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IL-9 Production by Regulatory T Cells Recruits Mast Cells That Are Essential for Regulatory T Cell-Induced Immune Suppression

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Both mast cells (MCs) and regulatory T cells (Tregs) have gained attention as immunosuppressive cell populations. To investigate a possible interaction, we used the Th1- and Th17-dependent model of nephrotoxic serum nephritis (NTS), in which both MCs and Tregs have been shown to play a protective role. Transfer of wild-type (wt) Tregs into wt recipients almost completely prevents development of NTS and leads to a profound increase of MCs in the renal draining lymph nodes (LNs). By contrast, transfer of wt Tregs into animals deficient in MCs, which are characterized by an exaggerated susceptibility to NTS, no longer exhibited protective effects. Blocking the pleiotropic cytokine IL-9, known to be involved in MC recruitment and proliferation, by means of a mAb in mice receiving Tregs abrogated protection from NTS. Moreover, transfer of IL-9–deficient Tregs also failed to protect from NTS. In the absence of Treg-derived IL-9, MCs fail to accumulate in the LNs, despite the fact that IL-9 deficiency does not alter the general suppressive activity of Tregs. In summary, to our knowledge, we provide the first direct in vivo evidence that the nephroprotective, anti-inflammatory effects of Tregs critically depend on IL-9–mediated attraction of MCs into kidney-draining LNs. *The Journal of Immunology, 2011, 186: 83–91.

Tipping the balance between effector and regulatory cell populations is of critical importance in the pathogenesis of various autoimmune disorders. According to a current paradigm, the proinflammatory axis of Th1 and Th17 cells is counterbalanced by the cell populations Th2 cells and regulatory T cells (Tregs) (1).

CD4+CD25+Foxp3+ cells are thought to have a huge therapeutic potential as cellular immunosuppressants (2). In line with this idea, various groups, including our own, have demonstrated the therapeutic efficacy of Tregs in murine models of inflammation (3–5). It is generally accepted that the predominant target cell effect of Tregs is a direct cell-to-cell contact-dependent inhibition primarily mediated by membrane-bound TGF-β (6). Moreover, soluble factors such as IL-10 have also been attributed to the Treg-induced immune-inhibitory effects (7, 8). However, various research groups have provided evidence that Tregs also modify the function of nonlymphatic cell types, such as dendritic cells (9, 10), monocytes (11), endothelial cells (12), and mast cells (MCs) (13). The latter are also known to play a critical role for immune regulation in allergy and autoimmunity (14). Very recently, MCs have been demonstrated to exhibit immunomodulatory functions (15). They seem to exert either pro- or anti-inflammatory effects depending on the surrounding milieu (15).

For a more detailed analysis of the complex orchestration of these immunoregulatory networks, the murine model of acute nephrotoxic serum nephritis (NTS) has proven to be both informative and robust. The role of T cells, including Th1 and Th17 cells for NTS induction and maintenance, is well documented (16–19). We recently provided evidence that CD4+CD25+Foxp3+ Tregs have a therapeutic potential to control the onset and course of NTS (5). Moreover, Tregs predominantly migrate to lymph nodes (LNs), but not to the end organ, suggesting that lymphatic organs are the prime sites of their immunosuppressive action (5). This hypothesis is further supported by our latest observation showing that CCR7-deficient Tregs lose their immunosuppressive potential due to their inability to enter the LNs (20). Moreover, we and others clearly demonstrated that MCs limit kidney-damaging immune responses (21, 22), as MC-deficient KitW/KitW–mice display a profound exaggeration of NTS when compared with wild-type (wt) animals. Lu et al. (23) support the concept of an important immune-regulatory function of MCs by showing that they regulate allograft tolerance in a skin transplantation model. In this particular model, MCs have been described to be protective by interacting with Tregs (23).

In contrast to the immune-inhibitory function of MCs in acute inflammation models (21, 22), MCs seem to play a central role in the development of inflammation-induced tissue fibrosis in chronic kidney diseases, because their kidney-infiltrating numbers tightly correlate with the grade of renal fibrosis (24–27).

