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IFN- γ -Producing Dendritic Cells Are Important for Priming of Gut Intraepithelial Lymphocyte Response Against Intracellular Parasitic Infection¹

Magali M. Moretto,*[†] Louis M. Weiss,[‡] Crescent L. Combe,* and Imtiaz A. Khan^{2*†‡}

The importance of intraepithelial lymphocytes (IEL) in immunoprotection against orally acquired pathogens is being increasingly recognized. Recent studies have demonstrated that Ag-specific IEL can be generated and can provide an important first line of defense against pathogens acquired via oral route. However, the mechanism involved in priming of IEL remains elusive. Our current study, using a microsporidial model of infection, demonstrates that priming of IEL is dependent on IFN- γ -producing dendritic cells (DC) from mucosal sites. DC from mice lacking the IFN- γ gene are unable to prime IEL, resulting in failure of these cells to proliferate and lyse pathogen-infected targets. Also, treatment of wild-type DC from Peyer's patches with Ab to IFN- γ abrogates their ability to prime an IEL response against *Encephalitozoon cuniculi* in vitro. Moreover, when incubated with activated DC from IFN- γ knockout mice, splenic CD8⁺ T cells are not primed efficiently and exhibit reduced ability to home to the gut compartment. These data strongly suggest that IFN- γ -producing DC from mucosal sites play an important role in the generation of an Ag-specific IEL response in the small intestine. To our knowledge, this report is the first demonstrating a role for IFN- γ -producing DC from Peyer's patches in the development of Ag-specific IEL population and their trafficking to the gut epithelium. *The Journal of Immunology*, 2007, 179: 2485–2492.

Cell-mediated immunity involving T cells plays an important role in immune response and survival against infections with obligate intracellular pathogens such as *Encephalitozoon cuniculi*, a member of the Microsporidia order associated with disseminated infections in mammalian hosts (1–4). Infections acquired via oral route (i.e., a gastrointestinal infection) usually evoke a strong mucosal response. This finding was recently demonstrated in a study conducted in our laboratory where oral infection with *E. cuniculi* led to an early and rapid intraepithelial lymphocyte (IEL)³ response against this organism (5). IEL from infected mice exhibited a strong Ag-specific immunity and were able to protect naive immunocompromised SCID mice against a lethal challenge with *E. cuniculi*.

IEL are considered nonconventional lymphocytes located among the epithelial cells in the lumen of the gut and are comprised predominantly of CD3⁺ T cells (6). Although IEL are known to produce cytokines, their importance in protective immunity against mucosal pathogens has only been recently recognized (7–10). Priming of an Ag-specific IEL response is not yet under-

stood and the mechanism by which IEL provide protection against infectious agents remains somewhat enigmatic. In this study, factors essential for the induction of an Ag-specific IEL response were analyzed, and the mechanism by which they confer immunoprotection against an intracellular infection was evaluated.

Materials and Methods

Mice

Six- to 7-wk-old SCID, perforin knockout (pf^{-/-}), and IFN- γ knockout (IFN- γ ^{-/-}) mice on a C57BL/6 genetic background were obtained from the Jackson Laboratory. Age- and sex-matched C57BL/6 controls were obtained from the National Cancer Institute (Frederick, MD). Animals were housed under approved conditions under an approved Institutional Animal Care and Use Committee protocol at the Animal Research Facility of the Louisiana State University Health Sciences Center (New Orleans, LA) and George Washington University Medical Center (Washington, DC).

Parasites and infection

A rabbit isolate of *E. cuniculi* (genotype II), provided by E. Didier (Tulane Regional Primate Center, Covington, LA), was used throughout the study. The parasites were maintained by continuous passage in rabbit kidney (RK-13) cells obtained from the American Type Culture Collection and in mice infected as previously described (5).

Histopathology

Mice were euthanized using an approved carbon dioxide technique, and liver, spleen, and small intestine were fixed in 10% Formalin and processed for 5- μ m histological sections, which were subsequently stained with H&E using standard methods. Sections were also stained with chromotrope 2R (microsporidia) using the Kokoskin et al. (11) with a modification of the 1992 Weber microsporidia stain that uses a 10-min staining time in chromotrope 2R at 50°C. Stained slides were examined on a Nikon Microphot FXA microscope with a Nikon DS-L1 digital photography system.

CTL assay

The CTL assay was performed using the previously described procedure from our laboratory (5). Mouse peritoneal macrophages were harvested 2 days after i.p. injection of 1 ml of thioglycolate (Sigma-Aldrich), washed three times in PBS, and dispensed at a concentration of 5×10^4 cells/well into U-bottom 96-well plates. After an overnight incubation, these macrophages were infected with 2.5×10^5 *E. cuniculi* spores per well for 48 h

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³ Abbreviations used in this paper: IEL, intraepithelial lymphocyte; DC, dendritic cell; WT, wild type; pf, perforin; PP, Peyer's patch; CMA, concanamycin A; FasL, Fas ligand; CD95L, CD95 ligand; MLN, mesenteric lymph node.

and then washed extensively with PBS to remove extracellular parasites. Macrophages were then labeled with ^{51}Cr (0.5 $\mu\text{Ci}/\text{well}$) and incubated with IEL at various E:T ratios. Purification of IEL was conducted according to previously described protocol (5). The microtiter plate was then centrifuged at $200 \times g$ for 3 min and incubated at 37°C for 4 h, and samples were removed and assayed for released ^{51}Cr using scintillation counting. The percentage of lysis was calculated as (mean cpm of test sample – mean cpm of spontaneous release)/(mean cpm of maximal release – mean cpm of spontaneous release) $\times 100$.

When indicated, IEL were incubated with 10 nM concanamycin A (CMA; Sigma-Aldrich) for 2 h at 37°C or 10 $\mu\text{g}/\text{ml}$ anti-Fas ligand (FasL; eBioscience) for 20 min before their addition to the ^{51}Cr -labeled macrophages (12).

