The Journal of Immunology

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J Immunol 2000; 164:4812-4818; ;
doi: 10.4049/jimmunol.164.9.4812
http://www.jimmunol.org/content/164/9/4812

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The Journal of Immunology is published twice each month by
The American Association of Immunologists, Inc.,
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Print ISSN: 0022-1767 Online ISSN: 1550-6606.

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Regulation and Role of IFN-γ in the Innate Resistance to Infection with *Chlamydia pneumoniae*[1]

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By using mice genomically lacking IFN-γR, IL-12, perforin, and recombination-activating gene-1 (RAG-1), we analyzed the regulation and importance of IFN-γ in the control of infection with *Chlamydia pneumoniae*. IL-12 participates in resistance of mice to *C. pneumoniae*, probably by regulating the protective levels of IFN-γ mRNA. In turn, IFN-γ is necessary for the increased IL-12p40 mRNA accumulation that occurs in lungs during infection with *C. pneumoniae*, suggesting a positive feedback regulation between these two cytokines. In experiments including RAG-1$^{-/-}$/IFN-γR$^{-/-}$ mice we showed that IFN-γ produced by innate cells controls the bacterial load and is necessary for the increased accumulation of transcripts for enzymes controlling high output NO release (inducible NO synthase), superoxide production (gp-91 NADPH oxidase), and catalyzis of tryptophan (indoleamine 2,3-dioxygenase (IDO)), mechanisms probably related to bacterial killing. Adaptive immune reponses diminish the levels of IFN-γ and IL-12 mRNA and thereby the levels of inducible NO synthase, IDO, and gp91 NADPH oxidase trancripts. By using RAG-1$^{-/-}$/perforin$^{-/-}$ mice, we excluded the overt participation of NK cell cytotoxicity in the control of *C. pneumoniae*. However, NK cells and probably other innate immune cells release IFN-γ during the bacterial infection. ***The Journal of Immunology*****, 2000, 164: 4812–4818.**

Infection with *Chlamydia pneumoniae*, a Gram-negative obligate intracellular bacteria, will occur at least once in more than 50% of the human population world-wide causing, e.g., pneumonia, sinusitis, and bronchitis. In addition, the spectrum of *C. pneumoniae* infection has been suggested to extend to atherosclerosis and its clinical manifestations (1–4).

C. pneumoniae provokes a similar lung pathology in humans and rodents (5). A mouse model of infection has been used to study the immunological mechanisms of immunity. Immunity to *C. pneumoniae* proceeds in two stages: 1) an early response requiring IFN-γ to limit the growth of the bacteria, and 2) a later adaptive immune response that involves CD4$^+$ and CD8$^+$ T cells in protection (6). Still, the role that cellular and soluble components play during chlamydial infection is not fully elucidated, nor is the relative importance of the adaptive vs the innate arms of the immune system early after infection.

IFN-γ can be induced by IL-12 but also by other cytokines such as IL-2, IL-18, and IFN-$\alpha\beta$ (7). The relative prevalence of these different stimuli seems to depend on the nature of the infecting/immunizing agent (8). IFN-γ can be released by cells from both the innate and acquired immune system. IFN-γR-deficient mice show much higher susceptibility to *C. pneumoniae* compared with SCID or RAG-1$^{-/-}$ mice or with wild-type (WT)[3] controls, suggesting major involvement of innate cells in the release of IFN-γ (6).

Chlamydia is internalized by macrophages as well as by "non-professional" phagocytes, where it survives and replicates. In such cells, IFN-γ synergizes with bacterial products to activate various bactericidal or bacteriostatic mechanisms (9–11). IFN-γ is a strong activator of indoleamine 2,3-dioxygenase (IDO), which catalyzes decyclization of L-tryptophan, thereby limiting the availability of this amino acid to intracellular microorganisms (12–14). Induction of IDO has been positively correlated with inhibition of chlamydial growth in vitro (15–19). IFN-γ can also activate inducible nitric oxide synthase (iNOS), which catalyzes production of NO from L-arginine (20). Inhibition of chlamydial growth through induction of iNOS has also been reported (9, 21–23). Stimulation of neutrophils or monocytes with IFN-γ leads to induced transcription of the gp91 component of NADPH oxidase (ox) mRNA (24, 25). Activation of this multicomponent NADPH ox drives the respiratory burst of phagocytic cells catalyzing the release of O_2^-. The relative importance of these effector mechanisms in the control of *C. pneumoniae* infection in vivo is unknown.

We have studied in detail the involvement of IFN-γ in the control of infection with *C. pneumoniae*. A reciprocal control of expression between IL-12 and IFN-γ during *C. pneumoniae* infection was found. IFN-γ also induced in vivo gene expression of iNOS, IDO, and gp91 NADPH ox, all likely to be involved in destruction of *C. pneumoniae*. That IFN-γ indeed plays this central role in the innate control of infection with *C. pneumoniae* was finally demonstrated by using RAG-1 and IFN-γR single- and double-knockout mice. In contrast, perforin-mediated lysis plays no overt role in the innate resistance to *C. pneumoniae*.

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Received for publication November 8, 1999. Accepted for publication February 17, 2000.

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[1] This work was supported by European Union Biotechnology and Transdisease Vaccinology Program (contract no. ERBBIO4ACT960152), by Amgen, and by the Karolinska Institutet.

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[3] Abbreviations used in this paper: WT, wild type; IDO, indoleamine 2,3-dioxygenase; iNOS, inducible nitric oxide synthase; ox, oxidase; RAG-1, recombination-activating gene-1; IFU, inclusion-forming unit; OMP, outer major protein; i.n., intranasal(ly).

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Materials and Methods

Mice

Mutant mouse strains without IFN-γR (26), perforin (27), recombination-activating gene-1 (RAG-1) (28), and IL-12p40 (29) were generated by homologous recombination in embryonic stem cells. Mice (H-2^b) underwent five to nine backcrosses with C57BL/6 mice, which were used as controls. Mice were bred and kept under specific pathogen-free conditions and were maintained in isolation under negative pressure during experiments. They were used between 6 and 10 wk of age.