In this study, to our knowledge, we provide for the first time direct evidence that the Treg/MC interaction is also of critical importance for limiting endogenous inflammatory disease. As ex-

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Abbreviations used in this paper: Hpf, high-power field; ko, knockout; LN, lymph node; MC, mast cell; n.s., not significant; NTS, nephrotoxic serum nephritis; PAS, periodic acid Schiff; Treg, regulatory T cell; wt, wild-type.

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emphasized in a model of acute renal inflammation, Treg-induced immune suppression critically depends on the recruitment of MCs into kidney-draining LNs. This process is mediated by Treg-derived IL-9 and is a prerequisite for the prevention of end-organ damage by effector immune cells.

Materials and Methods

Induction of accelerated NTS

C57BL/6 mice (purchased from Charles River Laboratories, Sulzfeld, Germany), MC-deficient WBB6F1-KitW–/KitW– (KitW–/KitW–) mice, and congenic wt WBB6F1-KitW+/KitW+ (KitW+/KitW+) mice were purchased from The Jackson Laboratory (Bar Harbor, ME). IL-9–deficient mice have been backcrossed with C57BL/6 mice for eight generations (28). Eight- to 10-week-old male animals were used in all studies. Accelerated NTS was induced, as described previously (29). In brief, mice were preimmunized s.c. with 100 μl 2 mg/ml rabbit IgG (Jackson ImmunoResearch Laboratories, West Grove, PA) dissolved in IFA (Sigma-Aldrich, St. Louis, MO) and 0.01 g/ml nonviable desiccated Mycobacterium tuberculosis H37a (Difco Laboratories, Detroit, MI). After 3 d, heat-inactivated rabbit anti-mouse glomerular basement membrane antiserum was injected via the tail vein. All animal experiments were performed according to Austrian law (GZ 66.011/0.111-11/106/2008).

Detection of urinary albumin and creatinine

Urinary albumin was determined by a double-sandwich ELISA (Abcam, Cambridge, MA), as reported previously (29). Urinary creatinine was quantitated spectrophotometrically using a picric acid-based method (Sigma-Aldrich).

Histo- and immunomorphological evaluation of renal pathology and LNs

Formalin-fixed renal and LN tissue was embedded in paraffin and cut in 4-μm sections. Renal sections were stained with periodic acid Schiff (PAS). In all cases, a minimum of 50 equatorial glomerular cross sections was evaluated, as previously described (30).

LN sections were stained with Wright-Giemsa. The three-layer immunoperoxidase staining of frozen tissue 4-μm sections was used for the detection of macrophages and T cell subpopulations in the kidney, as well as in LN sections (29). Macrophages were stained with rat anti-mouse mAb (clone F4/80 or anti-CD68; both from Serotec, Oxford, United Kingdom). A semiquantitative scoring system for kidney-infiltrating macrophages was performed as follows: 0 = 0–4 cells stained positive; 1+ = 5–10 cells; 2+ = 10–50 cells; 3+ = 50–200 cells; and 4+ = >200 cells stained positive per low-power field. For the detection of CD4+, CD8+ T cells, and CD117+ cells, we used rat anti-mouse mAbs (clones YTS191.1 and KT15, both from Serotec, and clone ACK2 from ebiosciences, San Diego, CA, respectively). T cell and MC quantitation was performed by counting the number of positive cells in six adjacent high-power fields (Hpf) of renal cortex and medulla or of the LNs. Samples were blinded before evaluation. Fibrin deposition was evaluated on renal cryosections by using a FITC-conjugated fibrinogen mAb (Dako, Glostrup, Denmark).

**FIGURE 1.** Treg transfer increases MCs in kidney-draining LNs. Kidney-draining LNs of animals receiving either Tregs (white bar) or control T cells (black bar; n = 13 per group) were analyzed for MC infiltration 14 d after disease induction. A, Real-time PCR for the expression of MC tryptase is shown. Data are presented as x-fold increase as compared with LNs of healthy controls. B, Giemsa staining of LNs. The number of MCs per mm² is given. The data are expressed as mean ± SEM. *p < 0.05. C, A representative example of a Giemsa-stained LN section from a Treg-injected mouse 14 d after induction of NTS is shown. MCs are marked by arrows. Original magnification ×400.

