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A role for IFN γ in differential superantigen stimulation of conventional versus plasmacytoid DCs

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Abstract

Superantigens (SAGs) are known to play a role in food poisoning, toxic shock syndrome and have been identified as a potential mediator of autoimmunity. Although much is known about the effects of SAGs on T cells, by comparison few studies have investigated how SAGs influence innate immune cells. In particular no study has examined how SAGs affect murine plasmacytoid dendritic cells (pDC). We report that in vivo administration of Staphylococcal enterotoxin A (SEA) increased the number of pDCs in secondary lymphoid organs, and induced CD86 and CD40 expression. Similar to SEA activation of conventional DCs (cDCs), pDCs relied on T cells, but not on CD40. Nonetheless, pDCs strictly required IFN γ for upregulation of CD86 and CD40, but cDCs did not depend upon IFN γ for activation. Further, even though IFN γ deficient pDCs were not activated by SEA, they were still capable of producing wild type levels of IFN α in response to CpG oligodeoxynucleotide (ODN). The source of IFN γ for pDC activation was not T cells, nor did pDCs themselves have to synthesize or bind IFN γ , but the presence of IFN γ was essential. After SEA stimulation, IFN γ deficient mice fail to induce expression of the pDC dependent chemokines CXCL9, and demonstrated a defect in recruitment of pDCs to marginal zones of lymphoid organs. Thus, SEA exerts its combined effect on pDC activation, recruitment and chemokine induction through the action of IFN γ . This fundamental dichotomy of the effects of SAGs on pDCs versus cDCs show how a non-PAMP from bacteria, can selectively and indirectly stimulate innate cell subpopulations much in the same way that differential TLR expression influences cells of the innate immune system.

Keywords

Superantigen; Plasmacytoid DC; IFN γ

Introduction

A mammalian host recognizes different types of bacterial toxins resulting in profound stimulation of the innate or adaptive immune system. In particular, PAMPs, such as endotoxin, are pathogen byproducts that potently activate the innate immune system by binding pathogen recognition receptors such as TLR and Nod. Many different cells of the innate immune system express these receptors and stimulation through them results in proinflammatory cytokine production and enhanced phagocytic ability [1–4]. In contrast, superantigens (SAGs) like *Staphylococcal aureus* enterotoxins stimulate the adaptive immune system by activating T cells bearing specific TCR V β chains. This type of oligo clonal stimulation results in massive T cell

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expansion and cytokine production [5]. Nevertheless, these seemingly different pathogenic toxins inextricably link the innate and adaptive immune systems, and their combined effects lead to very powerful immune responses.

The effects of SAGs on T cells have been documented for well over a decade [5–7]. They mediate a variety of maladies including Toxic Shock Syndrome, food poisoning, and are implicated in several autoimmune diseases [8–10]. Based on functional and crystallographic structure studies it is established that SAGs bridge MHC class II molecules with the variable portion of the β chain of the TCR. Because this does not require MHC peptide specificity, but only specific TCR V β chains, the frequency of stimulated T cells is substantially enhanced over that of an experimental peptide MHC ligand. This results in release of proinflammatory cytokines by T cells, and is thought to be a major aspect in the etiology manifested by this type of toxin [11–13]. Perhaps, this emphasis on adaptive immunity has largely dissuaded investigations regarding the influence of SAGs on the innate immune system.

In recent years, however, the effect of SAGs on the innate immune system has been documented. One *in vivo* study showed that Staphylococcal enterotoxin B (SEB) stimulated dendritic cells to upregulate maturation or activation markers, but only in a T cell dependent manner [14]. This is in sharp contrast to PAMPs, which do not require T cells to stimulate dendritic cells or macrophages. A second study by our laboratory showed that SEA conditions DCs to secrete heightened cytokine levels in response to challenge with various PAMPs, whereas conditioning with LPS induced endotoxin tolerance [15]. Therefore, SAGs reveal how the adaptive immune system can initiate and condition cells of the innate immune system.

In this report, we define a split mechanism for differential DC activation by SAG. Similar to conventional CD11c^{hi} MHC II⁺B220[−] DCs, we found that pDCs increased in number and upregulated CD40 and CD86 in response to SAG. Moreover, activation of pDCs and cDCs were both T cell dependent, but CD40 independent. Nevertheless, in direct contrast to cDCs, pDCs required IFN γ for upregulation of CD86 and CD40. We demonstrated through the use of mixed bone marrow chimeras that pDCs neither needed to synthesize nor bind IFN γ , but the presence of IFN γ was essential for activation of pDCs. The lack of IFN γ , however, did not inhibit pDC synthesis of IFN α in response to TLR9 stimulation, but IFN γ was shown to be essential for production of the pDC-dependent chemokine CXCL9. Finally, we demonstrated that IFN γ was pivotal for the accumulation of pDC into the marginal zones of spleen and LNs. Therefore, bacterial SAG conditioned pDCs in a T cell and IFN γ -dependent manner leading to increased expression of costimulatory molecules, and support for traverse through lymphoid tissue.

Materials and Methods

Mice, reagents and in vivo treatments

C57BL/6 mice were purchased from Charles River – National Cancer Institute (Fredrick, MD). IFN γ ^{−/−}, IFN γ R^{−/−} and CD40^{−/−} breeder mice were purchased from Jackson Laboratory (Bar Harbor, ME). TCR $\beta\delta$ ^{−/−} mice were a kind gift from Dr.T.V. Rajan (Dept. of Immunology, UConn Health Center). All mice were maintained in the Central Animal Facility at the University of Connecticut Health Center in accordance with federal guidelines.

SEA (staphylococcal enterotoxin A) was purchased from Toxin Technology, Inc. (Sarasota, FL). Recombinant Murine Interferon- γ (IFN γ) was purchased from MBL International Corp. (Watertown, MA). Mice received 1 μ g of SEA intraperitoneal (i.p.), and all IFN γ treated mice received either 5 μ g or 15 μ g of recombinant murine IFN γ i.p.

