre×Stat30/0 mice were used for detection of IFN-γ, IL-4, IL-5, IL-6, TNFa, IL-10, and IL-17A (Biolegend, San Diego, CA), IL-13 (Bioscience, San Diego, CA), TGF-β, IL-2 (BD Systems, Minneapolis, MN). Circulating sheep globulin–specific IgG titers were analyzed by ELISA (for total IgG; Biozol, Eching, Germany; for IgG1, IgG2b, IgG2c, and IgG3; Invitrogen, Frederick, MD).
mice were stained for CD45 and CD4. Cells were then separated using their Foxp3-driven YFP expression by FACS sorting provided by the institutional HEXT FACS Sorting Core facility (performed on a BD ARIAIII Cytometer, Becton Dickinson). A total of $4 \times 10^5$ CD45$^+$CD4$^+$ YFP$^+$ effector T cells from Foxp3Cre mice were then cultured for 72 hours in anti-CD3 mAb (5 mg/ml; BD Biosciences) precoated 96-well plates in the presence of anti-CD28 mAb (1 mg/ml; BD Biosciences) either alone or in coculture with $1 \times 10^5$ CD45$^+$CD4$^+$YFP$^+$ Tregs from Foxp3Cre or Foxp3CreStat3fl/fl mice. Suppressive capacity was determined by cytokine ELISAs performed from the supernatants as recently described.29

Human Renal Immunohistochemistry

Tissue samples were obtained from six patients from our center with confirmed diagnosis of active cANCA vasculitis. Preimplantation biopsy specimens from five renal allografts were used as controls. Paraffin sections were pretreated (microwave 20 minutes with EDTA buffer; pH 8–9) and washed in 99% ethanol, followed by blocking for 10 minutes in horse serum (Vector S 2000, dilution 1:20). The slides were then incubated at 4°C with primary antibodies against Foxp3 (mouse monoclonal 236A/E7; Abcam; ab20034, dilution 1:900) and CCR6 (rabbit Sigma HPA014488, dilution 1:100) overnight. Secondary antibodies (biotinylated anti-mouse IgG, Vector BA 2000, dilution 1:200, 30 minutes at 37°C) and StreptABC-Complex (DakoCytomation K 0377 dilution 1:100) were added. After washing in PBS, staining with benzidine in citrate buffer (Benzidin Fluka 32750) was done. Detection of CCR6 with AP Polymer (Zytochem-Plus POLAP 100, dilution 1:1, 30 minutes) and staining (after washing in PBS) with new fuchsinaphthol AS-Bi phosphate substrate mix was performed, followed by nuclear counterstaining with hematoxylin. Numbers of Foxp3$^+$CCR6$^+$ cells and direct spatial association with Foxp3$^+$CCR6$^+$ cells were quantified by counting of at least 10 high-power fields per biopsy specimen (magnification, ×400) in a blinded fashion.

Figure 8. Human Tregs express CCR6 in a STAT3-dependent manner. (A and B) FACS analysis of blood leukocytes from patients with hyper-IgE syndrome due to dominant-negative STAT3 mutations (n=4) and healthy controls (n=5). Percentages (A) and mean fluorescence intensity (B) of the CCR6 or CXCR3 expression on Foxp3$^+$ Tregs and Foxp3$^+$ Th cells. Error bars represent SDs. Numbers in FACS plots represent percentages of gated events. *P<0.05; **P<0.01; ***P<0.001.

**Human PBMCs from Patients with STAT3-Dependent hyper-IgE Syndrome**

PBMCs derived from patients carrying heterozygous STAT3 mutations (Supplemental Table 1; reported previously35) or healthy controls at the Dr. von Haunersches Kinderspital in Munich, Germany. PBMCs from both groups were stored in 90% FBS and 10% DMSO at −196°C (nitrogen). Thawing cells were rapidly supplemented with 1 ml of prewarmed medium (RPMI+10% FBS) and washed to remove DMSO. For FACS analysis, cells were stained for surface markers CD45, CD3, CD25, CXCR3, and Foxp3. Foxp3 staining was performed using a Foxp3-staining kit following the manufacturer’s protocol (Ebioscience).

**Statistical Analyses**

Results are expressed as the mean±SD. Groups were compared by t test, and a P value <0.05 was considered to represent a statistically significant difference. In the case of multiple comparisons, one-way ANOVA was applied using Tukey post hoc testing.