IEL phenotyping for cytotoxic markers

IEL from infected wild-type (WT) animals were isolated at day 7 postinfection (p.i.) and labeled for CD95 ligand (CD95L), perforin, and granzyme B expression. Briefly, the cells were incubated for 4 h with Golgi stop, and labeled with anti-CD8 α FITC-conjugated Ab (eBioscience). After permeabilization and fixation with Cytotfix kit from BD Pharmingen, according to the manufacturer's protocol, the cells were incubated with anti-CD95L anti-perforin, or anti-granzyme B PE-conjugated Abs (eBioscience). The cells were analyzed on FACSCalibur (BD Biosciences).

IEL adoptive transfer

IEL from $\text{pf}^{-/-}$, $\text{IFN-}\gamma^{-/-}$, or WT donors were isolated at day 7 p.i. and transferred to naive SCID animals as previously described (5). Recipients were challenged by oral infection at 48 h post transfer with 2×10^7 *E. cucurbiti* spores. Transferred SCID mice were monitored for morbidity and mortality on a daily basis until the termination of the experiment. For one set of experiments, the CD8 subsets of IEL were purified from naive or infected WT mice before transfer to SCID mice. After IEL purification, the cells were separated in two subsets of CD8 β^+ and CD8 β^- cells by magnetic cell sorting with biotin-conjugated anti-CD8 β Ab, followed by anti-biotin-coated microbeads (Miltenyi Biotec), according to manufacturer's instructions (purity $\geq 94\%$). The different cell suspensions were adoptively transferred i.p. to SCID recipients. Two days after the transfer, the mice were challenged orally with 2×10^7 *E. cucurbiti* spores and the recipient mice were monitored as described.

For IEL trafficking experiments, SCID mice were adoptively transferred with IEL labeled with CellTrace CFSE proliferation kit, according to the manufacturer's instructions (Invitrogen Life Technologies). The animals were then challenged as described. Recipient mice were sacrificed 2 days post transfer, and IEL, spleen, and mesenteric lymph node (MLN) lymphocytes were isolated and analyzed for CFSE expression by flow cytometry.

Phenotypic analysis of IEL

WT and $\text{IFN-}\gamma^{-/-}$ mice were orally infected with 2×10^7 *E. cucurbiti* spores. At day 7 p.i., purified IEL were labeled for surface markers CD8 α , CD8 β , TCR $\alpha\beta$, and TCR $\gamma\delta$ (eBioscience) as previously described (5). Cells were acquired on a FACS Vantage (BD Biosciences).

DC purification and in vitro priming of IEL

DC from Peyer's patches (PP) were prepared according to an already published protocol (13). Briefly, PP from WT and $\text{IFN-}\gamma^{-/-}$ mice were enzymatically and mechanically disrupted. After a single-cell suspension was prepared, the cells were incubated with anti-CD11c-coated microbeads (Miltenyi Biotec) and separated on MACS separation columns according to the manufacturer's instructions. Cells were then stained with PE-labeled anti-CD11c and FITC-labeled anti-CD19 Ab (eBioscience). CD11c $^{+}$ /CD19 $^{-}$ cells were sorted on a FACS Vantage. Purified cells (96–98% pure as determined by flow cytometry) were plated at various concentrations ($5\text{--}20 \times 10^3$) and infected overnight with 20 *E. cucurbiti* spores/DC. After several washes, 10^4 purified IEL were added to the DC. At 72 h later, 1 μCi [^3H]thymidine was added to each well, and proliferative response was determined as described elsewhere (14).

When indicated, different concentrations of anti- $\text{IFN-}\gamma$ Ab (2 and 0.5 $\mu\text{g}/\text{ml}$; R&D Systems) were added to the DC culture overnight at the time of infection. The next day, the cells were washed and the IEL added as previously described.

In vitro IL-12 production by DC

DC were isolated from PP of $\text{IFN-}\gamma^{-/-}$ and WT mice as described. Cells were plated in 96-well plates at concentrations of 5×10^4 cells per well and infected with *E. cucurbiti* (20 spores/DC). After overnight incubation, su-

pernatants were collected and assayed for IL-12 production by cytokine ELISA kit (BioLegend), according to the manufacturer's instructions.

In vitro priming of splenic CD8 $\alpha\beta$ T cells by DC from PP

DC from PP of WT and $\text{IFN-}\gamma^{-/-}$ mice were isolated and infected as described. After overnight incubation, magnetically purified, splenic CD8 $\alpha\beta^+$ T cells ($>95\%$ pure) were added to the culture and 72 h later, cells were harvested and labeled with CFSE (25 μM) according to manufacturer's instructions (Invitrogen Life Technologies). Labeled cells were transferred to naive WT mice (2×10^6 cells/mouse) and 24 h later the recipients were sacrificed. Tissues (spleen, MLN, PP, and IEL) were analyzed for the presence of donor CFSE $^+$ CD3 $^+$ cells by FACS analysis.

In another series of experiments, splenic CD8 $\alpha\beta^+$ T cells were recovered after 3 days of incubation with DC from PP of WT or $\text{IFN-}\gamma^{-/-}$. The cells were labeled with anti-CD8 β allophycocyanin-conjugated and anti-CD8 α Pacific blue-conjugated Abs (eBioscience) and surface expression of CCR9 was evaluated by FACS analysis using FITC-conjugated Ab (R&D Systems). $\text{IFN-}\gamma$ production by these cells was analyzed after intracellular staining with PE-conjugated anti- $\text{IFN-}\gamma$ Ab (eBioscience). Data were acquired on FACS Aria (BD Biosciences) using Diva software.

