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DIFFERENTIAL CONTROL OF IMMUNE CELL HOMEOSTASIS BY FOXP3$^+$ REGULATORY T CELLS IN MURINE PERIPHERAL LYMPH NODES AND SPLEEN

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Foxp3$^+$ regulatory T cells (Tregs) hamper efficient immune responses to tumors and chronic infections. Therefore, depletion of Foxp3$^+$ Tregs has been proposed as therapeutic option to boost immune responses and to improve vaccinations. Although Treg-mediated control of T cell homeostasis is well established, Foxp3$^+$ Treg interaction with other immune cell subsets is only incompletely understood. Thus, the present study aimed at examining dynamic effects of experimental Foxp3$^+$ Treg depletion on a broad range of immune cell subsets, including B cells, natural killer cells, and myeloid cells. Striking differences were observed when peripheral lymph nodes (LN) and spleen were compared. B cells, for example, showed a massive and long-lasting accumulation only in LN but not in spleen of transiently Treg-depleted mice. In contrast, monocyte-derived dendritic cells, which are potent inducers of T cell responses, also accumulated selectively, but only transiently in LN, suggesting that this cell population is under very strict control of Foxp3$^+$ Tregs. In summary, the observations described here provide insights into the dynamics of immune cells after selective depletion of Foxp3$^+$ Tregs. This will allow a better prediction of the impact of Treg ablation in translational studies that aim at boosting immune responses and vaccinations.

Keywords: immune homeostasis, Foxp3$^+$ regulatory T cells, lymph nodes, spleen, lymphoid organs, immune cell subsets

Introduction

Foxp3$^+$ regulatory T cells (Tregs) are a subset of CD4$^+$ T cells specialized in immunosuppression and being fundamental for preservation of immune homeostasis [1]. Selective depletion of Foxp3$^+$ Tregs by diphtheria toxin (DT) injection in transgenic mouse models that express the DT receptor under control of the *Foxp3* gene locus considerably helped understanding different aspects of Treg biology [2, 3]. On the one hand, sustained Treg depletion in mice induces catastrophic autoimmunity, which elegantly confirmed the indispensible role of Tregs in maintaining immune homeostasis [3]. On the other hand, transient Treg ablation provided important insights into Treg function in regulating tumor immunity, autoimmune diseases, and immune responses against infections [4–6].

It is widely accepted that Foxp3$^+$ Tregs can control the activation and effector functions of various immune cells. In particular, Treg-mediated control of conventional CD4$^+$ and CD8$^+$ T cells is well established, with cytokine consumption, direct killing as well as inhibition of activation and effector function being the most important mechanisms [7]. Furthermore, Foxp3$^+$ Tregs were described to directly and indirectly regulate other immune cell subsets, such as conventional dendritic cells (DCs) and natural killer (NK) cells [8–12]. DCs are considered as the hub of the immune system; this innate cell type senses signals in the respective environment and migrates to secondary lymphoid organs to initiate and shape an adequate adaptive immune response [13, 14]. In the steady state, the size of the DC population seems to be tightly controlled by the size of the Foxp3$^+$ Treg population [8, 9]. A loss of Foxp3$^+$ Tregs leads to a gain of DCs, which in turn induces an expansion of Foxp3$^+$ Tregs. This feedback loop is controlled by Flt3 [8, 9]. NK cells are innate lymphocytes, which become activated, among others, by interleukin (IL)-2 and are able to limit virus spread and tumor growth before adaptive immunity is mounted [15]. The homeostasis of the NK cell population is also modulated by Tregs, and Treg-mediated inhibition of IL-2 production as well as IL-2 consumption

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plays a central role in NK cell homeostasis [10–12]. Furthermore, under inflammatory conditions, Tregs can efficiently suppress B cell responses [16–19]. Interestingly, although no B cell intrinsic role for Foxp3 was found, B cell development is defective in Foxp3-deficient mice [20, 21]. However, since these animals suffer from severe multi-organ autoimmune disease, it is difficult to distinguish between effects on B cells caused directly by Treg absence from those caused by systemic inflammation.

Since Foxp3$^+$ Treg depletion has been proposed as strategy to improve immunotherapy and vaccination in clinical settings, it is important to conclusively establish whether and how Foxp3$^+$ Tregs regulate homeostasis of critical immune cell subsets. Whereas the role of Foxp3$^+$ Tregs in keeping immune homeostasis by modulating the function of T cells, DCs, and NK cells is well established [8, 9, 22], only limited knowledge exists regarding the effects of Foxp3$^+$ Treg depletion on myeloid cells such as monocytes and neutrophils, and previously published studies lack careful kinetic analyses of the effects of Foxp3$^+$ Treg depletion on the immune cell subset homeostasis. Thus, the aim here was to analyze the dynamics of various immune cell subsets in major secondary lymphoid organs after selective depletion of Foxp3$^+$ Tregs. For this purpose, the depletion of regulatory T cells (DEREG) mouse model was applied, which allows for selective and transient ablation of Foxp3$^+$ Tregs *in vivo* without development of severe multi-organ autoimmune diseases [2]. Absolute numbers of DCs, NK cells, B cells, monocytes, and neutrophils in LN and spleen were determined by multicolor flow cytometry. The data presented here indicate a previously unrecognized role of Foxp3$^+$ Tregs in controlling various immune cell subsets in secondary lymphoid organs. Further, substantial differences in the response of different immune cell populations to Foxp3$^+$ Treg depletion were observed when comparing LN and spleen. Hence, this study describes new findings on the kinetics of different immune cell subsets after transient depletion of Foxp3$^+$ Tregs. It also provides information valuable to better design translational studies focusing on the use of Foxp3$^+$ Treg depletion to enhance wanted immune responses and to improve the outcome of vaccinations.

Materials and methods

Mice

DEREG mice and wild-type (WT) littermates (BALB/c background) were bred at the animal facility of the Helmholtz Centre for Infection Research (HZI). Eight to twelve-week-old male mice were used. All animal experiments were performed under specific pathogen-free conditions and in accordance with institutional, state, and federal guidelines in accordance with good animal practice as defined by Federation of European Laboratory Animal Science Association (FELASA) and the national animal welfare body Gesellschaft für Versuchstierkunde – Society of Laboratory Animal Science (GV-SOLAS) under supervision of the institutional animal welfare officer. The number of animals used was notified to the Lower Saxony State Office for Consumer Protection and Food Safety according to the German laboratory animal-reporting act (VersTierMeldV BGBl S. 2156; 04.11.1999).

