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*J Immunol* 2008; 181:1633-1643; doi: 10.4049/jimmunol.181.3.1633
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Type I IFN-Induced, NKT Cell-Mediated Negative Control of CD8 T Cell Priming by Dendritic Cells

Petra Bochtler,* Andrea Kröger,† Reinhold Schirmbeck,* and Jörg Reimann2*

We investigated the negative effect of type I IFN (IFN-I) on the priming of specific CD8 T cell immunity. Priming of murine CD8 T cells is down-modulated if Ag is codelivered with IFN-I-inducing polyinosinic-polycytidylic acid (pI/C) that induces (NK cell- and T/B cell-independent) acute changes in the composition and surface phenotype of dendritic cells (DC). In wild-type but not IFN-I receptor-deficient mice, pI/C reduces the plasmacytoid DC but expands the CD8+ conventional DC (cDC) population and up-regulates surface expression of activation-associated (CD69, BST2), MHC (class I/II), costimulator (CD40, CD80/CD86), and coinhibitor (PD-L1/L2) molecules by cDC. Naive T cells are efficiently primed in vitro by IFN-I-stimulated CD8 cDC (the key APC involved in CD8 T cell priming) although these DC produced less IL-12 p40 and IL-6. pI/C (IFN-I)-mediated down modulation of CD8 T cell priming in vivo was not observed in NKT cell-deficient CD1d<sup>-/-</sup> mice. CD8 cDC from pI/C-treated mice inefficiently stimulated IFN-γ, IL-4, and IL-2 responses of NKT cells. In vitro, CD8 cDC that had activated NKT cells in the presence of IFN-I primed CD8 T cells that produced less IFN-γ but more IL-10. The described immunosuppressive effect of IFN-I thus involves an NKT cell-mediated change in the phenotype of CD8 cDC that favors priming of IL-10-producing CD8 T cells. In the presence of IFN-I, NKT cells hence impair the competence of CD8 cDC to prime proinflammatory CD8 T cell responses. The Journal of Immunology, 2008, 181: 1633–1643.

Dendritic cells (DC)<sup>3</sup> are rare cells of hemopoietic origin with a short half life that play key roles in the induction and regulation of specific T cell immunity (1, 2). Under steady-state conditions, DC are heterogeneous arising from different developmental lineages and expressing different states of maturation (2). DC represent <1% of the spleen cell population (3 × 10<sup>9</sup> DC per spleen). Conventional DC (cDC) dominate the splenic DC population. In contrast to lymph node, input from blood-derived, migratory DC into the splenic cDC population seems limited (2, 3) although this was recently questioned (4). Splenic cDC seem to be generated at a rapid rate from endogenous precursor cell pools along three sublineages, i.e., the dominant CD11c<sup>+</sup> CD4<sup>+</sup> CD8<sup>-</sup> CD11b<sup>+</sup> DEC205low 33D1<sup>+</sup> cDC (CD4 cDC) lineage, the CD11c<sup>-</sup> CD8<sup>+</sup> CD11b<sup>-</sup> DEC205<sup>high</sup> 33D1<sup>-</sup> cDC (CD8 cDC) lineage, and the double-negative (DN) CD4<sup>+</sup> CD8<sup>-</sup> CD11c<sup>+</sup> CD11b<sup>+</sup> cDC (DN cDC) lineage (2, 5). CD8 cDC are the main candidate APC for (cross)priming CD8 T cells (5). It is assumed that cDC development and activation in the spleen is an informative model for the study of lymphoid tissue DC. Plasmacytoid DC (pDC) are a unique subset of immature APC that secrete type I IFNs (IFN-I) upon infection with viruses (6). cDC and pDC are considered products of different differentiation pathways because they express different morphologies, recirculation patterns, surface markers, transcription factors, and cytokine profiles. pDC populations are heterogeneous in phenotype and number in different mouse strains (7–9) and may differentiate into cDC during virus infection (10). The mAbs 120G8 (8), 440c (11), and mPDCA-1 (12) have been extensively used to identify and selectively deplete murine pDC in vivo. The mAb 440c-defined marker was recently identified as Siglec-H (13) and the mAbs 120G8 and mPDCA-1 bind bone marrow stroma cell Ag-2* (BST2) (14).

IFN-I is spontaneously produced at low levels under steady-state conditions (15) and binds the heterodimeric IFN-I receptor (IFNAR) expressed by essentially all cells (16). Splenic DC (subset) development is largely independent of IFN-I as normal numbers of cDC and pDC are present in IFNAR-deficient mice (shown in this article). Virus infection or TLR3/mda-5 ligands trigger rapid IFN-I release that induces pleiotropic stimulation of the innate and specific immune system (17, 18). Positive and negative effects of IFN-I on CD8 T cell activation and development have been identified. IFN-I can directly drive activation and clonal expansion of effector/memory or bystander CD8 T cells (19–23) but can also negatively regulate the generation or maintenance of specific CD8 T cell immunity (24–30). Studying vaccine-induced, virus-specific CD8 T cell responses in IFNAR-deficient mice, we reported that IFN-I nonresponsiveness facilitates priming of CD8 T cell responses (31). We show in this study that CD8 T cell priming is down modulated in wild-type (wt) but not IFNAR<sup>-/-</sup> mice treated with the IFN-I-inducing agent polyinosinic:polycytidylic acid (pI/C). We assume in this report that a pI/C-induced effect observed in wt but not IFNAR<sup>-/-</sup> mice is IFN-I mediated.

T cell priming is DC dependent. We hence first analyzed the response of murine DC (subsets) to IFN-I. IFN-I accelerates (32) or inhibits (33) the generation of cDC from human CD14<sup>+</sup> blood precursors. IFN-I promotes the activation of DC (34, 35) but limits

<sup>*Department of Internal Medicine I, University of Ulm, Ulm, Germany; and †Helmholtz Centre of Infection Research, Braunschweig, Germany</sup>

Received for publication November 20, 2007. Accepted for publication May 8, 2008.