**Ethics**

Human studies were performed in accordance with the ethical principles stated by the Declaration of Helsinki. Kidney biopsy specimens from humans with ANCA vasculitis were evaluated after patients provided written informed consent and as approved by the local ethics committee (study pv3162; Hamburg University). PBMCs from patients with STAT3-dependent hyper-IgE syndrome and controls were isolated after written informed consent and as approved by the local ethics committee (Ludwig Maximilians University Munich, Germany).
ACKNOWLEDGMENTS

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DISCLOSURES

None.

REFERENCES


DISCLOSURES

None.

REFERENCES

the hyper-IgE syndromes: Immunologic and clinical key findings to differentiate hyper-IgE syndromes from atopic dermatitis. J Allergy Clin Immunol 126: 611–617 e611, 2010


40. Steinmetz OM, Stahl RA, Panzer U: Chemokines and B cells in renal inflammation and allograft rejection. Front Biosci (Schol Ed) 1: 13–22, 2009


42. Akira S: Roles of STAT3 defined by tissue-specific gene targeting. Oncogene 19: 2607–2611, 2000


This article contains supplemental material online at http://jasn.asnjournals.org/lookup/suppl/doi:10.1681/ASN.2013080904/-/DCSupplemental.
SUPPLEMENTARY FIGURE S1. Characterization of mice lacking Stat3 specifically in Tregs. (A) PCR from FACS sorted YFP+ Treg and YFP- Th confirms successful and specific Stat3-deletion in Tregs from Foxp3Cre x Stat3fl/fl mice. (B) Leukocyte composition in spleen and blood of naïve Foxp3Cre control and Foxp3Cre x Stat3fl/fl mice (n=6 and 7, respectively). (C) Activation status of Th and regulatory T cells in spleen and blood. (D) Quantification of spontaneous renal damage in naïve Foxp3Cre and Foxp3Cre x Stat3fl/fl mice determined by crescent formation and interstitial damage score. (E to I) Serum lipoproteins (E), glomerular and interstitial T cells (F), macrophages (G) and neutrophils (H) as well as interstitial Tregs (I) were determined and quantified. Circles represent individual mice, horizontal lines indicate mean values.
**SUPPLEMENTARY FIGURE S2.** Systemic T cell responses. (A) Splenic distribution of effector T cells (gated as indicated). (B) Antigen-specific splenic production of the indicated cytokines. Data derive from n=16 Foxp3<sup>Cre</sup> and n=14 Foxp3<sup>Cre</sup> x Stat3<sup>fl/fl</sup> mice. Error bars represent standard deviation.
SUPPLEMENTARY FIGURE S3. Systemic humoral immune responses. (A) Mouse anti-sheep globulin total IgG levels in sera from nephritic Foxp3<sup>Cre</sup> (n=16) or Foxp3<sup>Cre</sup> x Stat3<sup>fl/fl</sup> (n=14) mice. (B) Subclass analysis of mouse anti-sheep globulin IgG subclasses. ELISA data are shown as OD at 450nm in serial dilutions as indicated. Data are shown as mean SD. *P<0.05; **P<0.01, ***P<0.001.
SUPPLEMENTARY FIGURE S4. Renal and systemic immune responses in mice lacking Stat3 in CD4+ T cells. (A-D) Representative photographs (left, magnification x 400) and quantification (right) of glomerular and interstitial CD3+ T cells (A), glomerular (MAC2+) and interstitial (F4/80+) macrophages (B), glomerular and interstitial GR-1+ neutrophils (C), interstitial Foxp3+ Treg numbers and percentages (D). (E) Quantification of splenic production of indicated cytokines. (F) Mouse anti-sheep globulin total IgG levels from serum of nephritic CD4 Cre (n=9) or CD4 Cre x Stat3 fl/fl (n=10) mice. (G) Subclass analysis of mouse anti-sheep globulin IgG1, IgG2b, IgG2c and IgG3. ELISA data are shown as OD at 450nm in serial dilutions as indicated. Circles represent individual data points, horizontal lines indicate mean values. Error bars represent standard deviation. *P<0.05, **P<0.01, ***P<0.001.
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Supplementary Table 1: dominant-negative STAT3-mutations of the 4 HIES patients analysed in our study.