DT and Treg depletion

For Treg depletion *in vivo*, DEREG mice and WT littermates were injected intraperitoneally with 1 µg DT (Merck/Calbiochem) diluted in 100 µl sterile *phosphate buffered saline* (PBS) (Gibco). DEREG mice and WT littermates treated with PBS were used as controls (time point zero in each graph). For measurements, mice were sacrificed daily in the first week after DT injection as well as on days 10, 14, and 21. Cells from LN and spleen were isolated and analyzed.

Organ isolation and preparation of single cell suspensions

Mice were sacrificed by CO_2 asphyxia in compliance with the German animal protection law (TierSchG BGBI S. 1206; 18.05.2006). LN and spleen were taken. Single cell suspensions were prepared by mechanical squeezing of LN and minced spleens through 100 µm nylon meshes. Erythrocytes in spleen samples were lysed by incubation with ammonium–chloride–potassium (ACK) buffer for 4 min at room temperature. Lysis was stopped by diluting ACK buffer in a tenfold volume of PBS–bovine serum albumin (BSA). Centrifugation steps were performed at 4 °C and 450 g for 8 min. If not described otherwise, samples were subsequently maintained in PBS–BSA.

Antibodies

The following antibodies were used: anti-CD3 (17A2 and 145-2C11), anti-CD4 (RM4-5), anti-CD11b (M1/70), anti-CD11c (N418), anti-CD19 (MB19-1), anti-CD45R (RA3-6B2), anti-CD49b (DX5), anti-CD122 (TM-b1), anti-CD335 (29A1.4), anti-Ly6C (HK1.4), and anti-Ly6G (1A8). The amount of antibodies needed for each staining was previously defined by titration series. All antibodies were purchased from eBioscience, BioLegend, BD Biosciences, Santa Cruz Biotechnology, or R&D Systems.

Cell staining

Dead cells were discriminated using LIVE/DEAD® fixable dead cell stain kit (Invitrogen), following instructions by the manufacturer. Specific antibody staining was performed at 4 °C in the dark for 15 min (surface staining) or 30 min (intracellular staining using Foxp3/Transcrip-

tion Factor Staining Buffer Set from eBioscience). To reduce unspecific antibody binding, surface and intracellular staining were performed in the presence of anti-CD16/CD32 (2.4G2, BioXCell) antibodies and ChromPure rat IgG whole molecule (Jackson ImmunoResearch), respectively.

Flow cytometry data acquisition

Data was acquired on a BD LSR II SORP using BD FACSDiva software (BD Biosciences). Data were analyzed using FlowJo software (Treestar). Doublets and dead cells were excluded from flow cytometry analysis. Absolute cell numbers were calculated using a BD Accuri C6 (BD Biosciences) flow cytometer according to instructions by the manufacturer.

Statistics

The following calculation of *p* values was applied to the comparison of the effect of DT treatment on different immune cell subsets in WT and DEREG mice. Log2 Laplace-corrected values were considered, i.e., 1 was added to each of the original values and then the logarithm was calculated. The Laplace correction was necessary because there are zero values for which the logarithm is not defined. Under the assumption that the WT values followed roughly the same distribution over the whole period of time, the WT values were taken as an empirical distribution (most of the WT value distributions did not resemble a normal distribution). The smallest value of the available DEREG values at time point *t* was considered. When there are *n* DEREG values available at time point *t*, the *p* value is the probability that a random sample of size *n* from the WT values over the whole time period contains only values greater than or equal to the minimum of the considered DEREG values.

Results

Total number of leukocytes increases in LN but not in spleen upon Foxp3$^+$ Treg depletion

Transgenic DEREG mice express the DT receptor under the control of the *Foxp3* gene locus and therefore allow for selective and transient depletion of Foxp3$^+$ Tregs [2]. A single injection of DT leads to the rapid disappearance of Foxp3$^+$ Tregs from secondary lymphoid organs within 36–48 h followed by a fast and complete rebound within 4–5 days after DT injection *(Fig. 1)*. Importantly, the overall kinetic of Foxp3$^+$ Treg depletion and rebound was comparable between LN and spleen. Other mouse models of Foxp3$^+$ Treg depletion show similar kinetics, although the rebound quickness depends on the level of depletion [23, 24].

We analyzed the effect of a single intraperitoneal injection of DT into adult DEREG mice on the cellularity of secondary lymphoid organs, including LN (pool of inguinal, brachial, axillary, submandibular, and cervical LN) and spleen. Interestingly, Foxp3$^+$ Treg depletion resulted in a transient, but significant increase in the total number of leukocytes solely within LN, but not in spleen *(Fig. 2)*. Wild-type (WT) littermate controls did not show any effect of DT application. The number of total leukocytes peaked at day 6 after Foxp3$^+$ Treg depletion and returned to baseline levels 21 days after DT application. This observation indicates that the transient depletion of Foxp3$^+$ Tregs leads to a disturbed homeostasis of the immune cell compartment in LN, whereas the spleen seems to be unaffected.

DCs, NK cells, monocytes, and neutrophils display different accumulation kinetics in LN and spleen upon Foxp3$^+$ Treg depletion

A range of lymphoid and myeloid cells is present in LN and spleen under steady state conditions. Multicolor flow