**FIGURE 2.** Tregs in MC-deficient KitW–/KitW– and KitW+/ KitW+ mice. Fourteen days after NTS was induced in KitW–/KitW– (white bar) and KitW+/ KitW+ mice (black bar; n = 10 per group), LNs were evaluated for Treg infiltration by performing real-time PCR for the detection of Foxp3 (A) and by flow cytometric analysis for CD4+CD25+Foxp3+ cells (B). The real-time data are expressed as x-fold increase of Foxp3/β-actin compared with mRNA isolated from healthy control LNs (which was set as 1). C and D, Evaluation of the immune-suppressive potential of CD4+CD25+ Tregs from healthy KitW+/ KitW+ (C) and MC-deficient KitW–/ KitW– (D). Tregs from KitW+/ KitW+ or MC-deficient KitW–/ KitW– animals were coincubated with CD4+CD25− KitW+ cells in a 1:1 and 1:5 ratio. Proliferation was measured by [3H]thymidine incorporation after 7 d, and the percentage of proliferation compared with the respective control CD4+CD25− T cell population is shown. Moreover, both strains were subjected to Treg quantification, as shown by representative FACS stainings shown in the inserts in C and D.
Detection of circulating mouse anti-rabbit IgG

For detection of circulating mouse anti-rabbit IgG, 96-well plates (Greiner, Kremsmuenster, Austria) were coated with 100 μg/ml rabbit IgG (Jackson ImmunoResearch Laboratories) in carbonate/bicarbonate buffer (pH 9.5). After blocking with 1% BSA, plates were incubated with serial-doubling dilutions of mouse serum. Bound mouse IgG was detected by HRP-conjugated goat anti-mouse IgG (Dako).

Real-time RT-PCR

Total RNA was isolated using TRIzol (Sigma-Aldrich), according to a standard protocol. Thereafter, 2 μg total RNA was reverse transcribed using Superscript III Transcription Kit (Invitrogen, Carlsbad, CA) and random primers (Roche, Basel, Switzerland). Real-time PCR was performed on an ABI Prism 7700 (Applied Biosystems, Foster City, CA). For linear amplification of β-actin (reference gene) and Foxp3, SYBR Green Master Mix (Invitrogen) and primers, as described before (31), were used. For quantification of mouse MC tryptase 1, Cxcl-1, Cxcl-2, Cxcl-5, IFN-γ, IL-6, IL-17A, IL-10, Gata-3, and TGF-β1 TaqMan Mastermix (Applied Biosystems) and the gene expression assays Mm00600091_m1, Mm00433859_m1, Mm00436450_m1, Mm00436451_m1, Mm00801778_m1, Mm00446190_m1, Mm00439619_m1, Mm00439616_m1, Mm00484683_m1, and Mm03024-053_m1 (Applied Biosystems) were used.

Isolation and transfer of CD4+CD25+ Tregs

CD4+CD25+ Tregs were isolated from minced spleens and LNs obtained from C57BL/6 or from IL-9-deficient mice using magnetic bead separation (CD4+CD25+ Treg kit; Miltenyi Biotec, Bergisch Gladbach, Germany).

