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FcγRIIB Prevents Inflammatory Type I IFN Production from Plasmacytoid Dendritic Cells during a Viral Memory Response

Marcella Flores,†,‡ Claude Chew,†,‡ Kevin Tyan,†,‡ Wu Qing Huang,†,‡ Aliasher Salem,§ and Raphael Clynes†,‡

The type I IFN (IFN-α) response is crucial for viral clearance during primary viral infections. Plasmacytoid dendritic cells (pDCs) are important early responders during systemic viral infections and, in some cases, are the sole producers of IFN-α. However, their role in IFN-α production during memory responses is unclear. We found that IFN-α production is absent during a murine viral memory response, despite colocalization of virus and pDCs to the splenic marginal zone. The absence of IFN was dependent on circulating Ab and was reversed by the transgenic expression of the activating human FcγRIIA receptor on pDCs. Furthermore, FcγRIIB was required for Sendai virus immune complex uptake by splenic pDCs in vitro, and internalization via FcγRIIB prevented cargo from accessing TLR signaling endosomes. Thus, pDCs bind viral immune complexes via FcγRIIB and prevent IFN-α production in vivo during viral memory responses. This Ab-dependent IFN-α regulation may be an important mechanism by which the potentially deleterious effects of IFN-α are prevented during a secondary infection.

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The online version of this article contains supplemental material.

Abbreviations used in this article: BM, bone marrow; cDC, conventional DC; h, human; IC, immune complex; KO, knockout; pDC, plasmacytoid dendritic cell; ROI, region of interest; SeV, Sendai virus; WT, wild-type.

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Materials and Methods

Mice

C57BL/6 mice were purchased from The Jackson Laboratory (Bar Harbor, ME). FcyRIVb (FcγRIVb−/−), FcerIg (FcγRIVb−/−), and double-KO FcγRIVb (FcγRIVb−/−) mice, referred to in this article as FcγR-null mice, were purchased from Taconic Farms (Germantown, NY). Human (h)FcγRIIA transgenic mice were generated as described (21) and obtained from S. McKenzie (Department of Medicine, Cardeza Foundation for Hematological Research, Thomas Jefferson University). YFP-IFNβ mice were obtained from B. Reizis (Department of Microbiology and Immunology, Columbia University) with permission from R. Locksley (University of California, San Francisco). YFP-IFNβ hFcγRIIA mice were generated in the animal facility in the Institute of Comparative Medicine at the Columbia University Medical Center. All animal experiments were performed in compliance with institutional guidelines and approved by Columbia University's Institutional Animal Care and Use Committee.

FLT3L bone marrow cultures

Bone marrow (BM) cells (2 × 10⁶ cells/ml) were cultured for 7–9 d in RPMI 1640/10% FCS supplemented with 100 ng/ml murine Flt3 ligand (R&D Systems, Minneapolis, MN). It typically consisted of 80% conventional DCs (cDCs) and 20% pDCs.

Virus

SeV-Cantell strain was purchased from Charles River Labs and obtained from Vincent Racaniello (Columbia University).

Murine immune sera and immune complex formation

SeV antisera. Mice were immunized with either live or heat-killed SeV-Cantell by tail vein injection. Four to six weeks postinjection, mice were boosted, and sera were collected from mice 1 wk postboost. The blood of five C57BL/6 mice was pooled, and sera were frozen in aliquots.

For intracellular immune complex (IC)-trafficking studies, IgG was purified with protein A/G columns (Thermo Scientific, Rockford, IL). An ELISA was developed in-house to determine the presence of mouse anti-SeV IgG using SeV-coated plates.

For in vitro functional assays, SeV ICs were made using 5 μl live virus stock incubated with 5 μl antisera and 0.5 ml RPMI 1640 for ≥15 min in a 37°C water bath. SeV ICs or SeV alone was incubated overnight with purified BM pDCs, and IFN-α was read by ELISA.

For in vivo experiments, 20 μl anti-SeV immune sera was injected i.v. before, or after, or concurrently with 50 μl virus (i.v.), both into the tail vein.