Generation of RAG-$1^{-/-}$/IFN-γ$R^{-/-}$ and RAG-$1^{-/-}$/perforin$^{-/-}$ mice

Mice homozygous for the RAG-$1^{-/-}$ were crossed with animals homozygous for either IFN-γR or perforin deficiency. The progeny was intercrossed and F_2 was screened for the homozygous disrupted perforin or IFN-γRα genes by PCR analysis of tail DNA lysate according to standard protocols (26, 27) using the following primers: sense IFNγR, 5′-CCC ATT TAG ATC CTA CAT ACG AAA CAT ACG G-3′; antisense IFNγR, 5′-TTT CTG TCA TCA TGG AAA GGA GGG ATA CAG-3′; sense perforin, 5′-CCG GTC CTG AAC TCC TGG CCA C-3′; antisense perforin, 5′-CCC CTG CAC ACA TTA CTG GAA G-3′. RAG-$1^{-/-}$ homozygosity was determined by absence of IgG3 in sera and by absence of peripheral blood $CD4^+$ cells in F_2 mice.

Infection and infectivity assay

Mycoplasma-free *C. pneumoniae* isolate Kajaani was propagated in HL cells. Infected cells were sonicated, cell remnants were removed by centrifugation, and the bacteria were stored in small aliquots in sucrose-phosphate-glutamate solution at −70°C until used. The infectivity as measured by inclusion-forming units (IFU) of bacterial preparation was determined in HL cell culture. Mice were mildly sedated with metofane and were infected intranasally (i.n.) with 10^6 IFU diluted in 40 μl of PBS.

Animals were sacrificed, and right lungs were removed, minced, and mechanically homogenized in 2 ml of sucrose-phosphate-glutamate solution. Homogenates were centrifuged for 10 min at 500 × *g* to remove coarse tissue debris. Lysates were then diluted 10- and 100-fold in DMEM containing 5% FCS and streptomycin. The infectious titer was assayed by culturing 500 μl of duplicate dilutions of the lysates on confluent Vero cells grown on round 13-mm^2 coverslides in a shell vial. Inoculated cells were centrifuged for 1 h at 1600 rpm. Thereafter, supernatant was removed and DMEM containing cycloheximide and streptomycin was added. Cells were incubated at 35°C for 72 h, fixed with methanol, and stained with a FITC-conjugated *Chlamydia* genus-specific mAb (Pathfinder *Chlamydia* Confirmation System; Kallestad Diagnostics, Chaska, MN). Inclusion bodies were counted by fluorescence microscopy. The infectivity was expressed as IFU/lung.

Competitive RT-PCR assay

The accumulation of IFN-γ, IL-12p40, iNOS, gp91 NADPH ox, and β-actin mRNA in freshly extracted left lungs from infected mice was measured by competitive PCR assays as previously described (30). Competitor fragments with a different length but using the same primers as the target DNA were constructed using composite primers (31) and an exogenous DNA fragment or by subcloning of mutated (deleted or ligated) cytokine cDNA. Competitors were amplified by PCR, purified (Qiagen, Studio City, CA), and quantified in a spectrophotometer.

The primer sequences for the amplification of the cDNA were as follows: sense iNOS, 5′-CCC TTC CGA AGT TTC TGG CAG CAG CAG C-3′; antisense iNOS, 5′-GGC TGT CAG AGC CTC GTG GCT TTG G-3′; sense gp91 NADPH ox, 5′-CTT TGT CAT TCT GGT GTG GTT GG-3′; antisense gp91 NADPH ox, 5′-CCC CAT TCT TCG ATT TTG TCT GC-3′; sense IFN-γ, 5′-TGG ACC TGT GGG TTG TTG ACC TCA AAC TTG GC-3′; antisense IFN-γ, 5′-TCG ATC TTG GCT TTG CAG CTC TTC CTC ATG GC-3′; sense IL-12p40, 5′-CGT GCT CAT GGC TGG TGC AAA G-3′; antisense IL-12p40, 5′-CTT CAT CTG CAA GTT CTT GGG C-3′; sense β-actin, 5′-GTG GGC CGC TCT AGG CAC CAA 3′; antisense β-actin, 5′-CTC TTT GAT GTC ACG CAC GAT TTC-3′.

Ten- or 3-fold serial dilutions of the competitor were amplified in the presence of a constant amount of cDNA. Reactions were conducted for 28–45 cycles in a thermal cycler (Perkin-Elmer/Cetus, Norwalk, CT) using an annealing step at 60°C (except at 65°C for IL-12).

Southern blot of RT-PCR-amplified IDO transcripts

IDO transcripts were amplified from lung cDNA from individual mice containing similar titers of β-actin transcripts as measured in a competitive PCR assay. The primer sequences for amplification of IDO cDNA were as follows: sense IDO, 5′-CTG CCT CCT ATT CTG TCT TAT GC-3′; anti sense IDO, 5′-AGA CCA GAC CAT TCA CAC ACT CG-3′. After amplification (45 cycles, annealing step at 56°C), PCR products were subjected to electrophoresis in 1.5% agarose gels. The gels were denatured and neutralized, and a semidry transfer onto nylon membranes (Hybond-N; Amersham, Buckinghamshire, U.K.) was performed overnight at room temperature as described. After baking and prehybridization in a buffer containing 2× standard saline citrate phosphate/EDTA (SSPE), 5× Denhart's, and 0.5% SDS for 2 h at 62°C, membranes were hybridized with 5×10^6 cpm/membrane of [γ-^{32}P]5′-IDO probe (5′-TCT GGG AAT AAA ACA CGA GGC TGG CAA-3′) in prehybridization buffer overnight at 62°C. After hybridization, the membranes were washed twice with 0.2× SSPE and 0.5% SDS for 15 min at 62°C. Signal intensity of Southern blots was scanned using a PhosphorImager (Molecular Dynamics, Sunnyvale, CA).