Results

*IFN-}\gamma^{-/-} and $\text{pf}^{-/-}$ mice are susceptible to oral *E. cucurbiti* challenge*

Previous data from our laboratory demonstrated that oral *E. cucurbiti* infection induced a strong IEL response in the host (5). IEL from infected animals produced $\text{IFN-}\gamma$ and exhibited ex vivo cytotoxic activity against infected targets. In the present study, gene knockout mice were used to determine the importance of $\text{IFN-}\gamma$ production and cytolytic response in the protective immune mechanism against oral microsporidial infection. Mice lacking $\text{IFN-}\gamma$ or perforin gene were orally infected with 2×10^7 *E. cucurbiti* spores. Knockout mice started to die on day 13 p.i. and all the mice in $\text{IFN-}\gamma^{-/-}$ and $\text{pf}^{-/-}$ group succumbed to the infection by day 28 postchallenge. Conversely, as expected, WT mice survived till the termination of the experiment (Fig. 1A). Both $\text{IFN-}\gamma^{-/-}$ and $\text{pf}^{-/-}$ mice infected orally with *E. cucurbiti* were subjected to histopathological analysis. $\text{IFN-}\gamma^{-/-}$, $\text{pf}^{-/-}$, and WT mice were sacrificed on day 10 p.i. (representative tissue sections are shown in Fig. 1B). On necropsy, the spleen of WT mice was normal in size and appearance (Fig. 1B, i), whereas that of the $\text{pf}^{-/-}$ mice was one and a half its normal size and demonstrated mild effacement of the follicles (data not shown) and that of $\text{IFN-}\gamma^{-/-}$ mice was four times its normal size with marked effacement of the follicles and expansion of the red pulp (Fig. 1B, ii and iii). The liver of the WT and $\text{pf}^{-/-}$ mice was essentially normal with occasional inflammatory mononuclear cell foci (Fig. 1B, iv), whereas that of the $\text{IFN-}\gamma^{-/-}$ mice demonstrated numerous inflammatory foci (Fig. 1B, v and vi). Intestinal biopsy demonstrated a mild increase in PP size in both $\text{pf}^{-/-}$ and $\text{IFN-}\gamma^{-/-}$ animals compared with the control group (Fig. 1B, vii–ix). The increased inflammation in the tissues of knockout mice is suggestive of uncontrolled parasite replication and dissemination from the gastrointestinal tract following oral infection. The findings in the liver and spleen are similar to the pathology seen following i.p. infection of WT animals (1). These results suggest that both $\text{IFN-}\gamma$ production and the cytotoxic response are important for protection (and the prevention of widespread dissemination) following oral infection with *E. cucurbiti*.

Ag-specific cytotoxic activity of IEL is perforin-dependant

Data obtained suggested the importance of perforin-dependent cytotoxic activity in resolving oral *E. cucurbiti* infection. To further assess the molecules involved in cytotoxic pathway, we analyzed IEL for presence of cytotoxic molecules perforin, granzyme B, or CD95L by flow cytometry. IEL from WT mice were isolated at 7 days p.i., and intracellular staining was performed. As shown in

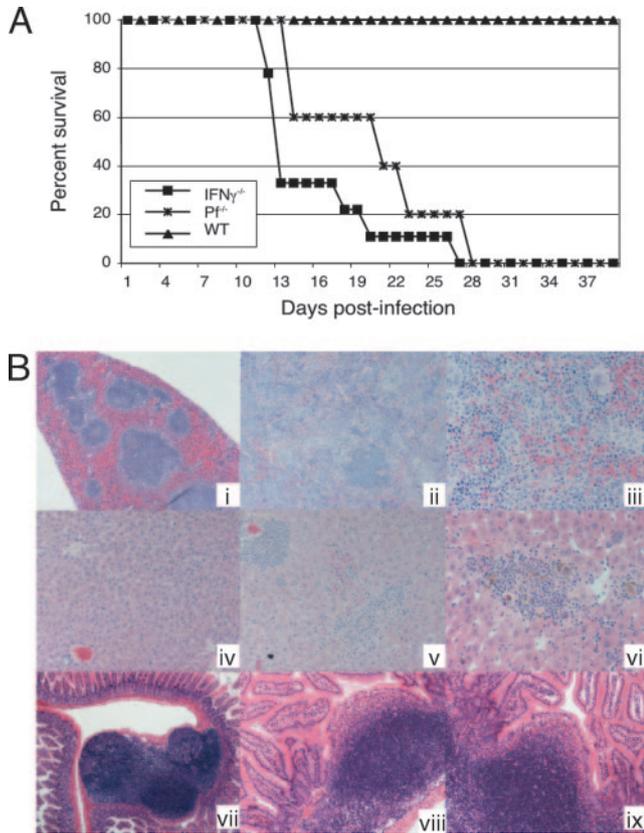


FIGURE 1. $IFN-\gamma^{-/-}$ and $pf^{-/-}$ mice are susceptible to oral *E. cucurbiti* infection. **A**, $IFN-\gamma^{-/-}$, $pf^{-/-}$, and WT mice ($n = 8$ mice/group) were orally infected with 2×10^7 *E. cucurbiti* spores. The animals were monitored daily for survival. The study was performed twice and data are representative of two separate experiments. **B**, Histopathology of infected mice. Spleen at necropsy at magnification $\times 10$ (i). WT mouse model demonstrating normal architecture. Spleen of $IFN-\gamma^{-/-}$ mouse at magnification $\times 20$ (ii) and $\times 40$ (iii), demonstrating effacement of follicles and increase in red pulp. Liver at magnification $\times 20$ (iv). WT mouse model demonstrating normal architecture. Liver $IFN-\gamma^{-/-}$ mouse at magnification $\times 20$ (v) and $\times 40$ (vi), demonstrating inflammatory foci. Mononuclear cell infiltration into the liver (vi) associated with a slight increase in the number of Kupfer cells in the adjacent areas. PP in the intestinal track of mice are shown (vii–ix). There is some increased activity of lymphoid aggregates. However, no organism was detected. PP from $IFN-\gamma^{-/-}$ mouse at magnification $\times 10$ (vii) and $\times 40$ (viii). PP at magnification $\times 20$ (ix) from a $pf^{-/-}$ mouse are shown.