**FIGURE 3.** MC-deficient animals with NTS are resistant to the anti-inflammatory Treg effects. KitW/KitW-v (gray and light gray bar) and Kit+/+ mice (black and white bar) received either 5 × 10⁶ Tregs (light gray and white bar) or control T cells (gray and black bar) and were subjected to NTS (n = 8 per group). A, Seven and 14 d after induction of NTS, albumin and creatinine in the urine were evaluated. The urinary albumin/creatinine ratio is given in mg/mg. B, Representative PAS staining from tissue sections of kidneys from the indicated groups 14 d after induction of NTS is shown. Original magnification ×400. KitW/KitW-v receiving either Tregs or control T cells display crescent formation as highlighted by black arrows. Only few crescent formations were seen in Kit+/+ control mice, but they were found to have, as KitW/KitW-v mice, PAS-positive deposits in their glomeruli (*) as well as infiltrating inflammatory cells. When Kit+/+ mice were treated with Tregs, no glomerular pathologies were detected. C, Semiquantitative analysis of PAS deposits in kidney sections according to the PAS score described in Materials and Methods is shown. D, T cell infiltration pattern in the indicated groups is shown by semiquantitative quantification of CD4- and CD8-stained tissue sections. The number of positive cells in 6 Hpf is given. E and F, Kidney infiltration patterns of F4/80+ (E) or CD68+ (F) cells, which were stained and counted according to the F4/80- and CD68-scoring procedure described in Materials and Methods. G, Rabbit anti-mouse titers were evaluated in the serum of respective mice 14 d after induction of NTS. All data are presented as mean ± SEM. *Provides the significance between Kit+/+ mice receiving either Tregs or control cells (p < 0.025). #Provides the significance between KitW/KitW-v and Kit+/+ mice receiving control cells (p < 0.025).
The real-time PCR data are expressed as a fold increase of the respective parameter/β-actin compared with the mean expression of Kit+/+ LNs (which was set as 1). All data are presented as mean ± SEM. *p < 0.05.

Flow cytometry of CD4+Foxp3+ cells

Cell suspensions from LNs, spleens, and kidneys were stained for Foxp3+ Tregs using costaining of CD4 (BD Biosciences, San Diego, CA) and Foxp3+ (eBiosciences) strictly adhering to the manufacturer’s instructions. Data collection and analysis were done on a FACS Calibur (BD Biosciences).

Treg suppression assays

Tregs and the respective CD4+CD25+ control T cell populations were isolated from the following: 1) IL-9–deficient and wt animals, or 2) KitW/KitW-v-deficient animal-derived Treg population versus wt Tregs. The responder to suppressor ratio was 5:1 and 1:1. The cells were stimulated by plate-bound anti-CD3 mAb (clone 17A2; BD Biosciences; coating concentration 5 μg/ml). As control, the respective T cell populations (i.e., Tregs and CD4+CD25+ T cells) were cultured alone. Proliferation was measured between days 5 and 7 by [3H]thymidine incorporation in a β-scintillator.

Statistics

When comparing two groups, the nonparametric Mann–Whitney U test was used and p < 0.05 was considered as significant. When comparing three groups, we performed the Kruskal–Wallis test. When significances were detected, the different groups were compared by the Mann–Whitney U test. The level of significance was corrected to the number of groups and p < 0.025 was considered to be significant. All statistical analyses were done using SPSS 13.0.1 for Windows (SPSS, Chicago, IL).

Results

MCs increase in LNs of Treg-treated mice after induction of NTS

C57BL/6 mice received either Tregs or CD4+CD25+ control T cells isolated from healthy C57BL/6 mice and were subjected to NTS. Fourteen days after induction of NTS, LNs of mice were evaluated for the infiltration of MCs. MC tryptase 1 was significantly elevated as compared with NTS animals receiving CD4+CD25+ control T cells (Fig. 1A). Accordingly, Giemsa-stained LN sections of Treg-injected NTS mice showed an almost 3-fold increase of MCs (Fig. 1B) with most of the MCs being located in the LN sinusoids close to the T cell area (Fig. 1C). In contrast to Treg-induced MC recruitment, Treg occupancy of LNs was not affected by MCs, as we did not detect any significant difference in LN Treg content between nephritic MC-deficient KitW/KitW-v when compared with the respective Kit+/+ controls suffering from NTS, as determined by quantification of Foxp3 mRNA (Fig. 2A) and by FACS costaining of CD4+Foxp3+ (Fig. 2B).

