In vivo and In vitro Regulation of Type I IFN Synthesis by Synergistic Effects of CD40 and Type II IFN

Jennifer A. Greene, Jennifer L. DeVecchio, Meetha P. Gould, Jeffery J. Auletta and Frederick P. Heinzel

*J Immunol* 2006; 176:5995-6003; doi: 10.4049/jimmunol.176.10.5995
http://www.jimmunol.org/content/176/10/5995

References

This article cites 36 articles, 19 of which you can access for free at:
http://www.jimmunol.org/content/176/10/5995.full#ref-list-1

Subscription

Information about subscribing to *The Journal of Immunology* is online at:
http://jimmunol.org/subscription

Permissions

Submit copyright permission requests at:
http://www.aai.org/About/Publications/JI/copyright.html

Email Alerts

Receive free email-alerts when new articles cite this article. Sign up at:
http://jimmunol.org/alerts
In vivo and In vitro Regulation of Type I IFN Synthesis by Synergistic Effects of CD40 and Type II IFN

Jennifer A. Greene,* Jennifer L. DeVecchio,‡ Meetha P. Gould,* Jeffry J. Auletta,† and Frederick P. Heinzel*‡†

During cognate interaction with CD40 ligand (CD154)-expressing T cells, Ag-presenting accessory cells are activated for increased cytokine synthetic and costimulatory function. We examined whether CD40 modulates in vivo innate immune function over time, hypothesizing that distinct cytokine responses evolve to delayed microbial exposure. C3H/HeN mice pretreated with activating anti-CD40 Ab (FGK45) produced 10-fold more serum IFN-γ and IL-12 p70 to delayed, but not synchronous, challenge with LPS. A novel finding was that LPS-induced IFN-α increased by 20-fold in mice pretreated for 24 h, but not 6 h or less, with anti-CD40. Anti-CD40-pretreated C57BL/6 RAG-2−/− mice similarly increased IFN-α responses to delayed LPS challenge, confirming mediation by innate immunity. Type I IFNR- and IFN-γ-deficient mice treated with anti-CD40 failed to expand serum IFN-α responses to LPS challenge. Combined pretreatment with anti-CD40 and anti-IFN-γ mAb showed that IFN-γ produced after anti-CD40 pretreatment, but before LPS challenge, was necessary for IFN-α synthetic enhancement. Anti-CD40 also increased polyinosinic-polycytidylic acid (poly(I:C))-inducible IFN-α by 5-fold in an IFN-γ-dependent fashion, but did not significantly increase IFN-α production to CpG or PamCys challenges. Poly(IC)-stimulated splenocytes from anti-CD40-pretreated mice produced 4-fold more IFN-α than controls and production associated with CD11c+ cells. Finally, rIFN-γ and anti-CD40 combined synergistically to increase poly(IC)-inducible IFN-α synthetic capacity in bone marrow dendritic cells. We conclude that innate immune production of IFN-α is cooperatively regulated by CD40 and IFN-γ acting on dendritic cells, suggesting a unique mechanism by which innate immune function evolves in response to specific adaptive immune signals. The Journal of Immunology, 2006, 176: 5995–6003.

Type I (IFN-α and IFN-β) and type II (IFN-γ) IFNs are inducible cytokines that mediate critical regulatory and anti-infective functions during an immune response (1). Both can be produced by the innate cellular immune system in response to TLR activation by viral and bacterial-derived pathogen associated molecular patterns. Defined groups of these molecules activate specific TLRs (2). Bacterial LPS and lipopeptide trigger TLR2 and TLR4, respectively, whereas microbial DNA containing unmethylated CpG motifs activates TLR9 and viral double-stranded or single-stranded RNA activate TLR3 or TLR7/TLR8, respectively (3, 4). Most TLR activations result in synthesis of immunoregulatory cytokines, some of which, such as IL-12 p70 and IL-18, critically regulate innate IFN-γ production by NK cells (5, 6). IFN-γ is also a product of IL-12-stimulated T cells in an ongoing adaptive immune response.

In contrast, type I IFN production is largely restricted to innate immune responses triggered by a few TLR operating through distinct mechanisms. For instance, TLR3 and TLR4 activate synthesis of IFN-α indirectly through MyD88-independent mechanisms that use Toll-IL-1 receptor domain-containing adapter inducing IFN-β and IFN-regulatory factor-3 as the critical signaling intermediates. This leads to IFN-β synthesis that then acts through the type I IFNR to self-induce high-level production of IFN-α. This model of type I IFN autinduction is characteristic of fibroblasts, macrophages, and myeloid dendritic cells responding to viral infection or endotoxemia (7, 8). In contrast, TLR7/8 and TLR9 activate MyD88-dependent and IFN-α/IFN-β-independent pathways for innate production of type I IFN after exposure to CpG oligonucleotide sequences or potent synthetic analogs of ssRNA, such as R848. This mechanism is restricted to a small population of plasmacytoid dendritic cells that are otherwise unresponsive to LPS and polyinosinic-polycytidylic acid (poly(I:C))3 (9).