PE-conjugated and APC- conjugated anti-mPDCA-1 (JF05-1C2.4.1) were purchased from Miltenyi Biotec (Auburn, CA). PE- conjugated anti-CD11c (HL3) and control ratIgG2b, FITC conjugated streptavidin and anti-CD11b (Mac-1), APC conjugated anti-CD11c (HL3), biotin-labeled anti-CD40 (3/23), anti-CD86 (GL1) and control ratIgG2ak were purchased from BD Biosciences (Mountain View, CA). PE-Cy5.5 conjugated anti-CD45.1 (A20) and anti-CD45.2 (104), PE- conjugated Ly-6G (RB6-8C5) were purchased from eBioscience (San Diego, CA).

Dendritic cell isolation and flow cytometric analysis

For analyzing the expression of maturation markers and for obtaining actual numbers of DCs, density gradient fractionation of innate APCs was followed as given in Swiggard et al. without DC adherence [16]. Briefly, spleens from mice were crushed mechanically with forceps and treated with 1 ml of a 3.3 mg/ml solution of Collagenase D (Roche Diagnostics Corporation, Indianapolis, IN) in MEM 2% fetal bovine serum (FBS, Gibco BRL, Grand Island, NY), 10 mM HEPES (Gibco). Splenic tissue was incubated for 30 min at 37°C, 5% CO₂. After treatment with 100µl 0.1 M EDTA for 5 min at room temperature, spleen tissue was disassociated through cell strainers and rinsed with Ca/Mg free BSS. The red blood cells were lysed and cells were resuspended in 2 ml PBS 35% BSA, added to Ultra-Clear centrifuge tubes (Beckman, Palo Alto, CA) with an additional 2 ml Ca/Mg Free BSS layered on top. After centrifugation at 9500×g for 15 min at 4°C, cells from the interface were collected and washed with BSS. Cells were resuspended in cell staining buffer (BSS, 3% FCS, 0.1% NaAz) and counted on a Z1 particle counter (Beckman Coulter, Miami, FL). For DC numbers, red blood cells were lysed and resuspended in cell staining buffer without the gradient centrifugation step.

For flow, cells were resuspended in cell staining buffer and nonspecific binding was blocked using a combination of 2.4G2 hybridoma supernatant, 5% heat inactivated normal mouse serum and approximately 10 µg/ml human IgG. Cells were incubated on ice with primary antibodies for approximately 30 min, washed, and resuspended in staining buffer. If a secondary incubation was necessary, cells were washed and resuspended in staining buffer, and incubated on ice with streptavidin-FITC for 30 min. The cells were analyzed on a FACSCalibur flow cytometer and data was analyzed using either CELL Quest software (BD Biosciences, Mountain View, CA) or FlowJo software (Tree Star Inc. Ashland, OR).

T cell purification and adoptive transfer

Splenocytes from either C57BL/6, IFN γ or IFN γ R^{-/-} mice were crushed through a cell strainer and run through a nylon wool column (PerkinElmer Life and Analytical Sciences, Boston, MA) for bulk removal of APCs. Ten million splenocytes enriched for T cells were adoptively transferred into TCR β ^{-/-} mice via the intravenous (i.v) route. One, 4 or 9 days after adoptive transfer into TCR β ^{-/-} mice, SEA was injected and 12h later the splenic DCs were analyzed for upregulation of maturation markers. Also, a Student's T test was performed for statistical analysis.

Flt3L treatment and pDC isolation

To obtain a high yield of pDCs, mice were treated with 10µg Flt3L (Kind gift from Amgen, Seattle, WA) per mouse. Five such treatments were administered every other day and at the end of the treatment schedule the mice were immunized with SEA or left unimmunized. At 12h after SEA challenge, pDCs from the spleen, PLN and MLN were isolated from these mice using MACS beads. The splenocytes and LN cells were pooled and subjected to density-gradient centrifugation. pDCs were then pre-enriched by depletion of T cells, B cells, NK cells, and myeloid cells with a cocktail of biotin-conjugated antibodies against CD3, CD19, CD11b (Mac-1), and CD49b (DX5). Subsequently, pDCs were positively selected from the pre-enriched DC fraction with CD45R (B220) MicroBeads (Miltenyi Biotec, Auburn, CA).

The purified cells were between 85% to 95% mPDCA-1⁺ CD11c⁺ and 3×10^5 purified cells were seeded into wells of a 96-well plate and then stimulated with complete tumor media (CTM) alone, 1 µg/ml ultra-pure LPS (Alexis Corporation), 1 µg/ml poly I:C (Sigma) or 1 µg/ml ODN (CpG oligodeoxynucleotide, ATA ATC GAC GTT CAA GCA AG from The Midland Certified Reagent Company, Midland, TX). Twelve hour supernatants were harvested and IFN-α levels were measured. The levels of mouse IFN-α were determined by ELISA. Briefly, 96-well plates were coated with rat anti-mouse IFN-α (mAb F-18, HyCult Biotechnology, Uden, The Netherlands) in carbonate buffer. After 12h incubation with the coating antibody, nonspecific binding was blocked using assay diluent.

Plates were then incubated with supernatants from pDC cultures and known concentrations of recombinant mouse IFN-α (HyCult Biotechnology) were used as a standard. After a 24h incubation with experimental samples and standards [17], plates were sequentially incubated for 1h with polyclonal rabbit anti-mouse IFN-α (PBL Biomedical Laboratories, New Brunswick, NJ) and for 1h with horseradish peroxidase-conjugated donkey anti-rabbit F(ab')₂ (Jackson ImmunoResearch). The reaction developed by using substrate solution TMB (eBioscience, San Diego, CA), and the reaction was killed with H₃PO₄. Optical density at 450 nm was measured and the concentration of IFN-α was calculated based on the standard curve.

Bone-marrow chimeras

Femurs and tibias were taken from IFNγ^{-/-} mice or IFNγR^{-/-} mice (CD45.2) and C57BL/6 (CD45.1). The bone marrow was flushed out with a syringe and passed through a 70 µm nylon mesh to generate a single-cell suspension. Red blood cells (RBCs) were lysed, and the cells resuspended in HBSS supplemented with HEPES, L-glutamine, penicillin, streptomycin, and gentamycin sulfate (HBSS-HGPG). Cells were washed twice and resuspended to a final volume of 30×10^6 cells/ml. CD45.1 recipient C57BL/6 mice were irradiated (2×500 rad) before bone marrow cells were transferred via the i.v route. The bone marrow cells transferred were a mix IFNγ^{-/-}/B6 or IFNγR^{-/-}/B6 in ratios of 1:1, 2:1 or 1:2. The total number of bone marrow cells that the CD45.1 B6 recipient received was 3×10^6 in all cases. The mice were allowed to rest for 8 weeks before use.