Fig. 2A, perforin, granzyme B, and CD95L expression were up-regulated after infection with *E. cucurbiti* compared with IEL isolated from naive mice. To establish that an Ag-specific cytotoxic response exhibited by IEL is exclusively mediated by the perforin/granzyme pathway, blocking assay was conducted. IEL from immune mice were isolated and an inhibition of cytotoxicity assay using CMA or anti-FasL Ab was performed. CMA is a known inhibitor of the perforin/granzyme pathway and anti-FasL Abs inhibit the Fas/FasL pathway (12). IEL were purified at day 7 p.i., incubated with either CMA or anti-FasL, and their cytolytic activity against infected macrophages measured. As expected, naive IEL did not exhibit any detectable lytic activity against the infected target cells (Fig. 2). On the contrary, immune IEL displayed close to 40% lysis of infected targets at an E:T ratio of 20:1, which upon incubation with CMA was reduced by 5-fold (8% vs 40%; $p = 0.0049$) (Fig. 2B). Treatment with anti-FasL did not affect the CTL response of immune IEL. This result suggests that the cytotoxic mechanism of IEL is mediated mainly by perforin.

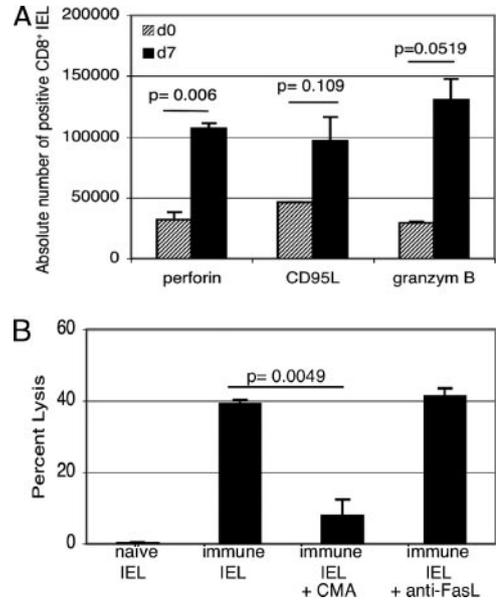


FIGURE 2. Cytotoxic activity of IEL against *E. cucurbiti*-infected target is perforin-dependent. IEL from WT mice were purified at day 7 p.i. ($n = 6$ mice/group). **A**, IELs were labeled for cytotoxic markers (perforin, CD95L, and granzyme B). Cells were isolated at day 7 p.i., and expression of perforin, granzyme B, and CD95L were analyzed by intracellular staining. **B**, Before incubation with ^{51}Cr -labeled uninfected and infected macrophages (E:T ratio of 20:1), IEL were treated with either CMA or anti-FasL Abs. After 4 h of incubation, the cytolytic activity was determined by radioisotope release into the culture supernatant. The experiment was performed twice with similar results. Data are representative of one set of experiments.

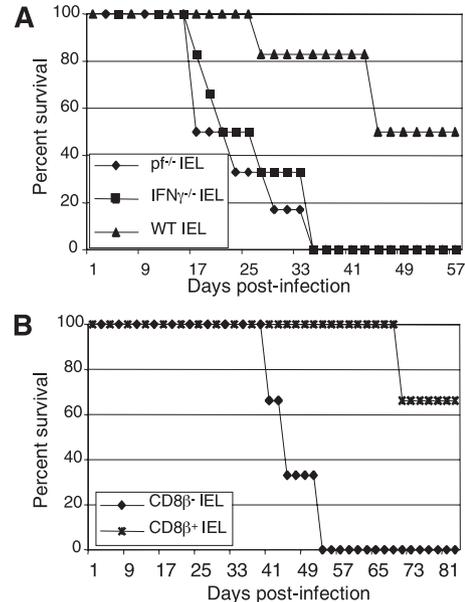


FIGURE 3. Adoptive transfer of IEL isolated from $pf^{-/-}$ and $IFN-\gamma^{-/-}$ mice fails to protect SCID mice against *E. cucurbiti* challenge. **A**, Adoptive transfer of IEL from $IFN-\gamma^{-/-}$ and $pf^{-/-}$ is unable to protect SCID mice against a lethal challenge with *E. cucurbiti*. IEL from naive and infected $pf^{-/-}$, $IFN-\gamma^{-/-}$, and WT mice ($n = 12$ mice/group) were isolated at day 7 p.i. A total of 5×10^6 IEL were injected i.p. into SCID mice ($n = 6$ mice/group). After 48 h, the mice were challenged orally with 2×10^7 spores of *E. cucurbiti* and the survival of the animals was monitored daily until the end of the experiment. **B**, IEL from WT mice were purified at day 7 p.i. $CD8\beta^+$ and $CD8\beta^-$ subset were separated and adoptively transferred to SCID recipients ($n = 6$ mice/group). The recipient animals were orally infected with 2×10^7 *E. cucurbiti* 2 days posttransfer. The survival of the animals was monitored daily until the end of the experiment.

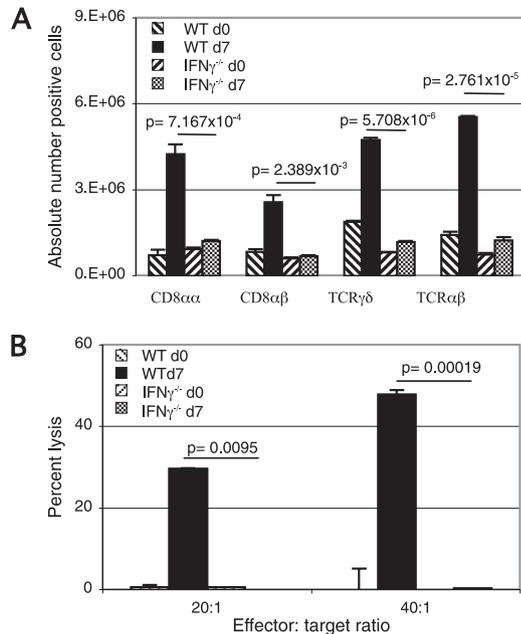


FIGURE 4. IFN- γ ^{-/-} mice exhibit a suboptimal IEL response against *E. cuniculi* infection. IEL from IFN- γ ^{-/-} and WT mice ($n = 6$ mice/group) were isolated 7 days p.i. **A**, Induction of IEL response in IFN- γ ^{-/-} mice following oral *E. cuniculi* infection. IFN- γ ^{-/-} and WT mice orally infected with *E. cuniculi* were sacrificed at day 7 p.i., and IEL were purified ($n = 4$ mice/group). Isolated IEL were assayed for CD8 $\alpha\alpha$, CD8 $\alpha\beta$, TCR $\alpha\beta$, and TCR $\gamma\delta$ expression by FACS analysis. Data represent the mean \pm SD of two individual sets of experiments. **B**, Cytotoxic activity of IEL against *E. cuniculi*-infected macrophages. IEL from IFN- γ ^{-/-} and WT mice were incubated with ⁵¹Cr-labeled uninfected and infected macrophages at different E:T ratio (20:1, 40:1). After 4 h of incubation, the cytolytic activity was determined by radioisotope release into culture supernatant. The experiment was performed twice with similar results and data are representative of one experiment.