NO release and measurement

NO was measured by the concentration of nitrite, the stable end product of NO synthase-generated reactive nitrogen intermediates. Peritoneal cells were adjusted to 10^6/ml in DMEM without phenol red. Cells were distributed in triplicate in V-shaped 96-well plates. A total of 0.5 mM L-N^G-monomethyl-L-arginine (Sigma, St. Louis, MO) was then added to some of the cultures. Supernatants were sampled after 48 h for the determination of nitrite concentration using the Griess assay.

Cytotoxic assay

A total of 5×10^3 ^{51}Cr-labeled yeast artificial chromosome-1 (YAC-1), RMA, or RMA-s target cells were added to 96-well V-bottom microtiter plates containing appropriate numbers of effector cells. The plates were incubated for 4 h at 37°C. After incubation, the plates were centrifuged, and the released ^{51}Cr in the supernatant was measured in a gamma counter. The percentage of specific lysis was calculated as follows: [(experimental release − spontaneous release)/(maximum release − spontaneous release)] cpm × 100.

Ab determinations

Outer membrane protein-2 is an abundant immunogenic protein of *C. pneumoniae*. The contents of anti-outer major protein-2 (anti-OMP-2) Abs in the sera from infected mice were measured by ELISA. The plates were coated overnight with 0.7 μg/ml Trx-albumin-binding protein-OMP fusion protein (6). After blocking with 1% BSA in PBS, sera from individual mice were then added at 1:100 or 1:400 dilutions. The plates were subsequently developed with alkaline phosphatase-conjugated rabbit anti-mouse IgG2a or anti-mouse IgG1 (The Jackson Laboratory, Bar Harbor, ME). The assay was standardized between plates by including the titration of a positive serum pool from *C. pneumoniae*-infected IFN-γ$R^{-/-}$ mice.

Results

We analyzed the involvement of IFN-γ in the resistance of mice against *C. pneumoniae*. IL-12 is one of the cytokines that triggers production of IFN-γ, and it is believed to be important in specific immunity by promoting Th1 cell differentiation. IFN-γ and IL-12 both play a relevant role in control of *C. pneumoniae* infection because lungs from IFN-γ$R^{-/-}$ or IL-$12^{-/-}$ mice contained higher numbers of bacteria than WT controls (Fig. 1*A*). Lungs from *C. pneumoniae*-infected IL-$12^{-/-}$ mice contained lower levels of IFN-γ mRNA transcripts than WT controls did (Fig. 1*B*). This indicates that IL-12 enhances the level of IFN-γ during infection. However, IL-$12^{-/-}$ mice displayed lower bacterial load than IFN-γ$R^{-/-}$ mice did, probably due to the remaining low, IL-12-independent IFN-γ release (Fig. 1*A*). IFN-γ is the main cytokine enhancing IgG2a switching. Sera from IL-$12^{-/-}$-infected mice do have diminished levels of IgG2a but normal IgG1 (isotype regulated by IL-4) anti-chlamydial OMP-2 Abs compared with WT mice, suggesting that diminished levels of IFN-γ during infection of IL-$12^{-/-}$ mice were not paralleled by an enhanced Th2 response (Fig. 1*C*).

To further investigate the protective role of IFN-γ in the innate resistance to *C. pneumoniae*, RAG-$1^{-/-}$/IFN-γ$R^{-/-}$ double knockouts were generated. The deletion of RAG-1 gene was confirmed by the absence of IgG3 in sera and by the absence of $CD4^+$

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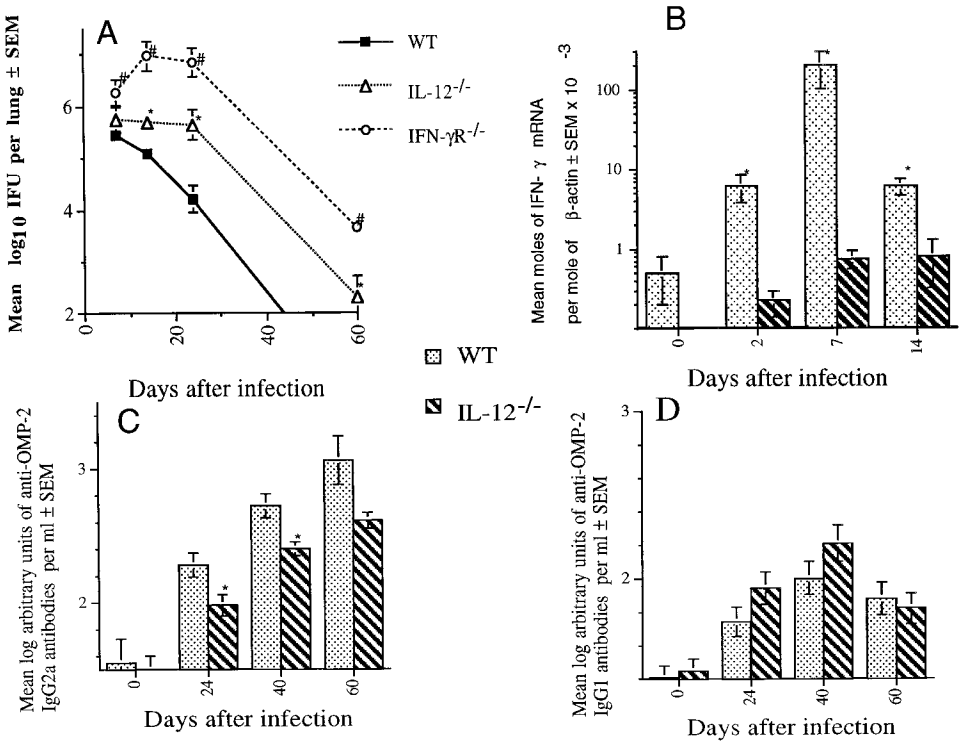