Adaptively transferred Tregs do not protect MC-deficient animals from NTS

We next tested whether adoptive Treg transfer can compensate for MC deficiency in KitW/KitW-v mice to further define whether Treg-mediated immunosuppression is preserved in the absence of MCs. Due to the well-known role of Kit signaling for thymic T cell development (32), we first excluded a numerical or functional deficit of Tregs isolated from KitW/KitW-v mice by performing standard Treg quantification by FACS (see representative FACS staining inserted into Fig. 2C, 2D) as well as proliferation and suppression assays with isolated Treg populations from the respective genetic backgrounds (Fig. 2C, 2D). We next transferred Tregs to NTS-subjected KitW/KitW-v and Kit+/+ animals. Notably, in contrast to the well-known protective effects of Tregs in wt animals with NTS, even high numbers of Tregs (5 × 106) were not able to confer protection from NTS in MC-deficient KitW/KitW-v mice. Accordingly, albuminuria, which is a marker for the severity of nephropathy, was significantly increased in the Treg group as compared with the control group. In summary, these results indicate that the presence of MCs is essential for Treg’s protective effects in NTS (Fig. 2).

Statistics

When comparing two groups, the nonparametric Mann–Whitney U test was used and p < 0.05 was considered as significant. When comparing three groups, we performed the Kruskal–Wallis test. When significances were detected, the different groups were compared by the Mann–Whitney U test. The level of significance was corrected to the number of groups and p < 0.025 was considered to be significant. All statistical analyses were done using SPSS 13.0.1 for Windows (SPSS, Chicago, IL).
of kidney damage, was markedly reduced by Tregs in wt nephritic animals, but remained unchanged in MC-deficient KitW/KitW−v mice with NTS when compared with control T cell-transferred animals, respectively (Fig. 3A). This observation was supported by histological data showing that hypercellularity and focal deposition of PAS-positive material were reduced by Tregs in wt animals, but was more pronounced in MC-deficient KitW/KitW−v mice. In the latter, adoptive Treg transfer did not affect morphological changes when compared with control T cell-injected animals as shown by histology (Fig. 3B) or semiquantitative evaluation of PAS deposition (Fig. 3C). Further analysis of kidney sections revealed that the increased disease susceptibility of MC-deficient KitW/KitW−v mice to NTS is also reflected by a significant increase of CD4+ and CD8+ T cells (Fig. 3D) as well as F4/80+ and CD68+ cells in the kidney (Fig. 3E, 3F). Of note, no F4/80+ cells were detected in healthy MC-deficient KitW/KitW−v and Kit+/+ mice (Supplemental Fig. 1). In line with the albuminuria and the histological data, Treg transfer in wt animals decreased inflammatory infiltrates (Fig. 3D–F), whereas adoptive Treg transfer did not alter T cell or macrophage infiltration into kidneys of MC-deficient KitW/KitW−v mice (Fig. 3D–F). To exclude an impaired production of anti-rabbit IgG of the respective animals, we quantified serum anti-rabbit IgG titers. Of note, we could not detect any significant alteration in the titres between the different groups (Fig. 3G), suggesting that the initial priming of B cells during immunization remains unaffected by either MC deficiency or Treg transfer.

To further evaluate the potential mechanism of MC-mediated immunosuppression, we evaluated the T cell response in the LNs 7 d after induction of NTS by performing real-time PCR for the mRNA expression of Th1, Th2, and Th17 markers. The Th1 cytokines IL-6 and IFN-γ and the Th17 cytokine IL-17A were significantly increased in LNs of KitW/KitW−v mice as compared with Kit+/+ mice. IL-10 was also found to be significantly increased in LNs of KitW/KitW−v mice. In contrast, other Th2 marker Gata-3 and TGF-B1 were not affected by MC deficiency (Fig. 4).

IL-9 links Tregs and MCs in NTS

We next focused on a potential candidate linking Tregs and MCs. Thus, we evaluated the functional role of IL-9 for Treg/MC interaction, as IL-9 functions as a key proliferation/differentiation factor and chemoattractant for MCs (33), produced by Tregs (23), and has been implicated to be a key cytokine regulating the interaction between Tregs and MCs (23). To address this particular question, NTS mice receiving Tregs were either i.p. injected with a blocking anti–IL-9 mAb or the respective isotype control Ab every second day. Systemic blockade of IL-9 reversed the protective effects of adoptively transferred Tregs in NTS, as shown by a significantly increased albuminuria when compared with the Treg/isotype control group (Fig. 5A). Again, this was accompanied by the respective histological changes, that is, increased PAS-positive deposits in the glomeruli (Fig. 5B) and decreased renal inflammatory cell infiltrates (Table I). Most importantly, the increased infiltration of MCs (quantified by semiquantitative staining of LNs for CD117 and by detection of MC tryptase 1 mRNA levels) in the LNs of Treg-treated mice was reduced in the anti–IL-9 mAb-treated animals to the levels of mice receiving control cells (Fig. 5C, 5D).

