Interferon-γ Receptor Signaling in Dendritic Cells Restrains Spontaneous Proliferation of CD4+ T Cells in Chronic Lymphopenic Mice

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In lymphopenic mice, T cells become activated and undergo lymphopenia-induced proliferation (LIP). However, not all T cells are equally sensitive to lymphopenia. Several lymphopenia-insensitive T cell clones were described and their non-responsiveness was mainly attributed to clone-specific properties. Here, we provide evidence for an additional, host-dependent mechanism restraining LIP of lymphopenia-insensitive CD4+ T cells. We show that such cells undergo LIP in lymphopenic mice lacking IFN-γ receptor (IFN-γR) expression, a process, which is promoted by the autocrine action of T cell-derived IFN-γ. Additionally, LIP of lymphopenia-insensitive CD4+ T cells requires an intact microflora and is accompanied by the massive accumulation of IL-6 and dendritic cells (DCs). Consistent with these results, IL-6 neutralization and the DC-specific restoration of IFN-γR expression are both sufficient to restrict LIP. Hence, the insensitivity of CD4+ T cells to lymphopenia relies on cell-intrinsic properties and a complex interplay between the commensal microflora, IL-6, IFN-γR+ DCs, and T cell-derived IFN-γ.

Keywords: CD4+ T cells, interferon-γ, lymphopenia, lymphopenia-induced proliferation (LIP), dendritic cells

INTRODUCTION

In lymphocyte-competent hosts, T cells continuously utilize homeostatic factors such as Interleukin-7 (IL-7) and self-peptide-MHC complexes and thereby limit their availability (1). Due to the lack of IL-7-consuming T cells, IL-7 accumulates in lymphopenic mice (2) and humans (3). IL-7 is a potent activation and survival signal for T cells and its overabundance promotes T cell responses (4). Consequently, the adoptive transfer of polyclonal naive CD4+ T cells into lymphopenic mice leads to their activation and subsequent lymphopenia-induced proliferation (LIP) (5, 6). However, LIP represents a mixed reaction in response to different stimuli. While IL-7 overabundance induces a comparably slow homeostatic proliferation (HP) of T cells, the commensal microflora triggers a rapid response referred to as spontaneous proliferation (SP) (7–11). Nevertheless, naive T cells undergoing LIP differentiate into interferon-γ (IFN-γ)-producing effector/memory T cells, which is frequently associated with autoimmunity (12, 13).
The degree of LIP varies strongly between T cell clones (14–16). For example, ovalbumin (OVA)-specific CD4+ TCR-transgenic (tg) OT-II T cells, contrary to pcDN4+ T cells, do not undergo LIP in irradiated hosts (14) and expand only moderately in fully lymphopenic Rag-deficient (Rag−/−) mice (10). TCR signal strength is a major factor that regulates the sensitivity of a T cell to lymphopenia (15, 16). It is affected by a complex interplay between TCR avidity and molecules modulating TCR signal transduction (15, 17, 18). Hence, cell-intrinsic mechanisms appear to determine whether a T cell is sensitive to lymphopenia or not. However, it remained unclear whether extrinsic mechanisms prevent LIP of lymphopenia-insensitive CD4+ T cells.

In the present study, we show that lymphopenia-insensitive OT-II T cells expand massively in IFN-γ receptor (IFN-γR)-deficient Rag−/− (Rag2Rko) mice, a phenomenon that is not observed in IFN-γ-deficient Rag−/− (Ragγ−/−) mice. LIP of OT-II cells is associated with a strong increase in systemic IL-6 and subsequent T cell accumulation. The lack of IFN-γ and IFN-γR expression by OT-II cells impaired LIP to some degree arguing for a growth promoting, autocrine effect of microflora is crucial for OT-II LIP in Rag−/− mice (19). Furthermore, we show that the commensal microflora is crucial for OT-II LIP in Ragγ−/− mice, which is accompanied by the massive expansion of dendritic cells (DCs). Finally, we show that IFN-γR expression exclusively in DCs is sufficient to restrict OT-II expansion, DC accumulation and IL-6 production in Ragγ−/− mice. In summary, we provide evidence that the suppression of CD4+ T cell activation in response to lymphopenia is determined by a combination of both, clone-specific properties and environmental factors such as the commensal microflora, IL-6 and IFN-γR expression by DCs.

