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Systemic bacterial infections affect dendritic cell development and function

Kristin Bieber [a], Manina Günter [a,b], Karina A. Pasquevich [c], Stella E. Autenrieth [a,b,*]

[a] Department of Internal Medicine II, University of Tübingen, Tübingen, Germany
[b] Research Group "Dendritic Cells in Infection and Cancer", German Cancer Research Center (DKFZ), Heidelberg, Germany
[c] Instituto de Investigaciones Biotecnológicas-(IIBio), Universidad Nacional de San Martín-CONICET, Argentina

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ABSTRACT

Dendritic cells (DCs) are critical in host defense against infection. DC depletion is an early event in the course of sepsis that may impair the host defense mechanisms. Here, we addressed whether DC depletion and dysfunction are pathogen-independent, mediated via pattern recognition receptors, and are due to impaired DC development upon systemic infection with the Gram-negative bacterium *Escherichia coli* and the Gram-positive pathogen *Staphylococcus aureus*.

Infection with *E. coli* and *S. aureus* led to reduced numbers of splenic DC subsets and of DC progenitors in the bone marrow (BM) with this effect persisting significantly longer in mice infected with *S. aureus* than with *E. coli*. The reduction of DC subsets and their progenitors was mainly TLR-independent as was the infection-induced monopoiesis. Moreover, *de novo* DC development was impaired in mice infected with *S. aureus*, and BM cells from *E. coli* or *S. aureus* infected mice favored macrophage differentiation *in vitro*. As a consequence of reduced DC numbers and their reduced expression of MHC II less $CD4^+$ and $CD8^+$ T cells, especially Th1 and IFN-γ producing $CD8^+$ T cells, could be detected in *S. aureus* compared to *E. coli* infected mice. These differences are reflected in the rapid killing of *E. coli* as opposed to an increase in bacterial load in *S. aureus*.

In summary, our study supports the idea that systemic bacterial infections generally affect the number and development of DCs and thereby the T cell responses, but the magnitude is pathogen-dependent.

1. Introduction

The mammalian immune system acts against microbes and prevents their invasion by constitutive innate immune barriers like mucosal and epithelial tissues. Infections with microbial pathogens provoke a concerted action of both antigen-non-specific innate immunity, like the activation of phagocytes, and antigen-specific adaptive immunity (Banchereau and Steinman, 1998; Medzhitov et al., 2012). Key features of the innate immune system include the ability to rapidly recognize pathogens and / or tissue injury and the ability to signal the presence of danger to cells of the adaptive immune system (Akira et al., 2006; Matzinger, 1994). In addition to neutrophils, DCs and circulating monocytes are becoming increasingly important components of anti-microbial host defense (Bieber and Autenrieth, 2015). Innate immune cells use a variety of receptors to recognize patterns shared between pathogens via pattern recognition receptors (PRRs). For instance, Toll-like receptor (TLR) 4 binds bacterial LPS from Gram-negative bacteria whereas TLR2 recognizes bacterial lipopeptides expressed by both Gram-positive and Gram-negative bacteria. On ligand binding, TLRs initiate signaling cascades via NF-κB and interferon regulatory factors (IRF) that result ultimately in the production of cytokines and chemokines (Kawai and Akira, 2010; Kumar et al., 2012; Moretti and Blander, 2014; O'Neill et al., 2013). The various innate sensing systems ensure the determination of the microorganisms location, viability, and pathogenicity (Iwasaki and Medzhitov, 2015). Thus, the innate immune response depends on the nature of the pathogen.

DCs are professional antigen presenting cells and play a crucial role in the maintenance of homeostasis and the initiation of protective immunity to infection (Merad et al., 2013). Classical DCs (cDCs) are the main DCs located in lymphoid and non-lymphoid tissues of mice and can be further differentiated by their distinct transcription factor and surface marker expression into cDC1 and cDC2 (Merad et al., 2013). In the peripheral tissues DCs are in an immature state and are specialized in antigen uptake. They rapidly endocytose endogenous and foreign

Abbreviations: DCs, dendritic cells; CDP, common DC progenitor; MDP, monocyte and DC progenitor; cMoP, common monocyte progenitor; HSCs, hematopoietic stem cells; BM, bone marrow; WT, wildtype; Ye, *Yersinia enterocolitica*; dpi, day post infection; Ec, *Escherichia coli*; Sa, *Staphylococcus aureus*.

* Corresponding author at: Department of Internal Medicine II, University of Tübingen, Tübingen, Germany.
E-mail address: Stella.Autenrieth@medizin.uni-tuebingen.de (S.E. Autenrieth).

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substances from the surrounding microenvironment. The activation of PRRs by their agonists leads to the maturation of DCs, meaning shut-down of endocytic capacities but increased processing and presentation of antigens engulfed at the site of infection via major histocompatibility complex (MHC) class II. In addition, DCs start to express high levels of co-stimulatory molecules and to produce pro-inflammatory cytokines, endowing mature DCs with their unique capacity to prime antigen-specific, naïve T cells (Guermonprez et al., 2002). In addition to T cell priming, DCs help to maintain the survival of naïve $CD4^+$ T cells and immune T cell memory (Brocker et al., 1997; Lanzavecchia and Sallusto, 2005).

The number of DCs has to be tightly controlled in order to maintain adequate T cell responses and to prevent autoimmunity. Infection and inflammation are common natural stressors for the immune and the hematopoietic system leading to the consumption of immune cells either by mobilization to the sites of infection / inflammation or by apoptosis (Baldridge et al., 2011). This, in turn, induces their replacement by hematopoietic precursors.

DCs originate from BM hematopoietic stem cells (HSCs), which develop into monocyte dendritic cell progenitors (MDPs) (Fogg, 2006; Varol et al., 2007). MDPs give either rise to monocytes, via common monocyte progenitors (cMoPs), monoblasts, and pro-monocytes (Hettinger et al., 2013;Serbina et al., 2009) or to common DC progenitors (CDPs) (Liu et al., 2009; Onai et al., 2007). CDPs differentiate into precursors of classical DCs (pre-DCs), which migrate via the blood to peripheral organs, where they develop into DCs (Naik et al., 2007; Onai et al., 2007; Schlitzer et al., 2015). However, the derivation of CDPs from MDPs is controversially discussed (Sathe et al., 2014). Hematopoietic stem and progenitor cells sense pathogens via TLR signaling (Nagai et al., 2006) as well as inflammatory cytokines and interferons produced upon infection leading to their expansion, mobilization, and differentiation (Baldridge et al., 2011; Nagai et al., 2006; Schmid et al., 2011). However, the mechanisms regulating DC differentiation from these progenitors during infection are poorly understood.