OVA was purchased from Worthington Biochemical Industries (Lakewood, NJ). CpGB–OVA fusion molecules were kindly provided by Susanne Bolte (Sorbonne Université, Paris, France).

Immunofluorescence and microscopy

Detecting SeV in the spleen. Splenocytes were harvested at various time points postinjection and analyzed as single-cell suspensions or placed into OCT compound and frozen at −80°C prior to sectioning (6-μm continuous sections) and staining as indicated. Thawed tissue sections were stained with the indicated Abs prior to fixing with 4% paraformaldehyde. SeV was stained using chicken anti-SeV Ab, followed by an anti-chicken secondary Ab, as described above.

Intracellular IC trafficking. Splenic pDCs were isolated from spleens by depleting CD19⁺ cells and then selecting for BST2⁺ cells using MicroBeads (Miltenyi Biotec, San Diego, CA). Images were acquired by confocal microscopy using a Nikon A1 confocal microscope with a 100X objective and analyzed by ImageJ. Integrated density above a certain threshold of Lamp1, LC3, and LysoTracker was measured at specific regions of interest (ROIs). Measurements at random ROIs were taken on images pivoted 90°, as described (23). Manders coefficients were determined using the ImageJ plugin IACOP (Just Another Colocalization Plugin) developed by Fabrice P. Cordelieres (Institut Curie, Paris, France) and Susanne Bolte (Sorbonne Université, Paris, France).

Statistical analysis

Differences between two groups were evaluated using the Student t test. ANOVA was used to compare three or more groups with the Tukey multiple-comparison posttest.

Results

IFN-α production is absent during secondary exposure to SeV

Type I IFN’s potent innate antiviral properties also have potentially deleterious local and systemic toxic effects that could cause unnecessary cellular activation and immunopathology if left unbridled during resolving or secondary infections. This suggests that a host would benefit by restricting IFN production to a first line of defense (i.e., before an effective adaptive immune response develops).

To investigate whether IFN-α is produced during a memory response to virus delivered i.v., a cohort of mice was immunized with SeV and then rechallenged 14–21 d later (memory response). A similar cohort of mice received only a single viral immunization (primary response). Both groups of mice were analyzed for circulating IFN-α levels in the sera over the next 2 d. During a primary challenge, IFN-α was detected in the sera as early as 5 h postinjection and began to wane by 24 h. In contrast, the SeV memory response failed to produce any detectable IFN throughout the 48-h period (Fig. 1A).

Given the importance of pDCs in the production of type I IFN during certain viral infections, we sought to determine whether pDCs were involved in IFN-α production during a naive response to SeV. We used a YFP-IFNβ reporter mouse to indirectly measure IFN-β production. YFP-IFNβ reporter mice were injected via the tail vein with live SeV, and spleens were harvested 3 h post-injection. Beyond 3 h, our ability to accurately gate on pDCs was compromised as the result of IFN-dependent induction of the pDC lineage marker BST2 on other cells (24). In this analysis of splenocyte populations, we found that, in a naive setting, IFN production was limited to pDCs; cDCs (CD11c⁺ BST2⁻), macrophages (CD11b⁺ CD11c⁻), B cells (CD19⁺), and cells negative for all markers (the vast majority of which are T cells) did not produce detectable YFP in response to SeV (Fig. 1B). In contrast, by backgating on YFP⁺ cells, CD11c⁺ BST2⁺ pDCs were identified as the sole producers of IFN-β during a naive viral challenge at 3 and 4 h (Fig. 1C).

The induction of other inflammatory cytokines by SeV also was differentially regulated between a primary and memory response. In particular, when measured in the sera 3 h after exposure, IL-6, like IFN-α, was limited to the primary response, whereas MCP1 and IFN-γ were significantly induced in both the primary and memory responses (Fig. 1D).

A lack of type I IFN during a viral memory challenge might be explained by an efficient and rapid clearance of virus before it is detected by pDCs. Yet Western blot analysis of splenocytes shows
that the presence of SeV is remarkably similar between a primary and memory response. Although about half of the initial inoculum is cleared in a memory versus a primary setting, the kinetics of the virus are similar in that they peak at 1 h and persist through 48 h compared with no treatment (Fig. 1E).