FIGURE 1. *A*, Course of *C. pneumoniae* infection in IL-12$^{-/-}$, IFN-γR$^{-/-}$, and WT mice (8–16 mice per time point and group). Mice were sacrificed at the indicated time points after i.n. infection with 10^6 *C. pneumoniae*. The mean of the $\log_{10}$ transformed IFU titers per lung is depicted. Bars indicate SE. ∗, Differences vs WT mice are significant ($p < 0.05$, Student's *t* test); #, differences vs WT and IL-12$^{-/-}$ mice are significant ($p < 0.05$, Student's *t* test). *B*, Diminished IFN-γ mRNA accumulation in lungs of IL-12$^{-/-}$ mice after infection with *C. pneumoniae*. Total RNA was obtained from lungs of IL-12$^{-/-}$ or WT mice after 7 or 14 days and transcribed into cDNA. Equal aliquots of cDNA from four to eight individual mice per group were then amplified with IFN-γ or β-actin primers in the presence of 3-fold serial dilutions of the respective competitors. The moles of IFN-γ (*C* and *D*) per mole of β-actin mRNA are depicted. ∗, Differences vs IL-12$^{-/-}$ mice are significant ($p < 0.05$, Student's *t* test). *C* and *D*, Levels of IgG2a (*C*) and IgG1 (*D*) anti-*C. pneumoniae* OMP-2 Abs after infection of IL-12$^{-/-}$ mice. Titers of anti-OMP-2 IgG1 and Ig2a were measured by ELISA in sera from individual WT and IL-12$^{-/-}$ mice (eight mice per group) after infection with *C. pneumoniae*. The mean $\log_{10}$ arbitrary units of anti-OMP-2 IgG2a (*C*) or IgG1 (*D*) per ml of sera ± SEM is depicted. ∗, Differences vs IL-12$^{-/-}$-infected mice are significant ($p < 0.05$, Student's *t* test).

cells in peripheral blood (data not shown). The disruption of IFN-γRα gene was detected by PCR and confirmed by the lack of detectable nitrite levels in supernatants of IFN-γ-stimulated macrophages (Fig. 2*A*). RAG-1$^{-/-}$/IFN-γR$^{-/-}$ mice showed dramatically increased bacterial load during infection with *C. pneumoniae* compared with RAG-1$^{-/-}$ controls. All RAG-1$^{-/-}$/IFN-γR$^{-/-}$ mice died within a month after infection (Fig. 2*B*). Deaths were seldom recorded during infection of IFN-γR$^{-/-}$ mice (data not shown). The enhanced susceptibility of RAG-1$^{-/-}$/IFN-γR$^{-/-}$ mice was associated with diminished levels of iNOS, gp91 NADPH ox, and IDO mRNA compared with those of RAG-1$^{-/-}$-infected controls (Fig. 3). IL-12p40 mRNA accumulation was also dramatically diminished in lungs from RAG-1$^{-/-}$/IFN-γR$^{-/-}$ mice 2 and 14 days after infection with *C. pneumonaie* compared with that of RAG-1$^{-/-}$-infected controls, indicating that IFN-γ is a major inducer of IL-12p40 expression during infection with *C. pneumoniae* (Fig. 4).

Confirming previous results, RAG-1$^{-/-}$ mice displayed susceptibility similar to that of WT animals early (7 and 14 days) after infection, whereas at later stages WT mice cleared infection more

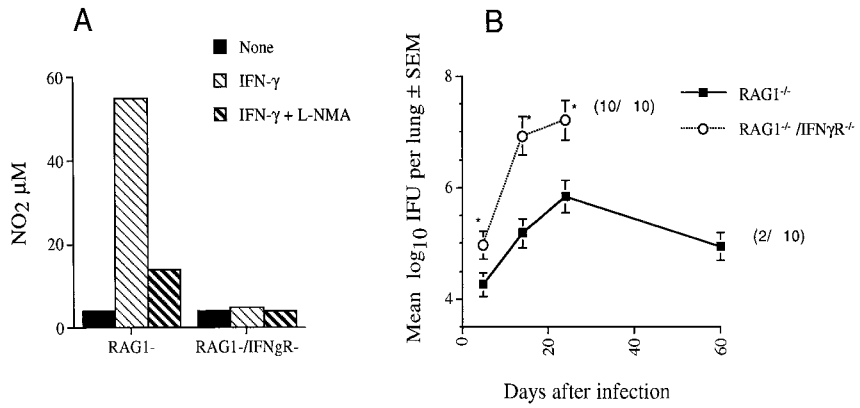

FIGURE 2. *A*, Cells from RAG-1$^{-/-}$ IFN-γR$^{-/-}$ mice do not release NO after IFN-γ stimulation. Peritoneal cells from RAG-1$^{-/-}$/IFN-γR$^{-/-}$ or RAG-1$^{-/-}$ mice were stimulated with 10 U recombinant IFN-γ per ml during 48 h in presence or absence of 5 mM L-N^G-monomethyl-L-arginine. The concentration of NO_2^- in the supernatant was measured by Griess assay. *B*, Course of *C. pneumoniae* infection in RAG-1$^{-/-}$/IFN-γR$^{-/-}$ and RAG-1$^{-/-}$ mice (eight mice per time point and group). Mice were sacrificed at the indicated time points after i.n. infection with 10^6 *C. pneumoniae*. The mean $\log_{10}$ IFU per lung are depicted; bars indicate SE; in parentheses is cumulative mortality at 30 days after infection; ∗, differences vs RAG-1$^{-/-}$ mice are significant ($p < 0.05$, Student's *t* test).

