

## Gamma Interferon Levels during *Chlamydia trachomatis* Pneumonia in Mice

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**Host defense against murine *Chlamydia trachomatis* (mouse pneumonitis agent [MoPn]) in a murine model was investigated. Gamma interferon (IFN- $\gamma$ ) was produced in the lungs by both MoPn-susceptible nude athymic (*nu/nu*) and MoPn-resistant heterozygous (*nu/+*) mice. In vivo depletion of IFN- $\gamma$  in *nu/nu* mice led to exacerbation of infection. Fluorescence-activated cell sorter analysis disclosed induction of GL3 antibody-positive cells (putatively  $\gamma/\delta^+$  T cells) in *nu/nu* mouse lung during infection with MoPn. Treatment of *nu/nu* mice in vivo with antibody to NK cells (anti-asialo GM1 antibody) or to  $\gamma/\delta$  cells (UC7-13D5) did not significantly decrease IFN- $\gamma$  production in the lung. However, treatment of severe combined immunodeficiency mice (which lack  $\gamma/\delta$  cells) with antibody to NK cells significantly reduced lung IFN- $\gamma$  levels.**

We have employed a murine model of T-cell-dependent pneumonia due to the mouse pneumonitis agent (MoPn) murine *Chlamydia trachomatis* in susceptible athymic *nu/nu* and resistant *nu/+* mice to investigate chlamydial cellular immunity (20, 22, 23). Because prior studies had suggested that gamma interferon (IFN- $\gamma$ ) was able to inhibit chlamydial replication in vitro and that production in the spleen was T cell dependent (3, 14), it was chosen as a likely T-cell-dependent cytokine for investigation in our model to explain a major portion of the difference between the susceptibilities of *nu/+* and *nu/nu* mice to MoPn. Treatment of *nu/+* mice in vivo with monoclonal antibody to IFN- $\gamma$  increased the susceptibility to MoPn, showing a role for IFN- $\gamma$  in host defense in the intact *nu/+* mouse (19). Therefore, we decided to reinvestigate the role of IFN- $\gamma$  measured in the lungs of *nu/+* and T-cell-deficient mice (primarily *nu/nu* but also severe combined immunodeficient [SCID]) during MoPn infection. (SCID mice are significantly more susceptible to MoPn than *nu/+* and *nu/nu* mice [18a].)

Male and female specific-pathogen-free *nu/+* and *nu/nu* mice on a BALB/c background were bred and maintained as in previous studies (10, 11). The BALB/c congenic SCID mice were obtained from the Gnotobiotic Research Laboratory at the University of Wisconsin Medical School, Madison, and were specific pathogen free. MoPn was maintained in HeLa cell culture (22). MoPn was harvested and frozen at  $-70^{\circ}\text{C}$  until use. Elementary bodies were prepared as previously described (22). The purified elementary bodies were killed under UV light before use in experiments examining IFN- $\gamma$  production by nonviable organisms. Quantitative culture of infected tissue was performed in McCoy cell monolayers and reported as inclusion-forming units per organ (IFU) (21). Mice were infected intranasally (10, 11). Whole-lung homogenates were prepared from both MoPn-infected and uninfected mice as in our earlier studies (10, 21). Measurement of IFN- $\gamma$  in lung supernatant material was by specific enzyme-linked immunosorbent assay (ELISA) employing IFN- $\gamma$  specific antibodies R4-6A2 and XMG 1.2