The failure of pDCs to respond to SeV during a memory response also may be explained by a lack of access to the virus. We and other investigators found pDCs in the red and white pulp in spleens of naive mice. In the white pulp, pDCs exist largely at the marginal zone and within the T cell area. More detailed analysis of pDCs at

FIGURE 1. IFN-α production is absent during secondary exposure to SeV. (A) WT mice, previously immunized with SeV, were rechallenged with SeV (memory) and compared with mice receiving a single injection (naive). IFN-α in the sera was measured at the indicated time points by ELISA. Repeated at least four times with a total of n = 12 mice in each group. (B) YFP-IFNb mice were injected as in (A); at 3 h post-SeV challenge, spleens were analyzed by FACS for YFP+ cells in pDCs (CD11cmid BST2+), cDCs (CD11c+ CD11b+), and B cells (CD19+ CD11b-). Each symbol (square, triangle, or circle) is a mouse obtained from pooled experiments. The experiments were repeated five times, with seven to nine mice total/group. (C) FACS analysis of YFP+ versus un gated cells. Representative sample from (B). (D) Cytokine bead assay analysis of sera 5 h post-SeV challenge, as in (A). The experiment was repeated twice with four to six mice in each group. (E) Western blot analysis of total splenocytes of primary and memory mice, as in (A). Representative of one mouse, as in (A). *p < 0.05, **p < 0.01, ***p < 0.001, ns, not significant.
the marginal zone showed that pDCs were interweaved between Marco+ and Moma-1+ cells, clearly delineating the zone by forming short chains of pDCs (Fig. 2).

To address spatiotemporal questions and pDC accessibility to injected virus during a memory response, previously immunized mice were rechallenged with SeV, and cross-sections of spleens were stained at various time points. We found that, during a memory response, SeV was largely present at the marginal zone associated equally between Marco+, Moma-1+ macrophages and marginal zone B cells at 20 and 35 min, moving eventually to follicular B cells at 50 and 75 min (Fig. 2A, 2C, left panel). En route to follicular B cells, SeV colocalized with pDCs at the marginal zone between 20 and 50 min. Surprisingly, this analysis indicated that only ~10% of SeV is present on pDCs during a memory response.

We wondered how this compared with a primary response where the interaction between pDCs and SeV is known to result in a strong IFN-α response. SeV was injected as above into the tail veins of naive mice, and spleens were harvested at the indicated time points. Interestingly, we found that, at early time points (20 min), SeV in a primary setting followed nearly the same route as in a memory setting, colocalizing with Marco+ and Moma-1+ cells. However, SeV trafficking differences between the memory and primary responses became evident by 40 min and more apparent by 75 min. SeV traveled further into the B cell area than in a memory setting and did not persist at the marginal zone (Fig. 2B, 2C, right panel). Interestingly, the early access to SeV by pDCs was nearly equivalent between the memory and primary settings (Fig. 2D).

Taken together, these data suggest that, unlike in a primary response, the interaction between SeV and pDCs during a memory response at the splenic marginal zone fails to induce an IFN response.

**Virus-specific Ab is required for IFN suppression during a memory response**

We found that, at 14–21 d postimmunization, mouse sera contained SeV-specific Ab, as determined by hemagglutination inhibition and an SeV-specific ELISA (data not shown). To investigate whether Ab was necessary for IFN suppression during a memory response, we repeated the primary and memory viral challenge using mice unable to produce virus-specific Ab. HELμMT mice express a transgenic BCR specific for hen egg lysozyme on an IgM-deficient background. Interestingly, and in complete contrast to WT mice, HELμMT mice did not suppress the IFN-α response during a memory rechallenge (Fig. 3A).

To more directly determine whether virus-specific Ab was required for IFN-α suppression, a cohort of naive mice was passively immunized by transfer of immune sera and then challenged with SeV as before; IFN-α was measured in the serum as described earlier. We found that immune sera was sufficient to prevent any type I IFN production in response to SeV (Fig. 3B) and flu (Supplemental Fig. 1). Lastly, colocalization of SeV and pDCs in the spleens of mice 30 min following injection of preformed SeV ICs suggests again that the inhibition of IFN by pDCs is not due to a lack of access by pDCs (Fig. 3C). Combined, these results suggest that inhibition of IFN production by pDCs during a viral memory response requires the presence of virus-specific Ab.