IEL from *pf*^{-/-} and IFN- γ ^{-/-} mice are unable to protect SCID recipients against oral challenge with *E. cuniculi*

To further establish that IFN- γ production and the cytotoxic effect of IEL are important for immune protection against oral *E. cuniculi* infection, adoptive transfer studies were performed. IEL isolated from *pf*^{-/-} or IFN- γ ^{-/-} infected mice were transferred to SCID recipients, which were subsequently challenged with *E. cuniculi* spores (Fig. 3A). As previously observed, immune IEL from WT mice were able to partially protect SCID mice against a lethal *E. cuniculi* challenge (5). However, IEL from both *pf*^{-/-} and IFN- γ ^{-/-} mice were unable to protect SCID recipients and all the animals were dead by day 35 p.i. In addition, none of the SCID recipients injected with naive IEL survived (data not shown). To analyze the migrating pattern of IEL in recipient animals, CFSE-labeled IEL isolated from *pf*^{-/-}, IFN- γ ^{-/-}, and WT animals were adoptively transferred to SCID mice. The recipient animals were challenged 1 day after transfer. Two days p.i., the animals were sacrificed, and the spleen, MLN, or IEL compartment was analyzed for the presence of CFSE⁺ cells. Similar to our previously published data (5), the majority of cells isolated from WT or knockout animals migrated back to the MLN and some of them were recovered from the IEL compartment. A very low number of CFSE⁺ cells was detected in the spleen (data not shown). These results suggest that elicitation of protective IEL response against oral *E. cuniculi* infection is dependant on IFN- γ and the cytotoxic function of these cells.

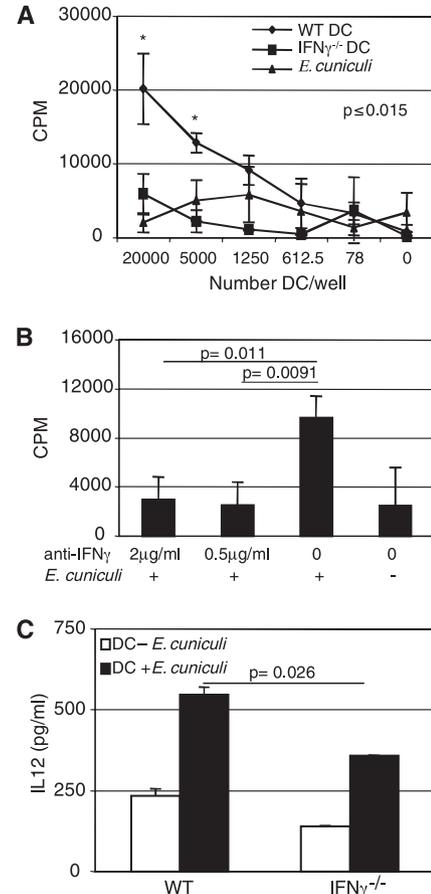


FIGURE 5. DC from PP of IFN- γ ^{-/-} mice are unable to stimulate naive CD8 $\alpha\beta$ IEL. Immature DC from naive IFN- γ ^{-/-} and WT mice ($n = 6$ mice/group) were isolated, plated, and infected overnight with *E. cuniculi*. The next day, naive IEL purified from WT mice ($n = 6$ mice/group) were separated and CD8 $\alpha\beta$ IEL were added to the culture. **A**, Presentation was measured as induction of proliferation of CD8 $\alpha\beta$ IEL. **B**, DC were treated with anti-IFN- γ or isotype control Ab at the same time that *E. cuniculi* infection and for the remaining of the experiment. **C**, IL-12 production in the supernatants of *E. cuniculi* primed DC from WT and knockout animals, after overnight incubation, was measured by ELISA. Experiments were performed twice with similar results and data are representative of one experiment.

Previous data from our laboratory demonstrated that most of IFN- γ -producing and cytotoxic IEL induced during *E. cuniculi* infection had a CD8 $\alpha\beta$ phenotype (5). As both of these properties appear to be critical for host survival against *E. cuniculi* infection, transfer of CD8 $\alpha\beta$ IEL alone should ensure the transfer of protection to naive host. To validate this hypothesis, CD8 β ⁺ and CD8 β ⁻ IEL from WT mice were purified and transferred to SCID animals. Twenty-four hours later, the recipients were challenged orally and monitored for morbidity and mortality. As shown in Fig. 3B, CD8 β ⁺ donor IEL conferred a high degree of protection to SCID recipients, and 80% of the animals that received these cells survived a lethal *E. cuniculi* challenge. Conversely, all the SCID mice injected with CD8 β ⁻ IEL from immune mice succumbed to infection.