Confocal Microscopy

Acetone fixed frozen spleen sections were incubated with purified hamster CD11c (N418), APC-conjugated B220 (RA3-6B2) and biotin labeled mPDCA-1 (JF05-1C2.4.1) or lymph node sections were incubated with Alexa fluor 488 conjugated B220 (RA3-6B2), APC-conjugated CD8α (53-6.7) and biotin labeled mPDCA-1 (JF05-1C2.4.1). After 1h at 37°C with primary antibodies, the sections were washed thrice with PBS. They were then incubated with secondary antibodies streptavidin alexa fluor 546 and/or goat anti-hamster alexa fluor 488 (Molecular Probes, Eugene, OR) for 1h at 37°C. Both the primary and secondary antibodies were diluted in 2 % FCS, 2% goat serum and PBS. The sections were washed thrice with PBS and mounted with Immu-Mount (Thermo Shandon, Pittsburgh, PA). The stained sections were finally analyzed using Zeiss LSM 510 meta confocal microscope (Carl Zeiss Inc. Thornwood, NY). The images were analyzed with IMARIS software (Bitplane Inc. St. Paul, MN). For the LN sections, quantitative data was obtained by counting mPDCA-1⁺ stains in each 14x field. For the spleen sections, quantitative data was obtained by converting the mPDCA-1⁺ staining into a gray-scale image. The intensity value of the gray-scale image was calculated using the histogram function in Adobe Photoshop (Adobe Systems Inc. San Jose, CA).

Real-Time PCR

CXCL9 mRNA expression was analyzed by quantitative real-time PCR. Total cellular RNA was extracted from cells of the peripheral and mesenteric LNs using RNeasy mini-kit (Qiagen Sciences, Maryland). RNA was reverse transcribed using SuperScript II RNase H⁻ (Invitrogen

Life Technologies, Carlsbad, CA). Real-time PCR assays used a final volume of 40 μ l containing 20 μ l iQ SYBR Green Supermix (Bio-Rad Laboratories), 4 μ l gene-specific primers (640 nmol), 5 μ l cDNA and 11 μ l H₂O. The starting concentration of cDNA was 1 μ g but 8- and 64-fold dilutions were also completed to ensure quality control of the real-time assay. Primers specific for the amplification of murine HPRT and CXCL9 were synthesized by Invitrogen life technologies (Carlsbad, CA). CXCL9 primers were obtained using the Beacon Designer software (Premier Biosoft International, Palo Alto, CA).

HPRT: sense strand 5'-CTCCTCAGACCGCTTTTTC-3' and anti-sense strand 5'-TAACCTGGTTCATCATCGCTAATC-3'. CXCL9: sense strand 5'-CCTAGTGATAAGGAATGCACGATG-3' and anti-sense strand 5'-TGTTTGAGGTCTTTGAGGGATTTG-3'. The cycling protocol was as follows: 95°C 3 min, followed by 45 cycles of 95°C denaturation for 15 s and gene-specific annealing and extension at 59°C for 70 s. A melt-curve was generated at the end of each real-time assay to check for non-specific amplification of cDNA. Hypoxanthine PhosphoRibosylTransferase (HPRT) amplification was the control and the fold difference in cDNA levels were quantitated using the ddCT method. Real-time PCR was performed using the Bio-Rad iCycler.

Chemokine Microarray

Chemokine cDNA microarray was performed according to manufacturer's instruction (Superarray Bioscience Corporation, Frederick, MD). Briefly total RNA was extracted from the PLNs. On average 2.5 μ g of total RNA was converted to cDNA and then labeled with biotinylated-UTP. This biotin labeled cDNA probe was denatured and hybridized with murine chemokine cDNA fragments printed on a nylon membrane. Hybridization was analyzed by chemiluminescence detection technique and the data was interpreted using GEM Express Analysis Suite.

Results

Plasmacytoid Dendritic cells undergo in vivo maturation after SEA treatment

Similar to other reports [18,19], pDCs were identified as Gr-1⁺ B220⁺ CD11c^{int} and CD11b⁻ (Fig. 1a). Twenty four hours after SEA treatment C57BL/6 mice displayed an increase in the proportion of pDCs accumulating in the secondary lymphoid organs including MLN, PLN and spleen. Specifically, the percentage increased by at least 1.5 fold in the PLN and 2 fold in the MLN (Fig. 1b, upper panel), there was also a corresponding increase in the absolute numbers of pDCs in both of these organs (Fig. 1b, lower panel). However, the number of pDCs in the spleen did not increase until 48h after SEA injection. This difference between the percentage increase at 24h and no change in absolute numbers may reflect strong adherence of DCs and T cells to tissue as we have seen in the past [20]. Conventional DCs (cDCs) identified as CD11c^{hi} MHC II⁺B220⁻, were also analyzed after SEA treatment. Similar to pDCs there was an increase in the percentage of cDCs (data not shown), and a corresponding increase in absolute numbers of cDCs in the MLN and PLN 24 h after SEA treatment (Fig. 1c), but the spleen did not show this effect until 72 h. In the PLN and MLN, the absolute numbers of cDCs increased by at least 2.4 fold at 24 h (Fig. 1c).