**Transgenic expression of hFcγRIIA on pDCs permits IFN-α production during a memory response**

We previously described the dominant expression of FcγRIIB on murine pDCs and that the limited expression of FcγRs prevented Ag presentation of OVA ICs to T cells (25). We designed a series of experiments to directly examine a role for FcγRIIB in the inhibition of IFN-α through the use of the KO mouse. Although we show that, in both a memory response and by directly injecting SeV ICs, there is an increase in IFN-α in the sera in the absence of FcγRIIB, we could not determine whether pDCs were responsible for this effect because the FcγRIIB-KO mouse is not pDC specific (Supplemental Fig. 2A, 2B). In vitro experiments coculturing WT and FcγRIIB-KO pDCs with SeV ICs showed that both WT and FcγRIIB-KO pDCs fail to produce IFN-α; however, the latter is due to the inability of FcγRIIB-KO pDCs to acquire ICs (Supplemental Fig. 2C, 2D).

Being unable to determine the direct requirement for FcγRIIB in vivo, we determined whether expressing the activating FcγRIIA would reverse the IFN suppression seen in WT responses. Transgenic mice expressing the hFcγRIIA receptor, an activating Fc receptor present only in primates, were compared with WT mice in a memory SeV response. Analysis of the pDCs from these transgenic mice showed the transgene expressed on pDCs (25). In contrast to WT mice, FcγRIIA-transgenic mice responded with limited, although significant, IFN-α concentrations in the sera (Fig. 4A). To determine whether pDCs were responsible for producing the IFN-α measured in sera, YFP-IFNβ mice were crossed to FcγRIIA-transgenic mice, and the memory experiment was repeated. Importantly, we found that, endowed with the presence of an activating FcγR, pDCs are capable of inducing an IFN response in a memory setting and, again, were the only cell involved in the response at 3 h (Fig. 4B). Furthermore, direct injection of SeV ICs into hFcγRIIA-transgenic mice resulted in IFN-α production, contrasting the suppressed response in WT mice (Fig. 4C).

To directly examine the interaction between SeV ICs and pDCs, we turned to an in vitro system. BM-expanded and isolated pDCs were enriched and incubated with SeV ICs overnight and sampled for IFN-α production. Although both WT and hFcγRIIA pDCs responded equally to virus alone (Fig. 4D), only the transgenic, activating FcγR bearing pDCs produced IFN in response to viral ICs (Fig. 4E). This was not the result of an increased IC binding potential by the transgenic pDCs over the WT, because both bound SeV ICs equally (Fig. 4F, 4G).

Thus, taken together, the data suggest that the prevention of an IFN-α response to SeV ICs is not because neutralizing Ab renders the complex inert; rather, the dominant expression of FcγRIIB on murine splenic pDCs prevents IFN-α production during viral memory responses.

**Splenic pDC IC binding occurs via FcγRIIB and prevents trafficking to TLR9 signaling compartments**

SeV ICs did not induce IRF-7 translocation to the nuclei in WT pDCs (data not shown), suggesting that the block to IFN-α production occurred upstream, potentially by derailing trafficking of the IC cargo to the canonical TLR signaling endosome. We reported previously that murine pDCs bind and internalize OVA ICs via FcγRIIB exclusively (25) and fail to reach the proteolytic endosome required for Ag processing. Therefore, we similarly assessed the intracellular trafficking of OVA ICs and TLR ligand–containing ICs (OVA-CpG ICs), which may interact directly with endosomal TLRs in pDCs and be internalized via an FcγRIIB-independent manner.

