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J Immunol 2005;175;421-432

The Journal of Immunology is published twice each month by
The American Association of Immunologists, Inc.,
9650 Rockville Pike, Bethesda, MD 20814-3994.

Print ISSN: 0022-1767 Online ISSN: 1550-6606.

Listeria-Infected Myeloid Dendritic Cells Produce IFN-β, Priming T Cell Activation[1]

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The intracellular bacterium *Listeria monocytogenes* infects dendritic cells (DC) and other APCs and induces potent cell-mediated protective immunity. However, heat-killed bacteria fail to do so. This study explored whether DC differentially respond to live and killed *Listeria* and how this affects T cell activation. To control for bacterial number, a replication-deficient strain, *Lmdd*, defective in D-alanine biosynthesis, was used. We found that DC internalize both live and heat-killed *Lmdd* and similarly up-regulate the expression of costimulatory molecules, a necessary step for T cell activation. However, only live *Lmdd*-infected DC stimulate T cells to express the early activation marker CD69 and enhance T cell activation upon TCR engagement. Infection with live, but not heat-killed, *Lmdd* induces myeloid DC to secrete copious amounts of IFN-β, which requires bacterial cytosolic invasion. Exposure to high concentrations of IFN-β sensitizes naive T cells for Ag-dependent activation. *The Journal of Immunology,* 2005, 175: 421–432.

The intracellular, Gram-positive bacterium *Listeria monocytogenes* (*Lm*)[4] induces such a potent T cell-mediated immune response that it is one of the main models for studying T cell immunity in mice and is being developed as a vaccine vector to deliver Ags derived from infectious agents or tumors (1–4). One of the reasons for the powerful immune response to *Lm* may be that *Lm* infects professional APCs: monocytes, tissue macrophages, and dendritic cells (DC). DC play a pivotal role in directing T cell responses. To activate naive T cells, DC must undergo a maturation process, in which DC up-regulate the expression of MHC, CD40, CD80, and CD86 surface molecules and cytokines. DC maturation can be induced by a variety of stimuli, including ligation of TLR, which recognize molecular patterns of infectious agents (5–7). TLR2 on DC binds to the *Lm* cell wall components, lipotechoic acid, and peptidoglycan (8), and TLR9 recognizes bacterial unmethylated CpG DNA (9).

DC are heterogeneous, and involvement of different DC subpopulations may dictate the outcome of subsequent immune responses. $CD11c^+$ DC that also express CD11b, but not CD8α, are termed myeloid DC (mDC). A second subset, plasmacytoid DC (pDC) (10), lack CD11b, but express B220, and are thought to be the major source of the type I IFNs in vivo (11). However, a recent report suggests that under certain conditions, viruses, such as lymphocytic choriomeningitis virus or influenza, can induce the production of high levels of type I IFN by non-pDC (12).

Immunization with killed *Lm* does not induce protective immunity (13). Similarly, adoptive transfer of DC infected with live *Lm*, but not with heat-killed (HK) *Lm*, protects against subsequent *Lm* infection (14). This is despite the fact that HK bacteria express a broad spectrum of immunostimulatory molecules capable of binding and activating TLRs. Why HK *Lm* fail to induce protective immunity remains unknown. Production of cytokines, such as IFN-γ and IL-12, or CD40 signaling may be important in inducing protective immunity after live *Lm* infection (15–17). Although immunization with HK *Lm* primes memory *Lm*-specific CD8 T cells, it does not induce them to differentiate into effector T cells and is much less efficient at activating their clonal expansion (18). The underlying mechanism for this differential effect remains to be defined. Although the effect of *Lm* infection on macrophages has been studied extensively (19–22), few efforts have focused on how DC respond to *Lm* infection (23).

Because DC are believed to be the key professional APC capable of priming naive T cells (24), we compared the effects of treatment with live and HK *Lm* on mouse mDC. To minimize possible differential effects that might be due to increased numbers of live bacteria, an attenuated nonreplicating strain, *Lmdd*, deficient in D-alanine (D-Ala) biosynthesis (25), was used in this study. *Lmdd* does not replicate in the absence of exogenous D-Ala, but is nonetheless able to stimulate T cell immunity if D-Ala is provided during inoculation (25, 26). Both HK and live *Lmdd* similarly activate DC to up-regulate costimulatory molecules and secrete most cytokines. However, supernatants derived from DC cultured with live *Lmdd*, but not HK *Lmdd*, activated polyclonal T cells via a rapidly produced soluble factor independently of MHC-TCR ligation. This soluble factor was identified as IFN-β. Microarray analysis, comparing genes expressed by DC treated with either live or HK *Lmdd*, and intracellular cytokine staining confirmed the induction of type I IFNs only by live *Lmdd*-treated DC. IFN-β acts as a T cell commitment factor; it significantly decreases the dose-response threshold of naive T cells for subsequent activation by the TCR and enhances T cell priming. Using mutant bacteria unable to escape the phagolysosome, we found that cytosolic invasion is required to induce mDC to produce IFN-β.

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Received for publication January 7, 2005. Accepted for publication April 29, 2005.

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[1] This work was supported by National Institutes of Health Grant AI056922 (to J.L.) and T32HL066987 (to A.S.).

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[4] Abbreviations used in this paper: *Lm*, *Listeria monocytogenes*; DC, dendritic cell; BMDC, bone marrow-derived DC; D-Ala, D-alanine; HK, heat killed; IPTG, isopropyl-β-D-thiogalactoside; IRF, IFN-regulated factor; LLO, listeriolysin O; mDC, myeloid DC; MFI, mean fluorescence intensity; pDC, plasmacytoid DC.

0022-1767/05/$02.00

Materials and Methods

Mice and cells

Wild-type BALB/c and C57BL/6, and β_2-microglobulin$^{-/-}$, CIITA$^{-/-}$ mice in the H-2^b background were obtained from The Jackson Laboratory. OT-1 mice (27) were provided by H. Eisen (Massachusetts Institute of Technology Center for Cancer Research, Cambridge, MA). MyD88$^{-/-}$ mice (28) were provided by M. Boes (Harvard Medical School, Boston, MA). Mouse bone marrow-derived DC (BMDC) were generated as previously described (29) by culture in GM-CSF and IL-4, followed by positive immunomagnetic selection using CD11c Ab-coated microbeads (Miltenyi Biotec). The selected BMDC were >98% CD11c$^+$ DC by flow cytometric analysis. These DC are mDC and CD11c$^+$ and B220$^-$, and express high levels of CD11b and MHC class II and moderate levels of CD80 and CD86 (see Fig. 1*A*; data not shown). The pDC were generated by culturing mouse bone marrow cells in Flt3 ligand-conditioned medium for 9 days. The macrophage cell line RAW264.7 was obtained from American Type Culture Collection, and the mDC cell line DC2.4 (30) was provided by K. Rock (University of Massachusetts Medical School, Worcester, MA). Cells were cultured in RPMI 1640 medium containing 2 mM L-glutamine, 100 U/ml penicillin G, 50 μg/ml streptomycin sulfate, 50 μM 2-ME, and 10% FBS, unless otherwise indicated.

Bacteria

L. monocytogenes strain *Lmdd* was provided by F. Frankel (University of Pennsylvania, Philadelphia, PA) (25). DP-L3885 (inducible listeriolysin O (LLO)), DP-L1942 (*Act-A*$^-$), and DP-L2612 (*hly*$^-$) strains were provided by D. Higgins (Harvard Medical School, Boston, MA) (31, 32). Bacteria were grown in brain-heart infusion medium (BD Biosciences) supplemented with 100 μg/ml D-Ala and washed to remove D-Ala before use. HK bacteria were prepared by treatment at 60°C for 1 h. In some experiments, *Lmdd* were killed by treatment with 100 μg/ml gentamicin for 1 h, by sonication (10^{10} CFU/ml bacteria in PBS were sonicated three times for 20 s each time at 4°C; Heat Systems), or by physical lysis using 425- to 600-μm diameter glass beads (Sigma-Aldrich). For CFSE (Molecular Probes) staining, *Lmdd* were incubated with 1.5 μM CFSE in PBS at room temperature for 15 min, followed by three washes. Cells were infected with 5–10 CFU of bacteria/cell. *Lm*-treated cells were added to T cells 4 h after culture at 37°C.

Generation of culture supernatants

Supernatants harvested at the indicated time or 12 h after *Lmdd* treatment from DC plated at 10^6 cells/ml in serum-free RPMI 1640 were passed through a 0.2-μm pore size filter and stored at −80°C before use. In some experiments, *Lmdd*-infected cells were treated with the indicated concentration of cycloheximide 1 h after infection, then cultured overnight before harvesting supernatants. Supernatants were desalted using Econo-Pac desalting columns (Bio-Rad) to remove cycloheximide before addition to T cell cultures.

Microarray assay and RT-PCR

On day 7 of culture, mouse BMDC were enriched using anti-CD11c-conjugated magnetic beads and treated with medium, LPS (1 μg/ml), 10 CFU of HK or live *Lmdd*/cell for 6 h before extracting total RNA using the Qiagen RNeasy Mini kit. Approximately 10 μg of each sample was used to make labeled probes and was prepared for hybridization to mouse expression set A and B oligonucleotide arrays (Affymetrix), performed at the Microarray Core facility, Dana-Farber Cancer Institute. RNA was extracted and analyzed as described previously (33) using samples from two independent experiments. For RT-PCR, 1 μg of total RNA was used to generate cDNA using the TaqMan RT kit (Applied Biosystems). IFN-β and β-actin primers were previously described (34): *ifnb*: forward, 5′-ctggagcagctgaatggaaag; reverse, 5′-cttgaagtccgccctgtaggt; and *β-actin*: forward, 5′-aggtgtgatggtgggaatgg; reverse, 5′-gcctcgtcacccacatagga. Two-step PCR was performed for 35 cycles at 95°C for 10 s and 62°C for 15 s.