Once produced, IFNs provide a regulatory link between innate immune activation and the intensity and phenotype of an ensuing adaptive immune response. Both IFN-γ and type I IFNs enhance accessory cell Ag presentation and costimulatory function, thereby accelerating T cell responses to Ag (10, 11). These distal immunoregulatory effects of LPS, poly(I:C), and CpG contribute to their effectiveness as adjuvants when incorporated into experimental and clinical vaccine preparations (11). Both IFNs also separately and directly modulate T cell differentiation, partly by up-regulating the IL-12R and downstream signaling functions that favor development of IFN-γ-producing CD4 and CD8 T cells (12, 13). Depending on the circumstances, type I IFNs can also impair IL-12 production, resulting in a paradoxical attenuation of cellular immunity during viral infection (14) or when used for therapy of autoimmune diseases (15).
We studied whether regulatory signals generated as part of an adaptive immune response, including T cell-derived cytokines and costimulatory ligands, reciprocally modulate innate cellular immunity to evolve new functions. T cells that express CD40L on their surface stimulate CD40-bearing dendritic cells and macrophages for greater function in support of both innate and adaptive immune responses. CD40L-conditioned dendritic cells previously or simultaneously exposed to microbial stimuli, such as Toxoplasma gondii or CpG, increase both their T cell-activating functions and produce higher quantities of regulatory cytokine, including IL-12 p70 (16, 17). We hypothesized that the immune-enhancing effects of CD40 agonists in vivo are progressive, causing the innate cellular immune system to evolve quantitatively and/or qualitative changes in cytokine response to delayed Toll agonist exposures. For these studies, mice were pretreated with the CD40-activating mAb, FGK45, a reagent previously shown to generate CD40L-comparable and immune-enhancing responses in vitro and in vivo (18–20). Anti-CD40 pretreatment not only amplified cytokine responses to delayed LPS exposure, but also broadened the diversity of the LPS-inducible cytokine repertoire to include a novel 20-fold expansion in IFN-α synthesis. We show that this was mediated by synergistic effects of both CD40 and CD40-induced IFN-γ acting on dendritic cells, identifying a novel mechanism for regulation of type I IFN by specific and sequential combinations of innate and adaptive immune signals.

Materials and Methods

Reagents

Anti-CD40 mAb, clone FGK45 (21), and neutralizing anti-mouse IFN-γ XM1G1.2 were purchased from Bio Express and affinity-purified rat IgG was purchased from Sigma-Aldrich. Recombinant murine IFN-γ was obtained from PeproTech. Salmonella enteritidis LPS was obtained from Sigma-Aldrich, phosphorothioated CpG oligonucleotide 1826 (TCCATGACCTCTCGACCTT 5’ to 3’) from Oligos Etc., bacterial lipopeptide Pam,CysSerLys, from EM C Microcollections, poly(I:C) from Amersham Biosciences, and R848 from InvivoGen. The LPS reagent used in this study was shown to be TLR4 dependent, as it only induced endotoxin tolerance in C3H/HeN and TLR2 knockout (KO) C57BL/6 mice, but not in TLR4-deficient C3H/HeJ mice. Both the anti-CD40 Ab and CpG and R848 reagents were proven free of LPS contamination by Limulus lysate assay (E-Toxate; Sigma-Aldrich).

Mice

Four- to 6-wk-old female C3H/HeN mice were purchased from Charles River Laboratories and C57BL/6 and C57BL/6 IFN-γ KO mice from The Jackson Laboratory. IFN-α/IFN-β KO 129S6/SvEv mice were bred at Case Western Reserve University (CWRU) from breeders provided by Dr. R. Fairchild (Lerner Research Institute, Cleveland Clinic Foundation, Cleveland, OH). Control 129S6/SvEv mice were obtained from Taconic Farms and IFN-α/IFN-β null genotype was verified by PCR analysis of tail DNA using primers and protocols obtained from B&K Universal. All mice were housed at the CWRU Animal Facility under specific pathogen-free conditions. For pretreatment, mice were injected once i.p. with either 0.2 mg of FGK45 or 0.2 mg of normal rat IgG. Twenty-four hours after pretreatment, mice were challenged by i.p. injection with either 200 μg of LPS (LD50) or PBS. Because anti-CD40 pretreatment increased the apparent toxicity of LPS challenge, mice were euthanized no later than 6 h after challenge. All procedures were approved by the CWRU Institutional Animal Care and Use Committee.