Defective induction of IEL response in IFN- γ ^{-/-} mice following *E. cuniculi* infection

These studies demonstrate an important role for IFN- γ in IEL-mediated protection against *E. cuniculi*. To further understand the

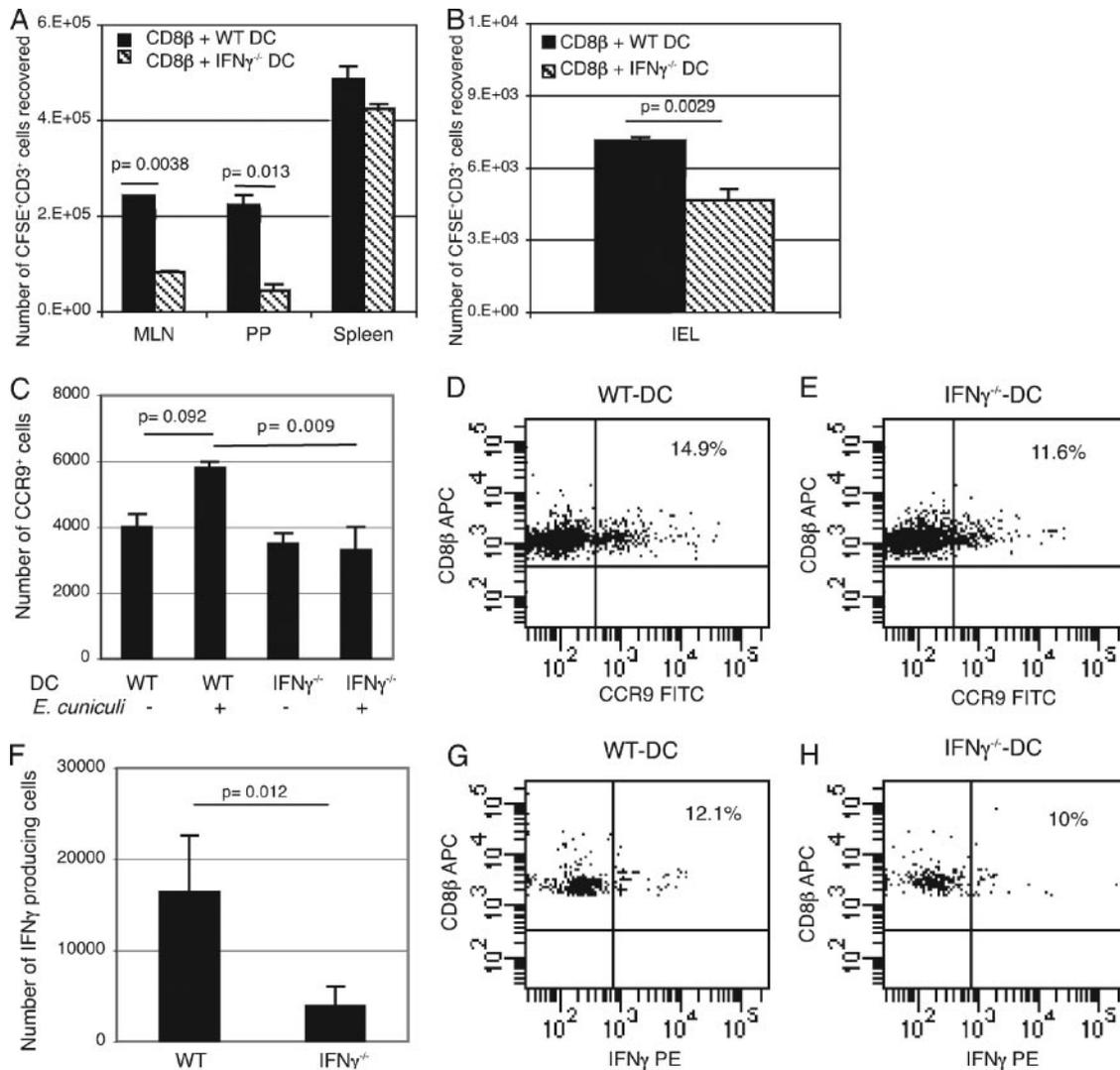


FIGURE 6. Splenic $CD8\alpha\beta^+$ T cells primed with DC from $IFN-\gamma^{-/-}$ mice have impaired ability to traffic to the gut. *A* and *B*, Immature DC from PP of naive $IFN-\gamma^{-/-}$ and WT mice ($n = 6$ mice/group) were isolated and infected with *E. cucuruli*. After overnight incubation, purified $CD8\beta^+$ splenocytes from naive WT mice ($n = 4$ mice) were added to the culture. Seventy-two hours later, cells were harvested and labeled with CFSE before transfer to WT recipient animals ($n = 2$ mice/group). Subsequently after 24 h, the recipients were sacrificed and cells from MLN, PP, spleen (*A*) and IEL (*B*) recovered and analyzed for the presence of CFSE-labeled $CD3^+$ cells by flow cytometry. *C–H*, $CD8\beta^+$ splenocytes isolated from naive mice were primed with PP DC from WT or $IFN-\gamma^{-/-}$. After 72 h, $CD8\beta^+$ cells were labeled for CCR9 expression (*C–E*) or $IFN-\gamma$ production by intracellular staining (*F–H*). Data in dot plots are gated on double positive $CD8\alpha^+CD8\beta^+$ T cells primed with DC from WT (*D–G*) or $IFN-\gamma^{-/-}$ (*E–H*) mice labeled with allophycocyanin-conjugated anti- $CD8\alpha$ and PE-conjugated anti-CCR9 (*D* and *E*) or $IFN-\gamma$ (*G* and *H*). Percentage represents surface expression of CCR9 (*D* and *E*) and $IFN-\gamma$ production (*G* and *H*) in $CD8\alpha\beta$ cells. Experiments were performed twice with similar results and data are representative of one experiment.

role of $IFN-\gamma$, a phenotypic analysis of IEL from infected $IFN-\gamma^{-/-}$ and WT mice was performed at day 7 p.i. Similar to our previous observations, WT mice showed a strong induction of $CD8\alpha\alpha$, $CD8\alpha\beta$, $TCR\alpha\beta$, and $TCR\gamma\delta$ IEL in response to *E. cucuruli* infection (Fig. 4*A*). However, a significantly lower increase ($p < 0.05$) in all the subpopulations of IEL was observed in the infected $IFN-\gamma^{-/-}$ mice.