### MATERIALS AND METHODS

#### Mice and Adoptive T Cell Transfer

Thy1.1+ B6.PL-Thy1a/Cy and Thy1.2+ B6.129S7-Rag1tm1Mom/J (Rag−/−), C57BL/6J (B6), B6.SJL-PtprcPep8/B6/Joyl (CD45.1+), B6.129S7-Ifngtm1Hsd (IFN-γ−/−), B6.129S7-Ifngtm1Aog (IFN-γR−/−), B6.Cg-Tg(TcraTcrb)425Cbn/J (OT-II) (expressing a transgenic TCR specific for the chicken ovalbumin (OVA)-derived, I-Aβ-restricted peptide OVA323–339), B6.Cg-Tg(Igα-EGFP-CRE-DTR-LUC)2Gjh/Crl (CD11c-GCDL) (19) and pCAGfoxPSTOPloxP-IFNγR-ires-GFP (IFN-γRlo) transgenic mice (20) were housed under specific pathogen-free conditions. Mice were crossed to generate Thy1.1+2/CD45.1+2-disparate Rag−/−OT-II (OT-IIWT), Rag−/−IFN-γR−/−OT-II (OT-IIγRko), and Rag−/−IFN-γ−/−OT-II (OT-IIγko) T cell donors. Lymphoproliferative Rag−/− (RagWT), Rag−/−IFN-γ−/− (RagγRko), Rag−/−IFN-γR−/− (RagγRko), and Rag−/−IFN-γ−/− × CD11c-GCDL × IFN-γRlo (RagγRko × IFN-γRCD11c−/−) mice served as T cell recipients. For the adoptive transfers shown in Figures 2A,B, B6 or CD45.1+ mice served as non-lymphopenic controls. For T cell transfers, single cell suspensions were prepared from spleens and lymph nodes of donor mice by forcing the organs through metal sieves. To lyse erythrocytes, cell suspensions were incubated with Ammonium-Chloride-Potassium lysis buffer for 90 s and subsequent addition of RPMI with 10% FCS. After washing with PBS/2mM EDTA, cell suspensions were resuspended in PBS and filtered through 40 µm cell strainers (BD and Corning, Durham, NC). Single cell suspensions were counted, stained with fluochrome-labeled antibodies for 30 min at 4°C and analyzed by flow cytometry to determine the frequency and activation state of OT-II cells (Supplementary Figure 1). Cell suspensions containing 1.6–10 × 105 naive CD4+ OT-II T cells were injected i.v. into the tail vein of recipient mice. For CFSE labeling, donor single cell suspensions (2.2–3.2 × 107 cells/ml) were incubated with 7.5 µM CFSE (Biolegend) in PBS for 20 min at 37°C. Subsequently, cells were washed twice with ice cold PBS or RPMI/10% FCS and were resuspended in PBS prior to injection. Cell suspensions containing 7.5–8 × 105 CFSE− OT-II T cells were injected i.v. into the tail vein of recipient mice. Ten to thirteen days after transfer, spleens and lymph nodes were isolated and single cell suspensions were prepared as described. Erythrocyte lysis was performed with spleen cell samples. Cells were counted and directly stained with fluochrome-labeled antibodies for 30 min at 4°C after blocking FcR with purified anti-CD32/CD16 monoclonal antibodies (2.4G2 ATCC® HB-197™). To neutralize IL-6 in vivo, mice were i.p. injected with 500 µg of anti-IL-6 (MP5-20F3; BioXCell) 2 days prior to OT-II transfer. Treatment was repeated every third day. Control mice received 500 µg control IgG1 (HRPN; BioXCell). To deplete the commensal microflora, mice were treated with 0.5 g/l vancomycin, 1.0 g/l metronidazole, 1.0 g/l ampicillin, and 1.0 g/l neomycin sulfate via the drinking water 4 weeks prior to and during the experiment (21). Mice treated with antibiotics did not show any obvious clinical symptoms. At the day of analysis, however, their cecum was enlarged indicating successful depletion of the commensal microflora.