Sepsis is defined as a systemic inflammatory response syndrome associated with a life-threatening organ dysfunction caused by a suppressed adaptive immune response amongst others due to apoptosis of immune cells (van der Poll et al., 2017). More and more sepsis cases are caused by commensal bacterial pathogens like *Staphylococcus aureus* (Sa) or *Escherichia coli* (Ec) localized in the upper respiratory or the gastrointestinal tract, respectively (Ramachandran, 2013). A strongly suppressed immune response is associated with the morbidity of septic patients (Boomer et al., 2011; Hotchkiss et al., 2013). Moreover, reduction and impaired DC function was shown in sepsis patients (Grimaldi et al., 2011; Guisset et al., 2007; Hotchkiss et al., 2002) as well as mouse models (Autenrieth et al., 2010, 2012; Ding et al., 2004; Efron et al., 2004; Tinsley et al., 2003) leading to sepsis-induced immunosuppression.

Animal and human data suggest that systemic DC depletion is an early event in the course of sepsis that may impair the host defense mechanisms (Autenrieth et al., 2012, 2010; De Trez et al., 2005; Efron et al., 2004; Grimaldi et al., 2011; Hotchkiss et al., 2002). We previously showed that infection-induced depletion of DCs is associated with reduced numbers of hematopoietic stem and progenitor cells (HSPCs) of the myeloid lineages as general effect of systemic bacterial infections (Pasquevich et al., 2015). Whether this is in general TLR-dependent and if other pathogens evoke similar impairment of DC development is addressed in this study. To address this question, we analyzed the effect of a non-pathogenic Ec and a pathogenic Sa strain in systemic infections with high or lethal infectious doses, respectively, on DC subsets and their precursors. For this purpose, wild-type (WT), TLR2- and TLR4-deficient mice were used to study their role in immune cell activation, since Ec is mainly recognized by TLR4 and Sa by TLR2 (Kimura-Takeuchi et al., 1992; Schreiner et al., 2013; Takeuchi et al., 2000).

2. Materials and methods

2.1. Mice and infection

The experiments were performed with 6 12-week-old female C57BL/6JRj mice (Janvier, France). $TLR2^{-/-}$, $TLR4^{-/-}$ (Spiller et al., 2008), CD11c.DOG (Hochweller et al., 2008) and OT-II mice (provided by Manfred Kneilling) on C57BL/6 background were bred under specific pathogen-free conditions. Animal procedures were carried out according to protocols approved by the Regierungspräsidium Tübingen (Permit Numbers: IZ1/10, IZ1/12, M1/14).

Mice were injected with 200 µL PBS or 5×10^7 colony forming units (CFU) of Ec JM83 or $1.5–3 \times 10^7$ CFU of Sa USA300 or 5×10^6 CFU of Sa USA300 (3 dpi in $TLR2^{-/-}$ mice) in 200 µL PBS into the tail vein. The bacterial load in the organs was assessed by serial dilutions plated on Müller-Hinton or Columbia agar plates, respectively.

2.2. α-IFN-γ and DT treatment

WT mice were treated intraperitoneally with 250 µg rat-α-mouse IFN-γ antibody (clone XMG1.2; BioXCell) or rat IgG1 isotype control antibody (clone HRPN; BioXCell), respectively, one day before and immediately prior to intravenous infection with Sa.

For systemic DC depletion CD11c.DOG mice were injected intraperitoneally with 8 ng / g bodyweight of diphtheria toxin (Sigma) in PBS one day prior to infection.

2.3. Cell preparation and flow cytometry

Preparation of single cell suspensions from spleen was performed as previously described (Autenrieth et al., 2012). BM cells were harvested from femurs and tibias. Blood was collected by cardiac puncture into a heparin-coated (Braun) 1 mL syringe. Plasma was obtained by centrifugation with 3500 rpm for 10 min at 4 °C. Total cell numbers were determined by trypan blue exclusion. Antibodies used are listed in Table S2. All washing and incubation steps were performed with FACS buffer (PBS (Life technologies) containing 1 % FBS (Sigma-Aldrich), 0.09 % NaN_3 (Sigma-Aldrich) and 2 mM EDTA (Merck)). Blocking of Fc receptors was done before staining by incubating cells at 4 °C for 15 min with anti-FcγRII / III mAb. Zombie NIR or Zombie Aqua (BioLegend) was used to exclude dead cells. Samples were acquired on an LSR Fortessa flow cytometer (BD Biosciences) and further analyzed using FlowJo software (BD Biosciences).

2.4. Bone marrow-derived DC (GM-DCs) and macrophage (GM-Macs) generation

RPMI-1640 medium (Sigma-Aldrich) supplemented with 10 % FBS (Sigma–Aldrich), 2 mM l-glutamine (Gibco), 100 U / ml penicillin / streptomycin (Gibco), 50 µM 2-mercaptoethanol (Roth),1 mM sodium pyruvate (Biochrom) and 1 x non-essential amino acids (Biochrom) was used in all cell culture experiments. BM antigen-presenting cells (APCs) were prepared using GM-CSF as previously described (Armbruster et al., 2016; Schreiner et al., 2013). Briefly, 2×10^6 bone marrow cells, flushed from the femurs and tibias of C57BL/6 mice, were seeded in 100 mm dishes in 10 mL medium containing 200 U / ml GM-CSF. After 3 days, an additional 10 mL of fresh medium containing 200 U / ml GM-CSF was added to the cultures. On day 6 half of the culture supernatant was replaced by fresh medium containing GM-CSF. At day 8, the slightly attached cells were used for the experiments described in this report.

2.5. Determination of cytokines

IFN-γ ELISA (eBiosciences) with peripheral blood plasma was performed according to the manufacturer's protocols.

2.6. Statistics

First, the data were analyzed for normal distribution and if the standard deviations were equal. If this was the case three groups were analyzed using ANOVA followed by Holm Sidak multiple comparison test. In case the standard deviations were not equal the data were analyzed by Welch ANOVA with Dunnett's test for multiple comparisons. In case variances were not normally distributed, the data were analyzed using the non-parametrical Kruskal-Wallis test with Dunn's multiple comparison test for three group comparisons or two-tailed Mann Whitney test for comparison of two groups. Differences were considered as statistically significant if $P < 0.05$ (*), $P < 0.01$ (**), $P < 0.001$ (***), $P < 0.0001$ (****). Statistical analyses were performed using GraphPad Prism 8 software (GraphPad Software, Inc., La Jolla, CA). The experiments with different infection durations (1 or 3 days) as well as those with different mouse strains (WT, TLR2$^{-/-}$ or TLR4$^{-/-}$)