To rule out the possibility that IL-9 mAb antagonizes IL-9 secreted from various cell types [e.g., Th9 (34), Th17 cells (35)] in this complex inflammation model, we next sought to define the functional significance of Treg-derived IL-9 in this particular model by using Tregs isolated from IL-9 knockout mice for adoptive transfer. Tregs from IL-9–deficient animals failed to reduce albuminuria, histological changes (Fig. 6A–C), or the renal cellular infiltrates (Table II), as well as the amount of the mouse anti-rabbit IgG titers remained unaltered (Table II). Moreover, the increase of MCs in the LNs of NTS mice receiving wt Tregs was blunted in NTS animals that received IL-9–deficient Tregs (Fig. 6D). Of note, no significant difference in the deposition of fibrin in the glomeruli of the three different groups was detected (Fig. 6E). Additionally, we excluded that IL-9–deficient Tregs per se are characterized by a defective immunosuppressive potential. Tregs derived from IL-9–deficient animals exert a comparable target cell inhibition when cultured with wt responder T cells (Fig. 6F). Finally, we evaluated chemokines known to be involved in the recruitment of MCs. Interestingly, Cxcl-1 was found to be significantly decreased in the LNs of mice receiving IL-9–deficient Tregs as compared with mice receiving wt Tregs or control cells (Fig. 7A). In contrast, Cxcl-2 and -5 were found to be up-regulated in LNs 7 d after NTS induction, but remained unaffected by any of the treatment modalities (Fig. 7A). Comparable results were found in mice treated with the IL-9–blocking mAb (Fig. 7B).

Discussion

Using the well-described murine complement–, Th1 and Th17 T cell-mediated NTS model (29), in this study, to our knowledge, we provide the first evidence that the nephroprotective effects of adoptively transferred Tregs depend on MC recruitment to the kidney-draining LNs, which is linked by the cytokine IL-9.

In line with various disease models (reviewed in Ref. 36), Tregs have been shown to be essential in the regulation of NTS (5). The importance of this cell population in renal disease such as human Goodpasture’s disease or kidney transplantation has also been convincingly demonstrated (37, 38). MCs have long been thought to only exert effector cell functions because they contain an armada of proinflammatory cytokines and chemokines stored in their granules. But more recently, it has been discussed that they can also function as an immunosuppressive cell population (15). In line, MC-deficient mice subjected to NTS were found to develop significantly increased disease indices as compared with respective wt control animals (21, 22). In the recent past, the interaction between Tregs and MCs gained attention in various

| Table I. The role of systemic IL-9 blockade on renal inflammatory cell infiltrates after Treg transfer |
|-----------------|-----------------|-----------------|
| Groups   | Control | Tregs | Tregs + Anti–IL-9 |
| CD4+ cells (no. per 6 Hpf) | 54.8 ± 9.6 | 14.2 ± 3.5a | 21.8 ± 3.4 |
| F4/80+ cells (score) | 1.05 ± 0.27 | 0.45 ± 0.08ab | 0.75 ± 0.07 |
| CD68+ cells (score) | 1.75 ± 0.40 | 0.55 ± 0.11a | 0.88 ± 0.21 |

The infiltration of CD4+, F4/80+, and CD68+ cells was evaluated in the kidneys of the respective animals (n = 5 per group) 14 d after induction of NTS. The data are presented as mean ± SEM.