First, to assess the FcγR dependence of OVA-CpG IC binding, we compared the IC binding potential of WT, FcγRIIB-deficient, and FcγR-null splenic pDCs by confocal microscopy. We found that, in the absence of FcγRIIB, OVA IC binding was significantly reduced on splenic pDCs and equaled that of the FcγR-null pDCs (Fig. 5A, 5B) confirming previous experiments (Supplemental Fig. 2D). The addition of a TLR9 moiety (CpG B) on the Ag did not alter the FcγRIIB dependence (Fig. 5B, right panel), strongly suggesting that WT pDCs exclusively bind ICs through the inhibitory FcγRIIB.
FIGURE 2. Splenic pDCs interact with SeV at the marginal zone. Memory (A) and primary (B) mice were compared for SeV (green) colocalization at the indicated time points with various cellular populations (red). Sendai virus was detected using chicken anti-SeV followed by anti-chicken Alexa 488 or Alexa 594. Cellular subsets were detected by using anti–moma-1 biotin with streptavidin Alexa 488, anti-Marco PE, anti-BST2 Alexa 488, and CD45R Alexa 647. All images are pseudocolored with SeV in green and cell subset in red. Original magnification $\times 40$. One representative experiment from two experiments is shown. (C and D) Quantification of (A) and (B), respectively, for each cell population. Between 5 and 10 sections were quantified for each time point and cell population. One representative experiment from two experiments is shown with similar patterns. (D) Expansion of the white box in (A) (left panel). Quantification of 20 and 35 min (right panel). SeV was detected using chicken anti-SeV followed by anti-chicken Alexa 594 and anti-BST2 Alexa 488. Original magnification $\times 86.6$ was achieved with an optical zoom. n.s., not significant.
We next sought to examine the intracellular trafficking pathways that may prevent viral ICs from reaching a TLR signaling compartment in pDCs. OVA ICs and OVA-CpG ICs were incubated with splenic pDCs, and colocalization with various intracellular markers was assessed by immunofluorescence and confocal microscopy.

IFN-α production by TLR9 ligands requires engagement of IRF7 in Lamp2α, LysoTracker+ endosomes (26). We examined the trafficking of ICs in splenic pDCs and found that OVA ICs incubated with WT splenic pDCs were not colocalized with LysoTracker, as was expected from previous work (25). However, the transgenic expression of activating FcγRIIA significantly increased colocalization of ICs and LysoTracker (Fig. 5C, 5D), and the results were confirmed with studies using Lamp1 (Fig. 5E, 5F).

Interestingly, when CpG was present in the cargo, WT pDCs delivered more ICs to LysoTracker. Furthermore, the FcγRIIA-dependent increase in the colocalization of ICs to acidic compartments was supported by Lamp1 (Fig. 5E, 5F). Thus, FcγRIIB-mediated internalization of ICs access a spatiotemporal endosomal pathway distinct from activating FcγRs that inefficiently access the acidic LAMP1α, IRF7+ IFN-α signalosome, thus preventing IFN-α production.

Recently published work identified a noncanonical IFNogenic pathway involving the LC3-associated phagosome. In these studies using BM-derived murine pDCs, the investigators found that Ab-coated beads were internalized via LC3, an autophagy-related protein, and that the recruitment of LC3 was Fcγ-chain dependent (27); both were functionally required for IFN-α production. We performed a similar experiment using splenic pDCs and soluble ICs. Recruitment of LC3 was measured by calculating the integrated density of LC3 fluorescence at each IC particle (ROI). We controlled for background fluorescence by measuring the same parameters and ROIs on images pivoted 90°. Our experiments yielded results consistent with an LC3-mediated internalization through activating FcγRs (Fig. 5G, 5H). However, we found that FcγRIIB did not recruit LC3 above background when using either WT or γ-chain–deficient pDCs. Indeed, only when an activating FcγR was transgenically expressed did we detect LC3 recruitment on splenic pDCs. Furthermore, the presence of a TLR moiety coupled to OVA did not change LC3 recruitment in the WT pDCs, supporting the conclusions of Henault et al. (27) that LC3 recruitment is initiated by the IgG component of the IC and not by its cargo. Our results show that ICs internalized through the inhibitory FcγRIIB do not lead to LC3 recruitment.