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1 This work was supported by Grant Re549/10-3 from the Deutsche Forschungsgemeinschaft (to J.R.).

2 Address correspondence and reprint requests to Dr. Joerg Reimann, Department of Internal Medicine I, University of Ulm, Albert Einstein Allee 11, Ulm, Germany. E-mail address: joerg.reimann@uni-ulm.de

3 Abbreviations used in this paper: DC, dendritic cell; cDC, conventional DC; DN, CD4<sup>-</sup> CD8<sup>-</sup> double negative; pDC, plasmacytoid DC; IFN-I, type I IFN; IFNAR, IFN-I receptor; wt, wild-type; pI/C, polyinosinic-polycytidylic acid; MCMV, murine cytomegalovirus; w/v, weight to volume ratio; FCM, flow cytometry; αGalCer, α-galactosyl-ceramide; NF, nonfractionated; BST2, bone marrow stroma cell antigen-2.

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their CD40 ligation-triggered IL-12 production (36–38). IFN-I induces expression of IL-15/LIF-15Rα by murine DC thereby enhancing their ability to (cross) prime CD8 T cell responses (39, 40). Naïve T cells and resting NK cells acquire functionality through interaction with IFN-I-stimulated DC (41). IFN-I up-regulates IDO in murine CD19 DCs that trigger potent T cell suppressive functions (42). IFN-I thus generates stimulatory or inhibitory DC. It is unclear whether these stimuli activate alternative signaling pathways in the same DC (subset) and/or target different DC subsets. We analyze in this report the pI/C (IFN-I)-induced, acute changes in the same DC (subset) and/or affect different DC subsets.

IFN-I may mediate its effects on DC either directly or indirectly (through other cells of the immune system). pI/C depletes splenic DC subsets. It is unclear whether these stimuli activate alternative signaling pathways in the same DC and/or affect different DC subsets. We analyze in this report the pI/C (IFN-I)-induced, acute changes in the same DC (subset) and/or target different DC subsets.

Materials and Methods

Mice

WT H-2β C57BL/6J mice (B6) mice and mutant or transgenic RAG1−/−B6 mice, IFN-I receptor 1-deficient (IFNAR−/−) B6 mice (43), CD1d−/−B6 mice, Aα−/−B6 mice, and TCR transgenic OT-I and OT-II B6 mice on the RAG1−/− background were bred and kept under standard pathogen-free conditions in the animal colony of Ulm University. Female and male mice, 8–12 wk of age were used. All animal experiments were performed according to the guidelines of the local Animal Use and Care Committee and the National Animal Welfare Law.

pI/C injection

Mice were injected once i.p. with the double-stranded polyribonucleotide pI/C (cat.no.27-4729-01; Amersham Biosciences) at a dose of 1 mg/mouse. Mice were injected once i.p. with the double-stranded polyribonucleotide pI/C (cat.no.27-4729-01; Amersham Biosciences) at a dose of 1 mg/mouse.

IFN-β treatment and murine cytomegalovirus (MCMV) infection of DC in vitro

DC (104–200 µl well) were cultured either in the presence of 10 IU/ml recombinant, murine IFN-β or after in vitro infection (for 1 h at 37°C) with 3 × 104 PFU MCMV. Supernatants were harvested from these cultures after an 18-h incubation.

Determination of type I IFN levels in serum or supernatant

An antiviral assay (using mouse L929 cells) was used to determine IFN-I concentrations (44). To confirm the specificity of the antiviral activity detected, a neutralizing mAb against mouse IFN-I was added before virus infection of the indicator cells.

Vaccination of mice and detection of specific CD8 T cell frequencies

Mice were immunized i.n. with the hepatitis B surface Ag S encoding pC/DNA vaccine (50 µg per mouse) as described (45, 46). The specific CD8 T cell response to the K1-restricted S190 –197 (VWLSVIWM (S2)) epitope of this Ag was determined at different time points post vaccination. Spleen cells (1 × 107 cell/ml) from immunized mice were incubated for 4 h in UltraCulture medium (cat.no.BE12-725F, Cambrex BioScience) with 2.5 µg/ml S2 peptide (JPT) in the presence of 5 µg/ml brefeldin A (cat.no.15870; Sigma-Aldrich). Cells were surface stained with allophycocyanin-conjugated anti-CD8 mAb (cat.no.555305, BD Biosciences), fixed with 2% paraformaldehyde, permeabilized in PBS containing 0.5% weight to volume ratio (w/v) BSA, 0.5% w/v saponin, and 0.05% w/v NaN3, and stained with FITC-conjugated anti-IFN-γ mAb (cat.no.55441, BD Biosciences). Alternatively, cells were washed twice in FACS buffer (PBS/0.3% w/v BSA; 0.1% w/v NaN3) and incubated for 30 min at 4°C with allophycocyanin-conjugated anti-CD8 mAb and the PE-conjugated K1/S2 tetramer (provided by the National Institute of Allergy and Infectious Diseases Tetramer Facility). Cells were washed twice and the frequency of IFN-γ CD8 or tetramer+ CD8 T cells/106 splenic CD8 T cells was determined by flow cytometry (FCM). Mean numbers of (~SEM) IFN-γ+ or tetramer+ CD8 T cells in the splenic CD8 T cell population are shown.