#### Flow Cytometry

The following antibodies and reagents were used: anti-CD4 (RM4-5; Biolegend/eBioscience), -CD11c (N418; BD/Biolegend), -CD44 (IM7; Biolegend), -CD45.1 (A20; Biolegend), -CD62L (MEI-14; Biolegend), CD127 (A7R34; BD/Biolegend), -KLRG-1 (2F1; Biolegend/eBioscience), -Ki67 (SolA15; eBioscience), -I-Aβ (AF6-120.1; Biolegend), -Thy1.1 (OX-7; Biolegend), -TCR Vα2 (B20.1; Biolegend), streptavidin-BV510 (Biolegend) and streptavidin-PE (Biolegend). For intranuclear staining of Ki67, cells were first stained with the indicated antibodies directed against cell surface molecules. Afterwards cells were fixed with the Foxp3/Transcription Factor Staining Buffer Set (eBioscience) according to the manufacturer’s instructions and subsequently incubated with anti-Ki67 for 30 min at 4°C. Samples were measured on LSRFortessa flow cytometer (Becton Dickinson) and analyzed by FlowJo and 10 software (FlowJo, LLC). To calculate the fold expansion of OT-II cells or DCs, the respective cell populations were quantified. For each experiment a mean value was calculated for the RagWT group. Finally, cell numbers of individual mice, including RagWT mice, were calculated in relation to the mean value of the RagWT group. Relative mean fluorescence intensities (MFIs) and relative frequencies of OT-II cells or DCs were calculated in analogy.
IFN-γ and IL-6 Detection

Blood (supplemented with EDTA) was centrifuged 10 min at 500 × g and 4°C. The supernatant was centrifuged again 10 min at 900 × g and 4°C to obtain the plasma that was analyzed by an IFN-γ or IL-6 specific ELISA (eBioscience) according to manufacturer's instructions.

Statistical Analysis

Statistical analysis and graphical representations were done using Prism 5 software (GraphPad Software). Statistical significance was determined using a non-parametric two-tailed Mann-Whitney U-test. *p ≤ 0.05; **p ≤ 0.01; ***p ≤ 0.001; ****p ≤ 0.0001.

RESULTS

Host IFN-γR Expression Restrains Commensal-Driven OT-II LIP

We have shown that host IFN-γR signaling restricts LIP of CD8+ T cells (22). Whether this mechanism prevents LIP of CD4+ OT-II T cells was unclear. To address this issue, naive CD4+ T cells from Rag-/- OT-II TCRβ mice (OT-II WT cells) were...
OT-II-Derived IFN-γ Promotes SP in an Autocrine Fashion

T cell-intrinsic IL-6 signaling promotes the expansion of IFN-γ-producing effector/memory CD4+ T cells under lymphopenic and non-lymphopenic conditions (30, 31). Consequently, the blockade of OT-IIWT activation and subsequent SP in αIL-6-treated RagγRko mice (Figures 3B–D) correlated with a strong reduction of plasma IFN-γ levels (Figure 3E).

Since IFN-γ directly promotes CD4+ T cell responses (32–34), we hypothesized that OT-II-derived IFN-γ supports SP in RagγRko mice in an autocrine fashion. To test this hypothesis, IFN-γ-deficient OT-II (OT-IIγRko) cells were transferred into RagγRko and RagWT mice. After 11–12 days, OT-IIγRko frequencies, cell numbers and relative expansion rates were determined. As shown in Figure 4A, some expansion of OT-IIγRko cells was detectable in RagγRko. This was associated with the up-regulation of CD44, KLRG-1, and Ki67 (Figures 4B,C). Importantly, however, OT-IIγRko cells expanded less well in RagγRko mice (~10-fold; Figure 4A) than OT-IIWT cells (~50-fold; Figure 1A) suggesting a growth-promoting effect of autocrine IFN-γ.

To further test this possibility, equal numbers of OT-IIWT and OT-IIγRko cells were co-transferred into RagγRko and RagWT mice. OT-IIWT cells expanded ~60-fold while OT-IIγRko cells expanded only ~20-fold (Figure 4D). Thus, SP of OT-IIγRko and OT-IIγRko cells occurs in RagγRko mice. Compared to OT-IIWT cells, OT-IIγRko and OT-IIγRko expansion was less pronounced suggesting that OT-II-derived IFN-γ promotes SP in an autocrine fashion. However, we cannot exclude a contribution of host-derived IFN-γ, which accumulates in IFN-γR-deficient mice due to lack of its consumption (22).