a p < 0.025 between the control T cell and the Treg group

b p < 0.025 when the Tregs and Tregs + anti–IL-9 group are compared.
disease models (13, 39–41). Supporting a possible interaction, we observed that NTS mice receiving Tregs displayed significantly increased MC infiltrates in the kidney-draining LNs. Intriguingly, Treg transfer cannot overcome the lack of MCs in our hands. Gri et al. (13) first provided evidence that Tregs might interact with MCs in allergic disorders by limiting the degranulation of MCs in vitro. Favoring our hypothesis, in vivo Treg depletion resulted in an increased anaphylactic reaction. Other reports found MCs to have the potential to regulate Treg function in vitro (39–41). They found MCs either to suppress (39, 40) or enhance (41) Treg function by various mechanisms. The difference in the in vitro experiments might be explained by variations in the surrounding conditions, which seem to be of crucial importance for the function of MCs. In line, MC-deficient mice were found to differentially develop various inflammatory disease models. Although they were found to be protected from inflammatory arthritis (42) or experimental allergic encephalomyelitis (43), they developed increased disease indices when subjected to NTS (21, 22), contact

**FIGURE 6.** Treg-derived IL-9 is critical for MC-dependent nephroprotection in NTS. NTS mice received either wt Tregs (white bar), control T cells (black bar), or Tregs isolated from IL-9-deficient animals (gray bar). A, The albumin/creatinine ratio in the urine was evaluated 7 d (n = 7–9) and 14 d (n = 5–7) after induction of NTS. B, Representative PAS-stained kidney sections from the indicated groups 14 d after induction of NTS are shown. Original magnification ×400. Mice receiving either control cells or IL-9–deficient Tregs displayed crescent formations and large PAS-positive deposits in their glomeruli, whereas Treg-treated mice were found to have only marginal PAS-positive deposits and no crescent formations. C, Quantification of PAS deposition by the PAS score is given for the respective groups. D, The kidney-draining LNs were evaluated for MC infiltration by real-time PCR for MC tryptase 1 mRNA. The data are expressed as x-fold increase of MC tryptase 1/β-actin compared with healthy control LNs (which was set as 1). All data are presented as mean ± SEM. *p < 0.05. E, Renal cryosections of the three groups were stained for fibrin deposition. No significant differences were observed. Representative pictures are shown. Original magnification ×400. F, Evaluation of the immune-suppressive potential of CD4+CD25+ Tregs from IL-9–deficient animals. Tregs from wt or IL-9–deficient animals were coincubated with CD4+CD25+ T cells in a 1:1 ratio. Proliferation was measured by [³H]thymidine incorporation after 7 d, and the percentage of proliferation compared with the respective control CD4+CD25+ T cell population is shown. The data are presented as mean ± SEM; *p < 0.025; n.s., not significant. Two independent experiments were performed.
IL-9 has been implicated as a crucial factor in the regulation of MC recruitment and their effector functions (28, 33). IL-9 is secreted from various cell types, namely Th9 (34), Th17 cells (35), and Tregs (23). Recent reports have suggested that IL-9 is an important factor driving Th17 T cell differentiation and supporting Treg function (35, 45). However, the exact role of IL-9 produced by Tregs in an in vivo model remains elusive to date. We in this study show that Treg-derived IL-9 is the central mediator linking the nephroprotective Treg effects in NTS to the induction of protective MC responses. This is clearly demonstrated by the observation that Tregs lacking IL-9 failed to suppress NTS. In contrast to Tregs from IL-9R–deficient animals (45), Tregs derived from IL-9–deficient animals exert a comparable target cell inhibition when cultured with wt responder T cells. The most likely reason for this observation is that IL-9 produced by the wt responder T cell population supports Treg function, whereas in vivo the Treg-derived IL-9 is the critical factor regulating MC accumulation in the inflammatory LNs. Whether Treg-derived IL-9 promotes the recruitment and/or the proliferation of MCs remains to be determined. Previous studies provided evidence that inflammation results in a remarkable MC hyperplasia, which is the result of both a limited expansion of mature MCs and a more dramatic expansion of MC progenitors accompanied by their maturation within the inflamed tissue, to which they are recruited (reviewed in Ref. 46). CXCR-2 expressed on MCs has been implicated in this recruitment process in an inflammatory model of the intestine (47). In our model, only one CXCR-2 ligand, namely KC/Cxcl-1, was regulated by either IL-9–blocking Ab or transfer of IL-9–deficient Tregs. In contrast, other CXCR2 ligands such as Cxcl-5/Cxcl-2 and MIP-2/Cxcl-5 remained unaltered in the absence of either systemic or Treg-derived IL-9. Whether the regulation of KC/Cxcl-1 is reflecting decreased MC infiltration or whether it is the key regulator in the recruitment of MCs by Treg-derived IL-9 needs to be explored in future studies.