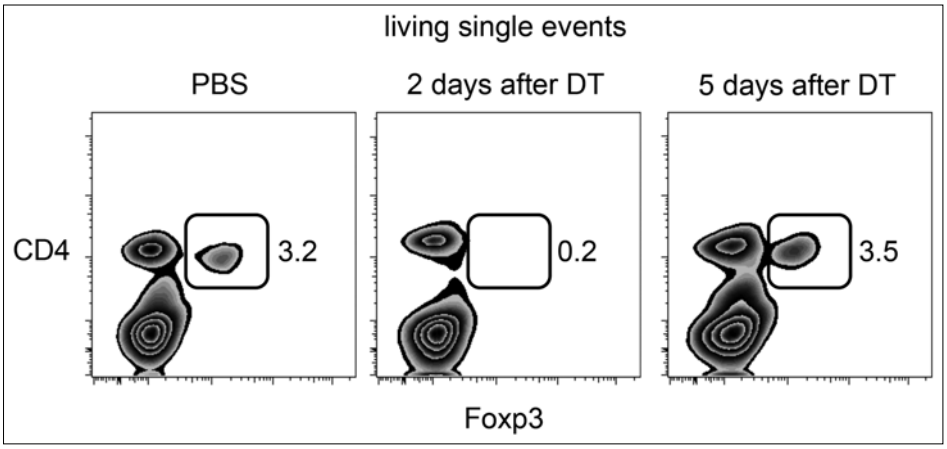

Fig. 1. Single DT injection in DEREG mice leads to quick depletion and rebound of Foxp3$^+$ Tregs. Zebra plots show splenocytes from DEREG mice receiving either a single injection of PBS or DT on day 0 and submitted to flow cytometry analysis either on day 2 or 5 after treatment. Only living single events were considered. Balloons represent gates, and numbers are the percentage of Foxp3$^+$ Tregs among all events. Data are representative for at least four mice per time point in two independent experiments

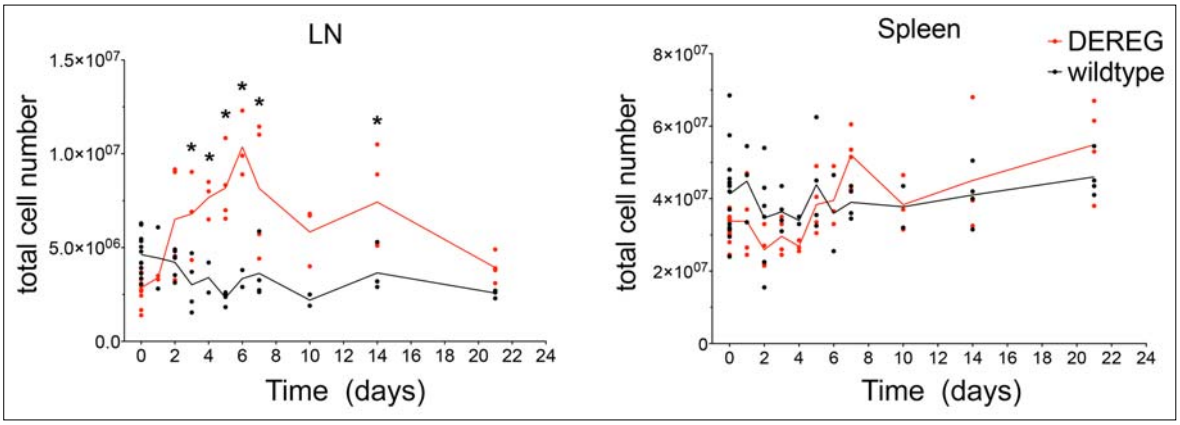

Fig. 2. Total number of leukocytes increases in LN but not in spleen upon Foxp3$^+$ Treg depletion. Left and right panels show total number of leukocytes in LN and spleen, respectively. Mice were treated with DT at day 0. Red symbols show data from DEREG mice while black symbols show data from WT mice. Values for individual mice (circles) as well as lines connecting means are plotted. Data were pooled from at least two independent experiments per time point (n = 2–14). *p < 0.05 at indicated time point

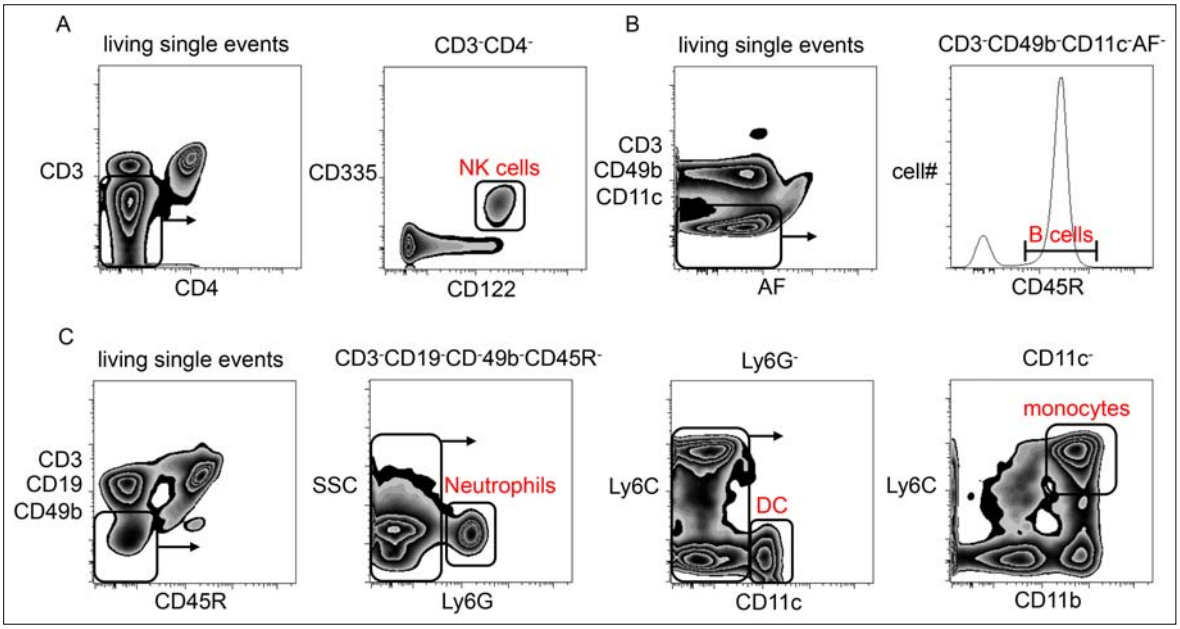

Fig. 3. Multicolor flow cytometry allows for characterization of the response of a range of immune cell subsets to Foxp3$^+$ Treg depletion. Balloons represent gates, and arrows denote when further gating was performed. Only living single events were considered. A: NK cells were defined as CD3$^-$CD4$^-$CD335$^+$CD122$^+$ events. B: B cells were CD3$^-$CD49b$^-$CD11c$^-$AF$^-$CD45R$^+$ events. C: Neutrophils were CD3$^-$CD19$^-$CD49b$^-$CD45R$^-$Ly6G$^+$ events. DCs were CD3$^-$CD19$^-$CD49b$^-$CD45R$^-$Ly6G$^-$Ly6C$^-$CD11c$^+$ events. Monocytes were CD3$^-$CD19$^-$CD49b$^-$CD45R$^-$Ly6G$^-$CD11c$^-$Ly6C^{++}CD11b$^+$ events. AF: autofluorescence