To analyze increased expression of the activation markers CD86 and CD40 on pDCs and cDCs, pDCs were marked as mPDCA-1⁺ CD11c^{int} and cDCs as mPDCA-1⁻ CD11c⁺, as shown in the dot plots (Fig. 2a). This strategy permitted direct analysis of pDCs without the B220 and Gr-1 markers, but all B220⁺ Gr-1⁺ cells were mPDCA-1⁺ (data not shown). The kinetics of CD86 upregulation on pDCs and cDCs after 6, 12, 24 or 48h with SEA is shown in figure 2b. After treatment with SEA, CD86 upregulation was observed as early as 6h on both pDCs and cDCs. The expression levels peaked by 12h, but 48h after SEA treatment CD86 expression

dropped to the level of naïve mice. The quantitated time kinetics of CD86 and CD40 MFI on pDCs and cDCs is shown in figure 2c. At peak expression, CD86 was up regulated by 1.4 fold on both pDCs and cDCs over their naïve counterparts. CD40 expression levels peaked 24h after SEA treatment and at this time point CD40 expression was upregulated by 1.4 fold on pDCs and by 1.25 fold on cDCs. Lastly, the MFI of the IgG isotype between the normal and 12 h SEA treated samples from the data in this paper demonstrated that on average the CD86 MFI was 49.1% higher in the SEA-treated over its own IgG isotype control, and 28.9% higher in the normal. Therefore, these data cannot be explained by non-specific binding of staining antibodies by the SEA treated cells.

T cells are required for pDC maturation after in vivo SEA treatment

Because SEA increased costimulatory molecule expression on the pDCs, it was reasoned that their stimulation may involve the adaptive immune system. Therefore we designed an experiment to test if T cells played any role in the upregulation of costimulatory molecules on pDCs. Both wildtype and TCR $\beta\delta$ deficient mice were challenged with SEA and 12h later we analyzed splenic pDCs and cDCs for CD86 upregulation. The histogram for CD86 expression, shown in figure 3a, indicates that in the absence of T cells there is very little activation of both pDCs and cDCs (bottom panels). The CD86 MFI for pDCs and cDCs, in both wildtype and TCR $\beta\delta$ deficient mice is given (Fig. 3b). The pDCs in naïve, wildtype and TCR $\beta\delta$ deficient mice had a CD86 MFI of approximately 250. After SEA treatment, while the wildtype CD86 MFI on pDCs increased from 250 to 325, the TCR $\beta\delta$ deficient CD86 MFI on pDCs only had a marginal increase from 247 to 256. We observed a similar trend with the cDCs, where the wildtype CD86 MFI on cDCs increased from 309 to 425 after SEA treatment. However, in the TCR $\beta\delta$ deficient mice, the CD86 MFI on cDCs, like the pDCs, only showed a marginal increase from 300 to 340 after SEA treatment. Data from figures 3a and 3b revealed the absolute requirement of T cells for not only cDC activation, but for pDC activation as well.

Since the above experiments were done in TCR $\beta\delta$ deficient mice, there was a possibility that the absence of pDC and cDC activation after SEA challenge was due to an inherent defect in these mice. To test this possibility, we transferred T cells from wildtype mice into TCR $\beta\delta$ deficient mice to see if pDC and cDC activation would be rescued. One group of TCR $\beta\delta$ deficient mice received 1×10^7 splenocytes enriched for T cells and the other group received 19 fold less. One day after T cell transfer, the mice were challenged with SEA. Twelve hours after challenge splenic pDCs and cDCs were analyzed for CD86 expression. While the group that received 1×10^7 splenocytes was able to rescue pDC and cDC activation, 19 fold fewer cells were not able to do the same. We also wanted to test if homeostatic proliferation of T cells enhanced the activation status of pDCs and cDCs in TCR $\beta\delta$ deficient mice. For this we transferred 1×10^7 splenocytes enriched for T cells, but waited 4 or 9 days before SEA challenge. As before, 12h after SEA challenge the activation status of splenic pDCs and cDCs were analyzed. We found that 4 days after transfer permitted optimal activation of both pDCs and cDCs post SEA treatment, but homeostatic proliferation alone of the transferred T cells was not sufficient to activate the DCs. Thus, T cell stimulation was central for the activation of pDCs.

IFN γ is required for pDCs, but not for cDCs, to fully upregulate CD86 after in vivo SEA treatment

We reasoned that T cells could be influencing pDC activation through the CD40-CD40L interaction or through the action of soluble mediators like cytokines. To test these possibilities, we challenged wildtype, CD40 or IFN γ deficient mice with SEA and analyzed the activation status of splenic pDCs and cDCs. In both CD40 and IFN γ deficient mice, cDC activation was comparable to that of wildtype (Fig. 4a, right panels). However, though CD40 deficiency did not affect pDC activation, the absence of IFN γ prevented pDC activation (Fig. 4b, left panels).

The CD86 MFI for pDCs and cDCs in IFN γ deficient, wildtype and IFN γ R deficient mice is given (Fig. 4b). The CD86 MFI for naïve cDCs in IFN γ deficient, wildtype and IFN γ R deficient mice was approximately 319, and after challenge with SEA increased to 404 in IFN γ deficient mice, 430 in wildtype and 388 in IFN γ R deficient mice. However, pDC CD86 MFI in IFN γ deficient mice was not affected and only had a marginal increase from 260 to 277, while the wildtype pDCs showed an increase from 248 to 317. The pDCs in IFN γ R deficient mice also showed a defect in activation, with their CD86 MFI increasing from 243 to 268 after SEA challenge. The results from figures 4a and 4b suggest that IFN γ played a key role in regulating pDC activation after SEA challenge.

To test if T cells were the direct source of IFN γ for activating pDCs, we transferred 1×10^7 splenocytes enriched for T cells, from wildtype, IFN γ and IFN γ R deficient mice into TCR $\beta\delta$ deficient recipients. Four days after T cell transfer, we challenged the mice with SEA and 12h later we analyzed the splenic pDCs and cDCs for CD86 upregulation. T cells from wildtype, IFN γ and IFN γ R deficient mice were all capable of activating pDCs (Fig. 4c). Thus, the T cells were not the direct source of IFN γ responsible for activating pDCs.