ELISAs

Cytokine concentrations in serum and conditioned culture medium were determined for IFN-γ and IL-6 using Ab kits from BD Pharmingen as previously described (19). Serum IL-12 p70 was measured using ELISA kits purchased from R&D Systems. IL-18 was measured using an ELISA obtained from Medical and Biological Laboratories that detects mature IL-18 with minimal cross-reactivity to precursor forms of IL-18. Serum IFN-α was measured using an ELISA purchased from PBL Biomedical Laboratories that has no cross-reactivity with mouse IFN-β or IFN-γ.

Tissue homogenization

Freshly harvested tissue (5 g) was placed in 10 ml of PBS containing 0.5% Triton X-100 and protease inhibitor mixture (Sigma-Aldrich). The tissue was homogenized using a Virtis blender, clarified by centrifugation at 12,000 × g for 10 min, and stored at −70°C.

Spleen cultures and splenocyte subset separations

Splenocytes were prepared and cultured at 106 cells/ml in tissue culture medium/Nutridoma medium (1:1 mix of RPMI 1640 and DMEM with supplemental 10 mM HEPES (pH 7.4), 1 mM t-glutamine, t-arginine, nonessential amino acids, 50 μM 2-ME, 100 μg/ml penicillin/streptomycin and 1% Nutridoma). TLR agonists were added as previously described (22). Where indicated, splenocytes (106 total cells each) were incubated separately with magnetic beads specific for mouse CD11c, CD11b, or DX5 (Miltenyi Biotec) and applied to magnetized LS columns in degassed wash buffer (PBS, 0.5% BSA, 5 μM EDTA (pH 7.4)) per manufacturer’s instructions. Unlabeled cells were eluted using 5 ml of wash buffer and labeled cells were recovered by washing of columns after removal from the magnetic field. The extent of depletion and the composition of the magnetically selected cells were confirmed by FACS analysis. Preselection and postdepletion cell populations were separately suspended at 1 × 106 cells/ml in tissue culture medium/Nutridoma culture medium with or without 33 μg/ml poly(I:C) and cultured for 24 h. Supernatants were assayed for IFN-α.

Dendritic cell cultures

Using a published methodology (23), C57BL/6 bone marrow cells were harvested from femurs, passed through a 70 μm cell strainer, centrifuged at 200 × g for 10 min and RBC lysed using ACK buffer (150 mM ammonium chloride, 10 mM potassium carbonate, and 0.1 mM EDTA adjusted to pH 7.4). Cells were washed in HBSS and resuspended at 107 cells/ml in dendritic cell medium: (RPMI 1640/10% FBS containing 1 mM sodium pyruvate, 10 mM HEPES, 50 mM 2-ME, and 100 μg/ml each of penicillin and streptomycin). Recombinant mouse Flt3L (Bio Express) was added to a final concentration of 300 ng/ml. Media were changed every 4 days and nonadherent cells were gently washed free of culture plates on day 10, counted, centrifuged, and resuspended to 106 cells/ml dendritic cell medium without Flt3L. Cells were cultured at 2 × 105 cells/well in round-bottom 96-well cluster plates and incubated with medium, rIFN-γ (10 ng/ml), or anti-CD40 (5 μg/ml) alone or in combination for 4 h before adding TLR agonists.

Statistics

Tests for significant differences were determined using the parametric Student t test for independent samples. Statistical significance was defined as p < 0.05.

Results

Pretreatment with anti-CD40 enhances systemic cytokine responses to LPS challenge 24 h later

C57BL/6 mice were pretreated with either 0.2 mg of rat IgG or anti-CD40 IgG and challenged with 0.2 mg (8 mg/kg) of S. enteritidis LPS, either at the time of anti-CD40 treatment or 24 h later. Compared with LPS challenge of rat IgG-pretreated mice, LPS administered 24 h after anti-CD40 pretreatment increased serum levels of IL-12 p70, IFN-γ, and IFN-α by >20-fold (p < 0.05) at 5 h postchallenge (Fig. 1A). In contrast, injection of anti-CD40 and LPS at the same time did not increase serum cytokine levels relative to LPS-only controls and decreased cytokine production to a second LPS challenge 24 h later. In a repeat study (Fig. 1B), in vivo exposure to anti-CD40 of >6 h was needed to increase LPS-induced production of IFN-α.

We confirmed that anti-CD40-enhanced serum cytokine levels measured at 5 h were not simply a result of altered kinetics of cytokine appearance. Groups of five C3H/HeN mice were pretreated once i.p. with either 0.2 mg of anti-CD40 mAb or affinity purified control rat IgG, challenged at 24 h with LPS challenge, and sera were obtained at 2, 4, and 6 h afterward (Fig. 1C). Levels of IFN-α, IFN-γ, IL-12 p70, IL-6, and IL-18 in anti-CD40-pretreated mice were 4- to 20-fold increased compared with those of rat IgG-pretreated mice throughout the time course studied. Anti-CD40 also
increased LPS-induced TNF-α by 40-fold (0.12 ± 0.06 to 4.86 ± 0.31 ng/ml; p < 0.05) and IL-1α by 10-fold (0.36 ± 0.16 to 3.19 ± 0.26 ng/ml; p < 0.05) at 1 and 4 h after LPS, respectively. Endotoxin-induced serum cytokines are dramatically and persistently elevated in anti-CD40-pretreated mice.