cytometry was applied to analyze immune cell composition in LN and spleen of Foxp3$^+$ Treg-depleted mice. The gating strategy allows identification of NK cells, B cells, DCs, monocytes, and neutrophils *(Fig. 3)*. When analyzed over time, DCs and NK cell numbers showed a slight and transient increase upon Foxp3$^+$ Treg depletion within spleen, whereas the effect was more pronounced and long lasting in LN *(Fig. 4)*, supporting previous observations [8–12]. Interestingly, the increase in NK cell numbers preceded the increase in DC numbers, with NK cells achieving high levels already on day 3 after DT application, while DCs reached maximum numbers as early as day 4. Nevertheless, the decline in both cell populations in LN was comparable, starting on day 6 and reaching close to baseline levels by day 10. When monocyte and neutrophil numbers were analyzed, a slight and transient increase upon Foxp3$^+$ Treg depletion was observed in the spleen, whereas the effect was more pronounced and long lasting within LN *(Fig. 5)*. It is worthy to mention that WT littermate controls treated with DT also showed a rapid increase in splenic neutrophil numbers; however, this response was only very short-lived. Together, these observations suggest that Foxp3$^+$ Treg depletion influences homeostasis of DCs, NK cells, monocytes, and neutrophils in an organ-specific manner.

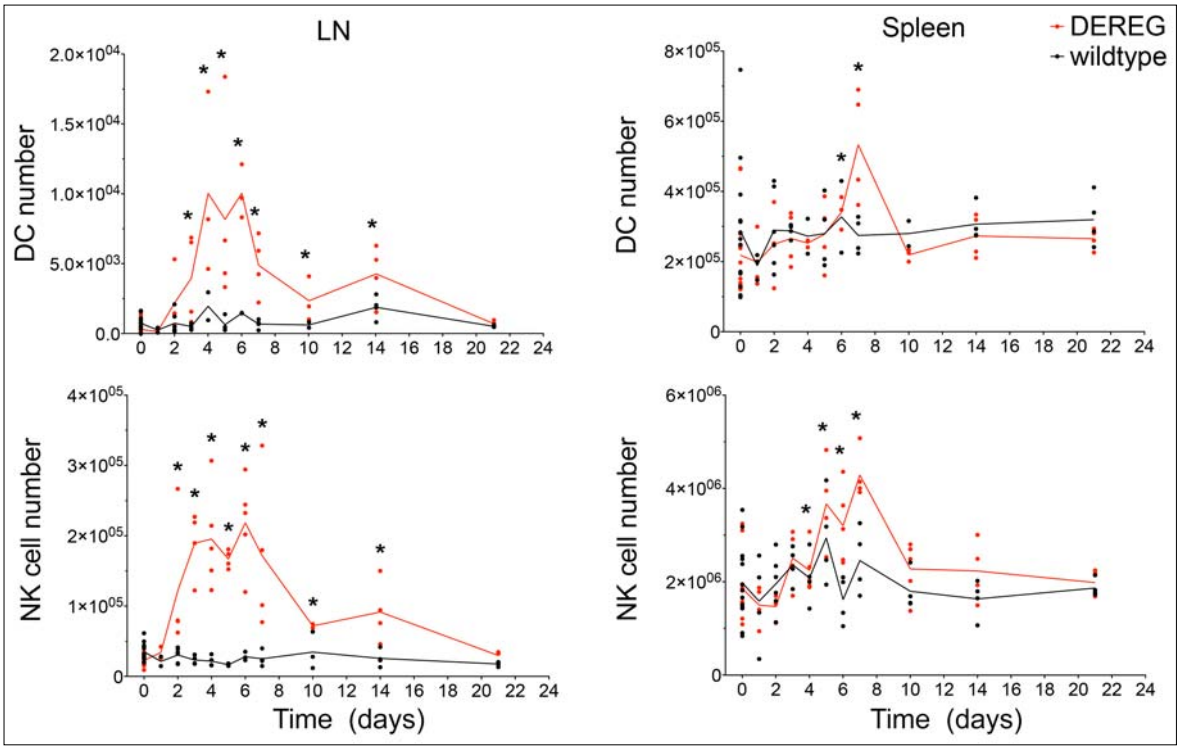

Fig. 4. DCs and NK cells transiently accumulate in the LN and spleen upon Foxp3$^+$ Treg depletion. Left and right panels show the number of indicated cell populations in LN and spleen, respectively. Upper panels show data for DCs, and lower panels for NK cells. DEREG mice (red) and WT littermates (black) were treated with DT at day 0. Values for individual mice (circles) as well as lines connecting means are plotted. Data were pooled from at least two independent experiments per time point (n = 2–14). *$p < 0.05$ at indicated time point