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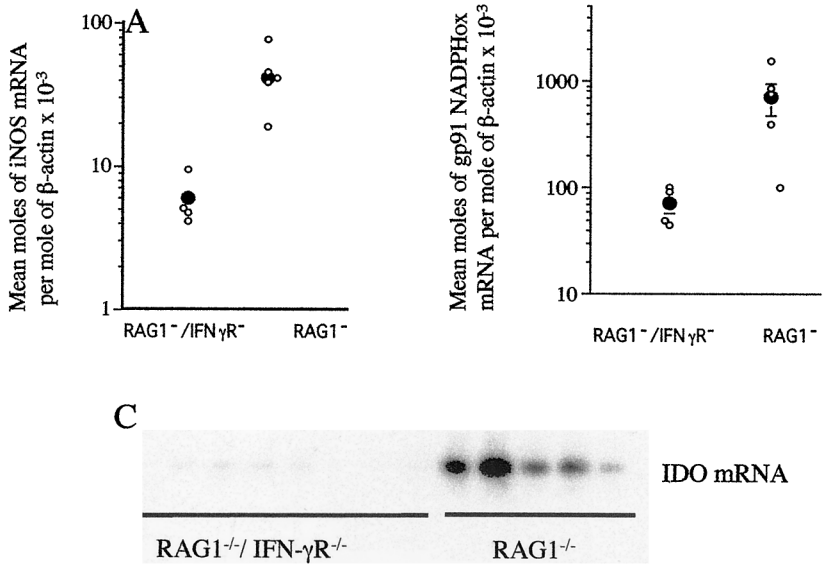

FIGURE 3. Diminished iNOS, gp91 NADPH ox, and IDO mRNA accumulation in lungs of RAG-$1^{-/-}$/IFN-$\gamma R^{-/-}$ mice after infection with *C. pneumoniae*. Total RNA was obtained from lungs of RAG-$1^{-/-}$/IFN-$\gamma R^{-/-}$ or RAG-$1^{-/-}$ mice after 14 days of infection and was transcribed into cDNA. Equal aliquots of cDNA from four to six individual mice per group were then amplified with iNOS, gp91 NADPH ox, or β-actin primers in the presence of 3-fold serial dilutions of the respective competitors. The moles of iNOS (*A*) and gp91 NADPH ox (*B*) per mole of β-actin mRNA are depicted. Differences in iNOS and gp91 NADPH ox mRNA in lungs of infected RAG-$1^{-/-}$ vs RAG-$1^{-/-}$/IFN-$\gamma R^{-/-}$ mice are significant ($p < 0.05$, Student's *t* test). *C*, Southern blot analysis of RT-PCR-amplified IDO transcript levels in lungs from individual RAG-$1^{-/-}$ and RAG-$1^{-/-}$/IFN-$\gamma R^{-/-}$ mice at 0 or 14 days after i.n. infection with 10^6 *C. pneumoniae*. Samples contained similar levels of β-actin cDNA as quantified by competitive PCR.

rapidly (Fig. 5*A*). We next asked whether the early efficient control of infection by RAG-$1^{-/-}$ mice reflected a dominating participation of innate immune responses in the WT mouse or compensatory increases of the innate mechanims in the RAG-$1^{-/-}$ mice due to the lack of the adaptive part of the protective immune responses. IFN-γ and IL-12p40 mRNA accumulations were increased in lungs from infected RAG-$1^{-/-}$ mice compared with those of WT infected controls (Fig. 5, *B* and *C*). The levels of iNOS, gp91 NADPH ox, and IDO mRNA in lungs from RAG-$1^{-/-}$ mice were also dramatically elevated compared with those of WT infected animals (Fig. 6). Thus, the relative resistance of RAG-1 mice early after infection is likely to be the result of an increase of IFN-γ and IL-12 mRNA levels that compensates for the absence of adaptive immune responses. Furthermore, this increase was positively correlated with transcripts levels of IFN-γ-inducible effector enzymes. The data suggest the presence of protective T and B cell-dependent immune responses early during infection of WT mice. At later time points after infection, when T and B cells are necessary for the control of infection, higher bacterial levels directly activating innate immune responses could also account for the increased titers of cytokine transcripts in RAG-$1^{-/-}$ mice.

NK cells are believed to provide the initial burst of IFN-γ in several protozoan and bacterial infections and might also mediate control of infection due to their cytotoxic ability. To study the involvement of NK cell cytotoxicity in the control of *C. pneumoniae* infection, RAG-$1^{-/-}$/perforin$^{-/-}$ mice were generated and infected with the bacteria. Perforin deficiency was confirmed by the lack of YAC-1 and RMA cytotoxicity (Fig. 7, *A* and *B*). Lungs from RAG-$1^{-/-}$/perforin$^{-/-}$ mice contained numbers of bacteria similar to those in RAG-$1^{-/-}$ controls during infection with *C. pneumoniae* (Fig. 7*C*).

We then analyzed whether NK-derived IFN-γ release contributed to control of infection with *C. pneumoniae*. For this purpose, mice were i.v. administered with anti-asialo GM-1 Abs (Fig. 8, *A* and *B*). Spleens from anti-asialo GM-1-administered mice showed a drastically diminished NK cytotoxic activity. Susceptibility to *C. pneumoniae* of RAG-$1^{-/-}$ mice was not altered by anti-asialo GM-1 administration (Fig. 8*C*). However, lungs from anti-asialo GM-1-treated and infected mice contained lower levels of IFN-γ mRNA in lungs compared with those of normal rabbit serum-infected controls (Fig. 8*D*).