Low levels of serum IFN-γ were observed at the time of LPS challenge (Fig. 1C) and we confirmed that anti-CD40 pretreatment independently generates circulating IFN-γ that peaks at 6 h and is sustained until 24 h afterward (Fig. 1D).

Anti-CD40-enhanced production of IFN-α, IFN-γ, and IL-12 p70 is not dependent on T or B cells

These studies did not exclude possible contributions by CD40-positive B cells or indirectly activated T cells as sources of
anti-CD40-expanded cytokine synthesis in response to Toll receptor activation (24). However, compared with their respective PBS-pretreated controls, anti-CD40-pretreated RAG KO mice generated as much or more LPS-induced IFN-γ and IFN-α as did anti-CD40-pretreated wild-type C57BL/6 (Fig. 2). The 2-fold increase in RAG2 KO production of IL-12 p70 compared with wild-type mice was not significant. These findings confirm that the cytokine-enhancing effects of anti-CD40 pretreatment are mediated by mechanisms not dependent on the presence of T or B cells.

**Anti-CD40-enhanced synthesis of IFN-α, but not IFN-γ and IL-12, is regulated by the type I IFNR**

IFN-α synthesis in endotoxemic mice is normally autoregulated by the IFN-α/IFN-βR responding to IFN-β stimulated through MyD88-independent pathways downstream from TLR4 (7). We confirmed that a similar pattern of type I IFN self-induction was essential for the augmented release of IFN-α in anti-CD40-pretreated mice (Fig. 3). Compared with anti-CD40 pretreated wild-type 129S6/SvEv mice that generated 100-fold increased levels of IFN-α after LPS challenge relative to control endotoxemic mice, type I IFNR KO mice failed to generate any significant increase in IFN-α relative to the amounts induced by LPS or anti-CD40 alone. Although type I IFN was released before IL-12 p70 or IFN-γ in this model, IFN-α/IFN-βR deficiency did not alter the LPS-induced increase in these cytokines in anti-CD40-pretreated mice.

**Anti-CD40 pretreatment increases IFN-α production through an IFN-γ-dependent mechanism**

Although IFN-γ is not known to regulate IFN-α synthesis, both type I and type II IFNRs share downstream signaling and RNA-transactivating components that can mediate similar in vivo LPS-sensitizing effects (25, 26). Because anti-CD40 injection significantly increased levels of circulating IFN-γ before LPS challenge (Fig. 1D), we tested whether the enhanced IFN-α synthetic response was IFN-γ dependent. Consistent with a novel regulatory role for IFN-γ over the quantity of type I IFN produced, IFN-γ KO C57BL/6 mice pretreated with anti-CD40 failed to increase their IFN-α response to LPS compared with PBS-pretreated controls (Fig. 4A). In contrast, wild-type C57BL/6 mice pretreated with anti-CD40 showed a 2-log increase in LPS-inducible IFN-α. The presence or absence of IFN-γ competency had no significant effect on anti-CD40-expanded production of IL-12 p70 (30% difference, p > 0.05), consistent with previous reports of IFN-γ-independent production of IL-12 p70 during endotoxemia (27). To specifically determine whether circulating IFN-γ induced by anti-CD40 was necessary for amplifying IFN-α production to later LPS challenge, C3H/HeN mice were pretreated with anti-CD40 with or without injection of neutralizing anti-IFN-γ IgG (0.5 mg) 2 h before. Mice cotreated with both anti-CD40 and anti-IFN-γ produced 11-fold less serum IFN-α after LPS challenge than did mice pretreated only with anti-CD40 (Fig. 4B). In contrast, anti-IFN-γ injected 2 h before LPS challenge failed to reduce the IFN-α response in anti-CD40-pretreated mice. These findings confirm a direct regulatory effect of anti-CD40-induced IFN-γ and indicate that these effects are mediated before LPS challenge.