Chromatography and mass spectrometry analysis

Supernatants derived from *Lmdd*-infected BMDC or DC2.4 cells cultured in serum-free RPMI 1640 were harvested, passed through a 0.2-μm pore size filter, concentrated, and separated on a Superdex-200 column. The apparent m.w. of fractions active in inducing CD69 expression on naive C57BL/6 splenocytes were determined by comparing their migrations with those of m.w. standards. Approximately 20 L of serum-free RPMI 1640 supernatant derived from *Lmdd*-infected DC2.4 cells was separated by sequential High S (Bio-Rad), heparin, hydroxyapatite, and Blue gel (Amersham Biosciences) chromatography. Active fractions were pooled, subjected to tryptic digestion, and analyzed by MALDI-TOF mass spectrometry at the Harvard Medical School Mass Spectoscopy Core facility.

Flow cytometry

Flow cytometry was performed using FACSCalibur and CellQuest software (BD Biosciences) with fluorophore-conjugated Abs to IL-6 (MP5-20F3), IL-12 (C15.6), TNF-α (MP6-XT3), I-A^b (AF6-120.1), CD11b (M1/70), CD11c (HL3), CD4 (GK1.5), CD8α (53-6.7), CD25 (clone PC61), CD40 (3/23), CD43 (1B11), CD69 (H1.2F3), CD62L (MEL-14), CD80 (16-10A1), and CD86 (GL1) from BD Pharmingen. Rat anti-mouse IFN mAb (F18) was purchased from Hycult Biotechnology. Rabbit anti-mouse polyclonal Abs against IFN-α and IFN-β were obtained from PBL Laboratory. For external staining, 2×10^5 cells/microtiter well were washed with FACS buffer (PBS containing 2% heat-inactivated FBS and 0.1% sodium azide) and incubated with an FcR-blocking Ab (BD Pharmingen) for 5 min, then incubated with saturating amounts of mAbs for 30 min at 4°C. For intracellular cytokine staining, cells were treated with 20 μM brefeldin A for the last 12 h of culture or as indicated, resuspended in 50 μl of FACS buffer, and permeabilized using the Fix and Perm kit according to the manufacturer's protocol (Caltag Laboratories). Saturating amounts of fluorochrome-conjugated Abs were added to the permeabilization buffer, and cells were incubated at room temperature for 15 min. Cells were washed and resuspended in FACS buffer with 1% formaldehyde for analysis.

T cell proliferation and ELISPOT assays

Splenocytes from naive C57BL/6 mice or CD8 T cells from OT-1 transgenic mice were harvested and exposed to 1000 U/ml or the indicated concentration of mouse rIFN-α (1.1×10^8 U/mg; PBL Laboratory) or IFN-β (1.2×10^7 U/mg; PBL Laboratory) or supernatant derived from *Lmdd*-infected DC for 3 h and washed. Splenocytes were cultured with the indicated concentrations of anti-CD3ϵ (BD Pharmingen). OT-1 CD8 T cells were cultured with irradiated splenocytes and the indicated concentrations of the cognate OVA peptide SIIFNEKL. Alternatively, splenocytes were directly cultured with the indicated concentration of anti-CD3ϵ in the presence of IFN-β or DC supernatant. T cell proliferation was measured by [^{3}H]thymidine incorporation. IFN-γ production was measured by ELISPOT, as previously described (35), using an immunospot counter (Cellular Technology).

Results

Both HK and live Lmdd *are similarly taken up by DC and induce DC maturation*

To begin to define the differences in the immune-stimulating effects of live or HK *Lmdd*, we analyzed the responses of mouse

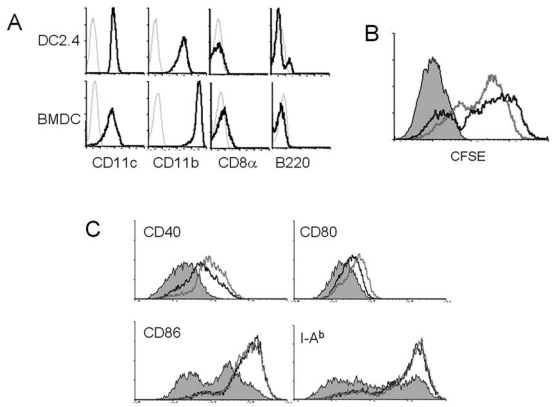

FIGURE 1. Both live and HK *Lmdd* are internalized by BMDC and induce DC maturation. *A*, DC2.4 and BMDC (generated by culture in GM-CSF and IL-4) are mDC. After 7-day culture, BMDC were harvested and enriched using anti-CD11c microbeads. DC2.4 and BMDC were stained with isotype control Ab (gray lines) or the indicated Abs (black lines). *B* and *C*, BMDC were pulsed with live (gray line) or HK (black line) *Lmdd* or cultured without treatment (filled gray histogram) for 5 h (*B*) or 24 h (*C*). *B*, Bacteria were stained with CFSE before incubation with BMDC and analyzed for fluorescence by flow cytometry; *C*, cells were analyzed for cell surface expression of the indicated activation markers.

BMDC and the DC cell line DC2.4 to live and HK *Lmdd*. Both BMDC and DC2.4 cells are myeloid lineage cells expressing CD11c and CD11b, but not B220 or CD8α (Fig. 1*A*). BMDC internalized both live and HK *Lmdd*. The number of internalized bacteria was similar 5 h after adding live or HK bacteria, as quantified by flow cytometry (mean fluorescence intensity (MFI), 798 HK vs 771 live *Lmdd*; Fig. 1*B*). Actin tails costained with CFSE-labeled bacteria were only evident in live *Lmdd*-infected DC (data not shown), suggesting that live *Lmdd* escape from the vacuole to the cytosol, whereas HK *Lmdd* remain in the vacuole (20). BMDC (Fig. 1*C*) and DC2.4 cells (data not shown) exposed to live or HK *Lmdd* for 24 h up-regulated the surface expression of molecules involved in T cell activation, such as CD40, CD80 (B7.1), CD86 (B7.2), and MHC class II (Fig. 1*C*). Although CD86 and the MHC class II molecule I-A^b were up-regulated to a similar extent, CD40 and B7.1 expression were slightly higher in live *Lmdd*-treated DC (CD40: MFI, 129 live vs 112 HK; CD80: MFI, 105 live vs 91 HK). Thus, both live and HK *Lmdd* were similarly internalized by DC, and both induced costimulatory molecules, with only subtle differences in magnitude.

HK and live Lmdd*-treated DC differ in the ability to activate T cells*

We next examined whether DC pulsed with live or HK *Lmdd* differentially activate T cells. Live bacteria-pulsed BMDC rapidly stimulated both CD4 and CD8 splenic T cells to express the early activation marker CD69, whereas HK *Lmdd*-treated DC had less of an effect (Fig. 2*A*). The reduced ability of HK *Lmdd* to activate DC capable of inducing CD69 expression on T cells was not due to heat inactivation of bacterial Ags or adjuvants, because *Lmdd* killed by other means (gentamicin, ultrasound, or glass beads) also did not efficiently activate DC (Fig. 2*A*). The *Lmdd*-infected DC2.4 DC line was also able to activate T cells to express CD69, whereas the infected RAW264.7 macrophage cell line had limited ability (Fig. 2*A*). We next examined whether T cells exposed to *Lmdd*-infected DC were fully activated. When CFSE-labeled CD8 T cells were cocultured with *Lmdd*-infected DC, they did not proliferate (Fig. 2, *B* and *C*), as determined by CFSE dilution. T cell activation markers, such as CD25 and CD43, were not up-regulated by 48 h (Fig. 2*C*), although CD62L expression was slightly down-regulated in T cells that had been cocultured with live *Lmdd*-pulsed DC (Fig. 2*C*). T cells did not