with peroxidase-conjugated streptavidin by a modification of a previously reported assay (13) and by bioassay for IFN activity as in our earlier studies (3). Levels of serum antibodies to MoPn were measured by microimmunofluorescence (23). In vivo neutralization of IFN- $\gamma$  was performed as in our previous studies with *nu/+* mice (19). *nu/nu* mice were given 1,000 neutralizing units of R4-6A2 intravenously on days 0, 1, 3, 5, and 7 of infection with MoPn, and mortality was monitored daily. Flow microfluorometry was used to detect GL3<sup>+</sup> cells in lungs. Minced lung preparations depleted of macrophages by adherence were obtained by the method of Eichelberger et al. (4) prior to incubation with antibody. Cell populations from the lungs ( $2 \times 10^6$ ) were treated with fluorescein-labeled antibody reactive for  $\gamma/\delta$  cells (GL3; PharMingen) for 30 min at  $4^{\circ}\text{C}$  after prior addition of irrelevant antibody to prevent nonspecific binding. Cells were analyzed within 1 day of staining in the Department of Microbiology Flow Cytometry Laboratory, University of Texas Health Science Center. The analysis gate was set for  $\gamma/\delta^+$  cells, and the expression of these molecules was analyzed with forward light scatter to exclude dead cells and erythrocytes.  $\gamma/\delta$  T cells were depleted in vivo employing 200  $\mu\text{g}$  of anti-T-cell receptor  $\gamma/\delta$  monoclonal antibody UC7-13D5 (PharMingen) given intraperitoneally on days  $-3$ ,  $0$  and  $+3$  of infection (6). NK cells were depleted in vivo by the same dosing schedule of anti-asialo GM1 antibody, which we have previously shown abrogated the increase of NK activity induced by MoPn infection in the lung in our model (24). Comparison of groups was performed by Student's *t* test with correction for unequal variance. The Mann-Whitney U test was used to compare nonparametric data. Fisher's exact test was used to compare mortality levels on specific days.

Figure 1 shows quantitative culture studies of lungs from groups of *nu/+* and *nu/nu* mice on days 0 through 28 postinfection with  $5 \times 10^2$  IFU of MoPn. Baseline cultures on day 0 were obtained 4 h postinfection and the levels of infection were 0, perhaps because the organisms were in the process of converting to the noninfectious intracellular form at that time, and infectious organism titers were below our level of detection. Only minor differences between the resistant *nu/+* and susceptible *nu/nu* mice were noted during

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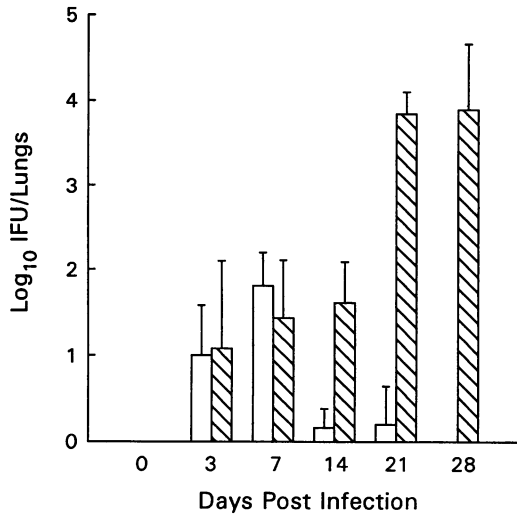


FIG. 1. Quantitative culture studies of lungs from groups of *nu/+* (open bars) and *nu/nu* (hatched bars) mice on days 0 through 28 postinfection with  $5 \times 10^2$  IFU of MoPn. Each group contained three to five mice.

the first 14 days of infection, with *nu/nu* mouse IFU being significantly higher for the first time at day 14 because of a fall in the *nu/+* mouse counts. After early relative control, quantitative cultures were dramatically higher in *nu/nu* mice on day 21 ( $P < 0.05$ , Mann-Whitney U test). Production of IFN- $\gamma$  in lungs was examined by ELISA to see whether this cytokine by itself could explain the differences in susceptibility in the two mouse groups at the time periods when they were observed (Fig. 2). Maximal IFN- $\gamma$  values at day 7 postinfection were equal in *nu/+* and *nu/nu* mouse lungs ( $P > 0.30$ ). *nu/nu* mouse data were not available at day 28 because of insufficient mouse survival. A repeat experiment examining days 14 and 21 postinfection showed similar results (data not shown). To confirm that IFN- $\gamma$  production was due to infection by MoPn rather than exposure to antigen, IFN- $\gamma$  levels were measured in *nu/nu* mice at days 3