Because IFN γ played an important role for pDC upregulation of CD86, it was reasoned that IFN γ may also play a role in regulating pDC cytokine production. pDCs are the primary producer of IFN α after viral infection or PAMP stimulation, in both murine and human models [19,21,22]. We tested if IFN γ or IFN γ R deficiency had any effect on the production of IFN α by pDCs. For this we treated 2 groups of mice with Flt3L for 10 days to increase pDC numbers [23], at the end of which one group received SEA and the other group was left unchallenged. Twelve hours after SEA challenge, pDCs were isolated from the spleen, PLN and MLN, and cultured with medium alone, LPS, ODN or poly I:C. The purified pDCs were cultured with these PAMPS for 12h, after which the supernatant from the cultures were assayed for the presence of IFN α . The cultures treated with nothing (medium alone), LPS or poly I:C had undetectable amounts of IFN α (data not shown). However, pDCs from unchallenged or SEA challenged mice, when cultured with ODN, produced 140 to 370 pg/ml of IFN α . The levels of IFN α did not vary significantly between naïve and SEA challenged wildtype, IFN γ or IFN γ R deficient mice (Fig. 4d). Thus, even though IFN γ had a significant effect on the activation status of pDCs, it did not affect their ability to produce IFN α .

The results from IFN γ and IFN γ R deficient mice underscored the importance of this cytokine for pDC activation. To test if pDCs themselves were the source of IFN γ or if they had to bind IFN γ to become activated, we designed a mixed bone marrow chimera experiment to address this question (Fig. 5a). Bone marrow cells were isolated from wildtype, IFN γ or IFN γ R deficient mice and mixed with cells from wildtype and IFN γ or wildtype and IFN γ R deficient mice in 1:1, 2:1 or 1:2 ratios and transferred the cells into irradiated wildtype recipients. This resulted in the generation of two kinds of bone marrow chimera, where one set of chimeric mice were reconstituted with wildtype and IFN γ deficient cells, while the other set was reconstituted with wildtype and IFN γ R deficient cells (Fig. 5a). Eight weeks after transfer, the recipient mice were analyzed for reconstitution by staining with the CD45.1 congenic marker. The cDCs and pDCs within each congenic population were then analyzed with CD11c and mPDCA-1 markers (Fig. 5b). With this experimental set-up we reasoned that if pDCs themselves had to make IFN γ for their activation, then the IFN γ deficient pDCs in the wildtype/IFN γ ^{-/-} chimeras would fail to become activated. However, if IFN γ from an exogenous source was sufficient to activate pDCs, then the IFN γ deficient pDCs would bind the IFN γ produced by the wildtype cells and become activated. Conversely, if the presence of IFN γ R on pDCs was important, then IFN γ R deficient pDCs in the wildtype/IFN γ R^{-/-} chimeras will not become activated as they would fail to bind IFN γ produced by wildtype cells.

To test these possibilities, we challenged the wildtype/IFN γ ^{-/-} and wildtype/IFN γ R^{-/-} chimeras with SEA and 12h later analyzed the pDCs from the wildtype and the knockout congenic populations. In the wildtype/IFN γ ^{-/-} chimera, the CD86 MFI of the IFN γ deficient pDCs increased from 259 to 301. The corresponding wildtype pDC CD86 MFI increased from 276 to 309. From this we concluded that the presence of IFN γ in the milieu was sufficient for pDC activation, although stimulation was not maximal. Next we tested if the IFN γ R on pDCs was important for its activation by analyzing the wildtype/IFN γ R^{-/-} chimera. After SEA challenge, the CD86 MFI of the IFN γ R deficient pDCs increased from 257 to 291 while its corresponding wildtype pDC CD86 MFI increased from 283 to 324. Though the IFN γ R deficient pDC activation was not as robust as the other groups, there was no absolute requirement for the presence of IFN γ R on pDCs (Fig. 5c).

To rule out the possibility that some factor, apart from IFN γ , might be playing a role in the activation of pDCs in IFN γ deficient mice, we decided to test if exogenous IFN γ by itself was sufficient to cause CD86 upregulation. To test this, wildtype mice were challenged with either 1 μ g of SEA or, 5 or 15 μ g of exogenous IFN γ . There was also one group of IFN γ R deficient mice that received 15 μ g of exogenous IFN γ as a negative control. Twelve hours after challenge, splenic pDCs from the SEA and IFN γ treated groups were analyzed for CD86 upregulation. As expected, SEA challenge induced pDC CD86 upregulation in wildtype mice while 5 μ g of IFN γ caused very minimal activation. However, 15 μ g of IFN γ was able to induce CD86 upregulation to levels comparable to that of wildtype challenged with SEA (Fig. 5d). Also, the IFN γ R deficient pDCs did not upregulate CD86 expression after IFN γ treatment. The pDC CD86 MFI of wildtype mice challenged with either SEA or 15 μ g of IFN γ is given in figure 5e. While SEA had the ability to induce pDC CD86 MFI from 270 to 350, 15 μ g of exogenous IFN γ induced CD86 upregulation from 286 to 332. Thus, exogenous IFN γ by itself enhanced pDC activation without PAMPs or superantigen.

IFN γ is required for pDC to appear in SEA primed lymphoid tissue

Since IFN γ played an important role in pDC activation, we investigated if this cytokine exerted its influence on pDC growth or movement. As a first step, it was determined if SEA challenge affected chemokine expression patterns, and if so, was there any difference in the expression patterns between wildtype and IFN γ deficient mice. For this, we challenged wildtype or IFN γ deficient mice with SEA and the next day extracted total RNA from the PLNs. The total RNA was reverse transcribed to cDNA and then labeled with biotinylated-UTP. This biotin labeled cDNA probe was denatured and hybridized with murine chemokine cDNA fragments printed on a nylon membrane. Hybridization was analyzed by chemiluminescence detection technique and raw data from one experiment is given (Fig. 6a). Among all the chemokines present in the membrane, we were particularly interested in CXCL9 which is known to be involved in pDC migration [24–26]. Thus, SEA challenge induced expression of CXCL9 in wildtype mice, but failed to do so in IFN γ deficient mice. The values of this chemokine, and its receptor CXCR3, relative to the housekeeping genes is given in table from (Fig. 6a). In wildtype mice the fold induction of this chemokine after SEA challenge, over naïve, was 3.9, 5.17 and 8.3 from 3 different experiments. In the IFN γ deficient mice, following SEA challenge, there was no expression of this chemokine (indicated as no value, n.v) over the housekeeping genes after exposure of the membrane for 30 seconds. This exposure time was optimal because longer exposure time caused the wildtype sample to become overdeveloped. Therefore, the SEA to naïve ratio, in the absence of IFN γ , was either close to zero or irrelevant.