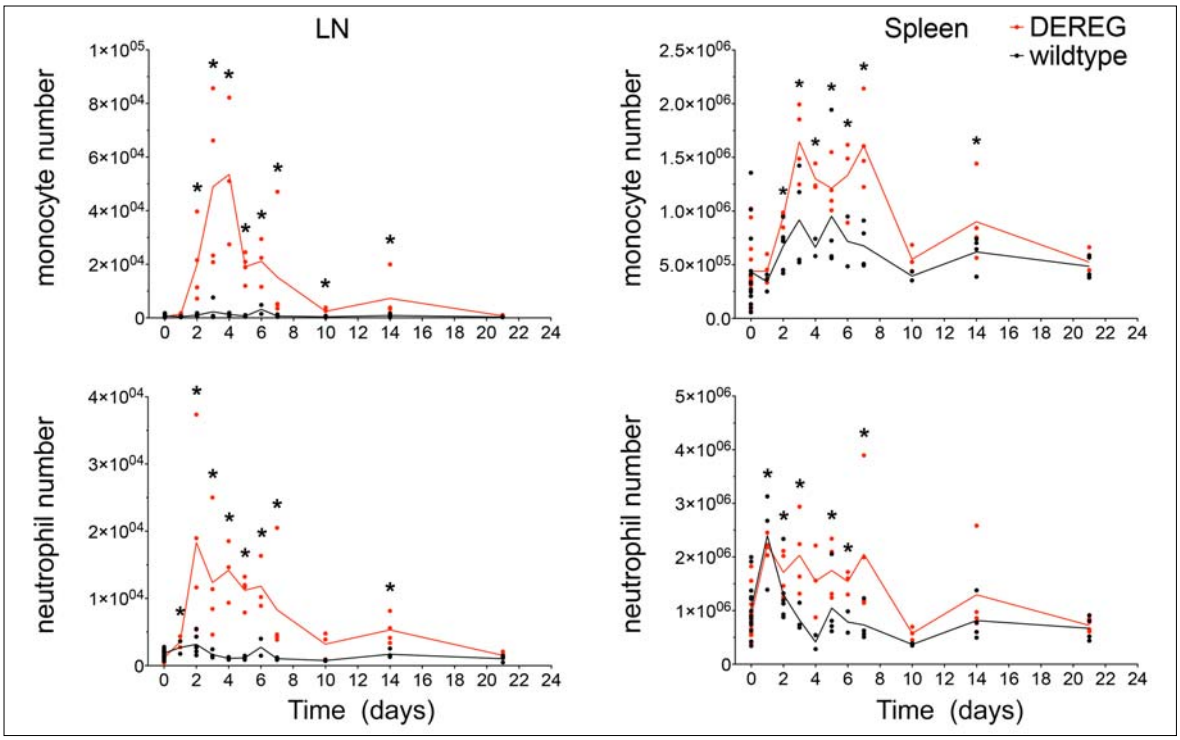

Fig. 5. CD11b$^+$Ly6C$^+$Ly6G$^+$ and CD11b$^+$Ly6C^{++}Ly6G$^-$ cells transiently accumulate in both LN and spleen upon Foxp3$^+$ Treg depletion. Left and right panels show the number of indicated cell populations in LN and spleen, respectively. Upper panels show data for CD11b$^+$Ly6C^{++}Ly6G$^-$ cells, and lower panels for CD11b$^+$Ly6C$^+$Ly6G$^+$ cells. DEREG mice (red) and WT littermates (black) were treated with DT at day 0. Values for individual mice (circles) as well as lines connecting means are plotted. Data were pooled from at least two independent experiments per time point (n = 2–14). *$p < 0.05$ at indicated time point

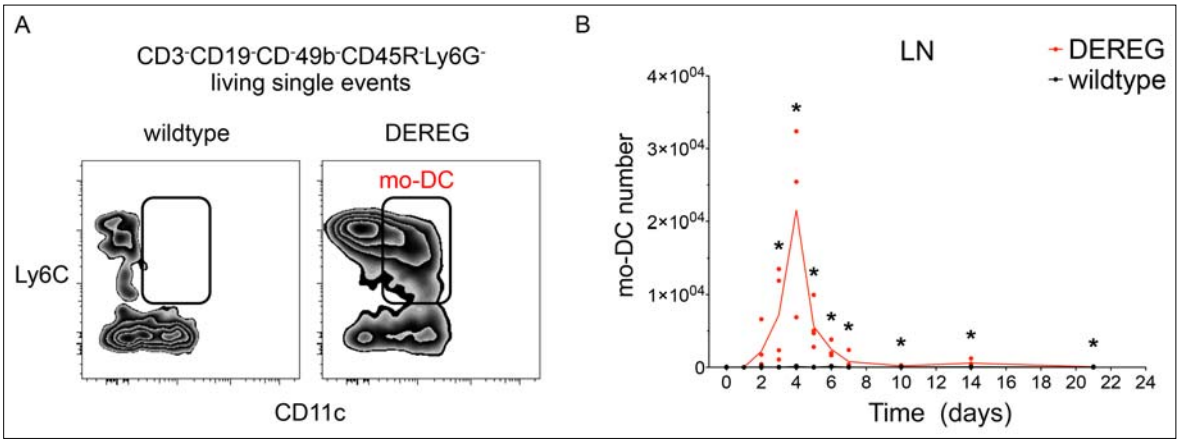

Fig. 6. mo-DCs appear punctually in LN upon Foxp3$^+$ Treg depletion. A: gating strategy for mo-DCs on day 4 upon DT is shown. B: the numbers of mo-DCs in LN are plotted. DEREG mice (red) and WT littermates (black) were treated with DT at day 0. Values for individual mice (circles) as well as lines connecting means are plotted. Data were pooled from at least two independent experiments per time point (n = 2–14). *$p < 0.05$ at indicated time point

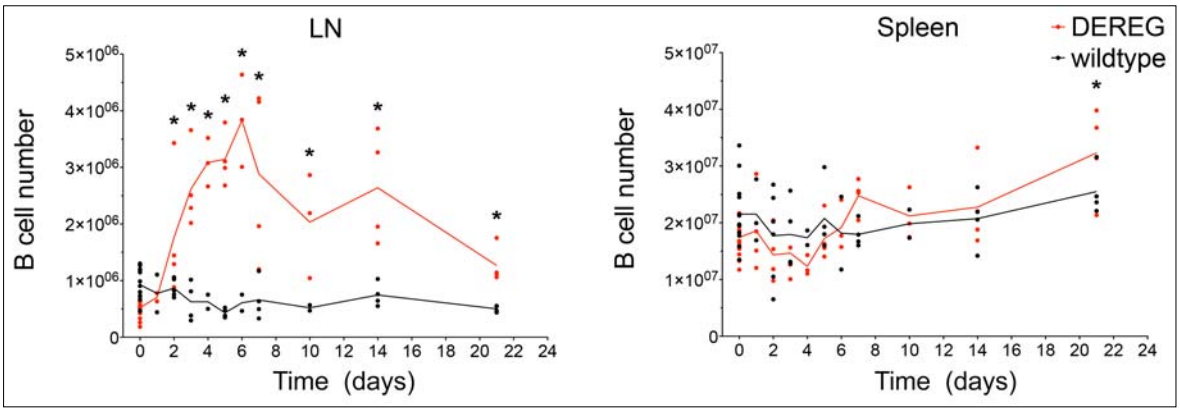

Fig. 7. B cells accumulate for at least 3 weeks in LN but not in the spleen upon Foxp3$^+$ Treg depletion. Left and right panels show the number of B cells in LN and spleen, respectively. DEREG mice (red) and WT littermates (black) were treated with DT at day 0. Values for individual mice (circles) as well as lines connecting means are plotted. Data were pooled from at least two independent experiments per time point (n = 2–14). *$p < 0.05$ at indicated time point