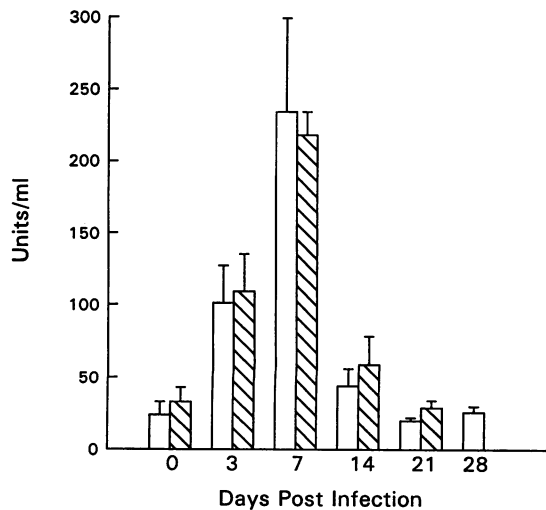


FIG. 2. Production of IFN- $\gamma$  in lungs (as detected by ELISA) at the same dose and time periods used for the quantitative culture results. Each group contained four to five mice. Open bars, *nu/+* mice; hatched bars, *nu/nu* mice.

and 7 after instilling either HeLa cell material containing no MoPn antigen or UV-killed MoPn. With either, levels of IFN- $\gamma$  remained at baseline. Levels with killed MoPn antigen were  $24 \pm 5$  U/ml at day 3 and  $23 \pm 8$  U/ml on day 7. Because the above assay measured IFN- $\gamma$  antigen but not bioactivity in lungs, we determined IFN- $\gamma$  levels by both bioassay and ELISA at day 10 after infection. The IFN- $\gamma$  bioassay confirmed the ELISA data, demonstrating IFN- $\gamma$  production by both *nu/+* and *nu/nu* mice, although with higher titers in the former ( $353 \pm 54$  versus  $237 \pm 83$  U/ml) at day 10 ( $P < 0.05$ , Mann-Whitney two-tailed test) in the bioassay. A mixing experiment in which recombinant IFN- $\gamma$  was quantitated in the bioassay with and without added minced uninfected lung material disclosed no inhibitor of the bioassay. Thus, these data in aggregate demonstrate that both *nu/+* and *nu/nu* mice had a significant IFN- $\gamma$  response during MoPn infection at a time that the infection was being temporarily controlled in the *nu/nu* mice and was apparently permanently controlled in the *nu/+* mice. To confirm that our *nu/nu* mice were still T cell deficient (1, 8), we measured levels of immunoglobulin G antibody to MoPn (a T-cell-dependent antibody) on day 14 postinfection with MoPn as in prior studies (23). Levels in 9 *nu/+* and 10 *nu/nu* mice were  $348 \pm 2$  and  $15 \pm 7$  U/ml ( $P < 0.0003$ , Mann Whitney two-tailed test), respectively, which is consistent with a profound Th2 (antibody-controlling) T-cell dysfunction in these mice.

To confirm the biological effect of IFN- $\gamma$  in *nu/nu* mouse lungs, we performed experiments in which *nu/nu* mice were given a specific monoclonal antibody to murine IFN- $\gamma$  (R4-6A2) in vivo during MoPn infection. In three separate experiments in which *nu/nu* mice in groups of 8 to 10 each were infected with  $10^3$  (experiment 1) or  $10^4$  (experiments 2 and 3) IFU of MoPn, significant exacerbation of mortality was seen (by two-tailed test in experiments 2 and 3 and one-tailed Fisher's exact test in experiment 1). Mortality levels at days 9 to 12 were 20, 20, and 50% in the three control groups and 63, 100, and 100% in those given antibody to IFN- $\gamma$ . R4-6A2 was not toxic to uninfected mice. Because our prior data had demonstrated intact NK cell function in the *nu/nu* mice, with significant stimulation during the first 10 days of MoPn infection (24), the NK cell seemed to be a plausible candidate for a source of IFN- $\gamma$  production in the *nu/nu* mice. Another possibility was the stimulation of  $\gamma/\delta^+$  cells, which are known to be present in *nu/nu* mice (4, 7). The latter were examined by fluorescent microscopy with the GL3 antibody. The baseline level in *nu/nu* mouse lungs was  $8\% \pm 1\%$  GL3 $^+$  cells at day 0 of infection. Values rose during infection to  $30\% \pm 2\%$  at day 7 and  $34\% \pm 3\%$  at day 14. In contrast, levels were  $<3\%$  in SCID mice at all time periods, which is consistent with the known SCID  $\gamma/\delta$  deficiency (2, 7). Studies are under way to further define the specificity of this observation by gating on only CD3 $^+$  cells and confirming the observations by molecular analysis. To test for an in vivo role for both NK and  $\gamma/\delta$  cells as possible producers of IFN- $\gamma$ , *nu/nu* mice were treated with antibody in vivo to deplete each type of cell. Anti-asialo antibody was used in doses we have shown previously to deplete MoPn-induced NK cell activity in the lung in our model (24). Anti- $\gamma/\delta$  monoclonal antibody UC7-13D5 was given in doses shown by others to deplete  $\gamma/\delta$  function in mice (6). These doses did not lead to a significant depletion of anti-GL3 $^+$  cells in the lung in our model, as shown by fluorescence-activated cell sorter analysis (data not shown). Three experiments were performed with these antibodies, employing four to five mice per group, with IFN- $\gamma$  levels in lungs on day