**FIGURE 2.** Anti-CD40 pretreatment enhances comparable LPS-inducible serum cytokine responses in wild-type and B and T cell-deficient mice. Groups of five C57BL/6 mice, either wild-type (RAG WT) or RAG2-deficient (RAG KO), were pretreated with rat IgG (−) or anti-CD40 Ab (+) 24 h before challenge with LPS or PBS as described above. Shown are the mean concentration and SEM of the indicated cytokines in nanograms per milliliter at 5 h after LPS challenge. As indicated by the asterisk (+), levels of IFN-α, IL-12 p70, and IFN-γ were significantly increased after LPS challenge of anti-CD40-pretreated RAG (+/+) and (−/−) mice compared with their respective controls treated with rat IgG (−) or anti-CD40 without challenge or with rat IgG-pretreated followed by LPS challenge (+, p < 0.05; Student’s t test). Brackets indicate nonsignificant (N.S.) differences between cytokine production in wild-type and RAG knockout after anti-CD40 pretreatment and LPS challenge.

**FIGURE 3.** Type I IFNR is necessary for expansion of IFN-α, but not IL-12 p70 and IFN-γ, production, in response to LPS. Groups of five 129S6 mice, expressing either wild-type (+/+ or IFN-α/IFN-βR knock-out (−/−) genotypes, were pretreated with either rat IgG or anti-CD40 IgG or, as indicated by + or −, respectively. Mice were challenged with either LPS (+) or PBS (−) 24 h later. Shown are the mean and SEM concentrations of IFN-α, IFN-γ, and IL-12 p70 in serum taken 5 h after LPS challenge. Brackets indicate that anti-CD40-expanded, LPS-induced IFN-α production was significantly decreased in IFN-α/IFN-βR-deficient mice compared with wild-type mice (+, p < 0.05), but that IL-12 p70 and IFN-γ levels were nonsignificantly (N.S.) changed as determined by Student’s t test.
**FIGURE 4.** IFN-γ is necessary, but not sufficient, for anti-CD40-mediated expansion of IFN-α synthetic capacity in vivo. A, Groups of five C57BL/6 wild-type (+/+) or IFN-γ-deficient (−/−) mice were pretreated with rat IgG or anti-CD40 IgG as indicated by + and −. All were challenged with LPS 24 h later. Shown are the mean and SEM concentrations of serum IFN-α and IL-12 p70 at 4 h after challenge. Anti-CD40-expanded LPS-inducible IFN-α production in wild-type mice, but IFN-γ −/− mice significantly underproduced IFN-α (*, p < 0.05). No significant difference (NS) was observed for anti-CD40-expanded IL-12 p70 production in these two mouse groups. B, Groups of five C3H/HeJ mice were pretreated with saline with or without LPS challenge. One group was anti-CD40 IgG pretreated (+) and LPS challenged. Two other groups of anti-CD40-injected mice were alternatively treated with 0.5 mg of neutralizing anti-IFN-α Ab. In contrast, neither the TLR2 agonist Pam3Cys nor a TLR9-active CpG oligonucleotide generated significant increases in circulating IFN-α in anti-CD40-pretreated mice (Table I), although the increase from PBS-pretreated control mice was only 5-fold for poly(I:C) due to higher levels of induction in control mice compared with LPS. Similar to results obtained using LPS, poly(I:C)-elicited IFN-α in anti-CD40-primed mice was dependent on IFN-γ activity, as demonstrated by a significant 56% reduction in cytokine release when anti-CD40 was administered with anti-IFN-γ Ab. In contrast, neither the TLR2 agonist Pam3Cys nor a TLR9-active CpG oligonucleotide generated significant increases in IFN-α production after anti-CD40 pretreatment relative to PBS-pretreated controls (<2-fold increase, p > 0.05). Anti-CD40 selectively expands LPS- and poly(I:C)-inducible IFN-α production by a common IFN-γ-dependent mechanism in vivo, but does not alter type I IFN synthetic capacity in response to at least two other heterologous TLR agonists.

IFN-γ treatment alone is insufficient to expand IFN-α synthetic capacity in vivo

To determine whether IFN-γ alone could substitute for anti-CD40 to increase LPS-inducible IFN-α production, groups of C3H/HeJ mice were pretreated once i.p. with either rat IgG or anti-CD40 or twice on successive days with 20 μg of rIFN-γ before challenge with LPS on the third day. Pretreatment with rIFN-γ was sufficient to increase LPS-induced serum levels of IL-18 at 3 h (Fig. 4C) and to increase TNF-α at 1.5 h relative to controls (1.3 ng ± 0.33 ng/ml compared with 0.12 ± 0.06 ng/ml; p < 0.05), however, relative to 100-fold increases in serum IFN-α present in anti-CD40-pretreated mice, rIFN-γ pretreatment only increased LPS-induced IFN-α levels ~4-fold over rat IgG-pretreated controls, an increase that was not statistically significant (p > 0.05). IFN-γ is therefore necessary, but not sufficient, to increase systemic IFN-α synthetic capacity after anti-CD40 pretreatment.