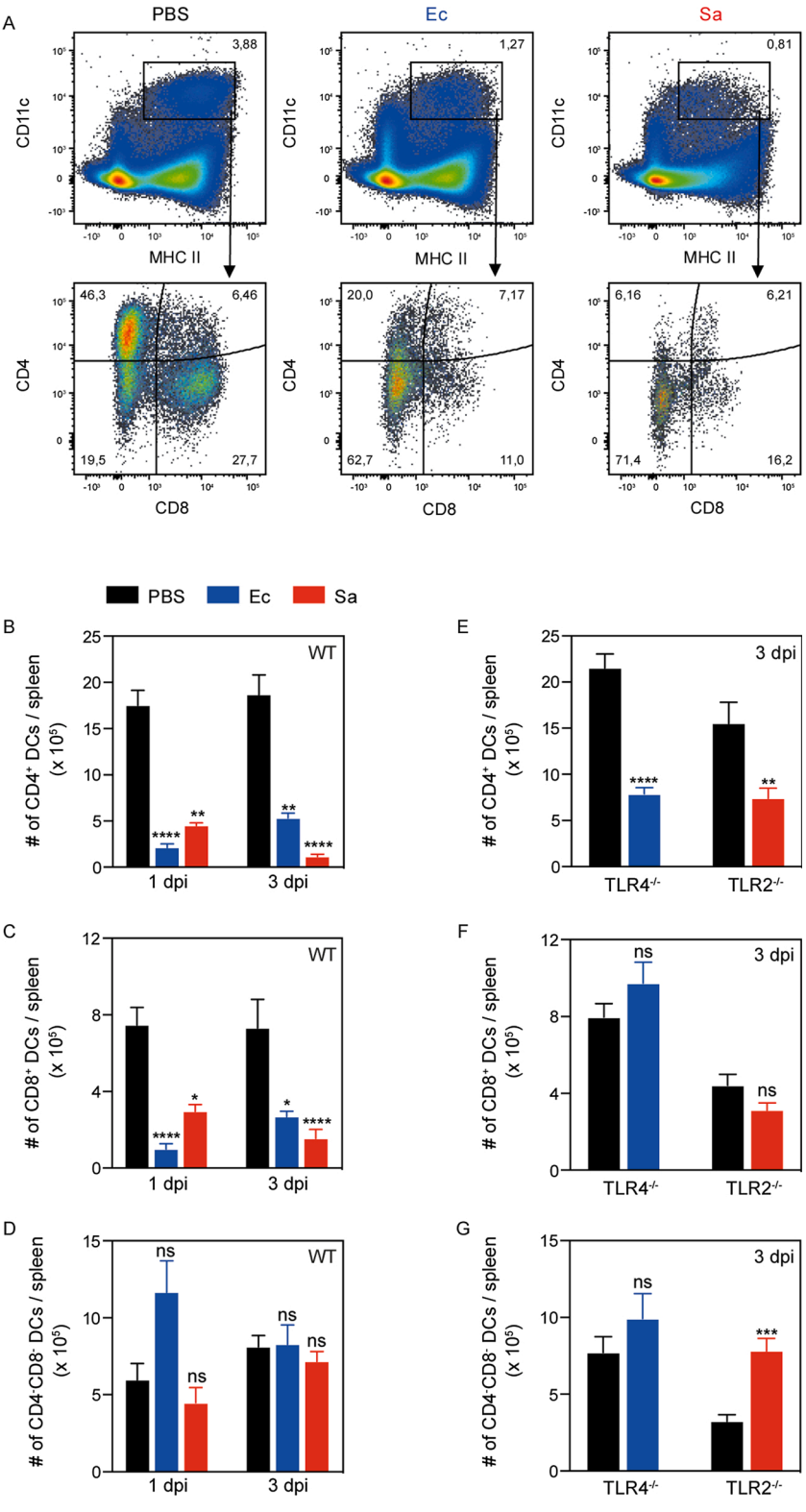

Fig. 1. Analysis of TLR-dependent effects of systemic bacterial infections on splenic DCs. WT (B—D), TLR4$^{-/-}$ (EG—), and TLR2$^{-/-}$ mice (E—G) were either treated with PBS or infected with Ec or Sa and splenocytes analyzed 1 and/or 3 dpi by flow cytometry. Representative gating scheme of splenic CD4^{+} DCs, CD8^{+} DCs and CD4^{-}CD8^{-} DCs from WT mice (A). Graphical summary of the absolute numbers of CD4^{+} DCs (B and E), CD8^{+} DCs (C and F) and CD4^{-}CD8^{-} DCs (D and G). Graphs show the mean ± SEM. Number of mice / experiment / groups are displayed in Table S1. WT mice were statistically analyzed by non-parametric Kruskal-Wallis test followed by Dunn's multiple comparison test. TLR4$^{-/-}$ and TLR2$^{-/-}$ mice were statistically analyzed by two-tailed Mann-Whitney-U-test. (*) P < 0.05, (**) P < 0.01, (***) P < 0.001, (****) P < 0.0001; ns = not significant.

were conducted independently of each other and therefore do not influence each other. The experiments were not randomized, and the investigators were not blinded to allocation during experiments and outcome assessment.

3. Results

3.1. Reduced numbers of splenic DC subsets upon systemic bacterial infections

To address whether systemic bacterial infection with Ec or Sa affects splenic DC numbers in a TLR-dependent manner, wildtype (WT), TLR4$^{-/-}$ and TLR2$^{-/-}$ mice were infected intravenously with these bacterial strains and the number of DC subsets was determined by flow cytometry. We analyzed TLR2 and TLR4 as they are activated by Sa or Ec, respectively (Schreiner et al., 2013; Takeuchi et al., 2000, 1999). In WT mice infected either with Ec or Sa for 1 and 3 days the frequencies (Fig. S1) and numbers of splenic cDC (gated as CD11c^{hi}MHC II^{+}) (Fig. 1A), mainly CD4^{+} (Fig. 1B) and CD8^{+} DCs (Fig. 1C), but not CD4-CD8- DCs (Fig. 1D) were significantly reduced by 60 % to 93 %.

Reduced frequencies and numbers of CD4^{+} DCs were also observed 3 dpi with Ec in TLR4$^{-/-}$ mice and with Sa in TLR2$^{-/-}$ mice, indicating a TLR-independent mechanism for this DC subset (Figs. 1E, S1D). In contrast, the number of CD8^{+} DCs 3 dpi with Ec or Sa was equal in TLR4$^{-/-}$ or TLR2$^{-/-}$ mice, respectively (Figs. 1F, S1E). Moreover, we observed an increase in the number of CD4^{-}CD8^{-} DCs upon Sa infection in TLR2$^{-/-}$ mice (Fig. 1G) indicating that CD4^{-}CD8^{-} DCs are TLR2-dependently decreased. These data show that systemic bacterial infections lead to dramatically reduced numbers of CD4^{+} and CD8^{+} DCs for up to 3 days post infection which is at least for CD8^{+} DCs TLR-dependent.