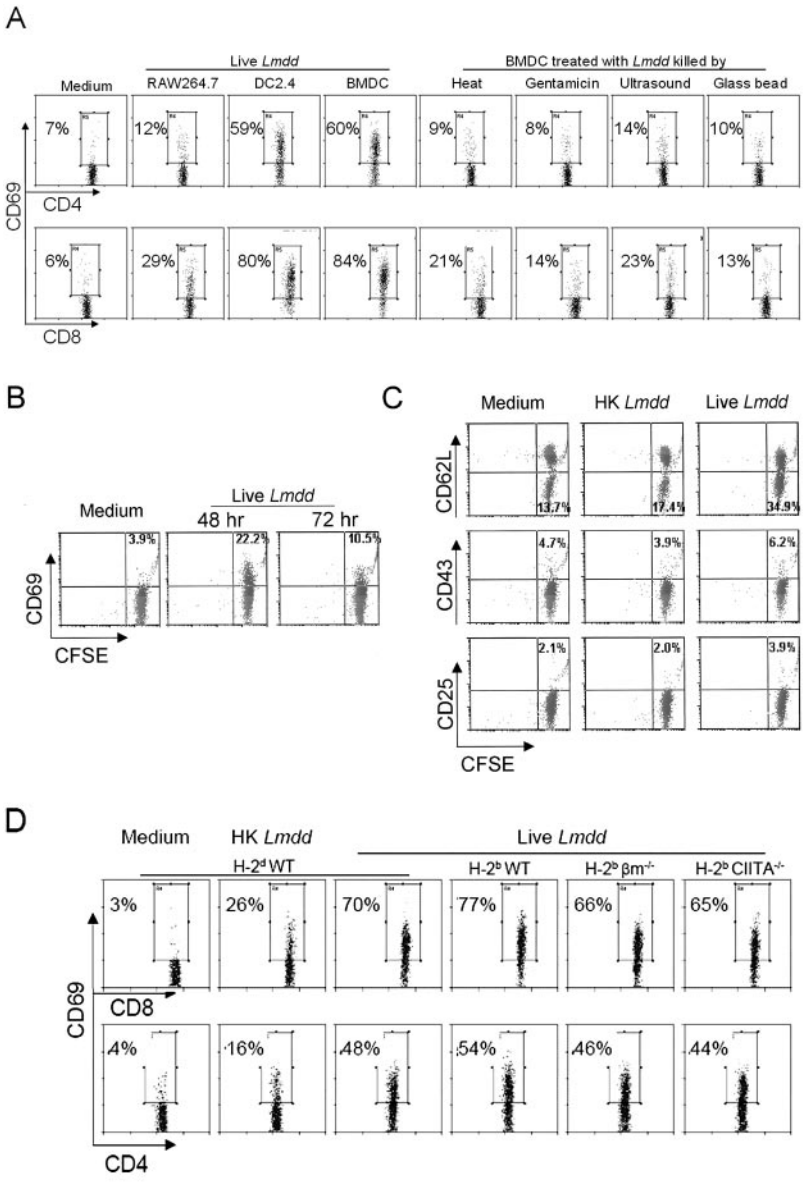

FIGURE 2. Live, but not dead, *Lmdd*-treated DC induce partial T cell activation, which is independent of TCR-MHC engagement. *A*, Live, but not dead, *Lmdd*-treated BMDC and DC2.4 cells activate CD4 and CD8 T cells to express CD69. $CD11c^+$ BMDC, RAW264.7 cells, or DC2.4 cells were treated with medium or live or killed *Lmdd* for 4 h, then cocultured with syngeneic splenic T cells overnight. CD69 expression by T cells was measured by flow cytometry on gated CD4 and CD8 T cells. *B* and *C*, Exposure to live *Lmdd*-infected BMDC activates CD69 expression and partial CD62L down-modulation on CD8 T cells, but does not trigger proliferation or expression of CD25 or CD43. CD8 T cells from naive mouse spleen were stained with CFSE, then cultured in medium or with bacteria-treated $CD11c^+$ DC. After 12 h, DC were removed, and T cells were cultured for another 48 h (*C*) or the indicated time (*B*). CD69 (*B*), CD25, CD43, and CD62L (*C*) expression on CD8 T cells was examined by flow cytometry. A positive control (*B*) for CFSE dilution shows reduced CFSE staining of CD8 T cells 72 h after exposure to anti-CD3ϵ. *D*, CD69 expression by CD4 and CD8 T cells in response to live *Lmdd*-infected DC occurs independently of TCR engagement, because allogeneic, class I- and class II-deficient BMDC are as effective as syngeneic wild-type BMDC. Naive H-2^b mice splenocyte-derived T cells were cocultured overnight with medium or HK or live *Lmdd*-treated BMDC from the indicated background mice. Surface CD69 expression on CD4 or CD8 T cells was determined by flow cytometry.

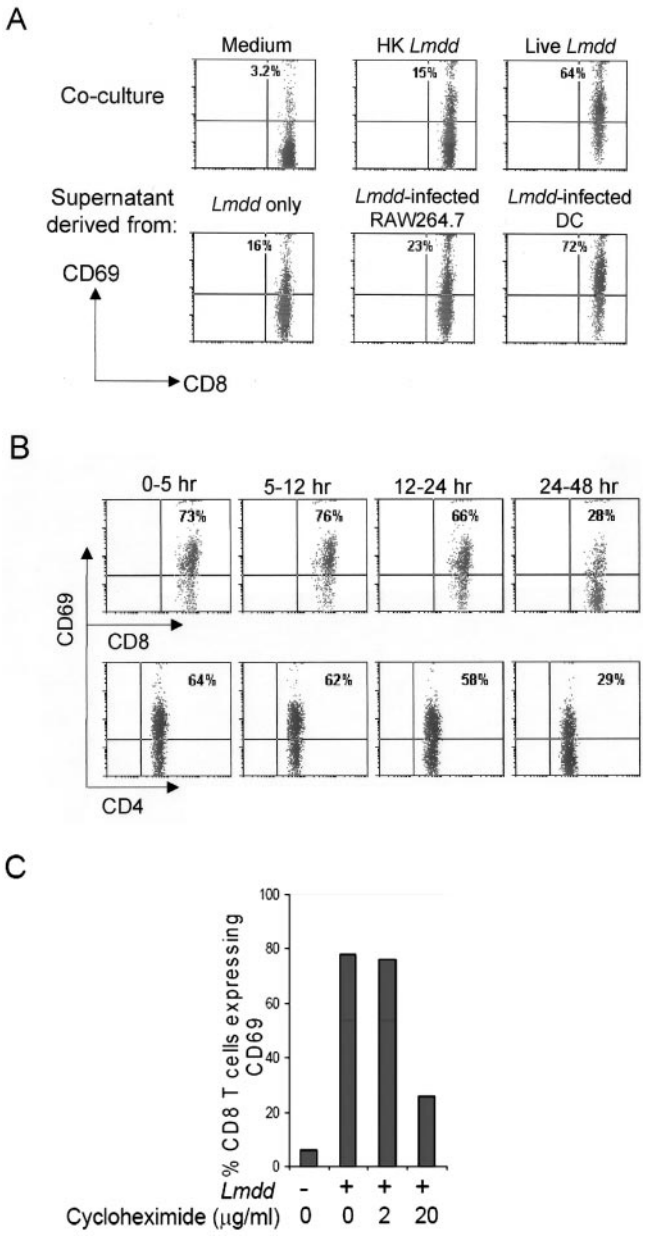

FIGURE 3. Newly synthesized soluble molecules secreted by live *Lmdd*-infected DC mediate T cell activation. *A*, Culture supernatant from BMDC infected with live *Lmdd* was as effective at activating CD69 expression on CD8 T cells as coculture with infected BMDC cells. T cells were cultured for 12 h with culture supernatant or *Lmdd*-infected BMDC before flow cytometric analysis of CD69 expression on T cells (*upper panel*). However, supernatant from live *Lmdd*-infected RAW264.7 macrophage cell line does not efficiently stimulate CD69 expression. *B*, The CD69-inducing soluble factor is rapidly secreted after *Lmdd* infection of BMDC. Supernatants were harvested from live *Lmdd*-infected BMDC at the indicated times, added to splenic T cells and cultured overnight. *C*, Secretion of the CD69-inducing soluble factor requires de novo protein synthesis. BMDC were treated with medium or live *Lmdd* in the presence of the indicated concentration of cycloheximide overnight. Desalted culture supernatants were then added to T cells. CD69 expression was examined by flow cytometry 12 h later.

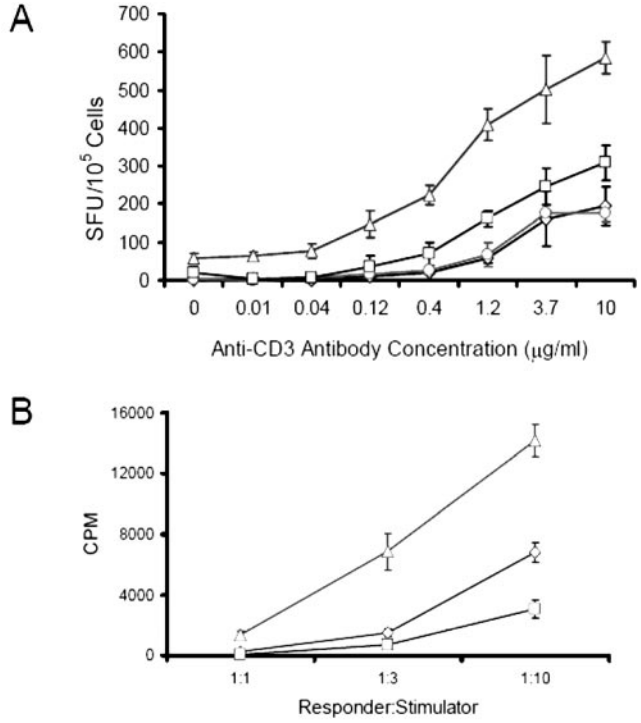

FIGURE 4. Live *Lmdd*-infected DC culture supernatants enhance Ag-dependent T cell activation. *A*, Splenocytes from naive mice were stimulated with the indicated concentration of anti-CD3ϵ in the absence (◇) or the presence of supernatants derived from untreated (○), HK (□), or live (△) *Lmdd*-treated BMDC, and the frequency of IFN-γ-producing cells was measured by ELISPOT. *B*, Supernatant from live *Lmdd*-infected BMDC enhances an allogeneic response. H-2^b splenocytes were exposed to *Lmdd*-infected BMDC supernatant or medium (□) for 3 h and either washed (◇) or not (△) before adding irradiated H-2^d splenocytes at the indicated ratio. Lymphocyte proliferation was determined by [^{3}H]thymidine incorporation 3 days later. T cell proliferation is enhanced more if cells remain exposed to the *Lmdd*-infected BMDC conditioned medium before and after encountering Ag.

produce IL-2, as determined by intracellular cytokine staining (data not shown). Therefore, T cell activation by *Lmdd*-infected DC was partial.