**Anti-CD40 pretreatment expands IFN-α production to poly(I:C), but not to Pam3Cys or CpG challenges**

We determined that CD40 pretreatment expanded IFN-α production in response to challenges with TLR agonists other than LPS. Both LPS and poly(I:C) challenges triggered similar levels of circulating IFN-α in anti-CD40-pretreated mice (Table I), although the increase from PBS-pretreated control mice was only 5-fold for poly(I:C) due to higher levels of induction in control mice compared with LPS. Similar to results obtained using LPS, poly(I:C)-elicited IFN-α in anti-CD40-primed mice was dependent on IFN-γ activity, as demonstrated by a significant 56% reduction in cytokine release when anti-CD40 was administered with anti-IFN-γ Ab. In contrast, neither the TLR2 agonist Pam3Cys nor a TLR9-active CpG oligonucleotide generated significant increases in IFN-α production after anti-CD40 pretreatment relative to PBS-pretreated controls (<2-fold increase, p > 0.05). Anti-CD40 selectively expands LPS- and poly(I:C)-inducible IFN-α production by a common IFN-γ-dependent mechanism in vivo, but does not alter type I IFN synthetic capacity in response to at least two other heterologous TLR agonists.

**Anti-CD40 expanded IFN-α production localizes to splenic CD11c+ cells**

IFN-α levels were assayed in homogenates of selected tissues obtained 2 h after LPS challenge of PBS- or anti-CD40-pretreated
mice. Anti-CD40-expanded IFN-α production was evident only in spleen tissue (Fig. 5, top panel). Based on this information, splenocytes from anti-CD40-pretreated mice were cultured with poly(I:C) and shown to produce 4-fold more IFN-α than did PBS-pretreated splenocytes (Fig. 5, middle panel). Unexpectedly, LPS did not stimulate IFN-α production in anti-CD40-pretreated splenocyte cultures and subsequent studies instead identified cellular sources of IFN-α using poly(I:C) as a mechanistically relevant substitute stimulus. In three studies, anti-CD40-pretreated splenocytes were depleted or enriched for CD11b+ or CD11c+ cells using Ab-coated magnetic beads and production of poly(I:C)-inducible IFN-α was shown to be segregated with the presence of CD11c+ cells (Fig. 5, bottom panel). IFN-α production did not sort according to enrichment for the NK cell marker DX5, ruling out CD11c+ NK cells as a source (data not shown). These findings were consistent with splenic dendritic cells as the source of anti-CD40-augmented IFN-α synthesis.

**Production of IFN-α by cultured dendritic cells is synergistically increased by combinations of IFN-γ and anti-CD40**

We tested whether IFN-γ and anti-CD40 were both required to expand poly(I:C)-inducible IFN-α production in dendritic cells obtained from Flt3L-expanded bone marrow cultures. Cells harvested from 10-day-old cultures contained ~60% myeloid (CD11c+/CD11b+) and 40% plasmacytoid (CD11c+low/CD11b+/B220+) dendritic cells, as originally described (24). Following a 4-h preincubation with medium, rIFN-γ, or anti-CD40 alone or rIFN-γ and anti-CD40 combined, the conditioned cells were stimulated with poly(I:C) for another 20 h. In five separate studies, poly(I:C) generated IFN-α levels that were highest in rIFN-γ/anti-CD40-preincubated cultures, at levels that were at least 6-fold greater than observed after either rIFN-γ or anti-CD40 preincubation (Fig. 6, top panels). LPS was an inconsistent stimulus for IFN-α in some studies, despite reproducibly triggering dendritic cell production of IL-12 p70 and IL-6. However, IFN-α-inducing activity was restored in subsequent studies using freshly prepared and sonicated LPS reagent (Fig. 6, bottom panel).

CpG proved to be a strong inducer of IFN-α in these cultures, showing similar levels of synergy for IFN-γ and anti-CD40. The pattern of cytokine induction for IL-12 p70 was also IFN-γ and CD40 synergetic, although induction was less restricted with regard to the specific TLR/pathogen-associated molecular pattern interaction, as LPS, peptidoglycan, R848, and CpG were all active stimuli. In contrast, the pattern of regulation over IL-6 production by IFN-γ and anti-CD40 was additive and all of the TLR agonists tested effectively induced IL-6 synthesis. These findings show an unexpected and strongly synergizing regulatory effect of IFN-γ and CD40 on the IFN-α- and IL-12 p70-productive capacity of dendritic cells that was not apparent for IL-6 production.