3.2. Reduced numbers of DC progenitors upon systemic bacterial infections is TLR-independent

Previously, we showed that the numbers of BM hematopoietic progenitors committed to the DC lineage were reduced upon systemic infection with the Gram-negative bacteria *Yersinia enterocolitica* (Ye) and Ec as well as with the Gram-positive Sa. For Ye this effect was TLR4- and IFN-γ-signaling dependent (Pasquevich et al., 2015). Here, we addressed (I) the long-term effect and (II) the TLR-dependency of the infection-induced reduction of DC progenitors observed with Ec, or Sa. Therefore, WT, TLR4$^{-/-}$, and TLR2$^{-/-}$ mice were infected intravenously with the aforementioned pathogens and DC progenitors, namely pre-DCs, CDPs, and MDPs were analyzed 1 and 3 days post infection (dpi) in the BM. The bacterial load in the spleen upon infection with

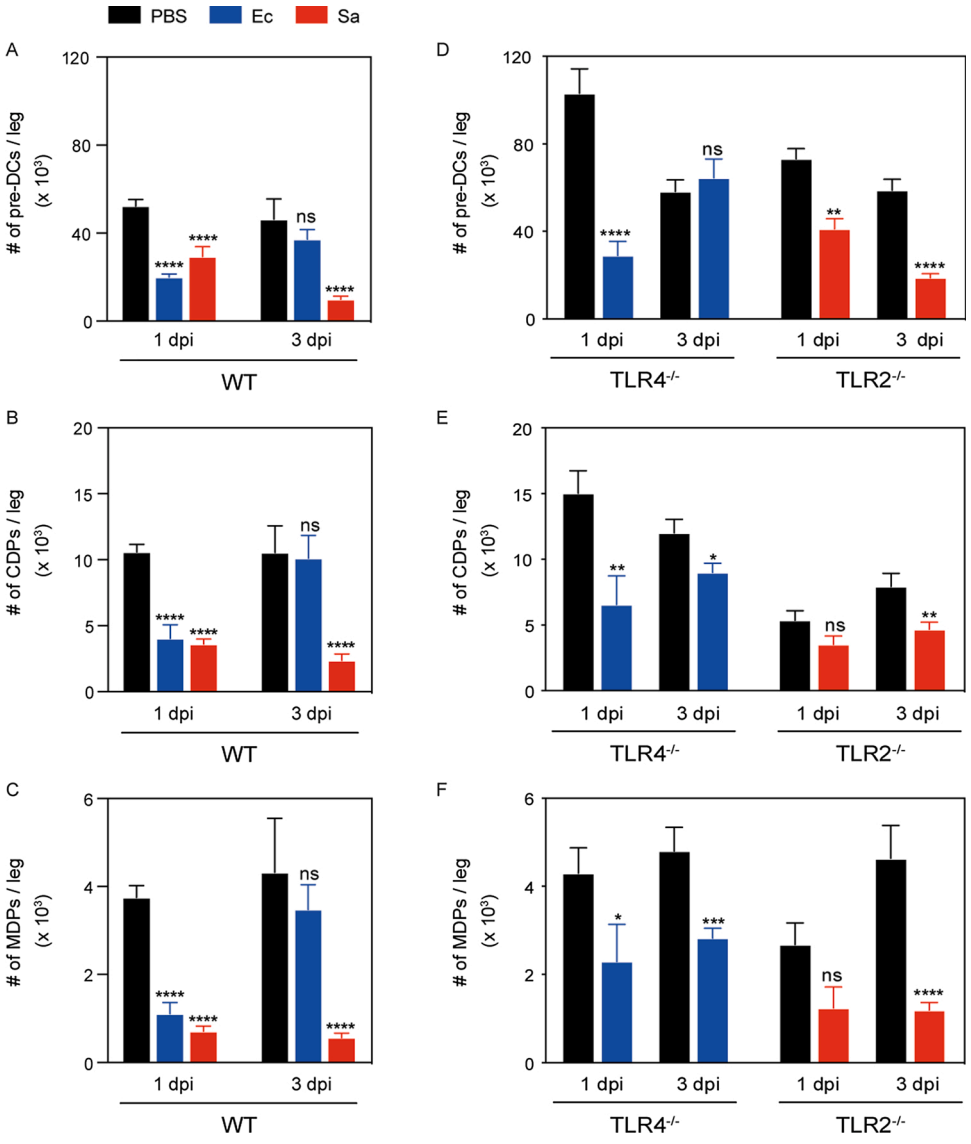

Fig. 2. Analysis of TLR-dependent effects of systemic bacterial infections on dendritic cell progenitors. WT, TLR4$^{-/-}$ or TLR2$^{-/-}$ mice were either treated with PBS or infected with Ec or Sa and BM cells analyzed 1 and 3 dpi by flow cytometry. Graphical summary of the absolute numbers of BM pre-DCs (A and D), CDPs (B and E), and MDPs (C and F). See Figure S3 for progenitor cell gating. Graphs show the means ± SEM. Number of mice / experiment / groups are displayed in Table S1. WT mice were statistically analyzed by non-parametric Kruskal-Wallis test followed by Dunn's multiple comparison test. TLR4$^{-/-}$ and TLR2$^{-/-}$ mice were statistically analysed by two-tailed Mann-Whitney-U-test. (*) $P < 0.05$, (**) $P < 0.01$, (***) $P < 0.001$, (****) $P < 0.0001$; ns = not significant.

either Ec or Sa was comparably high (log_{10} CFU approximately 4.5) in WT and TLR-deficient mice at 1 dpi (Fig. S2A&B) whereas the bacterial load in the BM slightly varied between log_{10} CFU 3.6 and 5.0 for Ec and log_{10} CFU 5.6 and 3.8 for Sa comparing the different mouse strains, respectively (Fig. S2C&D). Three dpi the bacterial load in the spleen and BM of Ec infected mice was significantly reduced in WT and TLR4-deficient mice compared to 1 dpi. In contrast, Sa infected WT mice still showed a high bacterial load in the spleen and BM, whereas the bacterial load in the spleen of TLR2-deficient mice was remarkably lower compared to WT mice (Fig. S2A-D). These data show that Ec infection is fast eradicated whereas Sa infection is not.

As previously described, WT mice showed significantly less BM DC progenitors (pre-DCs, CDPs, MDPs) 1 dpi with both strains in comparison to PBS-treated mice (Fig. 2A-C; Gating Figure S3) (Pasquevich et al., 2015). Three dpi with Ec the numbers of pre-DCs, CDPs, and MDPs were comparable to that of PBS-treated mice. In contrast, infection with Sa led to an even higher DC progenitor reduction of 75 % to 87 % in WT mice, indicating a long-lasting impairment of DC differentiation (Fig. 2A-C). This could be due to the differences in bacterial load but also to the pathogenicity of the pathogens.

Analysis of TLR-deficient mice showed that DC progenitor reduction observed at 1 dpi with the Gram-negative bacterium Ec was TLR4-independent for MDPs, CDPs and pre-DCs (Fig. 2D-F). Three dpi of TLR4-deficient mice with Ec reduction of MDPs and CDPs was less prominent, but still significant, whereas similar numbers of pre-DCs were detected in Ec infected and PBS-treated mice (Fig. 2D-F).

In contrast, TLR2-independent DC progenitor reduction upon Sa infection was observed 3 dpi for pre-DCs, CDPs, and MDPs, but only for pre-DCs at 1dpi (Fig. 2D-F). These data indicate that TLRs do not play a prominent role in the infection-induced reduction of DC progenitor cells.