IFN-γR+ DCs Restrain CD4+ T Cell SP in RagγRko Mice

Dendritic cells (DCs) producing elevated levels of IL-6 promote aberrant T cell activation and subsequent IFN-γ synthesis (35). Furthermore, the induction of EAE relies on the accumulation of IL-6-producing DCs (36). Under lymphopenic conditions, MyD88-dependent recognition of the commensal microflora is sufficient to induce IL-6 production by DCs thereby promoting SP of CD4+ T cells (10) similar to what we have observed in OT-IIWT-reconstituted RagγRko mice. Furthermore, DCs express high levels of MHCII, which is crucial for CD4+ T cell LIP (14, 37). Based on these data we speculated that DC responses were altered in RagγRko mice. When splenic CD11c+MHCIIhi DCs were quantified in OT-IIWT-reconstituted RagWT and
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**FIGURE 2** | OT-II LIP is more pronounced in spleen than in lymph nodes. (A,B) CFSE-labeled OT-II WT cells were adoptively transferred into Rag WT, RagγKO mice and (B) B6 mice. After 12 days, recipient (A) lymph nodes and (B) spleen were analyzed by flow cytometry. (A,B) Histograms show relative fluorescence intensities for CFSE after gating on CD4⁺ CD45.1⁺ OT-II WT cells and numbers indicate percentages. Bar diagrams show cell numbers and fold expansion of OT-II WT cells (mean (Continued)
Rag\textsuperscript{γRko} mice, their numbers were strongly increased in the latter (Figure 5A; + OT-II\textsuperscript{WT}). This was not the case in untreated Rag\textsuperscript{γRko} mice (Figure 5A; −OT-II\textsuperscript{WT}) suggesting that OT-II\textsuperscript{WT} activation is a prerequisite for DC accumulation in Rag\textsuperscript{γRko} recipients.

Whether the DC-specific restoration of IFN-γR expression is sufficient to block OT-II\textsuperscript{WT} SP and subsequent DC accumulation in Rag\textsuperscript{γRko} mice was tested next. For this purpose, we made use of a novel transgenic mouse line, allowing IFN-γR expression after the Cre-mediated deletion of a loxP-flanked DNA-Stop cassette (20). To activate this “switch-on” (IFN-γR\textsuperscript{RKO}) construct and express the transgenic IFN-γR specifically in DCs, IFN-γR\textsuperscript{SO} mice were crossed to CD11c-GCDL mice expressing Cre under the control of the CD11c promoter (19). Subsequently, CD11c-GCDL × IFN-γR\textsuperscript{SO} mice were crossed to Rag\textsuperscript{γRko} mice in order to generate T and B cell-deficient, fully lymphopenic Rag\textsuperscript{γRko} × CD11c-GCDL × IFN-γR\textsuperscript{SO} mice lacking IFN-γR expression on all cells except DCs. These mice are termed Rag\textsuperscript{γRko} × IFN-γR\textsuperscript{CD11c−ON} hereafter. Finally, OT-II\textsuperscript{WT} cells were transferred into Rag\textsuperscript{WT} mice, Rag\textsuperscript{γRko} × IFN-γR\textsuperscript{CD11c−ON}, and Rag\textsuperscript{γRko} controls. After 11–13 days, the numbers of splenic OT-II\textsuperscript{WT} cells were determined. As opposed to Rag\textsuperscript{WT} mice, OT-II\textsuperscript{WT} cells expanded strongly in Rag\textsuperscript{γRko} mice (Figure 5B). The values obtained with Rag\textsuperscript{γRko} × IFN-γR\textsuperscript{CD11c−ON} mice reached intermediate levels showing that IFN-γR expression by DCs is sufficient to restrain OT-II\textsuperscript{WT} SP. Similarly, DC expansion was most pronounced in OT-II\textsuperscript{WT}-reconstituted Rag\textsuperscript{γRko} mice, reached intermediate levels in Rag\textsuperscript{γRko} × IFN-γR\textsuperscript{CD11c−ON} mice and was least efficient in Rag\textsuperscript{WT} mice (Figure 5C; +OT-II\textsuperscript{WT}). On the contrary, CD numbers did not differ between untreated Rag\textsuperscript{WT}, Rag\textsuperscript{γRko} × IFN-γR\textsuperscript{CD11c−ON} and Rag\textsuperscript{γRko} mice (Figure 5C; −OT-II\textsuperscript{WT}) suggesting a causal link between OT-II\textsuperscript{WT} SP and DC expansion in Rag\textsuperscript{γRko} mice (Figures 5A,C).