Discussion

We have studied the relative importance of the innate vs the adaptive immune resistance of mice in the early infection with *C. pneumoniae*. Special emphasis was made on the role of IFN-γ. IL-12 has been shown to participate in the control of *Chlamydia psitacci* and *Chlamydia trachomatis* through IFN-γ-independent mechanisms (32–34). In the present study, IL-$12^{-/-}$ mice did also display increased susceptibility to infection with *C. pneumoniae*. This increase was paralleled by diminished levels of IFN-γ mRNA. In

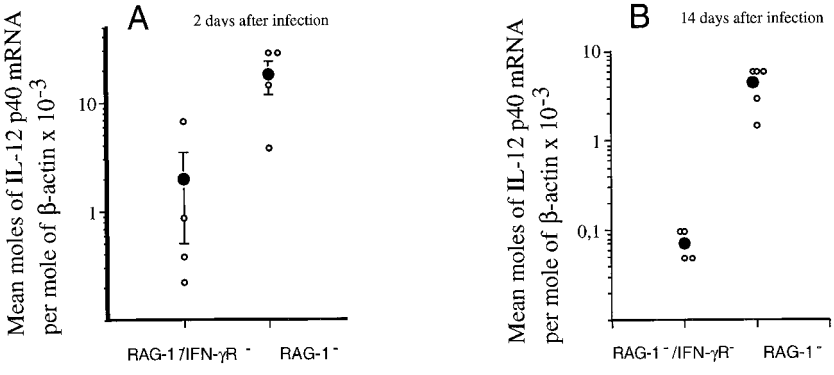

FIGURE 4. Levels of IL-12p40 mRNA in lungs from RAG-$1^{-/-}$/IFN-$\gamma R^{-/-}$ or RAG-$1^{-/-}$ mice after infection with *C. pneumoniae*. Total RNA was obtained from lungs of RAG-$1^{-/-}$/IFN-$\gamma R^{-/-}$ or RAG-$1^{-/-}$ mice after 2 (*A*) or 14 (*B*) days of infection and was transcribed into cDNA. The accumulations of IL-12p40 and β-actin mRNA in four to five individual mice per group were determined by competitive PCR. The mean moles of IL-12p40 per mole of β-actin mRNA are depicted. *, Differences vs control group are significant ($p < 0.05$, Student's *t* test).

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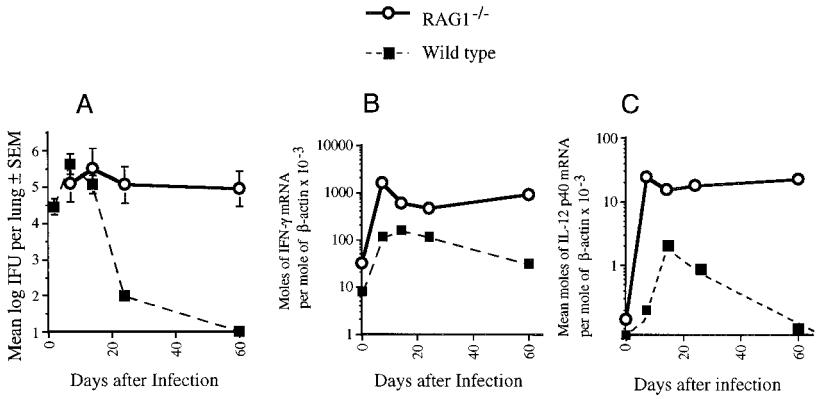

FIGURE 5. *A*, Course of *C. pneumoniae* infection in RAG-1$^{-/-}$ and WT mice (eight mice per time point and group). Mice were sacrificed at the indicated time points after i.n. infection with 10^6 *C. pneumoniae*. Mean IFU titers per lung are depicted. Bars indicate SE. Death was observed in one of nine RAG-1$^{-/-}$ mice, which were otherwise sacrificed 60 days after infection. *, Differences vs WT and RAG-1$^{-/-}$ mice are significant ($p < 0.05$, Mann Whitney U-Wilcoxon test). *B* and *C*, Total RNA was obtained from lungs of *C. pneumoniae*-infected individual RAG-1$^{-/-}$ and WT mice and was transcribed into cDNA. Equal aliquots of cDNA from three to four individual mice per group were amplified with IFN-γ, IL-12p40, or β-actin primers in the presence of 3-fold serial dilutions of the respective competitors. The mean moles of IFN-γ (*B*) and IL-12p40 (*C*) per mole of β-actin mRNA are depicted.

agreement, IgG2a-specific Abs were lower in IL-12$^{-/-}$ infected mice compared with WT mice. No compensatory increase of IgG1 Abs took place, thus no shift to a Th2 response was noted. The susceptibility of IL-12$^{-/-}$ mice to infection with *C. pneumoniae* was not as severe as that displayed by IFNγR$^{-/-}$ mice. Our data suggest that this might be due to a low, IL-12-independent IFN-γ expression, like during antiviral immunity (8, 35).

IFN-γ is known to enhance activation of IL-12p40 and p35 promoters (36). In our study, levels of IL-12p40 mRNA are dramatically diminished in RAG-1$^{-/-}$/IFN-γR$^{-/-}$ mice at 2 and 14 days after infection with *C. pneumoniae* compared with RAG-1$^{-/-}$ controls, suggesting that IFN-γ regulates IL-12 production during infection with *C. pneumoniae*. Taken together, our data suggest that a positive feedback loop controls IL-12p40 and IFN-γ mRNA levels during infection with *C. pneumoniae*. It remains to be demonstrated which of these cytokines acts as initiator of expression of the counterpart during infection with *C. pneumoniae*.

We demonstrate that IFN-γ is necessary for innate resistance against infection with *C. pneumoniae*. The difference in susceptibility of IFN-γR$^{-/-}$ mice compared with that of RAG-1$^{-/-}$/IFN-γR$^{-/-}$ mice suggests that IFN-γ-independent adaptive immune mechanisms also participate in protection. On the other hand, such protective adaptive immune responses may diminish IFN-γ and IL-12p40 gene expression early after infection, as suggested by the enhanced IFN-γ and IL-12p40 mRNA levels in RAG-1$^{-/-}$ compared with WT infected mice. For instance, IL-12 has been shown to induce IL-10 production by Th2 cells, which in turn inhibits IL-12 production by phagocytic cells (37). Other cytokines such as IL-4, IL-13, or TGF-β also have been shown to inhibit IL-12 and IFN-γ (7). Alternatively, an increased density of inflammatory cells in lungs could also account for the enhanced IL-12 and IFN-γ levels in RAG1$^{-/-}$ mice.