3.3. *Increased number of monocyte precursors in systemic bacterial infections is TLR-independent*

Emergency monopoiesis is a hallmark of systemic infection (Boettcher and Manz, 2016) and we previously showed that Ye infection induced early monopoiesis at the expense of DC development in a TLR4-dependent manner (Pasquevich et al., 2015). Analyzing monocyte progenitors, namely cMoPs (Hettinger et al., 2013), monoblasts and promonocytes, cMoPs were similarly decreased as MDPs 1dpi with Ec and Sa infection whereas a tremendous increase of monoblast and promonocyte numbers was observed in the BM (Fig. S4A-C). Similar findings were observed in Ec-infected TLR4- and in Sa-infected TLR2-deficient mice. Interestingly, monoblasts and pro-monocytes were still increased 3 dpi with Sa in WT and TLR2-deficient mice (Fig. S4A-F).

Next we used GM-CSF culture in order to investigate whether precursor cells of infected mice at 1 dpi have an intrinsic bias to develop into monocytes/macrophages (GM-Macs) or DCs (GM-DCs) (Gating Fig. S5A) (Helft et al., 2015). The number of GM-DCs generated per mouse was slightly, but not significantly, reduced upon Ec and Sa infection compared to PBS treatment (Fig. S5B), whereas the number of GM-Macs was significantly increased by 3-fold upon infection (Fig. S5C). Thus, systemic bacterial infections in mice with Ec or Sa affect the cell intrinsic developmental pathway of BM progenitors favoring macrophage development *in vitro*. Together, these data demonstrate that DC progenitor reduction and monopoiesis are general TLR-independent events upon systemic bacterial infections.

3.4. *Minor involvement of IFN-γ in infection-induced DC and DC progenitor reduction*

IFN-γ regulates proliferation of hematopoietic stem cells and is induced upon inflammation and infection (King and Goodell, 2011). Moreover, we previously showed that the Ye-induced DC progenitor reduction is IFN-γ dependent (Pasquevich et al., 2015). One dpi with Ec and Sa low to high levels of IFN-γ, respectively, were observed in the plasma of WT mice (Fig. 3A). To address whether IFN-γ is involved in DC depletion upon Ec and Sa infection WT mice were treated with α-IFN-γ or IgG control antibody one day prior to infection with Ec or Sa or treatment with PBS and DCs as well as their progenitors were analyzed at 1 dpi. DC numbers were significantly reduced by up to 50 % 1 dpi with Ec and Sa in IgG control antibody-treated mice, whereas in α-IFN-γ antibody treated mice this effect was less prominent (Fig. 3B). These data indicate a partial involvement of IFN-γ in infection-induced DC depletion in the periphery. Pre-treatment of WT mice with IgG control antibody revealed similar reduction of pre-DCs, CDPs, and MDPs 1dpi with Ec or Sa as shown in Fig. 2 with WT mice (Fig. 3C-E). In WT mice pre-treated with α-IFN-γ antibody the numbers of pre-DCs, CDPs, and MDPs upon infection with Ec and Sa were comparably low as to them of

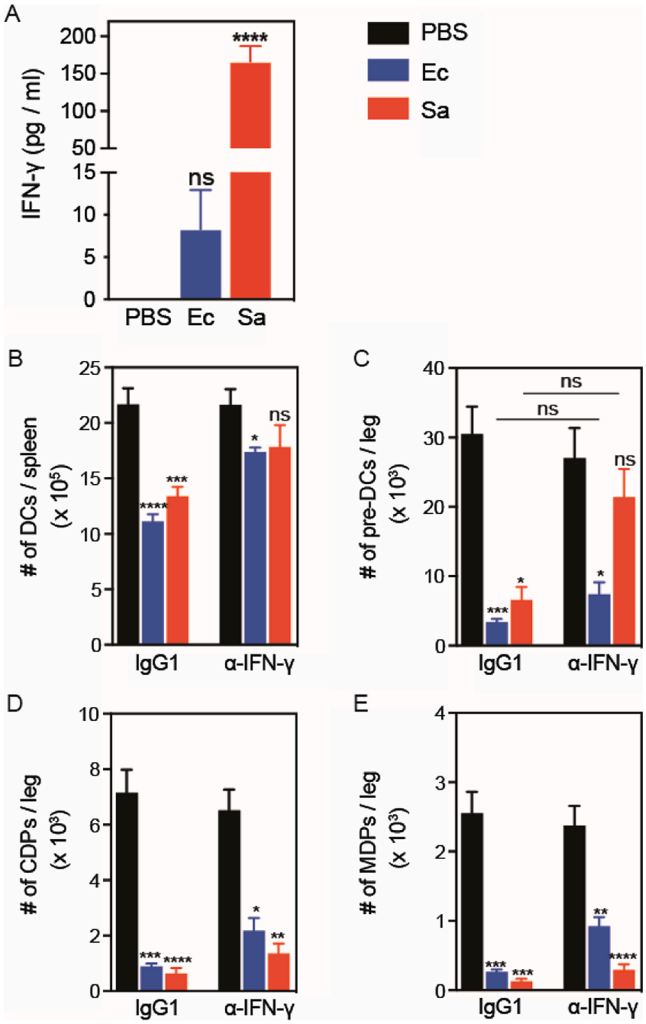

Fig. 3. Infection-induced reduction of DCs and pre-DCs upon systemic Sa infection is partially IFN-γ-dependent. IFN-γ concentration in plasma of mice 1 d after treatment with PBS, Ec or Sa (A). WT mice were either treated with IgG control or α-IFN-γ 1 d before and immediately before injection with PBS, Ec or Sa (B–E) and splenocytes and BM cells analyzed 1 dpi by flow cytometry. Graphical summary of the numbers of splenic DCs (B), BM pre-DCs (C), CDPs (D), and MDPs (E). Graphs show the means ± SEM. Numbers of mice / experiment / groups are displayed in Table S1. Groups were statistically analyzed by non-parametric Kruskal-Wallis test followed by Dunn's multiple comparison test (A), (C IgG), (D α-IFN-γ), Welch ANOVA followed by Dunnett's multiple comparison test (B α-IFN-γ), (D IgG), (E) or one-way analysis of variance (ANOVA) followed by Holm Sidak multiple comparison test (B IgG), (C α-IFN-γ). (*) $P < 0.05$, (**) $P < 0.01$, (***) $P < 0.001$, (****) $P < 0.0001$; ns = not significant.

IgG control antibody-treated mice (Fig. 3C-E), with the exception of pre-DC numbers in Sa infected mice, which were similar to PBS-treated mice (Fig. 3C). Thus, IFN-γ is only partially involved in the infection-induced reduction of DCs in the periphery and pre-DCs in the BM upon *S. aureus* infection.