Taken together, our data demonstrate that FcγRIIB on splenic murine pDCs is required to internalize ICs and that this internalization occurs outside of LC3α compartments and results in IC trafficking away from the LysoTracker+ endolysosomes where IRF7 and TLR9 signal. In contrast, ICs internalized through activating FcγRs access an LC3-mediated internalization pathway that leads to LAMP1+, LysoTracker+ endolysosomes, enabling MyD88 signaling and IRF7 nuclear translocation. In human pDCs, in which both activating and inhibitory FcγRs are coexpressed, as modeled in this study, the amplitude of the IFN-α signal would be balanced by the relative functional expression of these opposing receptors. This situation may be relevant to primates, including humans, in which the activating FcγRIIA is dominant. In mice, the exclusive expression of FcγRIIB should prevent untoward IFN-α production during recurrent or chronic exposure to a virus, in which the presence of adequate functional viral-specific neutralizing Abs enable benign clearance and limited viral replication and systemic spread.

Discussion
In the current study, we described one mechanism by which the innate IFN-α response is averted upon secondary exposure to virus. We determined that the presence of circulating virus-specific proteins.
FIGURE 4. IFN production in response to ICs is rescued by transgenic expression of FcγRIIA. (A) IFN-α concentrations in the sera of WT and hFcγRIIA-transgenic mice were compared during a memory response, using ELISA, at the indicated time points. Pooled experiments of 6–12 mice/group are shown. (B) Dual transgenic FcγRIIA+ YFP-IFNβ reporter mice were compared with WT YFP-IFNβ reporter mice at 3 h postrechallenge. The percentage of YFP+ pDCs (CD11c+ BST2+) was determined by FACS (n = 4–8 mice/group). (C) WT or hFcγRIIA-transgenic mice were injected with preformed SeV ICs. Sera were analyzed for IFN-α, using ELISA, at the indicated time points. Pooled experiments of at least six mice are shown. (D) BM-expanded WT or hFcγRIIA-expressing pDCs were incubated overnight with SeV, and the supernatant was analyzed for IFN-α. Repeated twice in duplicate and triplicate. (E) As in (D), except that enriched BM pDCs were incubated with SeV ICs. (F) Splenic pDCs from WT or hFcγRIIA mice were incubated with preformed SeV ICs for 30 min and analyzed by confocal microscopy. Sendai virus was detected using chicken anti-SeV followed by anti-chicken Alexa 594. B220 was detected with anti-B220 488. Image is pseudocolored with red for SeV, DAPI in blue, and B220 in white. (Figure legend continues)
Ab and the dominance of the inhibitory FcγR on pDCs are required for preventing the potentially toxic side effects of systemic IFN. Thus, the humoral immune memory response functions to temper the innate response upon re-exposure to virus.

Our work also describes a molecular pathway that differentiates activating and inhibitory FcγRs in their ability to traffic ICs for TLR sensing. We took several approaches, most of which were technically challenging, including using TLR9 Abs to demonstrate

FIGURE 5. Splenic pDCs internalize ICs via FcγRIIB and traffic particles to early endosomes. (A) A representative example of splenic pDCs binding OVA ICs. (B) Indicated ICs were incubated with splenic pDCs for 30 min and analyzed for IC binding by confocal microscopy. OVA IC and OVA CpG IC graphs were generated from at least two experiments. Each symbol represents the average number of IC particles/cell/image. (C) Representative example of WT and hFcγRIIA-transgenic splenic pDCs incubated with OVA ICs and stained for LysoTracker (green) and anti-rabbit OVA ICs (red). (D) Quantification of experiments provided in (C), showing integrated density of LysoTracker at IC+ ROI. Three pooled experiments are shown, with >150 IC particles analyzed. (E) Representative example of WT or hFcγRIIA-transgenic splenic pDCs incubated with OVA ICs and stained for LAMP1 (green) and DAPI (blue). (F) Quantification of experiments as in (E) for integrated density of LAMP1 at IC+ ROI. Two pooled experiments are shown. (G) Representative example of WT and hFcγRIIA-transgenic splenic pDCs incubated with OVA ICs and stained for LC3 (green) and anti-rabbit OVA ICs (red). (A), (C), (E), and (G), original magnification ×100. (H) Quantification of experiments as in (G) for integrated density of LC3 at IC+ ROI. Two pooled experiments are shown. *p < 0.05, **p < 0.01, ***p < 0.001. n.s., not significant.