Monocyte-derived DCs appear punctually only in LN upon Foxp3$^+$ Treg depletion

Monocytes respond to stimuli with an impressive plasticity. Upon acute inflammation, they can differentiate into monocyte-derived (mo-) DCs with the ability to migrate to LN draining inflamed tissues [25]. mo-DCs, characterized by the expression of CD11b and intermediate to high levels of Ly6C and CD11c, could be detected in LN of DT-treated DEREG mice (*Fig. 6A*, right panel), while they were completely absent from LN of DT-treated WT littermate controls (*Fig. 6A*, left panel) and from the spleen both before and after treatment (data not shown). The accumulation of mo-DCs in LN of Foxp3$^+$ Treg-depleted mice started at day 2, peaked at day 4 and rapidly declined close to baseline levels until day 7 *(Fig. 6B)*. Hence, Treg depletion led to a transient accumulation of mo-DCs selectively in LN.

B cells display a profound and long-lasting increase in numbers only in LN but not in spleen upon Foxp3$^+$ Treg depletion

Foxp3$^+$ Tregs were shown to control antigen-specific B cell responses [16–19], and high titers of autoantibodies can be found in Foxp3-deficient mice [20, 26]. Upon selective and transient depletion of Foxp3$^+$ Tregs, we observed here a considerable and long-lasting increase in B cell numbers in LN, while the spleen remained largely unaffected *(Fig. 7)*. The increase in B cell numbers started as early as 2 days after DT application and lasted at least until day 21. Given the observed expansion of B cells in LN, one could expect an increased incidence of autoimmunity in mice due to a potential production of self-reactive antibodies. However, no overt signs of autoimmunity were detected in these mice even 60 days after Foxp3$^+$ Treg depletion (data not shown).

Viewed as a whole, our results demonstrate that Foxp3$^+$ Treg depletion leads to dysregulated homeostasis of

different immune cell subsets, including B cells, NK cells, and various myeloid cells, in an organ-specific manner.

Discussion

In this study, the role of Tregs for keeping the immune system in balance was investigated. It was examined how the LN and splenic populations of different immune cell subsets respond to a transient depletion of Tregs. The results demonstrate that Treg-mediated regulation acts in a broad immunological context with important differences observed in LN and spleen.

$Foxp3^+$ Tregs are known to control the size of both DC and NK cell populations [8–12], so that a direct effect of Treg depletion on DCs and NK cells should be expected. Indeed, homeostasis of DCs and NK cells was disturbed in response to Treg ablation in the present study. DCs and NK cells accumulated rapidly and for around 1 week in LN, but showed a delayed and shorter accumulation in spleen. These organ-specific differences were not described before. The similar pattern of response observed for DCs and NK cells reinforces the observation of a direct interaction between these cell populations *in vivo* [27]. More specifically, DCs express the IL-15 receptor α chain, which is necessary for trans-presentation of IL-15, a survival and growth factor for mature NK cells in the periphery [28]. Accordingly, an increase in the number of DCs should lead to an increase in IL-15 trans-presentation followed by an increase in the NK cell population. Although the Treg–DC–NK cell axis was discussed before [29], future studies on the direct role of DCs in controlling the NK cell population *in vivo* in the absence of Tregs need to be performed.

Monocytes and neutrophils, defined as $CD11b^+Ly6C^{++}Ly6G^-$ and $CD11b^+Ly6C^+Ly6G^+$ cells, respectively, were shown here to accumulate upon Treg ablation in the lymphoid organs analyzed. Interestingly, the presence of cells expressing these markers during chronic inflammation was associated with an ineffective immune response [30, 31]. In agreement with their suppressive activity, such $CD11b^+Ly6C^{++}Ly6G^-$ and $CD11b^+Ly6C^+Ly6G^+$ cells in inflamed tissues are commonly called monocytic and polymorphonuclear myeloid-derived suppressor cells (MDSC), respectively [32–35]. Considering the accumulation of $CD11b^+Ly6C^{++}Ly6G^-$ and $CD11b^+Ly6C^+Ly6G^+$ cells observed here, it is tempting to speculate that these cells might be MDSC regulating aberrant immune responses in the absence of $Foxp3^+$ Tregs, which could be one reason for the lack of autoimmunity in DT-treated adult DEREG mice [2]. Indeed, proof of this hypothesis in future studies will be essential since the occurrence of immune suppressive cells after depleting Tregs might obstruct the intended, beneficial activation of the immune system during therapeutic applications targeting Tregs.

A surprising finding in this study was the transient appearance of mo-DCs upon Treg depletion selectively in LN. As noted above, mo-DCs are neither present in LN nor spleen before Treg depletion, but can be readily found in LN a few days after DT treatment in DEREG mice. Interestingly, in our model of transient Treg depletion, mo-DC accumulation paralleled Treg depletion and also occurred in a highly transient manner, suggesting that mo-DCs are under strict control of $Foxp3^+$ Tregs. This observation makes it tempting to speculate that mo-DC would accumulate in a chronic manner in a model of chronic Treg depletion, which also causes the development of catastrophic autoimmune disease [3]. The dynamics of mo-DC accumulation in LN upon Treg depletion might be of special relevance for the development of Treg targeting strategies aiming to boost immune responses during vaccinations. Previously, mo-DCs were described to express CCR7 as well as costimulatory molecules, such as CD40, CD80, and CD86, enabling their migration to LN as well as potent antigen presentation in the context of both MHCI and MHCII [38]. It would be intriguing to test whether mo-DCs accumulating in LN after Treg depletion can be used as targets for improving antigen presentation with the aim of boosting vaccination.