3.5. Prolonged impairment of DC development upon systemic infection with S.aureus

Due to the fact that DC and DC progenitor reduction was observed up to 3 dpi with Sa, but not with Ec infection, we addressed whether DC development is impaired for longer periods upon Sa infection. Therefore, CD11c.DOG mice were once injected i.p. with diphtheria toxin (DT) to ablate CD11c$^+$ cells. 24 h later the mice were injected i.v. with Sa or PBS (Fig. 4A). The absolute numbers of DC subsets in the spleen and DC progenitors in the BM were analyzed by flow cytometry 3 dpi. Depending on the DC subsets 50–80 % less DCs and pre-DCs were observed one day after DT treatment compared to CD11c.DOG mice without DT treatment (Fig. 4B–E) (Autenrieth et al., 2010). Three days later the number of cDC subsets in PBS-treated mice was similar or even slightly higher to that of CD11c.DOG mice without DT treatment, indicating that all splenic DC subsets were recovered at that time (Fig. 4B–D). In contrast, all splenic DC subsets and BM pre-DCs were still reduced by 90 % and 75 % 3 dpi with Sa compared to PBS-treated mice, demonstrating a prolonged impairment of DC development upon Sa infection (Fig. 4B–E).

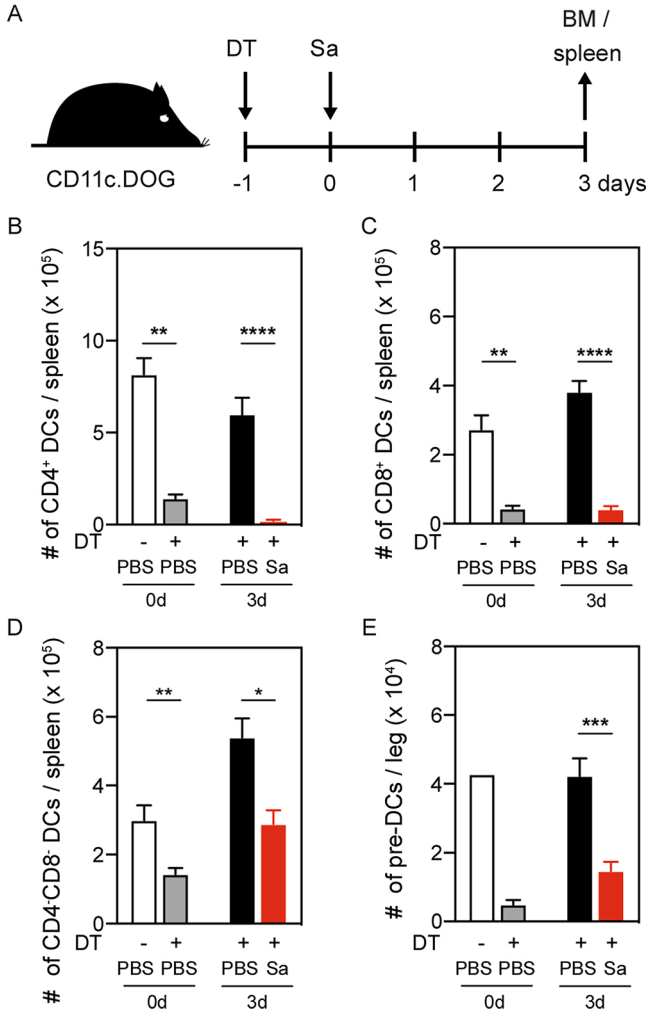

Fig. 4. Long-lasting effects on DC development upon systemic *S. aureus* infection. CD11c.DOG mice were once injected *i.p.* with diphtheria toxin (DT) to ablate DCs; PBS-treated mice served as controls. 24 h later mice were infected *i.v.* with Sa or treated with PBS and analyzed by flow cytometry 3 dpi (A). Graphical summary of absolute numbers of DC subsets CD4$^+$ DCs (B), CD8$^+$ DCs (C) and CD4$^-$CD8$^-$ DCs (D), and BM pre-DCs (E). Graphs show the mean ± SEM. Number of mice / experiment / groups are displayed in Table S1. Groups were statistically analyzed by two-tailed Mann-Whitney-U-test. (*) $P < 0.05$, (**) $P < 0.01$, (***) $P < 0.001$, (****) $P < 0.0001$; ns = not significant.

3.6. Impaired T cell responses upon infection with Sa

APCs are essential for the activation and polarization of CD4$^+$ T cells (Merad et al., 2013). We performed T cell proliferation assays using GM-DCs / GM-Macs from PBS-treated, Ec or Sa-infected mice as APCs and co-cultured similar numbers of these cells with Ovalbumin (OVA) T cell receptor transgenic CD4$^+$ T cells in the presence of OVA peptide for 3 days in order to address their capacity to induce T cell responses. CD4$^+$ T cell proliferation was similar between co-cultures of T cells with GM-APCs generated from PBS or infected mice (Fig. S6A). In contrast, the frequencies of IL-17$^+$CD4$^+$ T cells and IFN-γ$^+$CD4$^+$ T cells after co-culture with APCs from Sa-infected mice were 50 % and 30 % lower, respectively, the latter not being significant compared to APCs from PBS treated and Ec infected mice (Fig. S6B-C) (Gating Fig. S7). These data show that *in vitro* generated APCs from Sa-infected mice, although their phenotype is not significantly altered, have an impaired capacity to induce Th17 responses.

Next, we addressed the question whether the reduced numbers of DC subsets upon infection with Ec or Sa results in impaired CD4$^+$ and CD8$^+$ T cell responses *in vivo*. Therefore, splenic T cells were analyzed by flow cytometry 3 dpi of WT mice with Ec or Sa or upon treatment with PBS (Gating Fig. S7). Upon Ec infection the numbers of CD4$^+$ and CD8$^+$ T cells (Fig. 5A,B) and among them the numbers of Th1 (CD4$^+$IFN-γ$^+$T-bet$^+$ T cells), Th17 (CD4$^+$IL-17$^+$RORγt$^+$ T cells), regulatory T cells (CD4$^+$CD25$^+$FoxP3$^+$ T cells) and IFN-γ-producing CD8$^+$ T cells were slightly, but not significantly increased compared to PBS-treated mice (Fig. 5C–F). Interestingly, Sa infection revealed significantly less numbers of CD4$^+$ and CD8$^+$ T cells as well as Th1 (CD4$^+$IFN-γ$^+$T-bet$^+$ T cells) and IFN-γ-producing CD8$^+$ T cells, in the spleen of WT mice compared to Ec infected mice (Fig. 5A–D). These data indicate that systemic infection with Sa, but not Ec, affects the induction of a protective T-cell response. This could be due to an infection-induced low number of splenic DCs and/or an altered DC phenotype upon infection with Sa, as the remaining DC subsets express 45–60 % less MHC II on their surface compared with DCs from PBS-treated mice (Fig. 5G). These effects are not, or not as pronounced, upon infection with Ec, which again, as mentioned above, may be due to the different bacterial load and/or pathogenicity of Sa and Ec.