Because *Lmdd* infection introduces a great deal of foreign Ags and triggers DC maturation (Fig. 1), we examined whether activation of CD69 expression was dependent upon TCR engagement. T cells were cocultured with *Lmdd*-treated BMDC derived from MHC class I- or class II-null mice. CD69 expression on T cells was comparable after stimulation with wild-type or MHC-null DC (Fig. 2*D*). It was also comparable when T cells were cultured with *Lmdd*-infected allogeneic BMDC or syngeneic BMDC (Fig. 2*D*). These data suggest that live *Lmdd*-infected DC partially activate T cells through a TCR-independent process.

T cell activation by live Lmdd-*infected DC is mediated by a secreted soluble factor(s)*

Because the activation of T cells by live *Lmdd*-infected DC is independent of TCR-MHC engagement, we next determined whether it required cell-to-cell contact or was mediated by a soluble factor. Culture supernatant from live, but not HK, *Lmdd*-treated DC was fully able to induce CD69 expression on T cells (Fig. 3*A*). Supernatants derived from bacterial cultures without DC or from *Lmdd*-infected RAW246.7 macrophage cells had little capacity to activate CD69 expression (Fig. 3*A*), suggesting that the molecules that activate T cells were not produced by bacteria, but were secreted by DC after *Lmdd* infection. Secretion occurred rapidly within 5 h of infection, reached a peak by ~12 h, and gradually declined after 20 h of culture (Fig. 3*B*). This molecule(s) was synthesized de novo, because treating DC with cycloheximide almost completely inhibited its production (Fig. 3*C*). Cycloheximide

treatment did not affect cell viability, as assessed by forward and side scatter flow cytometry profiles, even at the highest concentration (data not shown).

Live Lmdd-*infected DC supernatant enhance Ag-dependent T cell activation*

To explore the effects of the soluble factors secreted by *Lmdd*-infected DC on Ag-dependent T cell activation, splenocytes from naive mice were pulsed with anti-CD3ϵ Ab in the presence or the absence of DC culture supernatant. *Lmdd*-treated DC supernatant significantly enhanced the numbers of T cells secreting IFN-γ after anti-CD3 treatment (Fig. 4*A*). At limiting concentrations of anti-CD3, the dose-response curve was shifted by about 1 log to the left in the presence of *Lmdd*-treated DC supernatant. HK *Lmdd*-treated DC supernatant slightly enhanced T cell activation, whereas supernatant from uninfected DC had no effect on promoting Ag-dependent T cell activation (Fig. 4*A*). In addition, T cell proliferation in response to stimulation by allogeneic splenocytes was significantly enhanced when T cells were continuously cultured in supernatant from *Lmdd*-infected DC (Fig. 4*B*). Pre-exposure of T cells to *Lmdd*-infected DC supernatant also improved T cell proliferation in response to allogeneic splenocytes (Fig. 4*B*), suggesting that the soluble factors secreted by live *Lmdd*-infected DC sensitized T cells for subsequent Ag-dependent activation. The continuous presence of supernatant, however, induced more proliferation than when T cells were washed before exposure to allogeneic splenocytes (Fig. 4*B*).

IFN-β expression is induced by live, but not HK, Lmdd-*treated DC*

To identify the secreted molecules produced by *Lmdd*-infected DC that activate T cells, we looked for differential production of proinflammatory cytokines, such as IL-12, IL-6, and TNF-α, by treated DC (23). HK or live *Lmdd* infection led to a comparable increase in IL-6 production (Fig. 5*A*). IL-12 and TNF-α production by live

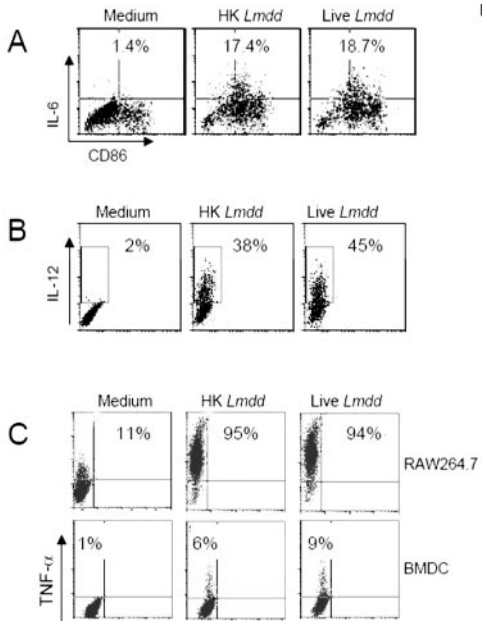

FIGURE 5. Live and HK *Lmdd* comparably induce DC to secrete IL-6, IL-12, and TNF-α. BMDC (*A–C*) or RAW264.7 (*C*) cells were untreated or treated with HK or live *Lmdd* for 16 h, then permeabilized and stained with the indicated Abs for flow cytometric analysis of intracellular cytokines. *B* and *C*, Analysis was performed on gated CD11c$^+$ cells. Brefeldin A was added for the last 12 h of culture.

Lmdd-treated DC was slightly higher than that by HK *Lmdd*-treated DC (Fig. 5, *B* and *C*, and data not shown). However, *Lmdd*-infected DC secreted much less TNF-α than infected RAW264.7 cells (Fig. 5*C*). Although *Escherichia coli*-infected DC have been reported to produce IL-2 (36), no IL-2 was induced after *Lmdd* infection (data not shown). Because the production of these cytokines was similar after HK or live *Lmdd* infection, none of these cytokines is probably responsible for the difference in T cell activation.

To identify the soluble DC factor capable of inducing CD69 expression and sensitizing T cell activation, *Lmdd*-infected DC supernatant was separated by chromatography, testing fractions for their ability to up-regulate CD69 expression on T cells. The apparent M_r of the soluble factor was determined by Superdex 200 gel filtration to be ~24–28 kDa (data not shown). *Lmdd*-infected DC supernatant was separated through sequential cation exchange High S, heparin, hydroxyapatite, and Blue gel columns. The final active fraction was subjected to tryptic digestion and MALDI-TOF mass spectroscopy analysis, and four peptides with sequences matching IFN-β were identified (data not shown). This suggested that IFN-β might be the soluble factor responsible for CD69 up-regulation. This result was unexpected, because IFN-β is thought to be produced mostly by pDC.

To determine whether IFN-β is the sought-after soluble factor produced by *Lmdd*-infected DC, we first looked by microarray analysis at whether mDC differentially up-regulate IFN-β mRNA expression after *Lm* infection (Table I). CD11c$^+$ BMDC were treated for 6 h with medium, LPS, or HK or live *Lmdd* before isolating mRNA. LPS-treated DC had limited capacity to activate T cells to express CD69 (data not shown). Twenty-seven genes were up-regulated by at least 10-fold after live *Lmdd* infection compared with untreated DC. Sixteen of these were also up-regulated at least 10-fold by treatment with HK *Lmdd*. The gene that showed the greatest modulation, however, was IFN-β, whose expression was increased 67-fold. Moreover, IFN-β expression was not enhanced by HK *Lmdd* and was only enhanced 4-fold by LPS. By semiquantitative RT-PCR, no IFN-β mRNA was detected in untreated BMDC, but expression was up-regulated in *Lmdd*-infected BMDC (Fig. 6*C*). Samples from LPS- and HK *Lmdd*-treated DC had detectable IFN-β expression, but substantially less than samples from *Lmdd*-infected DC. These data were also confirmed by quantitative real-time PCR (data not shown). Other genes that were up-regulated differentially by live *Lmdd* compared with both HK *Lmdd* and LPS were IFN-α genes 2 (increased 19-fold) and 5 (increased 8-fold). IFN-γ expression was up-regulated in BMDC to a similar extent by live *Lmdd* (6-fold) or LPS (8-fold), but only 2-fold by HK *Lmdd* treatment. In addition, a number of IFN-inducible genes and cytokine and chemokine genes and receptors as well as some genes with unknown function were up-regulated substantially by 6 h, but they were also up-regulated in HK *Lmdd*-treated and/or LPS-treated DC (Table I). The inducible IFN-regulated factor 7 (IRF7) gene was significantly up-regulated by treatment with live *Lmdd* (13-fold) or LPS (15-fold) and less so by HK *Lmdd* (4.5-fold; Table I), indicating that the positive feedback loop of type I IFN gene expression was rapidly activated by all these treatments (37, 38). The low levels of IFNs induced by LPS or HK *Lmdd* appeared to be sufficient to activate a wide array of IFN-inducible genes. However, although IRF3 mRNA remained unchanged, IRF3 protein translocated from the cytoplasm to the nucleus only in DC treated with live *Lmdd*, as determined by subcellular fractionation and immunoblotting (data not shown). IRF3 nuclear translocation is the key to inducing IFN-β expression (39).