Importantly, specific IFN-γR expression by DCs was sufficient to limit expansion of OT-II\textsuperscript{WT} cells and DCs as well as IL-6 up-regulation (Figure 5D) in Rag\textsuperscript{γRko} × IFN-γR\textsuperscript{CD11c−ON} mice.

The efficacy of CD4\textsuperscript{+} T cell responses correlates positively with the amount of IFN-γ available in the early phase of the response (32, 34). We have shown previously that IFN-γ accumulates in IFN-γR-deficient mice, most probably due to
the lack of its receptor-mediated clearance (22). Hence, elevated levels of steady-state IFN-γ may explain the rapid and strong induction of OT-II WT responses in RagγRko mice. To test whether decreased OT-II WT responses in RagγRko × IFN-γRCD11c−ON mice (Figure 5B) correlate with reduced steady-state IFN-γ levels, we compared plasma samples of untreated RagγRko and RagγRko × IFN-γRCD11c−ON mice. As shown in Figure 5E, IFN-γ levels were significantly lower in RagγRko × IFN-γRCD11c−ON mice. This suggests that IFN-γR+ DCs consume IFN-γ thereby reducing its availability for OT-II WT cells. This competition for IFN-γ would provide an explanation for the reduced levels of SP in RagγRko × IFN-γRCD11c−ON mice (Figure 5B).

**DISCUSSION**

T cell clones are not equally sensitive to lymphopenia-related activation signals (14–16). For example, ovalbumin-specific CD4+ T cells from OT-II TCRβ mice represent one of several T cell clones, which are resistant to lymphopenia-induced activation (14). It is well accepted that T cell clone-specific features such as CD5 levels correlate closely with the sensitivity to lymphopenia (15, 16, 38). Here, we provide evidence for an additional, recipient-dependent mechanism that restrains expansion of adoptively transferred CD4+ T cells. This mechanism relies on a complex interplay between the commensal microflora, IFN-γR+ DCs and CD4+ T cells.

The commensal microflora triggers IFN-γ production by various immune cells in the steady-state (39, 40). In IFN-γR-deficient mice, IFN-γ accumulates due to the lack of its consumption (22). Thus, elevated IFN-γ levels in RagγRko mice may provide early activation signals to OT-II cells initiating the rapid expansion we have observed. This interpretation is in accordance with our finding that both, OT-II WT expansion and steady-state levels of IFN-γ, were decreased in RagγRko × IFN-γRCD11c−ON mice. This suggests that IFN-γR+ DCs efficiently reduce amounts of circulating IFN-γ thereby restricting its availability for OT-II cells.