IFN-γ was necessary for accumulation of iNOS, gp91 NADPH ox, and IDO mRNA during *C. pneumoniae* infection of RAG-1$^{-/-}$ mice. The levels of these transcripts were accordingly positively associated with increased levels of IFN-γ and IL-12p40 mRNA in RAG-1$^{-/-}$ compared with those in WT animals. The iNOS mRNA level was increased in lungs from infected WT mice compared with the level in lungs from noninfected controls, but no increase of gp91 NADPH ox and IDO mRNA could be detected in the same tissues. Thus, IDO and NADPH ox are probably not involved in resistance to infection with *C. pneumoniae* in normal immunocompetent mice.

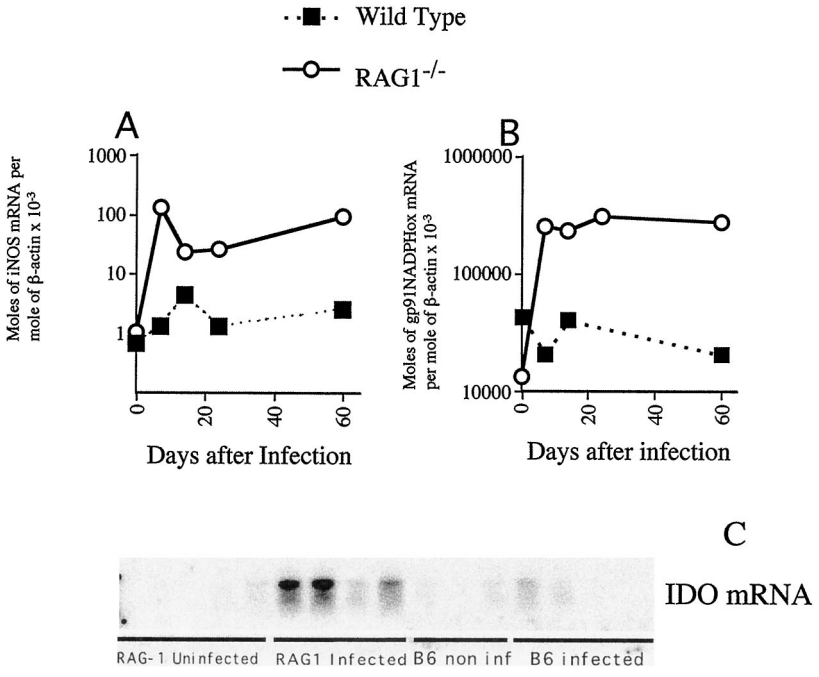

FIGURE 6. Total RNA was obtained from lungs of *C. pneumoniae*-infected RAG-1$^{-/-}$ and WT mice and transcribed into cDNA. Equal aliquots of cDNA from three to four individual mice per group were amplified with iNOS, gp91 NADPH ox, or β-actin primers in the presence of 3-fold serial dilutions of the respective competitors. The mean moles of iNOS (*A*) and gp91 NADPH ox (*B*) per mole of β-actin mRNA from two independent experiments are depicted. *C*, Southern blot analysis of RT-PCR-amplified IDO transcript levels in lungs from individual RAG-1$^{-/-}$ and WT mice at 0 or 14 days after i.n. infection with 10^6 *C. pneumoniae*. Samples contained similar levels of β-actin cDNA as quantified by competitive PCR.

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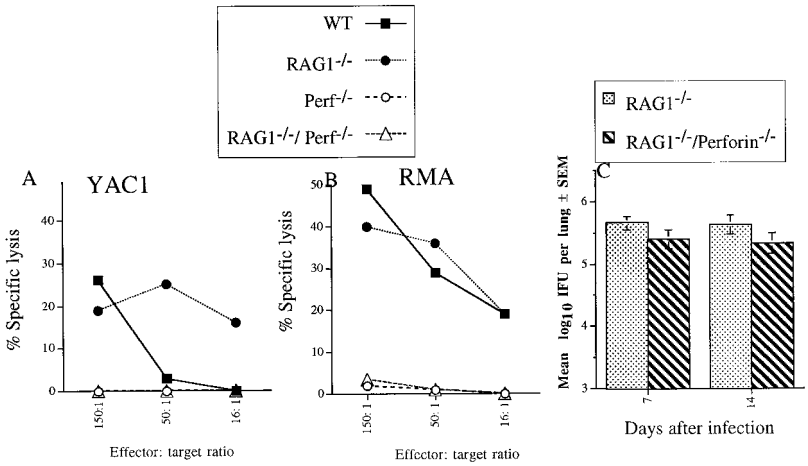

FIGURE 7. NK cell cytotoxicity is not necessary in the innate control of *C. pneumoniae* infection. *A* and *B*, Absence of NK cytotoxicity in RAG-1$^{-/-}$/perforin$^{-/-}$ (Perf$^{-/-}$) mice. The cytotoxic ability of spleen cells of WT, Perf$^{-/-}$, RAG-1$^{-/-}$, and RAG-1$^{-/-}$/Perf$^{-/-}$ mice was assayed using ^{51}Cr YAC-1 (*A*) or RMA (*B*) cells as targets. The precentages of lysis at different E:T ratios are depicted. *C*, Numbers of *C. pneumoniae* IFU in lungs of RAG-1$^{-/-}$ and RAG-1$^{-/-}$/Perf$^{-/-}$ mice (10 mice per time group) after 7 and 14 days of i.n. infection. The mean $\log_{10}$ IFU per lung and the SE are depicted.

Increased susceptibility to *C. pneumoniae* of both IFN-γR$^{-/-}$ and RAG-1$^{-/-}$/IFN-γR$^{-/-}$ mice compared with IFN-γR$^{+/+}$ controls was accompanied by a major shift in the inflammatory infiltrate toward an abundance of neutrophils over macrophages (data not shown). In accordance, mice given IL-12 show diminished levels of polymorphonuclear cells during infection with *C. psitacci* (34). Likewise, IFN-γ has been shown to promote the formation of inflammatory exudates rich in monocytes to the relative exclusion of granulocytes, through chemokine induction (38, 39). Whether the qualitative changes in the pathology in the present system are due to different levels of bacterial chemoattractants or to altered levels of IFN-γ-regulated adhesion molecules or chemokines (40) is a question that requires investigation.