To determine whether IFN-β protein is expressed within 6 h of *Lmdd* infection, as would be required by the kinetics of CD69

Table I. *Type I IFNs are differentially up-regulated in BMDC treated with live Lmdd compared to cells treated with HK Lmdd or LPS*[a]

Gene	Live *Lmdd*	HK Lmdd	LPS	Live/HK *Lmdd*
IFN-β, fibroblast	66.8	1.0	3.8	66.8
Chemokine (CXC motif) ligand 10	51.7	4.7	41.6	11.1
RIKEN cDNA 5033428E16 gene	19.7	2.0	13.6	9.9
RIKEN cDNA A630077B13 gene	25.2	2.6	15.9	9.5
Pleiomorphic adenoma gene-like 1	17.5	1.8	8.7	9.5
IFN-α family, gene 2	18.6	2.0	1.0	9.3
Thymidylate kinase family LPS-inducible member	32.4	3.7	25.3	8.8
RIKEN cDNA A630072M18 gene	21.0	2.6	3.5	8.2
RIKEN full-length enriched library, clone:G430091H17	26.0	3.6	12.1	7.3
IFN-stimulated protein	20.2	3.2	16.2	6.3
Schlafen 4	11.1	2.0	11.8	5.6
CD69 Ag	14.5	2.7	10.1	5.4
Chemokine (CXC motif) ligand 9	11.4	2.1	7.6	5.3
Phospholipase A1 member A	15.2	3.0	11.6	5.1
Viral hemorrhagic septicemia virus (VHSV) induced gene 1	26.4	5.2	19.5	5.1
IFN-induced protein with tetratricopeptide repeats 1	56.9	11.6	38.3	4.9
Nuclear protein 1	12.4	2.6	7.7	4.8
IFN-stimulated protein (15 kDa)	47.1	10.0	36.9	4.7
IFN-induced protein with tetratricopeptide repeats 2	38.1	8.6	31.3	4.4
Myxovirus (influenza virus) resistance 2	13.9	3.2	11.8	4.3
RIKEN full-length enriched library, clone:6330437C08	13.4	3.1	9.0	4.3
RIKEN cDNA 0910001B06 gene	10.5	2.5	6.0	4.1
IFN-α family, gene 5	8.1	2.0	1.0	4.0
2′–5′ Oligoadenylate synthetase-like 1	14.7	3.7	12.6	3.9
RIKEN cDNA 9130009C22 gene	16.4	4.3	9.9	3.8
Endothelin 1	21.8	5.9	8.9	3.7
Membrane-spanning 4-domains, subfamily A, member 4C	11.7	3.7	9.7	3.2
TNFR superfamily, member 5	34.6	11.1	36.8	3.1
cDNA sequence BC013672	9.7	3.2	6.7	3.1
ras homolog gene family, member E	6.3	2.0	2.8	3.1
IFN-γ	6.1	2.0	8.2	3.1
Tripartite motif protein 30	14.6	4.9	9.9	3.0
IFN-induced protein with tetratricopeptide repeats 3	8.9	3.0	8.3	3.0
IFN regulatory factor 7	13.4	4.5	14.9	3.0
TNF (ligand) superfamily, member 7	12.9	4.5	11.7	2.9
AXIN1 up-regulated 1	6.4	2.3	3.2	2.8
Max dimerization protein	6.6	2.3	4.8	2.8
Expressed sequence AW261460	17.4	6.2	14.4	2.8
Pellino 1	6.8	2.5	3.5	2.8
Ubiquitin-specific protease 18	12.8	4.6	9.9	2.8
IL-12b	22.9	8.6	26.6	2.7
Chemokine (CC motif) ligand 4	17.9	7.0	9.7	2.5
Heat shock protein 1A	6.2	2.6	5.7	2.4
5′-Nucleotidase cytosolic III	9.2	3.8	7.0	2.4
Chemokine (CC motif) receptor-like 2	10.8	4.5	7.5	2.4
IFN-activated gene 204	6.6	2.8	4.2	2.4
Membrane-spanning 4 domains, subfamily A, member 4B	7.1	3.0	6.5	2.4
2′–5′ Oligoadenylate synthetase 3	9.4	4.2	8.4	2.2
Hepcidin antimicrobial peptide	10.3	4.7	13.7	2.2
IFN-activated gene 205	9.4	4.3	7.3	2.2
IL-15	6.3	2.9	5.0	2.2
Ribosomal protein L35a	7.0	3.2	6.7	2.2
TNF (ligand) superfamily, member 9	8.5	3.9	6.5	2.2
DNA segment, Chr 11, ERATO Doi 759, expressed	8.1	3.8	6.4	2.2
Schlafen 1	8.0	3.7	7.9	2.1
RIKEN full-length enriched library, clone:9830116M05	7.7	3.6	8.6	2.1
Z-DNA-binding protein 1	8.1	3.9	7.4	2.1
RIKEN full-length enriched library, clone:B530033B21	6.6	3.2	6.1	2.1
Hypothetical protein LOC211526	6.7	3.3	5.2	2.0
Hypothetical protein LOC211526	8.0	4.1	7.1	2.0
IL-15R, α-chain	6.0	3.1	4.9	1.9

[a] Microarray analysis was performed on duplicate samples using RNA harvested 6 h after treatment. The mean fold increase in gene expression compared to untreated BMDC samples is shown for all genes that were up-regulated at least 6-fold. Genes are listed in order determined by the ratio of the increase in expression by live *Lmdd*-treated compared to HK *Lmdd*-treated BMDC. The last column gives the ratio of expression after BMDC treatment with live vs HK bacteria.

up-regulation (Fig. 3*B*), the mDC cell line DC2.4 was pulsed with HK or live *Lmdd*. Twenty-three percent of live, but not HK, *Lmdd*-treated DC2.4 cells produce IFN-β, and a smaller proportion (9%) stain for IFN-α, as determined by intracellular cytokine staining. Polyinosinic-polycytidylic acid (poly(IC)), a potent type I IFN inducer for pDC, failed to induce mDC to produce IFN-β (Fig. 6*A*), as previously reported (12). We next compared IFN-β production following *Lmdd* infection of mouse bone marrow-derived mDC

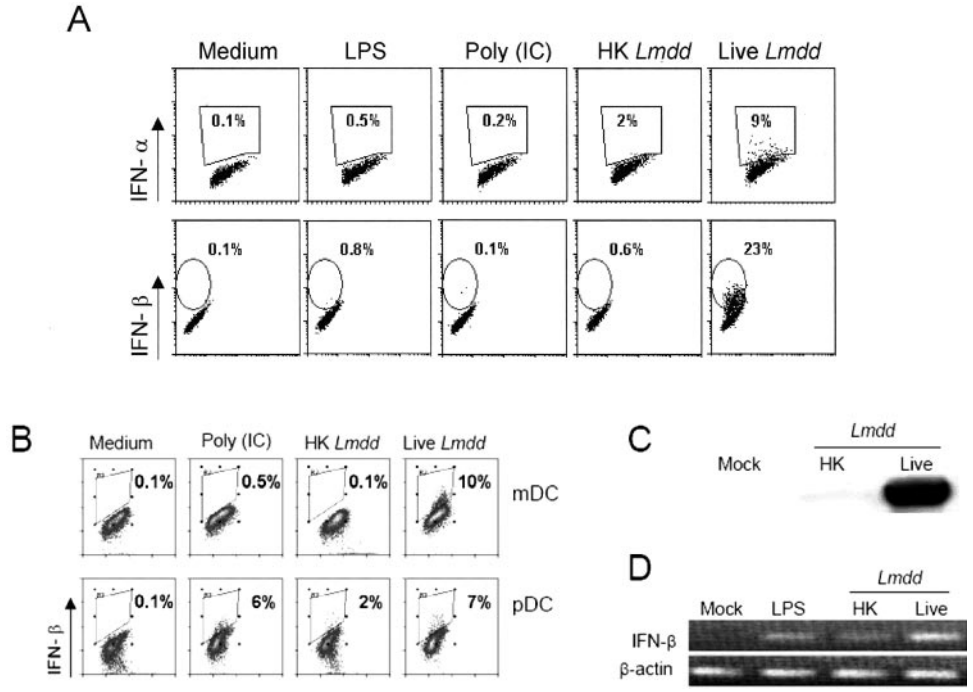

FIGURE 6. BMDC and DC2.4 cells up-regulate type I IFNs after live, but not HK, *Lmdd* infection. *A*, *Lmdd* infection, but not treatment with LPS, poly(IC), or HK bacteria, up-regulates type I IFN secretion by DC2.4 cells. DC2.4 cells were untreated or treated as indicated for 6 h in the presence of brefeldin A. IFN-α and IFN-β expression were examined by intracellular staining. *B*, Mouse bone marrow-derived mDC are as likely to produce IFN-β after *Lmdd* infection as bone marrow-derived cultures enriched for pDC (~30% B220$^+$) by culture in Flt3 ligand-conditioned medium. *C*, Supernatants from *Lmdd*-infected, but not HK *Lmdd*-treated, BMDC contain detectable IFN-β. Supernatants, harvested 6 h after treatment, were analyzed by immunoblot probed for IFN-β. *D*, *Lmdd*-infected DC express IFN-β mRNA. CD11c$^+$ BMDC were either untreated or treated with LPS, HK, or live *Lmdd* for 6 h before harvesting total RNA for RT-PCR analysis of IFN-β and β-actin expression.

with that produced by infected pDC generated by culture in Flt3 ligand conditioned medium. Approximately 10% of mDC produced IFN-β, as determined by intracellular cytokine staining, after live *Lmdd* treatment, which was comparable to IFN-β production by pDCs (7%) (Fig. 6*B*). As expected, poly(IC) stimulated IFN-β production by pDC, but not mDC. The proportion of IFN-β-producing pDC after poly(IC) or *Lmdd* infection was similar (6 and 7%, respectively). HK *Lmdd* activated IFN-β production by fewer pDC (2%) and, as expected, did not activate mDC. Immunoblotting (Fig. 6*C*) and RT-PCR (Fig. 6*D*) also showed that live, but not HK, *Lmdd*-treated bone marrow-derived mDC secreted IFN-β. Therefore, mDC are potent type I IFN producers after live, but not HK, *Lmdd* treatment.