Original magnification ×100. (G) Quantification of images as in (F) were analyzed for the average number of SeV particles/cell/image. Pooled data from two independent experiments, with >50 cells analyzed per group. *p < 0.05, **p < 0.01, ***p < 0.001. n.s., not significant.
that OVA-CpG ICs internalized by WT pDCs failed to colocalize with TLR9, whereas hFcγRIIA-expressing pDCs showed remarkable colocalization (data not shown). However, staining with TLR9-specific Abs was too weak for rigorous quantitative microscopy. In a second approach, we reasoned that pulsed pDCs with CpG-FITC (InvivoGen, San Diego CA) would delineate a pathway known to engage TLR9 and lead to IFN-α production. However, these experiments were also characterized by unreliable staining. Thus, we took advantage of well-described studies that reveal the requirement of acidification for TLR9 activation and IRF7 recruitment (26, 28) and capitalized on the highly reproducible staining that we achieved with LysoTracker, which reliably stains the acidic lysosomal organelles. Using this approach, we found that FcγRIIB on splenic pDCs was required for binding viral ICs and that subsequent internalization failed to deliver ICs to LysoTracker+ compartments. These results were confirmed by similar findings with Lamp1 and Lamp2. In contrast, the transgenic expression of an activating FcγR was sufficient to restore IFN-α production and delivered ICs to LysoTracker+ Lamp1 and Lamp2+ endolysosomes.

A recent study also described a noncanonical, autophagy-related pathway that is alternatively used by activating FcγRs for IFN-α induction by DNA-containing ICs (27). This pathway recruits LC3 to the IC in an IgG-dependent manner. Fig. 5 shows that LC3 was not recruited by WT splenic pDCs or by γ-chain–KO pDCs, but only when the activating FcγR was transgenically expressed. Taken together, these studies suggest that activating and inhibitory FcγRs are discriminating pathways that modulate TLR sensing by directing ICs either toward or away from TLR-sensing compartments. Furthermore, pDCs, by dominantly expressing the inhibitory FcγR, are prone to suppress the IFN-α response to ICs.

Our work supports the findings of Henault et al. (27) that LC3 is used by activating FcγRs to deliver cargo to TLR-sensing compartments. However, they showed that BM-expanded WT pDCs do recruit LC3 to internalized ICs in a γ-chain–dependent manner. Our studies consistently identified a lack of activating FcγRs on murine splenic pDCs. This apparent conflict could be due to the source of pDCs and contaminating cDCs. We found that expanding BM with FLT3L to obtain pDCs inconsistently altered FcγR expression compared with splenic pDCs. B220 and CD11b, markers used in the literature to isolate pDCs and cDCs from FLT3L cultures, were often not mutually exclusive or were suspected of containing a large percentage of cDC precursors, as has been reported (29). By using primary splenic pDCs to investigate the FcγRIIB-dependent requirement for binding, internalizing, and intracellular trafficking of ICs, we circumvented the problems that might arise by exposing dendritic cells to FCS and an inflammatory cytokine, such as FLT3L.

Our major finding that IFN-α production by splenic pDCs was prevented during a memory response might have been explained by a lack of access to the virus during transport through the spleen in a memory setting. However, we found that, in both primary and memory responses, pDCs had comparable access to the virus. Indeed, it may be possible that the same molecular mechanisms are directing traffic in both settings (i.e., complement by acting with natural IgG and IgM Ab in the primary response and with virus-specific IgG Ab in the memory) (30–33), which ultimately delivers virus to follicular B cells for trafficking and presentation by follicular dendritic cells.