FCM analysis

Cells were washed twice in FACS buffer, preincubinated with mAb 2.4G2 (cat.no.01241D; BD Biosciences) to block nonspecific Ab binding to Fc-receptors. washed, incubated for 30 min at 4°C with 0.5 µg of the relevant mAb per 106 cells, and washed again (twice). Cells were subsequently incubated for 20 min at 4°C with the second-step reagent Streptavidin-PerCP (cat.no.554064, BD Biosciences). Four-color FCM analyses were performed using the FACS Calibur (BD Biosciences). The forward narrow angle light scatter was used as an additional parameter to facilitate exclusion of dead cells and aggregated cell clumps. Data were analyzed using the FCS Express V3 (DeNovo) software. The following reagents and mAbs from BD Biosciences were used: FITC-conjugated mAb binding B220 (cat.no.553088), PE-conjugated mAb binding K1 (cat.no.553570), CD80 (cat.no.553769), PD-L1 (cat.no.558091), PD-L2 (cat.no.557796), CD4 (cat.no.553730), CD69 (cat.no.553237), CD25 (cat.no.553866), or PD-1 (cat.no.555189); biotinylated mAb binding CD40 (cat.no.553789), I-Ak (cat.no.553550), CD86 (cat.no.553690), ICOSL (cat.no.557788), CD11b (cat.no.553309), CD8α (cat.no.553029), 3D11 (cat.no.552776), CD69 (cat.no.553235), or CD28 (cat.no.553296); and APC-conjugated mAb binding CD11c (cat.no.550261). The PE-conjugated mAb mPDCA-1 (cat.no.130-091-962) was obtained from Miltenyi Biotec. The PE-conjugated mAb-binding TRAIL (cat.no.12-5951) and biotinylated mAb binding DR5 (cat.no.13-5993) were obtained from eBioSciences. The AlexaFlour 488-conjugated mAb 120G8 was a gift from Dr. Paul J. Zavody (Schering-Plough Research Institute, Kenilworth, NJ).

DimerX assay

Four µg davalent mouse CD1d-IgG1 fusion protein DimerX (cat.no.557599, BD Biosciences) were incubated overnight at 37°C and neutral pH with 100 ng α-galactosyl-ceramide (αGalCer) (cat.no.ALX-306-027; Axxora). The αGalCer-loaded CD1d-IgG1 dimers were incubated for 60 min at 20°C with PE-coupled anti-mouse IgG1 (cat.no.550083; BD Biosciences). Mouse NKT cells were labeled with 1 µg αGalCer-loaded dimers per 106 cells for 15 min at 4°C.

Isolation of DC, T, cells, and NKT cells

Spleen cells suspended in RPMI 1640 medium were carefully overlaid on Nycoprep (cat.no.1002380; Axis Shield) and centrifuged at 9,500 × g for 20 min. Cells at the interface were collected and depleted of B, NK, and T cells by MACS using PE-conjugated anti-CD3ε (cat.no.555064, from BD Biosciences) and anti-PE MicroBeads (cat.no.130-048-801), anti-CD19 MicroBeads (cat.no.130-052-201), and anti-NK (DX5) MicroBeads (cat.no.130-052-501). This was followed by positive MACS selection for CD11c+ cells (cat.no.130-052-001; Miltenyi Biotec). PE-conjugated anti-CD20205 (cat.no.CL8915PE) was obtained from Biozol. PE-conjugated mAb-binding TRAIL (cat.no.12-5951) and biotinylated mAb binding DR5 (cat.no.13-5993) were obtained from eBioSciences. The AlexaFlour 488-conjugated mAb 120G8 was a gift from Dr. Paul J. Zavody (Schering-Plough Research Institute, Kenilworth, NJ).

Stimulation of DC in vitro

DC (104–200 µl well) suspended in UltraCulture medium were stimulated with either irradiated CD40L-expressing 3558 transfectants (7 × 105/well) or 10 µg/ml LPS from E. coli (cat.no.L4391, Sigma-Aldrich). Supernatants were collected after 24, 48, and 72 h of incubation.
FIGURE 1. A. pI/C-induced IFN-I response in wt B6 mice. Appearance of IFN-I in the serum of wt B6 mice injected i.p. with 100 μg pI/C. Injected mice were bled at the indicated time points post injection to determine IFN-I serum levels. Mean values of two mice per time point (±SEM) are shown. B. Specific CD8 T cell response elicited by DNA vaccination in pI/C-treated or nontreated, wt, and IFNAR-deficient B6 mice. Mice were injected i.p. once with pI/C (100 μg/mouse) 18 h before i.m. pCI/S DNA vaccination. The specific response of CD8 T cells was determined at the indicated time points post vaccination by tetramer staining. Mean numbers of three mice per group per time point (±SEM) from one representative (of two independent) experiment(s) are shown.

Depletion of NK cells

NK cells were depleted by injecting 30 μl eAsGM1 Ab (cat.no.986-10001, Wako Pure Chemical Industries) on three consecutive days i.p. into mice.

Cocultures of DC, T cells, and NKT cells

DC suspended in 200 μl UltraCulture medium (supplemented with 2 mM L-glutamine, 50 mM 2-ME, and Abs) were pulsed for 2 h at 37°C with different concentrations of the Kb-binding OVA257-264 peptide SIINFEKL (recognized by the transgene-encoded TCR of OT-I mice) (47), the Aβ-binding OVA323-339 Peptide ISQAVHAAHAEINEAGR (recognized by the transgene-encoded TCR of OT-II mice) (48), the Kα-binding S208-215 ILSPFLPL control peptide from HBV (45), or the CD1d-binding glycolipid αGalCer. Cells were washed and cocultured with T and/or NKT cells: 10^4 DC/well were cocultured with 10^5 purified T cells/well or 3×10^4 purified NKT cells/well. For coactivation of NK and CD8 T cells, 0.5×10^5 DC/well were cocultured with 5×10^4 CD8 T cells/well and 2.5×10^4 NKT cells/well. In some experiments, 10^3 IU/ml IFNβ was added to the cultures. Ailuors of supernatants were collected after a 24–96-h incubation for cytokine determination.