The mechanism how MCs exert their immune-suppressive effects on kidney-damaging effector T cells remains elusive to date. Histamine secreted by MCs might be an interesting candidate, as exposure of dendritic cells to histamine induces a Th2 polarization (48, 49) and histamine injection itself inhibits NTS (50). Furthermore, it has been implicated that IL-10 or TGF-β both produced by MCs might contribute to the immunosuppressive state (51, 52). We found MCs to influence the T cell response in the LNs, as MC deficiency leads to increased mRNA expression of Th1 and Th17 markers, which is reminiscent to the effects seen in Treg-depleted animals (K. Eller and A. Rosenkranz, unpublished data) and which suggests that, at least in the model of NTS, both MCs and Tregs are endogenous immunosuppressive cells limiting inflammatory processes leading to renal tissue damage (5, 20). In summary, despite some limitations of the NTS model (e.g., that parts of the Ag presentation depend on the initial immunization process and that the Ag might not be renal specific, which might at least in part be modified by Treg transfer), we are confident that Tregs indeed recruit MCs to the local LNs, thus tipping the balance toward a more immunosuppressive milieu inhibiting the various inflammatory processes involved in renal inflammation and subsequent organ dysfunction. The excess of immune complex deposition in the kidney is not altered in the Treg-injected animals. This strongly supports the idea that the initial priming process resulting in anti-rabbit IgG production is not altered by Tregs, but that the Tregs are indeed primarily involved in regulating the systemic T cell immune response, which leads to amelioration of renal inflammation. The importance of Tregs localized to the regional LNs is supported by our recent data showing that CCR7-

**Table II. The role of Treg-derived IL-9 on mouse anti-rabbit IgG Ab titres and renal inflammatory cell infiltrates after Treg transfer**

<table>
<thead>
<tr>
<th>Groups</th>
<th>Control</th>
<th>wt Tregs</th>
<th>IL-9 ko Tregs</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mouse anti-rabbit IgG (OD450)</td>
<td>0.293 ± 0.040</td>
<td>0.354 ± 0.122</td>
<td>0.236 ± 0.060</td>
</tr>
<tr>
<td>CD4⁺ cells (no. per 6 Hpf)</td>
<td>30.0 ± 16.2</td>
<td>12.4 ± 4.3⁺</td>
<td>33.0 ± 5.5</td>
</tr>
<tr>
<td>F4/80⁺ cells (score)</td>
<td>3.38 ± 0.54</td>
<td>1.90 ± 0.26ᵇ</td>
<td>3.40 ± 0.17</td>
</tr>
<tr>
<td>CD68⁺ cells (score)</td>
<td>3.25 ± 0.28</td>
<td>1.80 ± 0.33(conv)</td>
<td>3.00 ± 0.37</td>
</tr>
</tbody>
</table>

Rabbit anti-mouse IgGs were evaluated in the serum of mice of the three different groups (n = 5 per group) 14 d after induction of NTS. The infiltration of CD4⁺, F4/80⁺, and CD68⁺ cells is given. The data are presented as mean ± SEM.⁺p < 0.025 when comparing the wt Tregs and IL-9–deficient Tregs group.ᵇp < 0.025 between the control T cell- and the Treg-injected group.

**ko**, knockout.
mediated occupancy of the kidney-draining LNs is required for their protective cues (20).

Thus, to our knowledge, our data provide the first evidence that the immunosuppressive effects of adoptively transferred Tregs depend on IL-9–mediated recruitment of MCs to the kidney-draining LNs in NTS. This model is in perfect agreement with our previous report showing that CCR7-mediated LN occupancy of Tregs is a prerequisite for their immune-suppressive potential and further adds a piece of information to the functional understanding of the in vivo anti-inflammatory effects of Tregs.

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Disclosures

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References