To establish that the lack of $IFN-\gamma$ leads to a failure of the Ag-specific IEL response in *E. cucuruli*-infected animals, functional assay was performed. IEL from WT and $IFN-\gamma^{-/-}$ mice were isolated at day 7 p.i. and assayed for Ag-specific cytotoxic activity. As shown in Fig. 4*B*, IEL from $IFN-\gamma^{-/-}$ mice exhibited a severe defect in induction of Ag-specific cytotoxic response as minimal lytic activity ($p < 0.01$) against infected targets was observed (Fig. 4*B*). Predictably, immune IEL from WT mice exhibited a strong cytotoxic activity against infected targets (30 and 45% at E:T ratio of 1:20 and 1:40, respectively). As expected, IEL

from naive WT and $IFN-\gamma^{-/-}$ mice were unable to lyse *E. cucuruli*-infected macrophages.

In vitro priming of IEL by DC from PP

It has been recently reported that $IFN-\gamma$ production by DC is very important for their ability to process Ags (15–19). Next, we determined whether the Ag-specific IEL response by DC can be induced and whether $IFN-\gamma$ is necessary for this process. As PP is a major site for induction of the mucosal immune response (20–22), DC from this site were isolated. Cells from naive $IFN-\gamma^{-/-}$ and WT mice were plated and infected overnight with *E. cucuruli* spores. Following extensive washing, IEL from WT mice were added to the culture and after 72 h, the proliferation of IEL was measured by overnight [3H]thymidine incorporation. As demonstrated in Fig. 5*A*, at a concentration of $5\text{--}20 \times 10^3$ cells per well, DC from WT mice induced a significant proliferation of IEL ($p \leq$

0.015). Conversely, IEL incubated with DC from IFN- $\gamma^{-/-}$ animals exhibited minimal proliferation, which was not significantly higher than the control wells containing no effector cells (Fig. 5A).

To establish the importance of IFN- γ in Ag-specific priming of IEL, DC from WT mice were incubated overnight with murine anti-IFN- γ Ab. IEL were added 24 h later, and proliferation was measured. As shown in Fig. 5B, anti-IFN- γ treatment of DC inhibited their ability to present *E. cuniculi* Ag to IEL as Ab treatment led to a 3-fold reduction in the proliferation of IEL ($p \leq 0.02$).

Because activated DC are known to release IL-12 in response to various infectious agents (23), in vitro IL-12 production by *E. cuniculi*-primed DC was measured. Purified DC from naive WT and IFN- $\gamma^{-/-}$ mice were cultured and pulsed with *E. cuniculi* spores. After 24 h of incubation, the supernatants were collected and assayed for IL-12 production by ELISA. As shown in Fig. 5C, compared with DC from WT mice, cells isolated from IFN- $\gamma^{-/-}$ animals release significantly less IL-12 in response to *E. cuniculi* stimulation ($p = 0.026$).

These studies demonstrated that IFN- γ produced by DC from PP acts in an autocrine manner and plays an important role in their activation. Recent studies using an OVA Ag model have demonstrated that DC from PP selectively imprint homing receptors on the splenic T cells, priming them and enabling them to migrate preferentially to the small intestine (24). To establish whether IFN- γ production by DC from PP is involved in the selective priming and subsequent migration of T cells to the gut, CD8⁺ splenocytes from naive animals were purified and incubated with *E. cuniculi* activated DC from PP of WT or IFN- $\gamma^{-/-}$ mice. After 72 h of incubation, the cells were collected and labeled with CFSE. A total of 2×10^6 labeled cells were administered to naive WT animals via i.p. route, recipients were sacrificed 24 h later, and tissues (spleen, MLN, PP, IEL) were assayed for the presence of donor CFSE⁺ CD3⁺ cells. As shown in Fig. 6, incubation of CD8⁺ T cells with DC from PP of WT mice led to greater migration to the gut ($p \leq 0.02$). Conversely, CD8⁺ T cells incubated with the DC of IFN- $\gamma^{-/-}$ animals had significantly reduced capability to traffic to this site, demonstrating the importance of IFN- γ produced by PP DC in the priming process of Ag-specific IEL response. To further assess the level of activation of the splenic CD8 β^+ before transfer, cells were analyzed for CCR9 expression, a chemokine receptor specific for gut tropism (25), and IFN- γ production. CD8 $\alpha\beta^+$ T cells from the spleen of naive animals were isolated and primed as described. After 72 h of incubation, the cells were harvested and labeled for surface CCR9 and intracellular IFN- γ expression, as described in *Materials and Methods*. CD8 $\alpha\beta^+$ T cells primed by DC from PP of IFN- $\gamma^{-/-}$ mice exhibited significantly lower level of CCR9 (Fig. 6, C–E) and reduced IFN- γ production (Fig. 6, F–H) when compared with cells primed by DC of WT mice. These studies demonstrate that IFN- γ plays a critical role in the initiation of IEL response during *E. cuniculi* infection.

Discussion

Previous studies from our laboratory reported a vigorous IEL response during *E. cuniculi* infection (5). The data presented in this study demonstrate that strong Ag-specific IEL immune response is dependent on IFN- γ -producing mucosal DC. IEL from mice lacking the IFN- γ gene failed to exhibit a robust IEL response and thus were unable to protect naive animals against a lethal challenge. Similarly, treatment of DC from WT mice with anti-IFN- γ Ab neutralized their ability to prime IEL population, and the cells subsequently failed to exhibit Ag-specific proliferation. Our observation also strongly suggest that IFN- γ is involved in the acti-

vation of DC in PP in an autocrine manner, which subsequently play an important role in the generation of Ag-specific IEL response in the small intestine. Moreover, IFN- γ production of DC from PP is important both for induction of Ag-specific IEL response and selective migration of primed T cells to gut compartment.

Although the involvement of Ag-specific cytotoxic IEL has been reported in various infectious disease models (10, 26, 27), the mechanism of killing by these IEL has not been described. Recent studies have demonstrated that CTL killing is dependent mostly on two mechanisms. The first mechanism is mediated by Fas-FasL interaction (28) and exocytosis of lytic granules and the second is via perforin, a molecule present in the cytoplasmic granules of the CTLs (29). Perforin release causes the formation of pores on the cellular membrane of target cells permitting the penetration of granzymes into their cytoplasm leading to the apoptosis of the target cell (30, 31). Although increase in all cytotoxic molecules was observed, perforin mediated lysis is apparently the main effector mechanism by which IEL lyse infected cells.