**Discussion**

Dendritic cells and macrophages constitutively express TLR that activate preprogrammed cytokine responses in response to specific sets of microbial molecules. The types and amounts of cytokine initiated by TLR activation are not fixed, but can be modified by the presence of costimulatory or cytokine signals. In this report, we confirm that the CD40L-mimetic Ab, F GK45, reprograms innate immunity for increased synthesis of IL-12 and IFN-γ in response to LPS, poly(I:C), and Pam3Cys. CD40-mediated expansion of innate immune function has been well-described, both in its use as a dendritic cell-maturational agent and as a signal synergizing with concurrent TLR engagement (16, 17). However, the central and novel findings of this study are that anti-CD40 also primes for a dramatic expansion of IFN-α production in response to either LPS or poly(I:C), that the reprogramming of IFN-α synthesis requires >6 h of exposure to anti-CD40 and that IFN-γ bioactivity present after anti-CD40 treatment is a necessary cofactor in up-regulation of IFN-α synthesis. Finally, we identify splenic dendritic cells as a probable source of increased IFN-α synthesis in vivo and confirm that anti-CD40 and rIFN-γ combine synergistically to dramatically increase IFN-α synthetic capacity in cultures of bone marrow-derived dendritic cell. Synergy was also observed for IL-12 p70 production, but not for IL-6 in response to the same TLR challenges, suggesting differing effects on distinct cytokine-specific synthetic pathways. On the basis of these findings, we propose that anti-CD40 promotes IFN-α synthesis both directly, by activating dendritic cell CD40, and indirectly, by triggering IFN-γ production during the requisite conditioning period. These findings are important in that they identify a new mechanism by which synergistic signals produced by activated T cells can regulate innate type I IFN responses.

To our knowledge, an IFN-γ-dependent mechanism for regulation of IFN-α synthesis has not been described. This may reflect the unusual context in which two distinct signals were required to achieve the regulatory effect. Although IFN-γ was essential for IFN-α up-regulation, treatment with recombinant mouse IFN-γ alone did not expand IFN-α synthetic capacity. We propose that anti-CD40 Ab and IFN-γ act together in support of high-level IFN-α synthesis in the in vivo model. Although anti-CD40 may
induce additional intermediary factors that critically interact with IFN-γ to alter cytokine synthetic capacity, this seems unlikely given the synergistic effects of rIFN-γ and anti-CD40 when they were directly applied to dendritic cell culture. We also show that IFN-γ synthesis after anti-CD40 pretreatment was necessary to alter IFN-α production. Serum levels of IFN-γ that peaked between 6 and 24 h after anti-CD40 injection were necessary for increased IFN-α synthesis, whereas IFN-γ produced after LPS challenge was not. This probably explains why only delayed, and not synchronous, LPS challenges demonstrated an increased IFN-α response. Separating the primary CD40 and secondary LPS challenge by 24 h also markedly increased serum IL-12 p70, IL-6, and IL-18 synthesis, although the role of IFN-γ as a critical anti-CD40-induced mediator of these responses requires further study.

The failure of simultaneous pretreatment with anti-CD40 and LPS to expand cytokine levels to a second LPS challenge 24 h later suggests that premature exposure to LPS had a dominant and negative effect on acquisition of new innate synthetic functions. This resembles the phenomenon of endotoxin tolerance. In this model, LPS causes macrophages to become unresponsive to subsequent TLR activation due to complex disruptions of signal transduction (28, 29). More specific to these studies, LPS separately depletes dendritic cells in multiple tissues, thereby removing a cell type critical to the systemic IL-12 p70 response (30). We previously showed that dendritic cell depletion by LPS prevented the cytokine-enhancing and dendritic cell preserving effects of CpG when the two TLR agonists were coadministered to mice (22). These new results reported here suggest that LPS-dominant and -negative regulatory effects similarly disrupt anti-CD40 expansion of cytokine synthesis by removing dendritic cells that are the source of anti-CD40-expanded IFN-α production.

The finding that dendritic cells were effective targets for synergistic IFN-γ/anti-CD40-conditioning defines a culture model in which the molecular mechanisms of synergy can be better defined. Although combined CD40L and CpG stimuli synergistically activate dendritic cell cytokine production in culture (31), we show that IFN-γ further amplifies cytokine synthetic capacity. Both myeloid and plasmacytoid dendritic cell subsets responded to the regulatory effects of anti-CD40 and IFN-γ. We first observed that superinduction of IFN-α in anti-CD40-pretreated mice was restricted to poly(I:C) and LPS challenges and depended on autoinduction through the type I IFNR. This was most consistent with a myeloid dendritic cell target. However, CpG-induced IFN-α was also markedly increased by anti-CD40/rIFN-γ in Flt3L-expanded dendritic cell culture, which is known to contain both myeloid and plasmacytoid dendritic cells (23). The lack of CpG-inducible IFN-α in anti-CD40-pretreated mice, relative to LPS or poly(I:C) stimuli, probably reflects the much decreased representation of plasmacytoid dendritic cells in C57BL/6 spleen compared with Flt3L-derived bone marrow dendritic cell cultures (32) or may indicate that there are additional cell populations in mouse tissue responding to TLR4 and TLR3 signals. The similar ability of IFN-γ and anti-CD40 to superinduce IFN-α by either MyD88-independent (LPS- and poly(I:C)) or by MyD88-dependent (CpG) stimuli suggests a regulatory mechanism that either nonspecifically increases TLR expression or that acts distal to the MyD88-distinct portions of TLR-induced signal transduction. Although IFN-γ is known to up-regulate TLR4 expression as a mechanism for increased cytokine synthesis (33), levels of splenic mRNA for TLR2, TLR4, and TLR9 were either unchanged or reduced at 24 h after anti-CD40 treatment. Furthermore, surface expression of TLR2 on splenic macrophages was unchanged by anti-CD40 pretreatment (data not shown). An alternative possibility is that activation of two distinct receptor families by IFN-γ and anti-CD40