We wanted to quantify the expression level of CXCL9 mRNA and also confirm if this chemokine was induced by IFN γ . This was accomplished by quantitative real time PCR, for which we isolated total RNA from the LN's of IFN γ or SEA challenged mice. The total RNA was reverse transcribed to cDNA and tested in real time PCR using CXCL9 primers. IFN γ

induced a 9 fold increase in CXCL9 expression relative to HPRT, while SEA induced a 17.5 fold increase (Fig. 6b). The results from the quantitative real time PCR support the cDNA microarray data and suggest that SEA-induced IFN γ increased CXCL9 expression in lymphoid organs and thereby might affect pDC migration. Since there was an increase in the proportion of pDCs 24h after SEA challenge (Fig. 1b), we examined spleen and LN sections of wildtype and IFN γ deficient mice at this time point. To determine if pDCs were recruited into lymphoid organs, 20 μ m LN sections from wildtype and IFN γ deficient mice were stained with B220 (green), CD8 α (blue) and mPDCA-1 (red) markers (Fig. 7a). Using confocal microscopy, the sections were then analyzed for an increase in mPDCA-1 staining. SEA was able to recruit pDCs to the wildtype, but not IFN γ deficient LN's, and the recruited pDCs localized close to the T and B cell margins. A magnified view of the pDCs from SEA treated wildtype LN's is also given (Fig. 7a). Isotype control staining for mPDCA-1 from SEA treated wildtype LN sections was negative. Quantitative analysis (as described in materials and methods) of the increased LN mPDCA-1 stains is given in figure 7b. The number of mPDCA-1 cells increased by close to 2 fold in SEA treated wildtype mice. A negligible increase was observed after SEA treatment in the IFN γ deficient mice, and those numbers were lower than naïve wildtype values.

Apart from the LN's, 30 μ m spleen sections from wildtype and IFN γ deficient mice were stained with B220 (blue), mPDCA-1 (red) and CD11c (green) markers. Plasmacytoid DCs were recruited to the splenic marginal zones of wildtype mice (Fig. 8a) after SEA challenge, and this was quantitated by an increase in mPDCA-1 staining. An enlarged view of the SEA treated wildtype panel is given (Fig. 8a). However, after SEA challenge mPDCA-1 staining had not increased in the spleen sections of IFN γ deficient mice. Isotype control staining for mPDCA-1 from SEA treated wildtype spleen sections was negative. Quantitative analysis (as described in materials and methods) of the increased splenic mPDCA-1 stains is given in figure 8b. The average intensity value of wildtype mPDCA-1 pixels increased by close to 3 fold after SEA challenge, while there was a negligible increase in SEA treated IFN γ mPDCA-1 staining.

Discussion

In this paper we investigated the effects of SAgS on murine pDCs and demonstrated that SAgS can induce pDC activation and accumulation in lymphoid organs. We demonstrated for the first time that the SAgS indirectly stimulate pDCs and activation is primarily mediated by IFN γ . This emphasizes the fact that *Staphylococcal aureus* enterotoxins not only induce massive T cell expansion but also serve to link the innate and adaptive immune system.

Intraperitoneal injection of SEA led to increased pDC numbers in lymphoid organs (Fig 1). Under the influence of infection or by potent inflammatory conditions, the recruitment of pDCs to the lymph nodes via the HEVs has been demonstrated [21,27,28]. This recruitment is thought to be the result of blood DC precursors homing into lymphoid organs to give rise to immature DCs. Thus, it was surprising that the pDC numbers in the spleen and LN's increased after just SAg stimulation in the absence of PAMPs. SEA not only increased pDC numbers but also resulted in activation of this cell type as determined by the upregulation of CD86 and CD40 (Fig 2). The effect of SAgS on CD11c⁺ DCs has been explored by one previous study, and it concluded that DC maturation in response to bacterial superantigens occurred in a T cell dependent fashion. This study also came to the conclusion that neither soluble mediators like IL-12p40, TNF- α , IL-4, IFN γ nor costimulatory molecules like CD40 played a role in DC maturation [14]. In our study we dissected out the requirements for cDCs versus pDCs. Our results for cDCs corroborate with what has been published before, but we further show that pDCs become activated and find that the requirements for pDC activation is different from that of cDCs.

SAGs cross-link MHC II to the TCR in order to stimulate T cell proliferation. It is possible that cDCs become activated when MHC II on their cell surface is cross-linked with TCR in the synapse on the T cells. This may help explain the T cell requirement for cDC activation. For example, the level of MHC II on pDCs is much lower than that of cDCs and pDC frequency is also less than that of cDCs [29]. Given these limitations, it is quite unlikely that pDCs directly present SAGs to T cells. However, as pDCs reside in the T cell areas of the lymphoid organs, they do become activated either directly or indirectly after SAG stimulus. This demonstrates that the DCs do not merely act as a platform to provide signal-one, but actively participate in the immune response against SAGs.

It was rather surprising to us, to see that IFN γ was a special requirement for pDC activation following SAG stimulation, and this requirement was specific for pDCs but not cDCs. The cellular source of IFN γ is still unclear. It is quite likely that macrophages or cDCs are the primary producers. Our results rule out the possibility that T cells could be the primary source of IFN γ as the T cells from IFN γ deficient mice were capable of activating TCR $\beta\delta^{-/-}$ pDCs (Fig. 4c). Recent studies have demonstrated reciprocal interaction between NK cells and pDCs, and NK cells are known to produce IFN γ upon stimulation [30]. However, depleting NK cells with the PK136 antibody did not have any effect on pDC activation (data not shown), thus ruling out the possibility of a role for NK cells. We were unable to identify the exact source of IFN γ , but it was clear that its presence in the milieu was crucial for pDC activation.