Upon ablation of $Foxp3^+$ Tregs, also B cells strongly accumulated in LN, but not in the spleen. This observation underlines the hypothesis that $Foxp3^+$ Tregs contribute to B cell tolerance, which has been suggested before [16, 17, 20, 26]. An argument against a role for Tregs in directly or indirectly controlling B cells is the fact that no overt signs of autoimmunity were observed in temporarily Treg-depleted mice kept for 2 months after treatment; however, it is highly likely that the very rapid rebound of $Foxp3^+$ Tregs has prevented B cells from developing a full response. Another explanation for the selective increase in the number of B cells in LN is that Tregs might control migration rather than activation–proliferation of B cells in this organ. B cells use similar pathways to pass through LN and spleen as T cells [39]. B cells in peripheral blood can interact with high endothelial venules (HEV) of LN, which eventually leads to transendothelial migration and LN entry. Similarly, B cells flowing with the blood stream within the marginal sinus and marginal zone of the spleen use similar molecular pathways to reach the splenic white pulp [39]. One important difference between LN and spleen in regard to lymphocyte migration is the exclusive presence of HEV in LN [40]. Interestingly, DCs were shown to control T cell entry to LN via modulation of HEV function [40, 41]. Since DC homeostasis is maintained by $Foxp3^+$ Tregs [8, 9], Tregs might be indirectly involved in HEV homeostasis. Indeed, Treg depletion in tumor-bearing mice leads to increased HEV formation within tumors [42]. This was associated with higher immune cell infiltration and enhanced tumor control. In case Tregs indeed control HEV homeostasis not only in tumors but also in LN, this could have effects on B cell migration specifically in LN but not in spleen. It would also explain some of the differences observed here with regard to differential accumulation of immune cell subsets in LN and spleen.

In conclusion, $Foxp3^+$ Tregs were shown here to be involved in controlling immune homeostasis in a broad man-

ner. Although further studies will be necessary for answering mechanistic questions, taken together, the data demonstrate that immune modulation is differentially achieved in LN and spleen. This might reflect an organ-specific role of Tregs as well as organ-specific anatomical organization and function. Understanding how this takes place might help to develop more efficient immunotherapies and vaccination strategies following Treg depletion to boost immune responses against chronic infections and tumors.

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References

1. Sakaguchi S: Naturally arising Foxp3-expressing $CD25^+$ $CD4^+$ regulatory T cells in immunological tolerance to self and non-self. Nat Immunol 6, 345–352 (2005)
2. Lahl K, Loddenkemper C, Drouin C, Freyer J, Arnason J, Eberl G, Hamann A, Wagner H, Huehn J, Sparwasser T: Selective depletion of $Foxp3^+$ regulatory T cells induces a scurfy-like disease. J Exp Med 204, 57–63 (2007)
3. Kim JM, Rasmussen JP, Rudensky AY: Regulatory T cells prevent catastrophic autoimmunity throughout the lifespan of mice. Nat Immunol 8, 191–197 (2007)
4. Klages K, Mayer CT, Lahl K, Loddenkemper C, Teng MW, Ngiow SF, Smyth MJ, Hamann A, Huehn J, Sparwasser T: Selective depletion of $Foxp3^+$ regulatory T cells improves effective therapeutic vaccination against established melanoma. Cancer Res 70, 7788–7799 (2010)
5. Feuerer M, Shen Y, Littman DR, Benoist C, Mathis D: How punctual ablation of regulatory T cells unleashes an autoimmune lesion within the pancreatic islets. Immunity 31, 654–664 (2009)
6. Blankenhaus B, Reitz M, Brenz Y, Eschbach ML, Hartmann W, Haben I, Sparwasser T, Huehn J, Kuhl A, Feyerabend TB, Rodewald HR, Breloer M: $Foxp3^+$ regulatory T cells delay expulsion of intestinal nematodes by suppression of IL-9-driven mast cell activation in BALB/c but not in C57BL/6 mice. PLoS Pathog 10, e1003913 (2014)
7. Wing K, Sakaguchi S: Regulatory T cells exert checks and balances on self tolerance and autoimmunity. Nat Immunol 11, 7–13 (2010)
8. Liu K, Victora GD, Schwickert TA, Guermonprez P, Meredith MM, Yao K, Chu FF, Randolph GJ, Rudensky AY, Nussenzweig M: In vivo analysis of dendritic cell development and homeostasis. Science 324, 392–397 (2009)
9. Darrasse-Jèze G, Deroubaix S, Mouquet H, Victora GD, Eisenreich T, Yao KH, Masilamani RF, Dustin ML, Rudensky A, Liu K, Nussenzweig MC: Feedback control of regulatory T cell homeostasis by dendritic cells *in vivo*. J Exp Med 206, 1853–1862 (2009)
10. Gasteiger G, Hemmers S, Firth MA, Le Floc'h A, Huse M, Sun JC, Rudensky AY: IL-2-dependent tuning of NK cell sensitivity for target cells is controlled by regulatory T cells. J Exp Med 210, 1167–1178 (2013)
11. Sitrin J, Ring A, Garcia KC, Benoist C, Mathis D: Regulatory T cells control NK cells in an insulitic lesion by depriving them of IL-2. J Exp Med 210, 1153–1165 (2013)
12. Gasteiger G, Hemmers S, Bos PD, Sun JC, Rudensky AY: IL-2-dependent adaptive control of NK cell homeostasis. J Exp Med 210, 1179–1187 (2013)
13. Banchereau J, Briere F, Caux C, Davoust J, Lebecque S, Liu YJ, Pulendran B, Palucka K: Immunobiology of dendritic cells. Annu Rev Immunol 18, 767–811 (2000)
14. Jenkins MK, Khoruts A, Ingulli E, Mueller DL, McSorley SJ, Reinhardt RL, Itano A, Pape KA: In vivo activation of antigen-specific CD4 T cells. Annu Rev Immunol 19, 23–45 (2001)
15. Cooper MA, Fehniger TA, Caligiuri MA: The biology of human natural killer-cell subsets. Trends Immunol 22, 633–640 (2001)
16. Ludwig-Portugall I, Hamilton-Williams EE, Gottschalk C, Kurts C: Cutting edge: $CD25^+$ regulatory T cells prevent expansion and induce apoptosis of B cells specific for tissue autoantigens. J Immunol 181, 4447–4451 (2008)
17. Ludwig-Portugall I, Hamilton-Williams EE, Gotot J, Kurts C: $CD25^+$ Treg specifically suppress auto-Ab generation against pancreatic tissue autoantigens. Eur J Immunol 39, 225–233 (2009)
18. Lim HW, Hillsamer P, Banham AH, Kim CH: Cutting edge: direct suppression of B cells by $CD4^+$ $CD25^+$ regulatory T cells. J Immunol 175, 4180–4183 (2005)
19. Lim HW, Hillsamer P, Kim CH: Regulatory T cells can migrate to follicles upon T cell activation and suppress GC-Th cells and GC-Th cell-driven B cell responses. J Clin Invest 114, 1640–1649 (2004)
20. Leonardo SM, Josephson JA, Hartog NL, Gauld SB: Altered B cell development and anergy in the absence of Foxp3. J Immunol 185, 2147–2156 (2010)
21. Riewaldt J, Duber S, Boernert M, Krey M, Dembinski M, Weiss S, Garbe AI, Kretschmer K: Severe developmental B lymphopoietic defects in Foxp3-deficient mice are refractory to adoptive regulatory t cell therapy. Front Immunol 3, 141 (2012)
22. Kerdiles Y, Ugolini S, Vivier E: T cell regulation of natural killer cells. J Exp Med 210, 1065–1068 (2013)
23. Pierson W, Cauwe B, Policheni A, Schlenner SM, Frankkaert D, Berges J, Humblet-Baron S, Schönefeldt S, Herold MJ, Hildeman D, Strasser A, Bouillet P, Lu LF, Matthys P, Freitas AA, Luther RJ, Weaver CT, Dooley J, Gray DH, Liston A: Antiapoptotic Mcl-1 is critical for the survival and niche-filling capacity of $Foxp3^+$ regulatory T cells. Nat Immunol 14, 959–965 (2013)
24. Suffner J, Hochweller K, Kühnle MC, Li X, Kroczek RA, Garbi N, Hämmerling GJ: Dendritic cells support homeostatic expansion of $Foxp3^+$ regulatory T cells in Foxp3.LuciDTR mice. J Immunol 184, 1810–1820 (2010)
25. Belz GT, Nutt SL: Transcriptional programming of the dendritic cell network. Nat Rev Immunol 12, 101–113 (2012)
26. Seo SJ, Fields ML, Buckler JL, Reed AJ, Mandik-Nayak L, Nish SA, Noelle RJ, Turka LA, Finkelman FD, Caton AJ, Erikson J: The impact of T helper and T regulatory cells on the regulation of anti-double-stranded DNA B cells. Immunity 16, 535–546 (2002)
27. Cooper MA, Fehniger TA, Fuchs A, Colonna M, Caligiuri MA: NK cell and DC interactions. Trends Immunol 25, 47–52 (2004)