Cytokine determination by FCM or ELISA

Cells were stained with PE-conjugated anti-CD8 mAb (cat.no.553033; BD Biosciences), fixed with 2% paraformaldehyde, permeabilized in PBS containing saponin, and stained with FITC-conjugated anti-IFN-γ mAb and allophycocyanin-conjugated anti-IL-10 mAb (cat.no.505010, BioLegend). Cells were washed twice and the frequency of IFN-γ+ IL-10+ CD8 T cells was determined by FCM.

Cytokines were detected in supernatants by conventional double-sandwich ELISA. The following mAbs (from BD Biosciences) were used for detection or capture: mAb R4-6A2 (cat.no.551216) and biotinylated mAb XMG1.2 (cat.no.554410) for IFN-γ; mAb JES6-1A12 (cat.no.554424) and biotinylated mAb JES6-5H4 (cat.no.554426) for IL-2; mAb 11B11 (cat.no.554434) and biotinylated mAb BV6-24G2 (cat.no.554390) for IL-4; and mAb C15.6 (cat.no.555129) and biotinylated mAb C17.8 (cat.no.554476) for IL-12 p40. We used OptEIA ELISA kits for detection of IL-10 and IL-6 (cat.no.55-52-52 and cat.no.55-52-40; BD Biosciences). Extinction was analyzed at 405/490 nm on a TECAN microplate reader (TECAN) using the EasyWin software (TECAN).

Results

pI/C injection acutely changes the splenic DC subset distribution and their phenotype in wt but not IFNAR−/− mice

pI/C induced a serum IFN-I response in wt but not IFNAR−/− mice that peaked 3–5 h post injection and was detectable for >24 h (Fig. 1A, data not shown). Confirming our previous report (31) vaccination of IFNAR-deficient mice induced 2- to 4-fold higher CD8 T cell responses than vaccination of wt mice, and pI/C treatment of wt B6 mice down modulated the vaccine-induced CD8 T cell response (evident by a >50% reduction of the number of specific CD8 T cells in the spleen 11 days post vaccination) (Fig. 1B). pI/C treatment had no effect on the vaccine-induced CD8 T cell response in IFNAR−/− B6 mice indicating that its modulatory effect was IFN-I-mediated (Fig. 1B). CD8 T cell response was reduced when pI/C was injected 24 h before, at the time of, and
FIGURE 3. Transient BST2 expression by splenic cDC following pI/C injection. A, mPDCA-1 and 120G8 surface expression by splenic cDC and pDC from wt B6 mice that were either nontreated or injected with pI/C (100 μg per mouse) 18 h previously. A representative example from seven individual mice per group analyzed independently is shown. B, Kinetics of appearance and disappearance of mPDCA-1 expression on the surface of cDC after pI/C injection. Splenic cDC were isolated from wt B6 mice injected with pI/C 3, 16, or 48 h previously. Data from representative individual mice (from three mice per time point analyzed in two independent experiments) are shown. C, FACS-purified cDC and pDC were treated in vitro for 18 h with IFN-α (103 U/ml). Surface expression of B220, PDCA-1, and 120G8 surface expression by cDC after pI/C injection. Splenic cDC were isolated from wt B6 mice (and their cDC or pDC subsets) per spleen as well as the percent CD4+, CD8+, or DN cDC in the (B220+) cDC populations from untreated (−) or pI/C-treated (+) wt B6 mice are shown. Pooled data from five independent experiments are shown. **, p < 0.01; ***, p < 0.001; ns, not significant.

24 h after vaccination (data not shown). IFN-I thus negatively modulates specific priming of CD8 T cells. As T cell priming is usually dependent on DC, we characterized pI/C (IFN-I)-stimulated, acute changes in subset distribution and phenotype of DC.

In individual spleens of wt and IFNAR−/− B6 mice, 3 × 10⁶ DC were found in the (CD3−) T cell−, (CD19−) B cell−, and (NK1+/DX5+) NK cell-depleted CD11c+ cell populations. CD11c− B220− cDC and CD11c− B220+ pDC were identified in these splenic DC populations, with a cDC/pDC ratio of 4:5 (Fig. 2A). Most splenic cDC showed a CD11cint phenotype while most splenic pDC showed a CD11chigh phenotype. A functional test (in vitro MCMV infection of cell sorter-purified cDC and pDC populations) confirmed that CD11cint B220+ pDC but not CD11c− B220− cDC produce abundant IFN-I after virus infection (Fig. 2A).
The numbers of splenic cDC and pDC in wt and IFNAR$^{-/-}$ B6 mice were similar but a moderate reduction in pDC numbers was often found in IFNAR-deficient mice (Fig. 2A). The number of pDC in the spleen of wt but not IFNAR$^{-/-}$ B6 mice was reduced by $>60\%$ 18 h after a single plC injection (Fig. 2A). RAG1$^{-/-}$ B6 mice (with no specific T or B cells) showed a similar splenic DC subset distribution and similar changes in response to plC injection (Fig. 2A). An IFN-I-dependent signal hence depletes splenic pDC.

plC injection moderately enhanced MHC class I (K$^b$) and class II (A$^b$) surface expression on pDC and cDC in wt B6 mice (Fig. 2C). cDC and pDC up-regulated surface expression of the costimulator molecules CD40 and CD86 and the coinhibitor molecule PD-L1. Expression of PD-L2 was induced only in cDC while ICOSL expression was not induced (Fig. 2C). These plC-induced changes in the surface profile were apparent in wt but not IFNAR$^{-/-}$ B6 mice, indicating that IFN-I mediates this in vivo response (Fig. 2C). IFN-I can thus enhance costimulation but also coinhibition of MHC-restricted Ag presentation by cDC.