Since pDC activation in the IFN γ R deficient mice was inhibited as well (Fig. 4b), we wondered if the presence of IFN γ R on the surface of pDCs was an absolute requirement. Data from the bone marrow chimera experiments demonstrated that pDCs did not have to make nor bind IFN γ , but its presence was pivotal for pDC activation (Fig. 5c). To ascertain the fact that no other factor apart from IFN γ was mediating these effects, we injected exogenous IFN γ into WT mice. Plasmacytoid DC activation following treatment with exogenous IFN γ confirmed that IFN γ by itself was capable of influencing pDCs (Fig. 5e.). One possibility to explain the role of IFN γ in this model is perhaps as a relay point for stimulation of other cytokines and chemokines that may then directly bind to pDCs and stimulate their activation. Perhaps TNF production by activated T cells induces macrophages to produce IFN γ thereby stimulating a storm of pro-inflammatory cytokines some of which have a propensity to bind pDCs.

Plasmacytoid DCs express the chemokine receptor CXCR3, and pDCs from CXCR3 deficient mice fail to enter inflamed LN's [25,31]. This receptor promotes pDC migration in response to IFN γ inducible chemokines like CXCL9 and CXCL10 [32,33]. Consistent with this data our chemokine microarray results revealed the presence of CXCL9 in SAG-stimulated wildtype, but not IFN γ deficient LN's. Real time PCR for CXCL9 also showed that both SEA and IFN γ had the capacity to induce CXCL9 mRNA expression. This suggested that SAG stimulation resulted in T cell dependent production of IFN γ , which regulated pDC activation and possibly pDC recruitment into lymphoid organs.

Data from confocal microscopy studies showed that in the absence of IFN γ , the ability of pDCs to be recruited to the lymphoid organs is severely restricted. This may be due to the lack of IFN γ , because of which CXCL9 is not induced and results in the failure to attract pDCs into LN's. However, in the wildtype mice pDCs were recruited, and they positioned themselves around the marginal zones in the spleen and along the T – B cell borders in the LN's (Fig 7 and 8). In steady-state conditions, pDCs were found in the T cell areas of both the spleen and in the LN's [27–29]. Why they move away from the T cell areas and closer to where B cells were present is not clear. However, one source of CXCL9 in the lymphoid organs may be B cells [34], and therefore it is possible that the pDCs line themselves up against a copious producer of the chemokine. This is speculative and may not be the only reason why the recruited pDCs position themselves in such a manner. If pDCs do interact with B cells; however, why

do they do so, and what is the functional outcome of this interaction? This process is not completely understood, but may relate to inducing Th responses, to provide T cell help for the B cells themselves.

These data demonstrate the extent to which SAGs can affect the innate immune system. By influencing pDC responses in such a manner it is clear that SAGs can no longer be viewed as just agents for inducing massive T cell responses. It is important for us to understand their effects on the innate immune system, particularly on DC subsets, in order to comprehend the biology of SAG induced acute and chronic disorders.

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Figure 1. The number of pDCs increases in lymphoid organs after SEA treatment

a.) To identify and quantify pDCs, tissue was collagenase treated and cells were stained for surface expression of Gr-1, B220, CD11c and CD11b. As an example from PLN, pDCs were identified as Gr-1⁺ B220⁺ CD11c⁺ and CD11b⁻ cells. b.) The percentage of pDCs in the MLN, PLN and spleens of 24, 48 and 72h SEA-treated mice are shown in the upper panels. The absolute number of pDCs in the MLN, PLN and spleens of 24, 48 and 72h SEA-treated mice are shown in the lower panels. Values calculated from three independent experiments (n=9) are presented as Mean $\pm$ SEM. c.) Conventional DCs expressing high levels of CD11c and no B220 were monitored as percentage (not shown), and by calculating absolute numbers. These data from two independent experiments (n=7) are presented as Mean $\pm$ SEM.

Figure 2. Kinetics of splenic pDC and cDC activation after SEA treatment

a.) Low-density cells were isolated from the spleen of naïve or 12h SEA treated mice and stained for surface expression of mPDCA-1, CD11c, B220 and CD86. CD11c^{int} mPDCA-1⁺ (pDC) and CD11c⁺ mPDCA-1⁻ (cDC) cells from naïve or SEA treated mice were analyzed for CD86 (or isotype control) expression. An example of gating and analysis strategy is shown.

b.) At indicated times after SEA treatment, CD11c^{int} mPDCA-1⁺ (pDC) and CD11c⁺ mPDCA-1⁻ (cDC) cells were gated and analyzed for CD86 expression. Black line indicates SEA treatment, filled histogram represents naïve mice and dotted gray lines represent isotype control. Data are from one representative experiment of three performed.

c.) The mean fluorescent intensity of CD86 and CD40 on pDCs or cDCs is given. Values calculated are from 3 independent experiments (n=3) and shown as combined data represented as Mean $\pm$ SEM. All data in timepoints 6–24 h were calculated to be at least $P < 0.0001$ in comparison to naïve.

Figure 3. T cells are required for pDC and cDC activation after SEA treatment

a.) Wild type or $\text{TCR}\beta\delta^{-/-}$ mice were injected with nothing or SEA and 12h later pDCs and cDCs were analyzed for CD86 expression. A representative histogram gated from $\text{CD11c}^{\text{int}} \text{mPDCA-1}^+$ for pDCs and gated from $\text{CD11c}^+ \text{mPDCA-1}^-$ for cDCs is shown. b.) The MFI of CD86 on pDCs or cDCs taken from wild type and $\text{TCR}\beta\delta^{-/-}$ mice is shown. Values calculated from 3 independent experiments ($n=6$) are presented as Mean $\pm$ SEM. c.) Splenocytes from wild type mice were enriched for T cells by passing them through a nylon-wool column. Ten million or 19 fold less, of the enriched T cells were adoptively transferred into $\text{TCR}\beta\delta^{-/-}$ mice and 1 day later received SEA. Twelve hours after SEA treatment, gradient fractionated pDCs and cDCs were analyzed for CD86 expression. d.) A second experiment transferring 10^7 T cells, but now waiting 4 or 9 days before SEA treatment was performed as well.