28. Marçais A, Viel S, Grau M, Henry T, Marvel J, Walzer T: Regulation of mouse NK cell development and function by cytokines. Front Immunol 4, 450 (2013)
29. Terme M, Chaput N, Combadiere B, Ma A, Ohteki T, Zitvogel L: Regulatory T cells control dendritic cell/NK cell cross-talk in lymph nodes at the steady state by inhibiting $CD4^+$ self-reactive T cells. J Immunol 180, 4679–4686 (2008)
30. Bronte V, Zanovello P: Regulation of immune responses by L-arginine metabolism. Nat Rev Immunol 5, 641–654 (2005)
31. Gabrilovich DI, Nagaraj S: Myeloid-derived suppressor cells as regulators of the immune system. Nat Rev Immunol 9, 162–174 (2009)
32. Youn JI, Nagaraj S, Collazo M, Gabrilovich DI: Subsets of myeloid-derived suppressor cells in tumor-bearing mice. J Immunol 181, 5791–5802 (2008)
33. Sawanobori Y, Ueha S, Kurachi M, Shimaoka T, Talmadge JE, Abe J, Shono Y, Kitabatake M, Kakimi K, Mukaida N, Matsushima K: Chemokine-mediated rapid turnover of myeloid-derived suppressor cells in tumor-bearing mice. Blood 111, 5457–5466 (2008)
34. Movahedi K, Guilliams M, Van den Bossche J, Van den Bergh R, Gysemans C, Beschin A, De Baetselier P, Van Ginderachter JA: Identification of discrete tumor-induced myeloid-derived suppressor cell subpopulations with distinct T cell-suppressive activity. Blood 111, 4233–4244 (2008)
35. Lesokhin AM, Hohl TM, Kitano S, Cortez C, Hirschhorn-Cymerman D, Avogadri F, Rizzuto GA, Lazarus JJ, Pamer EG, Houghton AN, Merghoub T, Wolchok JD: Monocytic $CCR2^+$ myeloid-derived suppressor cells promote immune escape by limiting activated CD8 T-cell infiltration into the tumor microenvironment. Cancer Res 72, 876–886 (2012)
36. Peter C, Wesselborg S, Herrmann M, Lauber K: Dangerous attraction: phagocyte recruitment and danger signals of apoptotic and necrotic cells. Apoptosis 15, 1007–1028 (2010)
37. Mebius RE, Kraal G: Structure and function of the spleen. Nat Rev Immunol 5, 606–616 (2005)
38. Cheong C, Matos I, Choi JH, Dandamudi DB, Shrestha E, Longhi MP, Jeffrey KL, Anthony RM, Kluger C, Nchinda G, Koh H, Rodriguez A, Idoyaga J, Pack M, Velinzon K, Park CG, Steinman RM: Microbial stimulation fully differentiates monocytes to DC-SIGN/$CD209^+$ dendritic cells for immune T cell areas. Cell 143, 416–429 (2010)
39. Lo CG, Lu TT, Cyster JG: Integrin-dependence of lymphocyte entry into the splenic white pulp. J Exp Med 197, 353–361 (2003)
40. Girard JP, Moussion C, Forster R: HEVs, lymphatics and homeostatic immune cell trafficking in lymph nodes. Nat Rev Immunol 12, 762–773 (2012)
41. Thomas RM, Sai H, Wells AD: Conserved intergenic elements and DNA methylation cooperate to regulate transcription at the il17 locus. J Biol Chem 287, 25049–25059 (2012)
42. Hindley JP, Jones E, Smart K, Bridgeman H, Lauder SN, Ondondo B, Cutting S, Ladell K, Wynn KK, Withers D, Price DA, Ager A, Godkin AJ, Gallimore AM: T-cell trafficking facilitated by high endothelial venules is required for tumor control after regulatory T-cell depletion. Cancer Res 72, 5473–5482 (2012)