NK cells have been suggested to play a role during genital but not pulmonary experimental infection with *C. trachomatis* (41, 42). We demonstrate by using RAG-1$^{-/-}$/perforin$^{-/-}$ mice that NK cytotoxicity does not play a relevant role in control of *C. pneumoniae*. Also, NK cell depletion in RAG-1$^{-/-}$ mice given anti-asialo GM-1 did not alter the bacterial load. However, pretreatment of infected mice with anti-asialo GM-1 diminished the level of IFN-γ mRNA in the lungs, which is in line with the fact that IFN-γ is also released by NK cells, as shown during infection with *C. trachomatis*. Pulmonary macrophages can secrete IFN-γ, as shown during infection with *Mycobacterium bovis* (43), and they are likely candidates to secrete IFN-γ in the RAG-1$^{-/-}$ anti-asialo GM-1-treated mice. Alternatively, a small number of IFN-γ-secreting NK cells surviving anti-asialo GM-1 treatment could account for the nonaltered susceptibility of the Ab-treated mice.

In conclusion, we show that IFN-γ-mediated protection is important for the control of infection with *C. pneumoniae*. IL-12 participates in resistance to *C. pneumoniae*, probably by enhancing IFN-γ mRNA. In turn, IL-12p40 mRNA is induced by IFN-γ. IFN-γ produced by innate cells can control the bacterial load and increases accumulation of transcripts of enzymes controlling high-output NO release, superoxide production, and catalyzis of tryptophan. Adaptive immune reponses may directly or indirectly diminish the levels of IFN-γ and IL-12 mRNA early after infection and thereby may alter the quality of the protective immune responses. NK cell cytotoxicity is not necessary for the control of *C.*

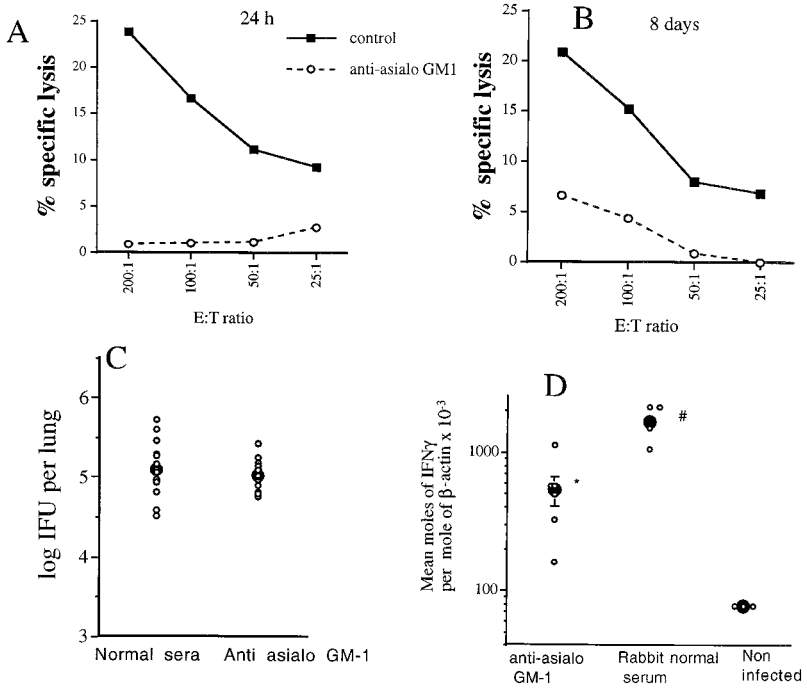

FIGURE 8. Effect of depletion of NK cell activity by anti-asialo GM-1 administration on the outcome of infection with *C. pneumoniae*. *A* and *B*, Mice were depleted of NK cells by i.v. administration of 20 μl anti-asialo GM-1 rabbit antiserum (Wako Pure Chemical, Osaka, Japan) diluted in 200 μl PBS. Control mice received 20 μl of normal rabbit serum. The antisera were given 24 h before infection and again 7 days after infection. The efficiency of the depletion was evaluated in ^{51}Cr-release assays. *C*, Levels of *C. pneumoniae* in lungs of SCID mice administered with 20 μl anti-asialo GM-1 at days 1 and 7 after i.n. infection with 10^6 *C. pneumoniae*. Mice were sacrificed 10 days after infection. The individual and mean IFU per lung are depicted. *D*, Effect of anti-asialo GM-1 administration on IFN-γ mRNA accumulation in lungs during infection with *C. pneumoniae*. Total RNA was extracted from lungs from anti-asialo GM-1 or normal rabbit serum-administered RAG-1$^{-/-}$ or WT mice 10 days after infection with *C. pneumoniae*. The accumulation of IFN-γ and β-actin mRNA in four to five individual mice per group was determined by competitive PCR. The mean moles of IFN-γ per mole of β-actin mRNA $\pm$ SEM are depicted. *, Differences vs mice administered with normal rabbit serum or those that were noninfected are significant ($p < 0.05$, Student *t* test); #, differences vs uninfected mice are significant ($p < 0.05$, Student's *t* test).

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pneumoniae, but NK cells and probably other innate immune cells release IFN-γ during infection with *C. pneumoniae*.

Acknowledgments

We thank Dr. M. Puolakkainen (Haartman Institute, Helsinki, Finland) for kindly providing *C. pneumoniae* stocks. IFN-γR$^{-/-}$ mice were provided by Dr. M. Aguet (Institute of Molecular Biology, University of Zurich, Zurich, Switzerland). We also thank Benedict Chambers for his comments to our manuscript.

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