Another important observation made in the current study is the in vitro proliferation of IEL in response to antigenic stimulation. It is generally believed that IEL exhibit phenotypic and functional characteristic of partially activated T cells and these cells do not proliferate as well as other cells (6, 32). Mosley et al. (33) demonstrated that IEL expressing TCR $\alpha\beta$ or TCR $\gamma\delta$ are largely unresponsive to proliferative signals mediated by the conventional stimulation of the CD3-TCR complex. Our findings are important since this report is the first of the in vitro proliferation studies of IEL in response to Ag-pulsed DC stimulation. It will be very interesting to determine whether a similar finding can be observed with other microbial agents in which IEL are known to play an important role in immune protection.

DC are the most potent professional APC with a unique ability to prime naive T cells (34, 35). They play an important role in the initiation and regulation of the immune response (6, 36). In the PP, DC are separated from the intestinal lumen by only a single layer of cells, the follicle associated epithelium retaining Ag-transporting microfold cells (M cells). In addition, a small number of DC are located directly within the follicle associated epithelium (37–39). Due to their location, these cells are likely to play a crucial role in the induction of immunity to infections acquired via an oral route. Moreover, detection of *Listeria* and *Salmonella* in DC from PP per oral infection suggests an important role for these cells in the mucosal protection against pathogens (40, 41). In the present study, we have demonstrated that DC from PP are important for the priming of IEL in vitro. Our data provide strong supportive evidence to the common belief that IEL are primed in the PP (36, 42) and then migrate to the epithelium where they can protect the mucosa against further infections (43). Moreover, recent studies with reovirus have demonstrated that intraduodenal infection in mice led to a rapid increase in precursor CTL population in the PP and IEL compartment (44). Also in the same study, it was demonstrated that adoptive transfer of lymphocytes from PP to SCID mice resulted in the appearance of a large number of CD8⁺TCR $\alpha\beta^+$ IEL. However, there are reports that most IEL populations develop independently of passage through the PP or MLN (45). The variation in these observations can be attributed to different developmental requirements for CD8 $\alpha\alpha$ and CD8 $\alpha\beta$ IEL. Although a recent report suggests that all gut IEL are from thymic origin (46), CD8 $\alpha\alpha$ IEL are believed to develop locally in cryptopatches independently of PP (47). Conversely, CD8 $\alpha\beta$ IEL seems to dependent on priming by DC from PP (24). Similar to these observations, in the current study we demonstrate that gut

immunity against *E. cucuruli* infection is mediated by CD8 $\alpha\beta$ population of IEL subset and that DC from PP seem to be important for initiating this response.

Priming of IEL by DC appears to be highly dependent on IFN- γ because neutralization of this cytokine abrogates the induction of Ag-specific response. Moreover, increase in IEL levels during *E. cucuruli* infection in IFN- $\gamma^{-/-}$ mice was severely compromised and cells from these animals failed to lyse infected target. Recent studies have reported a high level of intracellular IFN- γ expression in IL-12-stimulated DC (16, 17, 48). Similar to these findings, our study suggests that IL-12-dependent IFN- γ production by DC is important for stimulation of IEL response against oral *E. cucuruli* infection. Moreover, although, the role of IFN- γ in induction of the systemic CTL response has been described in various infectious disease models (14, 49, 50), this study is the first to describe the importance of IFN- γ in the generation of Ag-specific IEL response against an oral pathogen.

Our data demonstrate that DC from PP are important for the trafficking of Ag-primed CD8 $^{+}$ T cells to the gut compartment. When primed with *E. cucuruli*-pulsed DC from PP, a larger population of adoptively transferred splenic CD8 $^{+}$ T cells ended up in the small intestine (MLN, PP, and IEL compartment) of the donor mice. However, when primed with DC from PP of IFN- $\gamma^{-/-}$ mice, a reduced population of CD8 $^{+}$ T cells was able to migrate to the gut. Also, CD8 $^{+}$ T cells primed with DC from PP of WT mice exhibit an up-regulation of CCR9, a receptor for chemokine responsible for trafficking of lymphocytes to the gut (25). Conversely, no increase in CCR9 expression was observed on CD8 $\alpha\beta$ T cells when primed with DC from PP of IFN- $\gamma^{-/-}$ mice. These findings are supported by a recent study by Mora et al. (24) in which it was reported that DC from PP induce high levels of gut homing molecules on CD8 $^{+}$ T cells enabling them to migrate to the small intestine. The novelty of this report is it is the first to describe the ability of DC from PP to prime and induce a gut-homing ability on CD8 $^{+}$ T cells in an infectious disease model. Moreover, our observations go further in demonstrating the importance of IFN- γ in this process.

Our data presented in this study shed a new light on the induction and function of IEL in general and *E. cucuruli* infection in particular. Based on these findings, we hypothesize the following: oral *E. cucuruli* infection leads to an activation of DC in mucosal sites like PP and MLN. The CD8 $\alpha\beta^{+}$ T cell population in the gut is primed by these DC, and these T cells subsequently acquire homing receptors specific for mucosal T cells, enabling them to home to the epithelial mucosa. Cells of the intestinal epithelium are the first line of defense against oral pathogen. Ag-specific IEL due to their cytolytic activity keep the infection in control thus preventing it from disseminating to other tissues. The current studies raise several important questions regarding the priming of the IEL response. What is the mechanism by which IFN- γ induces an Ag-specific IEL response against *E. cucuruli* infection? Is IFN- γ involved in efficient Ag processing and presentation by DC? Are DC exclusive APCs able to present Ag to IEL? On-going experiments in our laboratory should help answer these questions and provide information that will be valuable for targeting these cells for developing therapeutic agents against oral pathogens.

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Disclosures

The authors have no financial conflict of interest.

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