![FIGURE 5. Anti-CD40-enhanced production of IFN-α localizes to spleen and segregates with CD11c+ cell populations. Top panel. Groups of three C3H/HeN mice were pretreated with rat IgG or with anti-CD40 followed by either PBS or LPS challenge. Two hours after LPS challenge, the indicated tissues were harvested, pooled, homogenized in the presence of protease inhibitors, and the clarified homogenates were assayed for IFN-α by ELISA. Shown are the means of duplicate assays for each tissue. Middle panel. Cultures of spleen cells from PBS or anti-CD40-pretreated C3H/HeN mice were incubated with increasing concentrations of poly(I:C) for 24 h and IFN-α was assayed. Bottom panel. Anti-CD40-pretreated spleen cells were depleted or enriched for CD11c and CD11b cells using magnetic beads; the eluted cells were adjusted to 10^7 cells/ml and incubated 24 h with medium alone or medium containing 30 μg/ml poly(I:C) as indicated in the legend. Shown are the mean concentrations of IFN-α in picograms per milliliter from triplicate assays. Relative to preselected control cells, the amounts of poly(I:C)-induced IFN-α in CD11c-depleted cultures were significantly reduced (\*, \( p < 0.05 \)) whereas IFN-α produced in CD11c-enriched cultures were significantly increased (†, \( p < 0.05 \)). Depletion with anti-CD11b beads or sham depletion with anti-human CD56 beads did not significantly affect IFN-α production relative to preselection control cultures.](http://www.jimmunol.org/content/images/full/6001fig5.jpg)
results in complementary signal cascades that synergistically enhance IFN-α production at the level of transcriptional regulation. A similar explanation has been proposed for recent observations of synergy between different TLR for cytokine production (34).

In summary, we report the novel finding that anti-CD40 pre-treatment promotes an IFN-γ-dependent up-regulation of LPS-induced IFN-α synthesis in amounts that approach those obtained in response to live viral infection (4). We propose that activation of CD40 on dendritic cells results in low-level synthesis of IFN-γ in vivo that, in combination with direct effects of anti-CD40, induced IFN-α panels, C57BL/6 marrow-derived dendritic cells were obtained from 10-day cultures and incubated for 4 h in medium, or medium containing 10 ng/ml rIFN-γ, 10 μg/ml anti-CD40 or both, as indicated by the legend. At 4 h, TLR agonists were added as indicated on the ordinate; medium: poly(I:C) at 30 μg/ml final concentration, LPS at 100 ng/ml, peptidoglycan (PGN) at 100 ng/ml, R848 at 5 μg/ml, or CpG ODN at 10 μg/ml. Supernatants were obtained after another 20 h of culture and assayed for IFN-α, IL-12 p70, and IL-6 as indicated on the abscissa. Asterisks (*) by the combined IFN-γ-anti-CD40 culture bar indicate a significant increase in cytokine concentration compared with the other three culture preconditions. For IFN-α and IL-12 p70, combined preconditioning resulted in cytokine concentrations that were synergistic, defined as being at least two times greater than the sum of concentrations present in IFN-γ and anti-CD40-preconditioned cells for the same stimulus. IL-6 production was additive for combined-compared with singly preconditioned cells. Bottom panel, Repeat study showing synergistic effects of anti-CD40 and rIFN-γ on LPS- and poly(I:C)-induced IFN-α production by bone marrow-derived dendritic cells. LPS was freshly prepared and sonicated before culture. Brackets (**) indicate significant increases in IFN-α for the indicated comparisons between anti-CD40/rIFN-γ-preincubated cells (p < 0.05). *, significant increases comparing anti-CD40/rIFN-γ-preincubated cells compared with cells incubate singly with media, rIFN-γ, or anti-CD40 (p < 0.01).

Acknowledgments

We thank Robert Fairchild for his provision of IFN-α/IFN-βR KO mice and Donald Anthony, Eric Pearlman, and Christopher King for critical reading and discussion of the manuscript. We also gratefully acknowledge Gopal Yadavalli and Lopamudra Das for valuable discussions.

Disclosures

The authors have no financial conflict of interest.

References