IFN-β secreted by Lmdd-*infected DC activates CD69 expression on T cells*

To determine whether IFN-β is responsible for inducing CD69 expression on T cells and sensitizing T cell activation, we verified that rIFN-β could replace the infected DC-conditioned medium and that anti-IFN-β could block its effects. Ab against mouse IFN-β almost completely blocked the ability of *Lmdd*-infected DC supernatant to induce CD69 up-regulation by T cells (Fig. 7*A*), whereas Ab against mouse IFN-α had little effect (Fig. 7*B*). Neutralizing Abs against mouse TNF-α, IL-12, and IL-6 had no effect (Fig. 7*C*). Recombinant mouse IFN-β induced the up-regulation of CD69 expression on T cells in a dose-dependent manner (Fig. 7*D*). A high concentration of IFN-β was required (~250 U/ml) to induce CD69 on half the CD8 T cells in the culture. The dose-response curve plateaued at ~1,000 U/ml. When the same experiment was repeated with rIFN-α, much more IFN-α than IFN-β was required for an equivalent effect on CD69 expression. Approximately 5,000 U/ml IFN-α activates 50% of CD8 T cells to express CD69, whereas no plateau was reached even at an IFN-α concentration of 20,000 U/ml, at which 70% of CD8 T cells express CD69 (data not shown). Therefore, IFN-β is at least 20-fold more potent than IFN-α at priming T cell activation of CD69 expression. CD69 induction is not due to endotoxin contamination, because LPS prepared from *E. coli* had no ability to induce CD69 expression by T cells even at a very high concentration (data not shown). These data demonstrate that IFN-β and, to a lesser extent, IFN-α are the soluble factors in *Lmdd*-infected DC-conditioned medium responsible for inducing CD69 expression by T cells.

Because neutralizing Abs to IFN-β largely blocked the up-regulation of CD69 expression on CD8 T cells, we compared the bioactivity of *Lmdd*-infected DC-conditioned medium to the standard curve obtained by treating CD8 T cells with rIFN-β to estimate the concentration of IFN-β secreted by 10^6 *Lmdd*-infected myeloid DCs to be ~3000 U.

Cytosolic invasion of Lm *is required to induce IFN-β secretion by mDC*

A previous report has shown that *Lm* escape from the phagolysosome is necessary for infected macrophages to secrete IFN-β (20). To explore whether cytosolic invasion is also required for mDC to produce IFN-β after *Lm* infection, T cell activation by mDCs infected with mutant strains of *Lm* was studied. LLO is required for *Lm* to escape from the phagolysosome. The LLO-deficient strain DP-L2126 is unable to escape from the vacuole to the cytosol. LLO expression in strain DP-L3885 requires isopropyl-β-D-thiogalactoside (IPTG) exposure (32). The *Act-A*-deficient strain DP-L1942 is released from the phagolysosome, but is incapable of cell-to-cell spread (40). DC2.4 cells were pulsed with DP-L3885 in

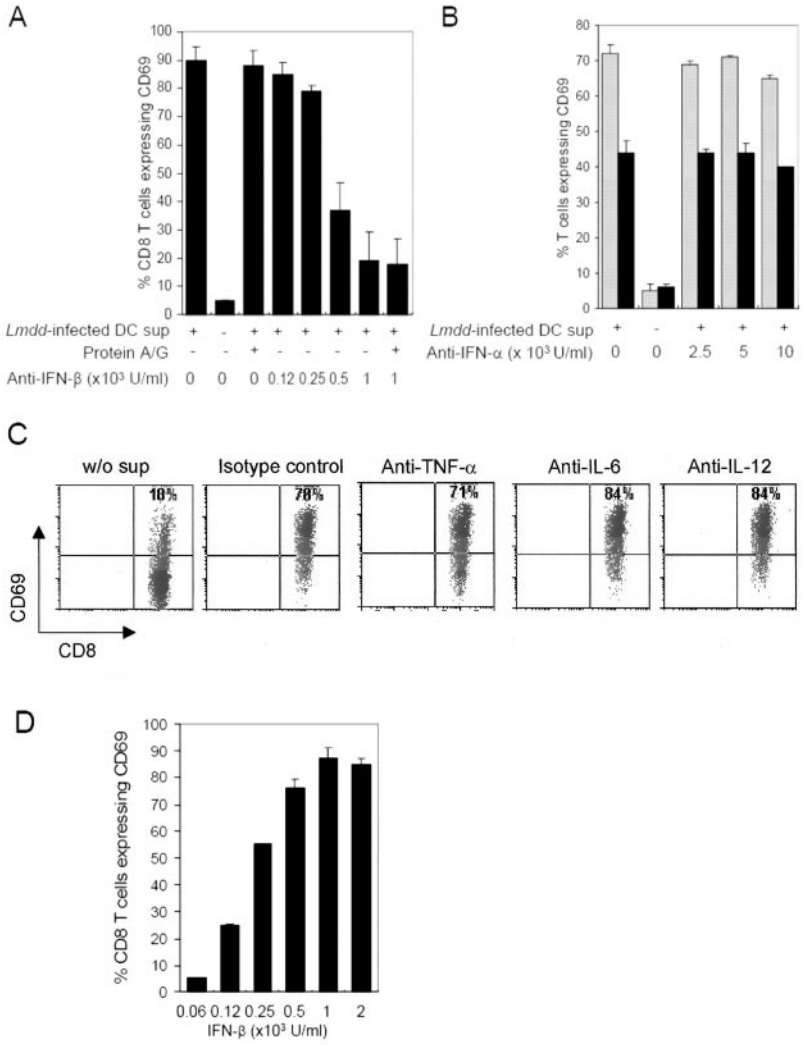

FIGURE 7. Type I IFNs are responsible for inducing CD69 expression. Supernatant from medium-treated or live *Lmdd*-infected BMDC was incubated with neutralizing Abs against IFN-β (*A*), IFN-α (*B*), or TNF-α, IL-6, or IL-12 (*C*). In the indicated lanes in *A* and in all conditions in *B* and *C*, protein A/G beads were added to remove Ab complexes. Depletion of IFN-β and, to a lesser extent, IFN-α inhibits the ability of *Lmdd*-infected BMDC supernatant to activate T cells to express CD69. B, ▨, CD8 T cells; ■, CD4 T cells. *D*, Recombinant mouse IFN-β at high concentrations activates CD69 expression on CD8 T cells. Cells were cultured overnight with the indicated concentrations of IFN-β before examining CD69 expression by flow cytometry. *A*, *B*, and *D*, Means and SDs from three independent experiments.

the presence or the absence of IPTG. Intracellular IFN-β staining showed that only infection with live *Lmdd* or DP-L3885 in the presence of IPTG induced IFN-β production, whereas treatment with HK *Lmdd* or DP-L3885 in the absence of ITPG did not (Fig. 8*A*). Furthermore, supernatants derived from DC that were pulsed with the *Lm* strains capable of cytosolic invasion, including *Act-A*

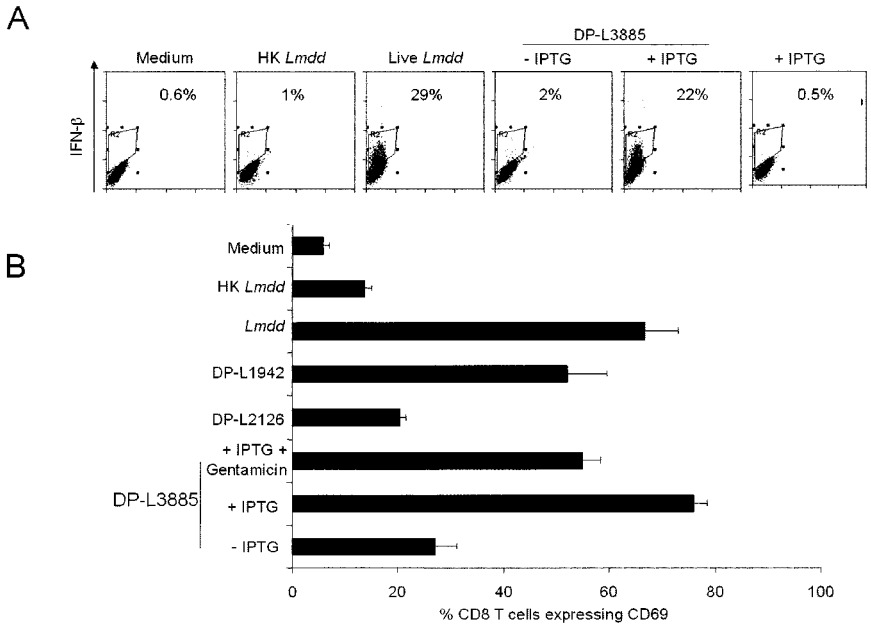

FIGURE 8. Cytosolic invasion by *Lm* is required for IFN-β production by infected DC2.4 cells. *A*, DC2.4 cells were untreated or treated with the indicated bacteria for 6 h in the presence of brefeldin A and analyzed for IFN-β production by intracellular Ab staining. *B*, CD8 T cells were analyzed for CD69 expression after overnight exposure to culture supernatants harvested from DC2.4 cells treated as described in *A*. Strain DP-L3885 with IPTG-inducible *hly* expression escapes to the cytosol only in the presence of IPTG, strain L2126 lacks *hly* and remains in the phagolysosome, and strain L1942, deficient in *ActA*, can escape to the cytosol, but cannot efficiently spread from cell to cell. Means and SDs from three independent experiments are shown.