IFN-α induces transient BST2 surface expression of cDC

The mAbs mPDCA-1 (12) and 120G8 (8) bind BST2 (14) expressed on the surface of CD11c$^{hi}$ B220$^+$ pDC but not CD11c$^{hi}$ B220$^-$ cDC from untreated (wt or IFNAR$^{-/-}$) B6 mice (Fig. 3A, data not shown). Almost all cDC in spleen, bone marrow, liver, and lymph nodes from wt (but not IFNAR$^{-/-}$) B6 mice expressed BST2 on the surface after plC injection (Fig. 3A, data not shown). BST2 surface expression by cDC was detectable already 3 h after plC injection, peaked at 16–24 h and disappeared by 48 h post injection (Fig. 3B). Repeated, daily plC injections (for 4 consecutive days) did not lead to stable BST2 expression by cDC (data not shown). IFN-β stimulates BST2 surface expression by purified cDC in vitro (Fig. 3C). CD11c$^{+}$ B220$^-$ mPDCA-1$^+$ cDC and CD11c$^{+}$ B220$^+$ mPDCA-1$^-$ pDC were sorted from (T, B, and NK cell-depleted) spleen cell populations of wt mice and cultured either without stimulation, or in the presence of murine IFN-β (Fig. 3C). During a 24- to 48-h culture period, pDC showed stable surface expression of B220 and BST2 and did not gain expression of the cDC marker CD11b (Fig. 3B, data not shown). cDC showed stable CD11b expression and did not express BST2, but acquired BST2 expression in response to IFN-I stimulation (Fig. 3C, data not shown). IFN-I induced transfer of BST2 molecules from pDC to cDC, or the generation of cDC from pDC are thus unlikely to explain BST2 surface expression by cDC. IFN-I stimulates surface expression of BST2 by almost all cDC, indicating that almost all cDC are IFN-I responsive. IFN-I acutely expands the splenic CD8 cDC subset

Under steady-state conditions, splenic cDC populations contain a major CD8$^+$ subset and minor CD8$^-$ and DN (CD4$^-$ CD8$^-$) cDC subsets (2). This subset distribution was found in wt, IFNAR-, and RAG1-deficient B6 mice (Fig. 4A). In untreated (wt and IFNAR$^{-/-}$) mice, CD8 and CD4 cDC were not activated (showed no or low CD69 and CD25 surface expression), expressed PD-L1 but not PD-L2, and showed the reported subset-specific maker expression (2, 5), i.e., CD8 cDC expressed the C-type lectin CD205 (recognized by mAb DEC205), but not the inhibitory receptor-2 DCIR2 (recognized by mAb 33D1) or the α$\alpha$ integrin CD11b; in contrast, CD4 cDC were CD205$^{low}$ but expressed DCIR2 and CD11b (Fig. 4A). Almost all cDC in spleen, bone marrow, liver, and lymph nodes from wt (but not IFNAR$^{-/-}$) B6 mice expressed BST2 on the surface after plC injection (Fig. 3A, data not shown). BST2 surface expression by cDC was detectable already 3 h after plC injection, peaked at 16–24 h and disappeared by 48 h post injection (Fig. 3B). Repeated, daily plC injections (for 4 consecutive days) did not lead to stable BST2 expression by cDC (data not shown). IFN-β stimulates BST2 surface expression by purified cDC in vitro (Fig. 3C). CD11c$^{+}$ B220$^-$ mPDCA-1$^+$ cDC and CD11c$^{+}$ B220$^+$ mPDCA-1$^-$ pDC were sorted from (T, B, and NK cell-depleted) spleen cell populations of wt mice and cultured either without stimulation, or in the presence of murine IFN-β (Fig. 3C). During a 24- to 48-h culture period, pDC showed stable surface expression of B220 and BST2 and did not gain expression of the cDC marker CD11b (Fig. 3B, data not shown). cDC showed stable CD11b expression and did not express BST2, but acquired BST2 expression in response to IFN-I stimulation (Fig. 3C, data not shown). IFN-I induced transfer of BST2 molecules from pDC to cDC, or the generation of cDC from pDC are thus unlikely to explain BST2 surface expression by cDC. IFN-I stimulates surface expression of BST2 by almost all cDC, indicating that almost all cDC are IFN-I responsive.

IFN-I acutely expands the splenic CD8 cDC subset

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4B, data not shown). Hence, the splenic cDC population is heterogeneous, the development of their typical subset distribution is IFN-I-independent, and most cDC are not activated under steady-state conditions.

In response to pI/C injection, the splenic CD8 cDC population of wt and RAG-deficient but not IFNAR-deficient mice expanded 2- to 3-fold while the splenic CD4 cDC population contracted ~2-fold (Fig. 4, A and C and 5D). No significant change in the numbers of cDC and DN cDC per spleen were found in pI/C-injected mice (Fig. 4C and 5, C and D). This was observed after injection of 1 μg or 100 μg pI/C per mouse though the cDC subset change induced by injection of the low pI/C dose was less striking (Fig. 5D). CD8 and CD4 cDC were activated (CD69high) and up-regulated surface expression of PD-L1 and PD-L2 in pI/C-treated wt but not IFNAR−/− mice (Fig. 4B, data not shown). The surface expression of the subset-specific makers CD205, CD11b, and DCIR2 was not changed after pI/C injection (Fig. 4B, data not shown). Thus, IFN-I changes the subset composition of the splenic cDC population and enhances surface expression of their activation, costimulatory but also coinhibitory molecules.