7 of infection measured by bioassay. The results were inconclusive, with no consistent differences found (data not shown). Similar studies were then conducted with SCID mice, which have intact NK cell function, like *nu/nu* mice (2, 7). Anti-asialo antibody significantly reduced IFN- $\gamma$  levels in SCID mouse lungs in four separate experiments on day 7 postinfection. Representative data from two experiments are  $175 \pm 27$  versus  $68 \pm 2$  U/ml and  $481 \pm 85$  versus  $205 \pm 43$  U/ml (each  $P < 0.05$ ; two-tailed test) in control and anti-asialo-treated mice, respectively. Uninfected SCID mice had IFN- $\gamma$  levels in the same range as uninfected *nu/nu* mice (data not shown). Thus, in the SCID model, IFN- $\gamma$  production in response to MoPn was clearly partly NK cell related.

These studies demonstrate that IFN- $\gamma$  is produced by both T-cell-intact (*nu/+*) and T-cell-deficient (*nu/nu* and SCID) mice in response to MoPn infection. Early non-T-cell-dependent production of this cytokine may be responsible for the early control of infection in *nu/nu* mice, which do not experience uncontrolled infection until IFN- $\gamma$  levels in the lungs decline. In SCID mice, the IFN- $\gamma$  production is due in part to NK cells. Why this was not similarly demonstrable in *nu/nu* mice when the same anti-asialo doses were employed is unclear but may be due to the fact that SCID mice are devoid of  $\gamma/\delta$  T cells while exhibiting intact NK cell function (2, 7). Thus, there might be less redundancy of production in the SCID model, making it easier to define a source. It is of interest that *nu/nu* mice were able in these studies to generate significant amounts of IFN- $\gamma$  in the lung while having a poor response in the spleen in our prior studies (3) (a finding that we continue to observe). This may be related to recruitment of IFN- $\gamma$ -producing cells to the site of active infection, which in our model is the lung. IFN- $\gamma$  production by *nu/nu* mice has been observed previously and could be by NK cells,  $\gamma/\delta$  T cells, or both (5, 15, 17).

The fact that both *nu/nu* and *nu/+* mice make IFN- $\gamma$  suggests that one must look beyond this cytokine to define the additional factor(s) (such as T-cell-dependent colony-stimulating factors [11]) that may be critical for ultimate control of the infection in the latter.

The fact that GL3<sup>+</sup> T cells are generated in response to MoPn is a potentially important observation. If the induction of  $\gamma/\delta$ <sup>+</sup> cells in response to MoPn is confirmed by our planned additional studies, it would be consistent with observations in other intracellular infection models (6, 7) and human chlamydial infection (16). This cell is of particular immunopathologic interest in chlamydial infection because the 57-kDa chlamydial hypersensitivity antigen is a stress response protein (12, 18) and thus is potentially recognized by  $\gamma/\delta$  T cells (9), which could react as well to host cell stress response proteins.

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