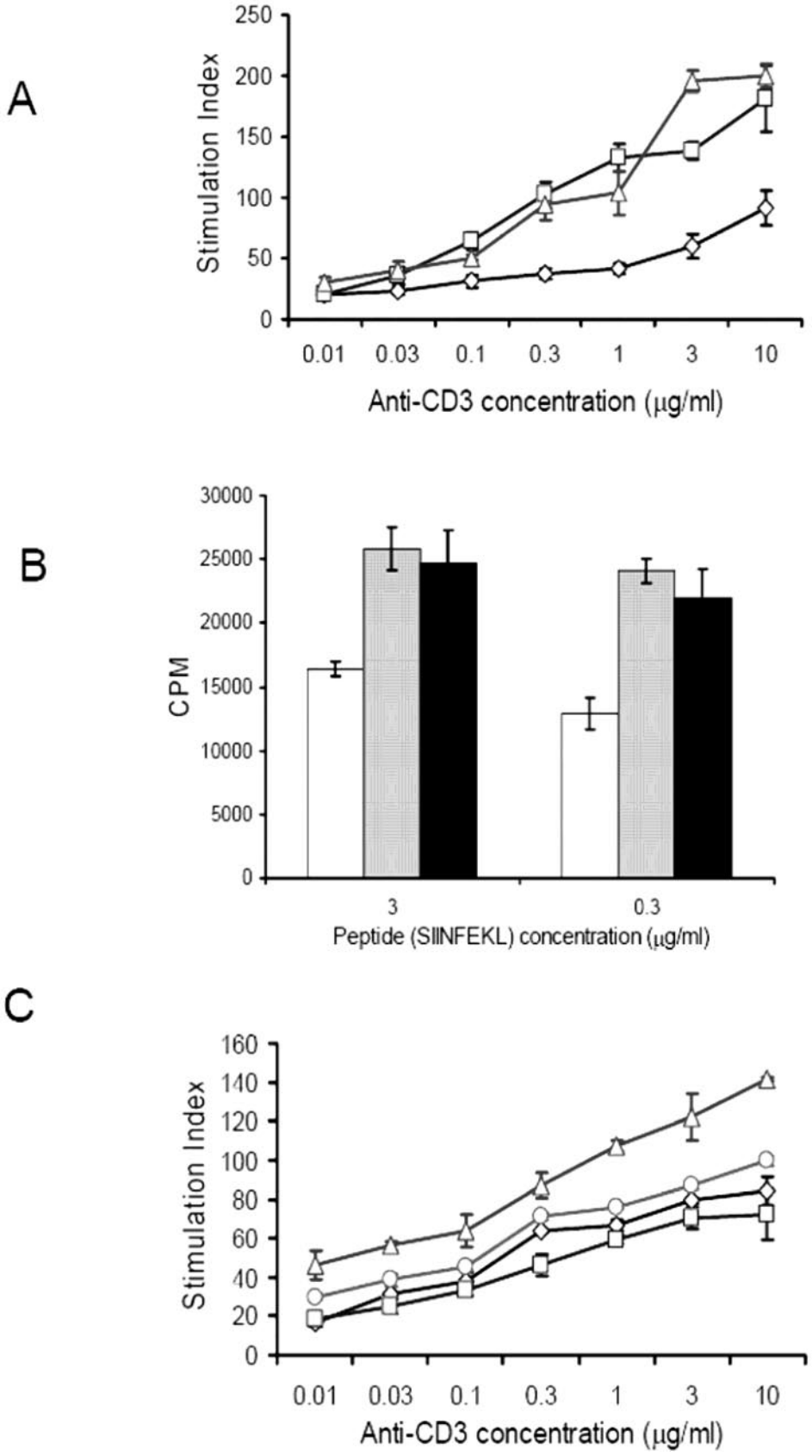

FIGURE 9. IFN-β sensitizes T cells for Ag stimulation. *A*, Pre-exposure to *Lmdd*-infected DC supernatant or rIFN-β similarly primes splenic T cells for activation by CD3ϵ Ab. Splenic T cells, positively selected with immunomagnetic anti-CD4 and anti-CD8 beads from naive mice, were pre-exposed to medium (◇), *Lmdd*-infected DC supernatant (△), or IFN-β (500 U/ml; □) for 3 h, washed extensively, and then cultured with the indicated concentrations of anti-CD3ϵ in the presence of irradiated splenocytes for 3 days before measuring proliferation. *B*, CD8 T cells from TCR transgenic OT-1 mice were similarly pre-exposed to medium (□), *Lmdd*-infected supernatant (▨), or IFN-β (500 U/ml; ■) for 3 h before adding the indicated concentration of OVA peptide (SIIFNEKL) and irradiated splenocytes. Proliferation 3 days later was similarly enhanced by *Lmdd*-infected DC conditioned medium or rIFN-β. *C*, *Lmdd*-infected DC-conditioned medium enhances T cell proliferation when added 1 day after TCR stimulation, but IFN-β does not. Splenocytes from naive mice were stimulated with anti-CD3ϵ for 1 day, then cultured in the absence (◇) or the presence of IFN-β (500 U/ml; □) or of supernatants derived from HK (○) or live (△) *Lmdd*-treated DC for another 2 days before measuring T cell proliferation.

mutant bacteria, were able to induce CD69 expression on T cells, whereas strains stuck in the phagolysosome were unable to stimulate DC to efficiently activate CD69 expression on T cells (Fig. 8*B*). A statistically significant increase in the proportion of T cells expressing CD69 compared with the background after HK *Lmdd* infection was only seen in strains capable of escaping to the cytosol (DP-L3885 with IPTG with or without gentamicin, $p < 0.001$; *Act-A*-deficient DP-L1942, $p < 0.02$). Therefore, cytosolic

invasion is needed to activate IFN-β secretion by infected DC and consequent T cell activation.

IFN-β primes T cells for antigenic stimulation

We found that brief exposure of naive T cells to the supernatant from live *Lmdd*-infected DC enhanced their Ag-dependent activation (Fig. 4), suggesting that IFN-β may prime T cells for subsequent antigenic stimulation. Therefore, we investigated whether rIFN-β had the same effect. Pre-exposure of T cells to 500 U/ml rIFN-β before CD3 Ab decreased the Ag response threshold for T cell activation in the same manner as *Lmdd*-infected DC conditional medium (Fig. 9*A*). IFN-β also sensitized splenic T cells from naive OT-1 transgenic mice for activation by the cognate OVA peptide (Fig. 9*B*). However, if *Lmdd*-infected DC culture supernatant or IFN-β was not added until 1 day after T cell activation by anti-CD3ε, the effect was different. Although the culture supernatant enhanced T cell proliferation to some extent, rIFN-β might have inhibited proliferation (Fig. 9*C*). This suggests that the effect of IFN-β is different on resting and already activated cells, and that the *Lmdd*-infected DC culture supernatant contains other T cell growth factors, as suggested by the microarray results (Table I).

Discussion

It has long been known that live *Lm* induce protective immunity, whereas killed bacteria fail to do so (13, 18). Unveiling the mechanism by which immune cells differentially respond to live vs dead *Lm* is important for understanding innate and adaptive immunity, and ultimately will help to design better vaccines against cancer and infectious diseases. Because DC are the key to priming immune responses, in this study we focused on differential effects of live and HK bacteria on DC activation and priming of T cells. We found that although live and HK *Lm* similarly cause DC to mature, as measured by up-regulating cell surface costimulatory molecules and MHC class II, only live bacteria-infected DC are able to activate CD69 expression on T cells efficiently and prime them for subsequent activation by the TCR. Using protein chemistry and microarray gene expression analysis, we identified the type I IFN, IFN-β, as a soluble factor rapidly produced in large amounts (~3000 U/10^6 cells) by BMDC upon infection by live *Listeria*, but not HK bacteria. Moreover, we were able to show that only high concentrations of type I IFNs are able to prime T cell activation. We therefore hypothesize that the exceptionally strong and protective immune response to *Listeria* is related to the amplification of T cell priming that occurs in the presence of high local concentrations of type I IFN secreted by infected DC.

The copious production of type I IFN in our studies was unanticipated, because our experiments were performed using mDC. Conventional wisdom holds that the major type I IFN-producing DC is the $B220^+CD11b^-$ pDC (11). However, in our experiments the source of DC was either the $B220^-CD11b^+$ mDC cell line DC2.4 or BMDC that had been cultured in GM-CSF and IL-4 and selected for CD11c expression. These cultured BMDC are mDC, contain <1% $B220^+$ cells, and are >98% $CD11b^+$ (Fig. 1*A*). Furthermore, *Lmdd* infection did not alter the BMDC expression of cell surface markers that distinguish mDC and pDC (data not shown). Although the DC2.4 cell line is fully myeloid, we were concerned that contamination of our BMDC with a few pDC might account for the IFN production we measured in the BMDC cultures. However, when BMDC were cultured in the presence of Flt3 ligand to generate cultures enriched in pDC (~30% $B220^+$), the bioactivity of the *Lmdd*-infected DC-conditioned medium was not enhanced (Fig. 6*B*). These data therefore suggest that mDC produce high levels of type I IFN when infected with *Listeria*. Production of type I IFN by mDC was also documented in a recent study that showed that mDC produce large amounts of type I IFN after infection with viruses such as lymphocytic choriomeningitis virus (12).