The pI/C-elicited changes in the splenic DC compartment are NK cell independent

The pI/C-induced changes in the splenic pDC and cDC compartments were independent of the specific T/B cell system (as they were observed in RAG1−/− B6 mice) and of NK T cells (as they were observed in CD1d−/− B6 mice, data not shown). IFN-I-stimulated DC activate resting NK cells (41, 49, 50) that up-regulate surface expression of CD69, CD86, PD-1, PD-L1, and TRAIL (Fig. 5A). Some IFN-I-stimulated pDC and few DN cDC up-regulated surface expression of DR5, the receptor for TRAIL (data not shown). It is hence conceivable that pI/C-induced TRAIL (NK cell)/DR5 (DC) interactions delete DC subsets. We found no evidence for in vitro killing of DC target cells by NK T or NK effector cells from pI/C-treated mice (data not shown). To test whether the pI/C-induced changes in the splenic DC compartment are NK cell dependent, we depleted mice of NK cells by repeated treatment with αAsGM1 Ab before injecting pI/C (at a low dose of 1 μg per mouse or a high dose of 100 μg). Ab treatment eliminated >95% of all splenic (NK1.1+ DX5+) NK cells (Fig. 5B). The pI/C-induced changes in surface phenotype and subset composition observed in the splenic DC compartment of pI/C-treated, NK cell-competent, and NK cell-deficient mice were identical (Fig. 5D, data not shown). NK cells are hence not critical for pI/C-induced changes in subset composition and surface marker profile of splenic DC.

Functional characterization in vitro of pI/C-activated cDC

Purified (FACS-sorted) splenic CD3− CD19− NK1− B220− CD11c+ nonfractionated (NF) cDC or their CD8− subset (sorted from these cDC in a subsequent step) from nontreated or pI/C-treated mice were cultured for 48 h (Fig. 6A). These cells were either not stimulated (groups 1, 4), or stimulated by CD40 ligation (groups 2, 5) or TLR4 ligation (groups 3, 6). Unstimulated cDC from nontreated or treated mice did not produce IL-12 p40, IL-10,
IFN-I-treated (NF or CD8) cDC was and the Kb-restricted, S2-specific CD8 T cell response was determined 12 days post priming by CD8/tetramer staining or specific ex vivo IFN-γ induction. Mean numbers of five mice per group (±SEM) from a representative (of three independent) experiment(s) are shown. B, Splenic dimer+ CD4 NKT cells from untreated (−) or pI/C-injected (+) wt B6 mice. C, Changes in the surface profile of splenetic and hepatic (dimer+) NKT cells from untreated (−pI/C) or pI/C-treated (+pI/C) B6 mice. Two representative individual mice (from four individual mice analyzed per group) are shown. D, Cytokine release of NKT cells stimulated by purified, αGalCer-pulsed, splenic CD8+ or CD8− cDC from untreated or pI/C-treated B6 mice. Cytokines were detected by ELISA in supernatants harvested from 18 h cocultures. Mean values of triplicates (±SEM) of one representative (of three independent) experiment(s) are shown.

To test whether the reduced production of proinflammatory cytokines but unimpaired IL-10 production of IFN-I-treated cDC reduces their immunostimulatory potency, we cocultured naive CD8 (OT-I RAG1−/−) or CD4 (OT-II RAG1−/−) T cells with purified, peptide-pulsed splenic NF or CD8 cDC from nontreated or pI/C-treated mice. Naive CD4 T cells cocultured with peptide-pulsed cDC from IFN-I-treated (NF or CD8) cDC showed reduced IFN-γ and IL-2 production (Fig. 6B, data not shown). In contrast, the >100-fold higher IFN-γ response of naive CD8 T cells primed by IFN-I-treated (NF or CD8) cDC was >3-fold enhanced (Fig. 6B). Hence, cDC from pI/C-treated mice do not show a defect in priming CD8 T cells in vitro.

NKT cell activation by IFN-I-stimulated cDC

pI/C-induced IFN-I limits priming specific CD8 T cell immunity in wt mice (Fig. 1B) but did not impair establishment of specific CD8 T cell immunity by the same vaccination protocol in NKT cell-deficient (CD1d−/−) B6 mice (Fig. 7A). NKT cells are hence critical in mediating down-regulation of CD8 T cell priming by pI/C. This confirms the report that CD1d-restricted NKT cells control the magnitude of the cell-mediated immune response to acute viral infection (51). Splenic DC from nontreated and pI/C-treated CD1d−/− B6 mice showed similar subset and surface marker profiles than wt mice, and in vitro priming of CD8 T cells by peptide-pulsed, splenic cDC (subsets) from pI/C-treated, wt or CD1d−/− B6 mice was similar (data not shown).

Two to 4% of the CD4 TCRβ T cells in the spleen of a young adult B6 mouse are (CD1d/αGalCer) dimer+ NKT cells (5.1 ± 2.1 × 105 cells per spleen). Treatment of mice with pI/C reduced the number of splenic NKT cells by ~30% (3.6 ± 1.0 × 105 cells per spleen) (Fig. 7B), pI/C (IFN-I) induced up-regulation of CD69, PD-L1, and CD86 surface expression in NKT cells (Fig. 7C). CD8 cDC from pI/C-treated mice inefficiently triggered cytokine responses of NKT cells. CD8+ or CD8− cDC obtained from either nontreated or pI/C-treated mice (or an 18 h preincubation culture with or without IFN-β)
FIGURE 8. NKT cells impair the competence of CD8 cDC to prime proinflammatory CD8 T cell responses in the presence of IFN-γ. A, Purified, splenic CD8 cDC from nontreated wt mice were nonpulsed, pulsed with 1 ng/ml or 100 ng/ml αGalCer, or 100 ng Kβ-binding OVA263–269 peptide SIINFEKL (OVA-kβ). These cDC (0.5 × 10⁴ DC/well) were cocultured with purified naive OT-I RAG1−/− CD8 T cells (5 × 10⁴ CD8 T cells/well) and NKT cells (2.5 × 10⁴ NKT cells/well) in the absence (−IFNγ) or presence (+IFNγ) of 10³ IU/ml IFNγ. Supernatants were collected after 4 days of incubation for cytokine determination. Mean values of triplicates (±SEM) of two independent experiments are shown. B, IFN-γ-/+ IL-10- CD8 T cells are primed in the presence of IFN-γ. CD8 T cells primed in the cultures described in A were harvested and stained for intracellular IFN-γ and IL-10. Cytokine expression of gated CD8⁺ T cells from a representative example of each group is shown (two independent experiments were performed). C, NKT cells change the priming competence of CD8 cDC in the presence of IFN-γ. Splenic CD8 cDC were isolated, nonpulsed or pulsed with 100 ng/ml αGalCer, washed, and cocultured in the presence (10³ IU/ml) or absence of IFN-γ with purified NKT cells. CD8 cDC were harvested from these 18 h cocultures, washed, pulsed with antigenic peptide, washed, and cocultured with naive OT-I RAG−/− CD8 T cells. IFN-γ and IL-10 release into the 72 h supernatant of these cocultures were determined by ELISA. Mean values (+SEM) of triplicates from one representative (of two independent) experiment(s) are shown. **, p < 0.01.