4. Discussion

Here, we showed that reduced numbers of DC subsets in the periphery and reduced numbers of DC progenitors combined with monopoiesis are general causes of systemic bacterial infection whereas the long-term effects on DC functions and T cell priming are dependent on the pathogenicity of the microbe.

Systemic infection with Ec or Sa reduced the numbers of DC subsets in the spleen which confirm data that systemic bacterial infections in humans and mice affect DC numbers at least partially in a TLR-dependent manner (Fig. 1) (Autenrieth et al., 2010; De Trez et al., 2005; Grimaldi et al., 2011; Pasquevich et al., 2015; Pène et al., 2009). It should be particularly emphasized that the reduced number of CD8$^+$ DCs after infection with Ec but also Sa was dependent on TLR4 or TLR2, respectively. This could be due to the fact that these receptors are more frequently expressed on CD8$^+$ DCs (Edwards et al., 2003) and apoptosis is induced in these cells via this pathway. The latter was shown for Ec and polymicrobial sepsis for CD8$^+$ DCs via TLR4 (De Trez et al., 2005; Efron et al., 2004). Interestingly, CD4$^-$CD8$^-$ DCs were reduced in a

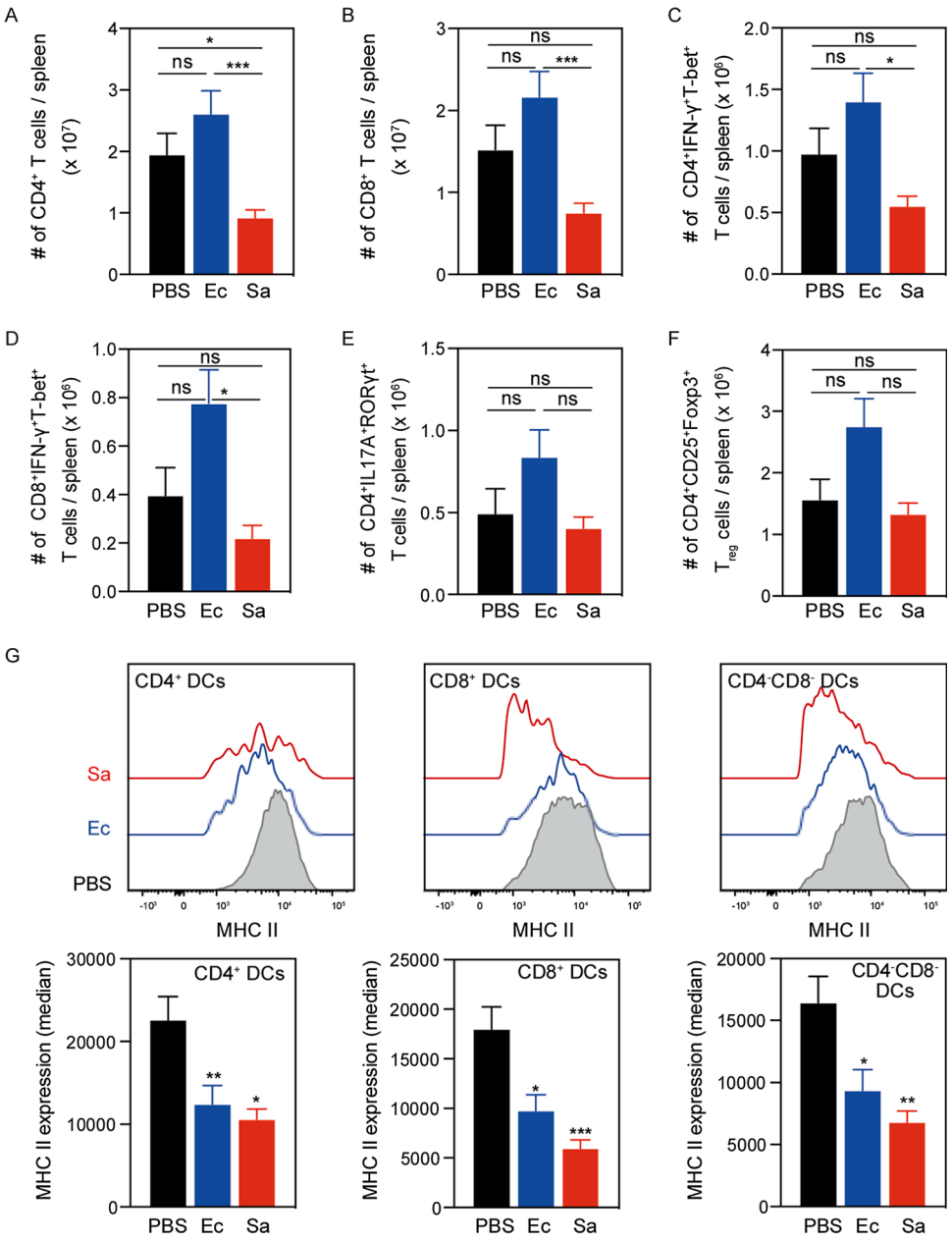

Fig. 5. Analysis of infection-induced effects on splenic T cells. WT mice were either treated with PBS or infected with Ec or Sa and splenocytes analyzed 3 dpi by flow cytometry. Graphical summary of absolute numbers of splenic $CD4^+$ T cells (A), $CD8^+$ T cells (B), $CD4^+IFN\text{-}\gamma^+T\text{-}bet^+$ (C), $CD8^+IFN\text{-}\gamma^+T\text{-}bet^+$ (D), $CD4^+IL\text{-}17A^+ROR\gamma t^+$ (E) and $CD4^+CD25^+Foxp3^+$ regulatory T cells (F). Representative gating scheme and graphical summary of the expression of MHC II on splenic $CD4^+$ DCs, $CD8^+$ DCs and $CD4^-CD8^-$ DCs (G). Graphs show the mean ± SEM. Number of mice / experiment / groups are displayed in Table S1. Groups were statistically analyzed by non-parametric Kruskal-Wallis test followed by Dunn's multiple comparison test (A—G). (*) $P < 0.05$, (**) $P < 0.01$, (***) $P < 0.001$; ns = not significant.

TLR2-dependent manner during infection with Sa, although the underlying mechanisms are not clear (Fig. 1G). One explanation could be that the $CD4^-CD8^-$ DCs may be the recently characterized inflammatory cDC2s (Inf-cDC2s), which are increased in the lymph node during infections via IFN- and TLR-signaling (Bosteels et al., 2020). To confirm this, very precise characterization of this cell population would need to be performed in future studies.