Other mechanisms that may have contributed to the prevention of an IFN response during a memory setting may include pDC "exhaustion" due to low-level viral exposure (34). We found by Western blot that SeV did not persist in the spleens of mice by day 14 (Fig. 1E), and in naive mice we found that weight was recovered by day 7 post-SeV injection, suggesting that they had cleared the virus by days 14 and 21 (data not shown). However, during the course of our studies, we found that there was a tendency for previously SeV-immunized mice to respond less robustly to unrelated stimuli (not statistically significant, data not shown). This tendency may have contributed to the absence of a response to SeV in memory mice. However, we also found that passive immunization with antisera was sufficient to suppress the IFN response in naive mice (Fig. 3B) and that the transgenic expression of activating FcγRs could reverse the IFN suppression in a memory setting with presumably equally exhausted mice (Fig. 4).

Our current study highlights the dominance of FcγRIIB on pDCs as a mechanism to prevent IFN-α production by TLR-bearing ICs. Systemic infections elicit IFN-α from pDCs and provide the host with a protective, potent, and immediate source of IFN-α. In the setting of a well-orchestrated adaptive memory response, this innate production of IFN-α is mitigated by circulating virus-specific Abs and through inhibitory FcγRs on pDCs. If pDCs express an FcγR repertoire equal to that of their cDC counterparts, secondary infections would lead to unnecessary IFN-α production and elicit an exacerbated immune response that could lead to untoward consequences of injury and/or autoimmunity.

Alternatively, a study by Honke et al. (33) highlights another possible mechanism for halting IFN-α production in a memory setting. They found that IFN-α resistance by MOMA-1+ macrophages during primary viral responses is required for the mounting of a protective adaptive immune response. By overexpressing Usp18, an inhibitor of IFNAR signaling, MOMA-1+ macrophages at the marginal zone capture virus and allow viral replication for presentation to T and B cells. Thus, during memory responses, a synergistic action to improve upon the memory response could be aided by viral IC presentation and the viral replication allowed by the absence of IFN.

The FcγRIIB-specific means of controlling pDC-derived IFN-α also may be important during chronic infections when circulating viral ICs may be present. In a classic model of chronic lymphocytic choriomeningitis virus infection, Zuniga et al. (34) found that pDCs were prevented from making IFN in response to CpG 30 d postinfection with lymphocytic choriomeningitis virus. This time point correlates with the presence of neutralizing Abs, as gauged by Berghaler et al. (35). It is possible then that, in this model of chronic infection, IFN-α production is suppressed as a result of circulating viral ICs being sensed through FcγRIIB on pDCs.

Human pDCs were shown to dominantly express the activating receptor FcγRIIA (36). Nevertheless, evidence that human pDCs, like mice, exhibit an Fc-dependent inhibition of IFN-α production was described previously (37, 38), and it may have important implications for vaccine development. In one prominent example, a Merck-designed adenovirus-based HIV vaccine (Ad5/HIV) showed an increase in HIV susceptibility in patients with pre-existing Abs to adenovirus (39). A separate study, using a systems analysis approach, identified the production of type I IFN as the most significant difference between those with and without pre-existing Abs to adenovirus (40). In another example, the whole inactivated influenza vaccine elicits a strong IFN-α production and a potent adaptive immune response (41), whereas the split vaccine mediates immunity through Fc receptors and prevents an IFN-α response (41, 42). Not surprisingly, the latter is far less immunogenic, and children without pre-existing flu Abs require two vaccinations for efficacy. These studies point to the perils involved in mounting an adaptive immune response in the absence of an innate immune response. In the case of the Ad5/HIV
clinical trial, the lack of IFN during immune priming could have resulted in the production of nonneutralizing Abs, through a lack of optimal B cell activation, and subsequent Ab-enhanced replication of HIV (43).

In our unpublished results and in published results (37, 44), we found that both humans and mice have mechanisms to prevent IFN-α production from pDCs when exposed to ICs. In humans, this mechanism is mediated by monocytes responding to ICs (44, 45). In the mouse, we described a mechanism mediated through FcγRIIB directly on pDCs. Understanding whether inhibition in the human is similarly controlled by the inhibitory FcγR on monocytes is difficult without a commercially available Ab specifically recognizing FcγRIIB. However, future work should include devising vaccine strategies including adjuvants that block the human is similarly controlled by the inhibitory FcγR on monocytes.

References


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Disclosures

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