were pulsed with glycolipid and cocultured with purified NKT cells. Only CD8⁺ cDC (that mediate most CD8 T cell priming) efficiently stimulated IFN-γ, IL-4, or IL-2 release by NKT cells. This response was strikingly down modulated when CD8 cDC were exposed to IFN-γ before coculture (Fig. 7D, data not shown). The release of cytokines assumed to play a role in NKT cell help for specific activation, clonal expansion, and/or Th1 polarization of DC-primed T cells (52–54) was hence inefficiently recalled by IFN-γ-stimulated CD8 cDC.

IFN-γ is required to impair the competence of CD8 cDC to prime proinflammatory CD8 T cell responses by NKT cells

We studied in vitro the role of IFN-γ and NKT cells on CD8 T cell priming by cDC. CD8 cDC from nontreated mice were pulsed (or not pulsed) with αGalCer and antigenic peptide and cocultured for 4 days with naive (OT-I RAG1−/−) CD8 T cells and purified, splenic NKT cells (from nontreated mice) in the presence or absence of IFN-γ (Fig. 8A). In the absence of IFN-γ, primed CD8 T cells produced substantial amounts of IFN-γ but only low amounts of IL-10, and IFN-γ production was enhanced in a dose-dependent manner by NKT cells activated in the same culture. In the presence of IFN-γ, CD8 T cells produced similar amounts of IFN-γ but greatly enhanced amounts of IL-10. NKT cell help in the presence of IFN-γ hence did not promote IFN-γ production but strikingly enhanced IL-10 production (Fig. 8A). IFN-γ and IL-10 were produced by CD8 T cells in these cultures (Fig. 8B). The presence of IFN-γ enhanced IL-10 production by CD8 T cells, especially when they were primed by CD8 cDC in the presence of “help” from NKT cells.

We used this system to test whether NKT cells (directly) influence CD8 T cells during priming or (indirectly) exert an effect on DC that changes their competence to subsequently prime CD8 T cell responses. CD8 cDC from nontreated mice were pulsed (or not pulsed) with αGalCer and cocultured for 18 h with purified NKT cells in the presence or absence of IFN-γ. CD8 cDC were harvested from these cocultures, washed, pulsed with antigenic peptide, washed, and cocultured with naive OT-I RAG−/− CD8 T cells. IFN-γ and IL-10 responses of CD8 T cells that had activated NKT cells in the presence of IFN-β were less effectively primed IFN-γ but more effectively primed IL-10 responses of CD8 T cells that they primed. In the presence of IFN-γ, NKT cells can hence impair the competence of CD8 cDC to prime proinflammatory CD8 T cell responses.

Discussion

When exposed to IFN-γ, almost all cDC subsets respond to IFN-γ. IFN-γ induces acute changes in the phenotype and subset composition of murine, splenic DC that are independent of NK, NKT, T, or B cells. In vitro assays showed that IFN-γ-stimulated CD8 cDC are: 1) deficient in their ability to produce IL-12 and IL-6 in response to CD40 or TLR4 ligation but not in their ability to produce the immunosuppressive cytokine IL-10; 2) deficient in their ability to specifically trigger IFN-γ, IL-4, and IL-2 release by NKT cells; but 3) competent to prime naive
CD8 T cell responses in vitro. In vivo, pI/C down modulates CD8 T cell priming in wt but not CD11c−/− B6 mice although IFN-I-induced changes in DC subset composition and surface profile are similar in both mouse lines. In the presence of IFN-I, NKT cells impair in vitro the ability of CD8 cDC to prime specific CD8 T cell immunity. These data point to an IFN-I-induced, NKT cell-mediated down modulation of the competence of CD8 cDC to prime antiviral CD8 T cell immunity.

In most studies, we injected low (1 µg/mouse) or high (100 µg/mouse) doses of pI/C to induce IFN-I in vivo. The TLR3 and md-5 ligand pI/C (17, 18) induces a transient IFN-I response readily detectable in the serum, pI/C-induced IFN-I was more efficient in inducing DC activation than injection of recombinant, murine IFN-β (data not shown), probably because injection of nonformulated IFN-I has a limited tissue distribution and a short half-life in vivo. Control experiments using IFNAR−/− B6 mice confirmed that we are investigating an IFN-I-mediated effect. Systemic IFN-I responses (comparable to responses induced by the viral mimic pI/C) are apparent early in acute virus infections. We thus study IFN-I-mediated effects operating early in virus infection that affect subsequent priming of specific immunity.

pDC in this study were CD11c+ B220− CD11b+ CD3− CD19− DX5/NK1− cells that rapidly release IFNα/β after MCMV infection and stably express (in an IFN-I-independent manner) BST2 serologically defined by the mAbs 120G8 and mPDCA-1. There is consensus that this is the murine pDC population. The mAbs mPDCA-1 and 120G8 bind BST2 (14) and have been extensively used to identify murine pDC and to deplete them in vivo for functional studies. Our data confirm the IFN-I-induced promiscuous expression of BST2 on most cell types (14) including cDC, lymphoid, and myeloid cells (data not shown). All cDC are hence responsive to IFN-I. The mAbs mPDCA-1 and 120G8 do not have the requisite specificity for pDC that they were proposed to have which poses severe limitations to their use in the specific identification and depletion of pDC. As almost all cDC rapidly (but transiently) express large amounts of BST2 on the cell surface after IFN stimulation. This will lead to overestimates of the functional competence of pDC in Ab-mediated depletion studies, especially under inflammatory conditions. IFN-I reduces splenic pDC populations that are inefficient in priming CD8 T cells. We therefore focused our subsequent studies on splenic cDC.