Figure 4. Plasmacytoid DCs, but not cDCs rely on IFN γ for activation

a.) Wild type, CD40^{-/-} and IFN γ ^{-/-} mice were injected with nothing or SEA and 12h later gradient fractionated pDCs and cDCs were analyzed for CD86 expression. Representative plots are shown. b.) The MFI of CD86 on pDCs or cDCs of wild type, IFN γ ^{-/-} and IFN γ R^{-/-} mice is given. Values calculated from 3 independent experiments (n=6) are shown as Mean $\pm$ SEM. c.) Splenocytes from wild type, IFN γ ^{-/-} or IFN γ R^{-/-} mice were enriched for T cells through nylon-wool columns and adoptively transferred into TCR β δ ^{-/-} mice. Four days after transfer into TCR β δ ^{-/-} mice, SEA was injected and 12h later gradient fractionated pDCs and cDCs were analyzed for CD86. Data are from 1 representative experiment of 3 performed (n=3). d.) Mice were treated with Flt3L to obtain a high yield of pDCs. Three hundred thousand purified pDCs from un-injected or 12h SEA treated mice were stimulated with nothing, LPS, ODN or poly I:C. The purified pDCs were between 85% to 95% mPDCA-1⁺ CD11c⁺. IFN- α levels were measured by ELISA. Data from wild-type mice are from 3 independent experiments and data from IFN γ ^{-/-} mice are from 2 independent experiments (Mean $\pm$ SEM). The data from IFN γ R^{-/-} mice is from one experiment. Each symbol represents results from an individual mouse.

Figure 5. pDCs need not make nor bind $IFN\gamma$, but $IFN\gamma$ causes pDC activation

a.) Schematic showing the generation of mixed BM chimeras. Bone marrow cells from $IFN\gamma^{-/-}$ or $IFN\gamma^R^{-/-}$ mice were mixed with CD45.1 wild type bone marrow cells and transferred into irradiated wild type mice. This gave rise to B6/ $IFN\gamma^{-/-}$ (I) or B6/ $IFN\gamma^R^{-/-}$ (II) mixed BM chimeras. b.) Representative FACS plot showing the analysis of wild-type or $IFN\gamma/IFN\gamma^R^{-/-}$ cells from a mixed chimera mouse. c.) The MFI of CD86 on the pDCs of wild type and $IFN\gamma^{-/-}$ or $IFN\gamma^R^{-/-}$ cells from mixed chimera mice. Values calculated from 4 independent experiments (n=7) are given as Mean $\pm$ SEM. d.) Wild type mice were injected with PBS, SEA (upper left) 5 (upper right) or 15 μ g (lower left) of $IFN\gamma$ and $IFN\gamma^R^{-/-}$ mice were injected with PBS or 15 μ g of $IFN\gamma$ (lower right). After 12h, gradient fractionated cells were analyzed for CD86 expression on pDCs. Data from the panels on the left are from 1 representative experiment of 3 performed, the data from the upper right panel is from 1 representative experiment of 2 performed while the data from lower right panel is from 1 experiment. e.) The MFI of CD86 on pDCs from wild type mice, left untreated, or treated with either SEA or $IFN\gamma$ is given. Twelve hours after SEA or $IFN\gamma$ treatment, gradient fractionated cells were analyzed for CD86 expression on pDCs. Values calculated from 3 independent experiments (n=4) are presented as Mean $\pm$ SEM.

Table 1

	Expt No	Gene	Values relative to housekeeping genes		Ratio
			Naive	SEA	SEA/Naive
C57BL/6	1	CXCL9	0.2	0.79	3.9
		CXCR3	0.24	0.12	0.5
	2	CXCL9	0.107	0.557	5.17
		CXCR3	N.V	N.V	-
	3	CXCL9	0.15	1.23	8.3
		CXCR3	0.002	0.0009	0.33
IFN γ ^{-/-}	2	CXCL9	N.V	N.V	-
		CXCR3	N.V	N.V	-
	3	CXCL9	0.28	N.V	-
		CXCR3	0.12	N.V	-

b

Figure 6. CXCL9 up-regulation after SEA treatment is dependent on IFN γ

a.) Wild-type and IFN γ ^{-/-} mice were left un-injected or injected with SEA 20h earlier. Total RNA obtained from the cells of PLN were labeled with biotinylated dUTP and hybridized with chemokine gene array. Table 1) Values obtained for CXCL9 and CXCR3 from chemokine microarray. N.V indicates no value or values below level of detection. b.) Wild-type mice were injected with SEA or IFN γ 12h earlier. Total RNA from the PLNs were reverse transcribed into cDNA and all samples were calculated in relation to the house keeping gene HPRT. Data from the PBS treated sample was normalized to 1-fold, and the data from IFN γ or SEA treated sample is shown as fold increase over PBS treated sample. Values calculated from three independent experiments (n=4) are presented as Mean $\pm$ SEM.

Figure 7. IFN γ is pivotal for pDC recruitment into lymph nodes after SEA treatment

a.) Wild-type or IFN γ $^{-/-}$ mice were injected with nothing or SEA. After 24h, frozen LN sections from these mice were stained with B220 (green), CD8 α (blue) and mPDCA-1 (red) or isotype control antibody. Data from LN sections are from 1 representative experiment of 2 performed.

b.) Number of mPDCA-1 $^{+}$ cells per 14x field of a LN section was counted, and the data represent numbers from 10 distinct fields from two combined independent experiments. In the B6 mice the SEA group was calculated to be at least $P < 0.03$ in comparison to naïve, but no statistical difference was observed in the same comparison in the IFN γ $^{-/-}$ mice.

Figure 8. IFN γ mediates pDC recruitment into spleen after SEA treatment

a.) Wild-type or IFN γ ^{-/-} mice were injected with nothing or SEA. After 24h, frozen spleen sections from these mice were stained with B220 (blue), CD11c (green) and mPDCA-1 (red) or isotype control antibody. Data from spleen sections are from 1 representative experiment of 3 performed. b.) Average intensity value of mPDCA-1⁺ pixels from spleen sections was obtained and the data represent numbers from 5 distinct fields from three combined independent experiments. In the B6 mice the SEA group was calculated to be at least $P < 0.003$ in comparison to naïve, but no statistical difference was observed in the same comparison in the IFN γ ^{-/-} mice