A possible explanation for the TLR-independent reduction of DCs and their progenitors upon Sa infection may be the pore-forming toxins or leukocidins LukAB, LukED and HlgAB, which are secreted by the Sa strain USA300 used herein (Berends et al., 2019; Spaan et al., 2017). Sa leukocidins interact with receptors expressed on DCs, such as CCR2 a receptor for HlgAB, CCR5 for LukED, and CD11b a receptor for LukAB (Merad et al., 2013; Sallusto et al., 1998; Vecchi et al., 1999). Based on the activity of leukocidins in mice (Spaan et al., 2017), it is most likely that LukED and HlgAB secreted by *S. aureus* trigger the described TLR-independent reduction of DCs in the spleen. For progenitor cells, CDPs have been described to express more CCR2 mRNA than MDPs (data generated by the Immunological Genome Project consortium ImmGen), which could explain that the number of CDPs in the BM of Sa-infected mice is lower than that of MDPs. HlgAB could be responsible for this effect. The cytolytic effects of LukAB are significantly stronger in the human system than in the mouse. Recently, it was shown that while all leukocidins can kill human monocyte-derived DCs, LukAB is most effective (Berends et al., 2019). This suggests that the cytotoxic effects of leukocidins on DCs may be even more pronounced in humans than in mice.

Moreover, similar to infection with *Y. enterocolitica* (Ye) or to LPS-induced endotoxemia BM DC progenitors were reduced in numbers upon infection with Ec or Sa, whereas monocyte progenitors were highly increased (Figs. 2 & S4) (Lasseaux et al., 2017; Pasquevich et al., 2015). Thus, among other studies, we provide further evidence that the reduction of DCs and their precursors in systemic infections is a common phenomenon (Bieber and Autenrieth, 2020; Poulin et al., 2018).

A possible explanation for altered hematopoiesis upon systemic bacterial infection is direct triggering of TLRs expressed on HSPCs

(Merad et al., 2013; Schmid et al., 2011; Takizawa et al., 2012), which is supported by the fact that bacteria are detected in the BM (Figure S2; (Takizawa et al., 2012)). The interaction may lead to a change in the expression of chemokine receptors and migratory behavior of progenitor cells as already shown upon *in vitro* stimulations (Schmid et al., 2011). Mechanistically, DC progenitor reduction and monopoiesis were TLR4-dependent for Ye, a TLR4 ligand, but TLR-independent for Ec and Sa infection. Moreover, results obtained with mixed BM chimeras upon Ye-infection argue against a direct impact of TLR4-signalling pathways on CDPs or MDPs (Pasquevich et al., 2015).

Alternatively, reduction of DC progenitors and monopoiesis may result from mediators produced by nonhematopoietic BM stromal cells in response to TLR-activation by changing their cytokine pattern in order to support hematopoiesis (Boettcher et al., 2012). Similarly, LPS-stimulated human BM stromal cell cultures enhance hematopoiesis and give rise to the full spectrum of myeloid colonies to support the replenishment of innate immune effector cells (Ziegler et al., 2015). However, we show here that DC progenitor reduction and monopoiesis are TLR4- and TLR2-independent in the case of Ec or Sa infection, respectively, arguing against a role of these TLRs in the induction of secondary mediators. The underlying molecular mechanisms remain to be resolved. In contrast to our results, monopoiesis upon *Listeria monocytogenes* infection was described as TLR-signaling dependent using MyD88$^{-/-}$Trif$^{lps2/lps2}$ mice in which signaling via TLRs is completely abolished (Serbina et al., 2009). In our setting bacterial recognition by other TLRs and non-TLR pattern-recognition receptors like Nod-like receptors or C-type lectin receptors, which are triggered by Sa and Ec (Askarian et al., 2018; Burberry et al., 2014) may change the BM microenvironment, leading to the production of cytokines, that may indirectly impact DC progenitor function.

In this direction a critical role of the IFN-γ pathway in HSC activation and expansion (Morales-Mantilla and King, 2018), as well as in differentiation of progenitors into myeloid cells has been demonstrated following infection with *Mycobacterium avium* (Baldridge et al., 2010), Malaria (Belyaev et al., 2013, 2010), *Ehrlichia* (MacNamara et al., 2011) or with BCG vaccination (Kaufmann et al., 2018). Our data indicate that IFN-γ is involved in DC and pre-DC reduction upon systemic infection with extracellular pathogens like Sa (Fig. 3) and Ye (Pasquevich et al., 2015), but not with non-pathogenic Ec. This could be due to lower production of IFN-γ caused by infection with Ec.

Infection with Sa profoundly affected DC development *in vivo* (Fig. 4), like it was shown for Ye infection (Autenrieth et al., 2010). Moreover, BM cells from Ec and Sa infected mice differentiated predominantly into macrophages (Fig. S5), supporting the findings of increased numbers of monocyte progenitors in the BM upon *in vivo* infections. Nevertheless, there is an effect of the BM microenvironment, as the *in vitro* effects are not as drastic as the *in vivo* impairment of DC development.

A major problem of systemic infections and sepsis is a permanent immune suppression leading to secondary infections and a severe course of the disease (Boomer et al., 2011; Grimaldi et al., 2011; Hotchkiss et al., 2013). We now show that this may be due to impaired DC development most likely combined with altered DC functions probably accounting for the insufficient T cell responses following infection with the pathogen Sa (Richardson et al., 2019; Schreiner et al., 2013). In contrast, infection with the self-limiting Ec has hardly any effect on the T cell responses, indicating differences regarding pathogenic and non-pathogenic bacteria. However, the difference in bacterial load in the spleen and bone marrow, respectively, could also be responsible for the observed differences in DC development and functions between Ec and Sa-infected mice. Further studies are needed to unravel the underlying molecular mechanisms of prolonged DC depletion, which may provide an approach to abrogate sepsis-induced immunosuppression.

In summary, our data provide evidence for a general induction of monopoiesis most likely at the expense of DC differentiation upon systemic infection with various pathogens, which could explain DC depletion in the periphery during sepsis. However, the severity of DC paralysis, as well as the underlying mechanisms seem to be pathogen-specific. Several studies provide evidence that hematopoietic progenitor cells are able to process signals from different PRRs simultaneously, resulting in a pathogen specific response (Pasquevich et al., 2015; Yáñez et al., 2011). Similarly, *in vivo* administration of the *C. albicans* cell wall component β-glucan or Candida itself induced myeloid cell differentiation amongst others via the mediators IL-1β and GM-CSF (Martínez et al., 2018; Mitroulis et al., 2018). The control of *Listeria* and Ec infections depends on inflammatory monocytes (Serbina et al., 2008). Although control of Sa infections is mainly based on neutrophils, recent studies show the recruitment of monocytes in Sa mouse models (de Oliveira et al., 2015; Svedova et al., 2016). Moreover, monocytes play a critical role in the pathophysiology of bacterial sepsis as they are the main source of inflammatory cytokines responsible for septic shock (van der Poll et al., 2017). Thus, differences in cytokine release triggered by different key elements of inflammatory signaling pathways, which depend on the nature of the infection could explain the various mechanisms underlying the infection-induced DC depletion and monopoiesis.

Author contributions

K.B. and S.E.A. conceived the study, designed the experiments and wrote the manuscript. K.B., M.G. and K.A.P. performed the experiments. K.B., M.G., K.A.P. and S.E.A. analyzed the data.

Declaration of Competing Interest

The authors declare no conflicts of interests.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.ijmm.2021.151517.

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