Establishment of splenic cDC and pDC populations is IFN-I-independent because we found similar numbers of splenic DC in IFNAR−/− mice (though pDC numbers were slightly but reproducibly reduced in IFNAR−/− mice). No overall changes of DC numbers were apparent in the spleen after pI/C injection although changes in the DC subset distribution were obvious. Size estimates of this population have to be considered with caution because only low numbers of CD11c+ DC are isolated from the spleen of individual mice. This is especially true if subsets of this small cell population are analyzed. We may have missed subtle changes in the numbers of the total splenic pDC or cDC, or the cDC subsets. It is unknown whether IFN-I induces local depletion or expansion of splenic DC subsets and/or changes the ability of DC to enter or to leave the spleen. DC development under steady-state and inflammatory conditions differs (2). IFN-I may stimulate either local generation and/or expansion of CD8 cDC from precursors, or induce rapid redistriution of particular DC subsets. IFN-I-treated DC subsets up-regulate CD69 expression that triggers their retention in lymph nodes through down modulation of sphingosine 1-phosphate receptor-1 expression (55). IFN-I-mediated trapping of pDC in lymph nodes may cause their depletion in the spleen.

IFN-I-induced changes in the surface marker profile and subset composition of splenic DC was observed in B6 mice deficient in T/B cells (RAG1−/−), NKT cells (CD11c−/−), and NK cells (treated with αAsGM1 Ab). Hence, IFN-I seems to target DC directly supporting the notion that immune modulation by this cytokine operates mainly through its direct effect on DC (subsets).

We studied the immunosuppressive effect of IFN-I on CD8 T cell priming in different steps in this report. First, IFN-I induced changes in the surface expression of MHC (CD40, CD80, CD86), costimulator, and (PD-L1/L2) coinnhibitor molecules of splenic DC subsets. These IFN-I-stimulated changes in the surface phenotype of DC can more efficiently costimulate or coinhibit priming of cellular immune responses and were therefore not considered helpful to explain the immunosuppressive effect of IFN-I on CD8 T cell priming. Second, IFN-I expanded the splenic CD8 cDC subset, the main APC for CD8 T cell priming (5, 56–58). We therefore placed particular emphasis on the analysis of this DC subset. Third, the functional analysis of IFN-I-stimulated CD8 cDC revealed that they were deficient in generating proinflammatory cytokine responses but displayed an intact IL-10 response to CD40 or TLR ligation. This confirmed published data (36, 37). It could be relevant for the immunosuppressive effect of IFN-I but, fourth, could not be shown in vitro when naive CD8 T cells were primed by IFN-I-treated CD8 cDC, and, fifth, could not explain why NK cells were required to detect the immunosuppressive effect of IFN-I on CD8 T cell priming in vivo. Sixth, IFN-I-treated CD8 cDC inefficiently stimulated in vitro the IL-2, IL-4, and IFN-γ response of NKT cells. IFN-I thus changes the specific (CD1d/glycolipid-dependent) response of NKT cells to CD8 cDC. It down modulates proinflammatory mediator production by NKT cells and possibly up-regulates their suppressor mediator production (the nature of these “suppressive” signals and the effect they transmit to CD8 cDC are unknown but under investigation). Seventh, specific in vitro coactivation of naive CD8 T cells and NKT cells by ligand-pulsed CD8 cDC in the presence of IFN-I supports priming of IL-10-producing CD8 T cells. Eighth, this involved a first step in which CD8 cDC activating NKT cells in the presence of IFN-I apparently change their phenotype in a way that subsequently results in their enhanced priming of IL-10-producing CD8 T cells.

We previously reported experimental conditions under which IFN-I favor development of IL-10-producing T cells. IFN-I supports priming of specific IL-10+ CD4 T cells, that limit CD8 T cell responses induced by vaccines (31). IFN-I is required to prime IFN-γ+ IL-10+ CD8 T cells by hepatocytes with NKT cell “help” (59). We describe in this report that IFN-I controls priming of IFN-γ+ IL-10+ CD8 T cell immunity by CD8 cDC in an NKT cell-dependent way. The changes induced by NKT cells in the competence of CD8 cDC to prime CD8 T cells in the presence of IFN-I remain to be resolved at the molecular level. We identified NKT cells as an important checkpoint that mediates some effects of IFN-I on the specific CD8 T cell immunity. NKT cells have been reported to “help” or “suppress” CD8 T cell priming. Dependent on the cytokine environment, NKT cells may provide “context sensitive” regulation for the priming of specific T cell immunity. This may explain the contradictory data on “positive” and “negative” regulatory effects of NKT cells reported in different models.

**Acknowledgments**

We greatly appreciate the expert technical assistance of Ina Sebald and Ellen Allmendinger. We are grateful for the gift of the mAb 120G8.
from Dr. Paul J. Zavadny, Schering-Plough Research Institute (Kenilworth, NJ) and of the PE-conjugated K5/S2 tetramer from the NIAID Tetramer Facility. We thank the referees for many helpful suggestions.

Disclosures

The authors have no financial conflict of interest.

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