Type I IFNs have multiple biological activities and play important roles in bridging innate immune responses and adaptive immunity (41). Both IFN-α and IFN-β trigger CD69 expression on T cells, but *Lm* infection triggered more IFN-β than IFN-α expression in mDCs. Ab-blocking experiments also suggested that most of the biological effect could be attributed to IFN-β. In this study we also demonstrated that IFN-β produced by *Lm*-infected DC acts as a commitment factor to decrease the Ag response threshold of T cells and enhance their priming. The initial exposure to IFN-β partially activates naive T cells, preparing them for subsequent Ag-specific activation.

Stimulation of type I IFN production by mDC and their priming of T cell CD69 expression and sensitization for TCR stimulation require *Lm* escape from the phagolysosome. HK bacteria remain in the phagolysosome. Using bacterial mutants lacking or with inducible expression of LLO, which is required for cytosolic invasion, we also found that the production of IFN-β and T cell activation by mDC correlate with the ability to escape the phagocytic vacuole. Portnoy and colleagues (20) previously reported production of IFN-β by *Lm*-infected macrophages via a mechanism that requires bacterial cytosolic invasion. They postulated a bacterial cytosolic sensor that signals the presence of cytosolic bacteria and triggers the secretion of IFN-β by macrophages. Our results suggest that a similar sensor and pathway are triggered by intracytoplasmic infection of mDC. The sensor remains to be identified. LPS activates a TLR4, MyD88-independent signaling pathway that induces IRF3 translocation and IFN-β production via the Toll/IL-1R domain-containing adaptor (42–44). However, we found that LPS has a very limited capacity to stimulate BMDC to produce IFN-β compared with live *Lmdd*, and LPS-activated DC do not efficiently stimulate T cells to express CD69. Supernatants from *Lmdd*-infected BMDC derived from mice deficient in MyD88, a key signaling molecule for TLR engagement, produce comparable amounts of IFN-β (data not shown). It is, moreover, unlikely that TLR family proteins are responsible, because these receptors are displayed on the cell surface and within endosomes, but not in the cytosol. The nucleotide-binding oligomerization domain and Nacht, leucine-rich repeat, and pyrin domain-containing protein family proteins that are present in the cytosol and are able to sense Gram-positive and Gram-negative bacterial cell wall components are attractive candidates for the unknown sensor (45–47).

In this study we found that pulsing bone marrow-derived mDC with either HK or live *Lmdd* induces DC to up-regulate costimulatory molecules on their surface and secrete proinflammatory cytokines, such as TNF-α, IL-6, and IL-12. DC maturation and induction of proinflammatory cytokines were probably stimulated by engagement of TLR receptors by both HK and live *Lmdd*. Because the differential effects of live and HK bacteria were also evident when bacteria were killed by other means (such as antibiotic treatment) that do not cause denaturation, differences in TLR engagement of pathogenic patterning molecules in the bacteria were probably minimal. It is, therefore, not surprising that dead *Lm* also induce DC maturation. Although slightly more CD40 and CD80 were detected on the surface of BMDC infected with live *Lmdd* than HK *Lmdd*, the expression of other cell surface markers (class II MHC and CD86) and that of inflammatory cytokine production were comparable. Therefore, these factors are unlikely to explain the large difference in T cell activation we observed. However, the difference in type I IFN expression was dramatic, and Abs to type I IFNs could abrogate the effect of *Lmdd*-infected BMDC supernatants on T cells.

Our conclusions differ from those in a recent paper that compared GM-CSF-cultured BMDC maturation, DC inflammatory cytokine production, and stimulation of T cells to produce IFN-γ in response to infection with wild-type and LLO-deficient *Lm* (23). That study also found that bacterial cytosolic invasion was critical for T cell activation, but found more substantial differences in costimulatory molecule expression and proinflammatory cytokine expression, which they interpreted as the distinguishing feature. Our use of a replication-defective *Lmdd* strain may account for differences between their study and ours (25). In fact, they had twice as much infection after 4 h using wild-type as *hly*$^-$ bacteria. Another difference is that the other study examined DC maturation 18 h after *Lm* infection, whereas we observed DC maturation after 24 h. Typically, costimulatory molecule up-regulation occurs relatively slowly compared with IFN-β induction, requiring 16–24 h (data not shown). Type I IFN production in response to bacterial invasion may accelerate DC maturation because it induces a positive loop of type I IFNs and IFN-inducible gene up-regulation (38) that enhances Ag presentation function and DC maturation (48). However, DC matured more slowly via TLR engagement may reach the same final state for efficient Ag presentation by 24 h. Accelerated DC maturation may lead to a more rapid and effective immune response. A final difference is that the myeloid DC used in the other study were generated from bone marrow by culture in GM-CSF, whereas we generated myeloid DC by culture in both IL-4 and GM-CSF. The combination of subtle differences in cytokines, timing, and bacterial replication may account for the different conclusions. However, which in vitro condition more faithfully recapitulates in vivo conditions is impossible to predict.

Both live and HK bacteria induce the rapid expression of multiple IFN-responsive genes in BMDC within 6 h, the time after *Lmdd* exposure at which we performed our microarray analysis. The downstream activation of these genes, many of which are also up-regulated by LPS engagement of TLR4, is probably triggered by low levels of IFNs generated after TLR signaling. Our results are similar to a gene expression analysis of *Lm*- or LPS-treated bone marrow-derived macrophages, which found that both bacterial invasion and TLR engagement were able to activate many IFN-responsive genes (49). However, high concentrations of type I IFN (~250 U/ml IFN-β, ~5000 U/ml IFN-α to activate CD69 on 50% of T cells) are needed to induce CD69 on T cells and prime T cells for antigenic exposure. These high concentrations of type I IFN decrease the Ag response threshold of naive T cells by ~1 log. Therefore, weak signals that might otherwise be ignored or produce an ineffective, tolerogenic, or even suppressive regulatory immune response can be activated in the presence of high levels of type I IFN. The partial TCR-independent activation of T cells by high concentrations of type I IFN may also play a part in the bystander T cell apoptosis and lymphocyte depletion observed after *Lm* infection, because these partially activated T cells are prone to spontaneous apoptosis (data not shown) (50). Although removing nonspecific T cells may make room for expansion of *Lm*-specific T cells, it might also interfere with maintaining effective memory.

Our results and those reported by Portnoy and colleagues (20) suggest that in addition to TLR engagement, there is a second alarm system in APCs, such as DC and macrophages, that is activated when bacteria invade the cytosol. This alarm triggers the nuclear translocation of IRF3 and the production of copious quantities of type I IFNs that enhance T cell sensitivity to antigenic stimulation. This alarm system may be triggered by intracellular pathogens to enlist the aid of the cells (T cells) most capable of eliminating intracellular pathogens. The induction of type I IFN production by mDC after infection with lymphocytic choriomeningitis virus and a mutant strain of influenza (12) suggests that this second alarm might also be triggered by some viral infections. It will be of interest to determine whether other viruses and cytosolic pathogens also trigger exuberant type I IFN expression. Because T cells are not important for eliminating extracellular pathogens, it makes sense that this response would not be elicited by most bacteria and would not be triggered by cell surface receptors, like the TLR system. Our data also suggest that mDC produce much more IFN-β than macrophages after live *Lmdd* infection, because supernatant from the infected RAW264.7 macrophage cell line barely activates T cells to express CD69 (Figs. 2*A* and 3*A*). Therefore, this second alarm system may function more effectively in DC than in macrophages.

This specialized type I IFN danger response may explain why *Lm* is such an effective vector for priming T cell responses to Ags. Because *Lm* causes serious disease in immunosuppressed individuals, pregnant women, and newborns, most investigators developing *Lm* as a vaccine vector agree that the bacterium must be attenuated before human use is contemplated (51). Our results suggest that attenuated bacteria unable to escape from the phagolysosome may be impaired in T cell priming and lead to ineffective vaccines. In fact, the vaccine strains that appear the most promising are able to invade the cytosol. Screening for type I IFN production may be a useful tool for predicting which attenuated bacteria are likely to be effective vaccine vectors.

Although this study suggests that type I IFN production in response to *Lm* cytosolic invasion should send a strong signal to enhance T cell immunity, mice deficient in the common type I IFNR are actually better able to handle *Lm* challenge (34, 52, 53). This is unexpected, because type I IFNs generally protect against other types of infection, especially by viruses. In fact, the harmful effect of type I IFNs may be peculiar to *Lm* infection (52, 54). Carrero et al. (52, 54) found that LLO, the bacterial pore-forming protein, acts as a bacterial toxin to induce T cell apoptosis, and that partially or fully activated T cells are particularly prone to LLO-induced apoptosis. Type I IFNs probably accelerate this process, because they induce partial activation of nonspecific T cells and facilitate full activation of specific T cells. This idiosyncratic effect of LLO on T cells and the induction of type I IFN need to be considered in developing *Listeria*-based vaccine vectors. Because of the pleiotropic effects of IFNs on different immune cells, however, the resistance of mice null for the type I IFN receptor to *Lm* may be due to more than one factor.

Acknowledgments

We thank Lianfa Shi, Manjunath Swamy, Howell Moffat, Pascale Cossart, and Jay Berzofsky for useful advice, and Fred Frankel and Darren Higgins for providing the strains of *Lm* used in this study.

Disclosures

The authors have no financial conflict of interest.

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