However, increased rates of OT-II expansion in RagγRko mice do not only rely on host-derived IFN-γ. As we have shown here, OT-II-derived IFN-γ acts in an autocrine manner. Hence, host- and OT-II-derived IFN-γ may synergize in promoting full-blown OT-II expansion in RagγRko mice. OT-II expansion is accompanied by the up-regulation of CD127, which would facilitate their IL-7-dependent survival (41–43) and provides one explanation for the accumulation of OT-II cells in RagγRko mice. Importantly, the accumulation of DCs and IL-6 correlates positively with the degree of OT-II expansion in RagγRko mice and might be interrelated. DCs produce IL-6 in response to the commensal microflora (10) and express MHCII, which are both required for CD4+ T cell expansion under lymphopenic conditions (10, 14, 37). Since (i) T cell-intrinsic IL-6R signaling is critical for CD4+ T cell responses (30, 31), (ii) IL-6 prevents apoptosis of naive and effector CD4+ T cells (44, 45), and (iii) counter-regulates DC function (35, 46–50) we suggest a direct, growth-promoting and/or anti-apoptotic effect of IL-6 on OT-II cells expanding in RagγRko mice. Although the T cell-stimulatory potential of DC-derived IL-6 is well established (10, 35, 36) recent findings identified multiple hematopoietic and non-hematopoietic cell types as potential IL-6 producers (36). Importantly, different IL-6 producers appear to regulate...
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FIGURE 5 | IFN-γR+ DCs restrain CD4+ T cell SP in RagγRko mice. (A–D) OT-IIWT cells were adoptively transferred into RagWT and RagγRko mice. After 11–13 days, recipient splenocytes were analyzed by flow cytometry. (A) Results of 2–6 independent experiments with a total of 10–25 mice were pooled to calculate the numbers and fold expansion of CD11c+ MHCIIhi DCs after reconstitution with OT-IIWT cells (+OT-IIWT). DC numbers from untreated RagWT and RagγRko mice were determined as well (-OT-IIWT). (B–D) Frequencies, cell numbers and fold expansion of OT-II cells/DCs as well as plasma IL-6 levels were analyzed in RagWT, RagγRko × CD11c-GCDL × IFN-γRSO (RagγRko × IFN-γRCD11c−ON) and RagγRko mice. Pooled results of 2 independent experiments with a total of 8 mice per group are shown. (E) Steady-state levels of IFN-γ were determined in plasma samples of 8–9 untreated RagγRko × IFN-γRCD11c−ON and RagγRko mice. (A–E) Graphs show mean values + SEM; *p ≤ 0.05; **p ≤ 0.01; ***p ≤ 0.001.

different aspects of the same CD4+ T cell response (36). Hence, it remains to be shown for our experimental system whether (i) DCs and/or other cell types up-regulate IL-6 expression in OT-II-reconstituted RagγRko mice, whether (ii) the elevation of IL-6 levels in these mice results from the accumulation of DCs producing constant amounts of IL-6, and whether (iii) there is a causal relationship between the cellular origin of IL-6 and its growth-promoting effect. As reported recently, definite answers to such questions would require the combined use of cell type-specific IL-6 reporter as well as conditional IL-6 knockout mice (36) and their integration into our experimental systems. However, this would be beyond the scope of this study and therefore remains an important task for the future.

From previous experiments we know that only effector, but not naive, OT-IIWT cells activate immature DCs (51). This suggests that IFN-γ-associated OT-II activation is an integral part of a self-amplifying loop in RagγRko mice, which involves the T cell-dependent accumulation of DCs, which in turn promote OT-II expansion. The lack of IFN-γR signaling in DCs increases their lifespan (52) and T cell-stimulatory potential (53) providing an additional explanation for the accumulation of DCs in RagγRko mice. In accordance with this interpretation, IFN-γR re-expression in DCs is sufficient to disrupt this self-amplifying loop and to down-modulate DC accumulation, IL-6 levels and OT-II cell expansion.

In summary, we demonstrate that the sensitivity of CD4+ T cells to lymphopenia is not only determined by cell-intrinsic properties but also by a complex interplay between CD4+ T cells, the commensal microflora and IFN-γR+ DCs. We postulate that T cell- and host cell-specific mechanisms have to cooperate to restrain spontaneous proliferation, the commensal-driven form of LIP. The molecular nature and the relative importance of either mechanism may vary for different T cell clones.

ETHICS STATEMENT

Animal experiments were performed according to institutional guidelines and were approved by the Landesverwaltungsamt Sachsen-Anhalt (Permit Number: 2-1155/2-1288 Uni MD).

AUTHOR CONTRIBUTIONS

LK, CF, DS, and KD performed and analyzed the experiments. LK substantially contributed to manuscript preparation. UK and ID analyzed and discussed the data. TB and TK provided
essential material, analyzed and discussed the data. TS designed and supervised the study, analyzed and discussed the data and wrote the manuscript with the help of the other co-authors.

**FUNDING**

This work was supported by the Deutsche Forschungsgemeinschaft [Sonderforschungsbereich TR36 (B2, B7), SFBB854 (B15), and DU 1112/5-1].

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**ACKNOWLEDGMENTS**

We thank E. Denks and J. Giese for excellent technical assistance and Natalio Garbi for CD11c-GCDL mice.

**SUPPLEMENTARY MATERIAL**

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2019.00140/full#supplementary-material
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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.