Humoral and Cellular Immunity in Secondary Infection Due to Murine *Chlamydia trachomatis*

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A murine model of pneumonia due to the mouse pneumonitis agent (MoPn [murine *Chlamydia trachomatis*]) in mice deficient in CD4⁺ T-cell function (major histocompatibility complex [MHC] class II function [class II^{-/-}], CD8⁺ T-cell function (β_2 -microglobulin deficient, MHC class I deficient [Beta_{2m}^{-/-}]), B-cell function (C57BL/10J-Igh^{tm1Cgn} [Igh^{-/-}]), and gamma interferon (IFN- γ) (C57BL/6-Ifg^{tm1} [Ifg^{-/-}]) or interleukin-4 (C57BL/6J-Igh^{tm1Cgn129} [IL4^{-/-}]) production was employed to determine if each of these mechanisms was critical to resistance against reinfection by *C. trachomatis* or if alternate compensatory mechanisms existed in their absence which could potentially be exploited in vaccine development. Resistance to reinfection with MoPn was heavily dependent on CD4⁺ T cells. CD4 T-cell-deficient MHC class II^{-/-} mice were very susceptible to reinfection with MoPn, showing the critical importance of this cell to resistance. These mice lacked antibody production but did produce IFN- γ , apparently by mechanisms involving NK and CD8⁺ T cells. Neutralization of IFN- γ in these mice led to a borderline increase in susceptibility, showing a possible role (albeit small) of this cytokine in this setting. Tumor necrosis factor alpha (TNF- α) was also present at increased levels in these mice. Igh^{-/-} B-cell-deficient mice which produce no antibody to MoPn were only modestly more susceptible to reinfection than immunized B-cell-intact controls, showing that antibody, including lung immunoglobulin A, is not an absolute requirement for relatively successful host defense in this setting. Levels of lung IFN- γ and TNF- α were elevated in Igh^{-/-} mice compared to those in controls. IL-4^{-/-} mice (deficient in Th2 function) could develop normal resistance to reinfection with MoPn. Conversely, normal mice rendered partially IFN- γ deficient by antibody depletion were somewhat impaired in their ability to develop acquired immunity to MoPn, again indicating a role for this cytokine in host defense against rechallenge. Of most importance, however, congenitally IFN- γ -deficient Ifg^{-/-} mice (which have elevated levels of other cytokines, including TNF- α and granulocyte-macrophage colony-stimulating factor) are paradoxically more resistant to MoPn rechallenge than controls, showing that IFN- γ is not an absolute requirement for acquired resistance and implying the presence of very effective compensatory host defense mechanism(s). In vivo depletion of TNF- α significantly increased MoPn levels in the lungs in these mice. Thus, resistance to reinfection in this model is flexible and multifactorial and is heavily dependent on CD4⁺ T cells, with a probable role for IFN- γ and TNF- α and a possible modest role for Th1-dependent antibody. Since IFN- γ was dispensable in host defense, the highly effective mechanism or mechanisms which can compensate for its absence (which include TNF- α) deserve further study.

Despite extensive study, the important host defense mechanisms against *Chlamydia trachomatis* remain incompletely defined. While it is apparent from studies of primary infection in athymic mice that T cells are critical to recovery from this infection (3, 30), the relative roles of cellular and humoral immunity remain controversial. In models of murine pulmonary and genital primary infection due to the mouse pneumonitis agent (MoPn [murine *C. trachomatis*]), although non-T-cell modalities of cell-mediated immunity are stimulated (29), host resistance to initial infection is strongly T-cell dependent in that BALB/c-background athymic mice are much more susceptible than euthymic mice and cannot clear the infection (10, 30). CD4⁺ T cells play a more important role than CD8⁺ T cells in host resistance to primary infection due to MoPn in mice on both BALB/c and C57BL/6 backgrounds (15, 17). Since CD4⁺ T cells play an important role in both cellular and humoral immunity, it is of interest that mice rendered B-cell

deficient by treatment with anti- μ antibody and unable to produce significant amounts of antibody against MoPn (19, 25) are able to clear the infection in the genital model and control it in the pulmonary model in primary infection, suggesting an important role for Th1-dependent cellular immunity in primary infection due to *C. trachomatis*. This is consistent with data showing a significant role for gamma interferon (IFN- γ) in host defense in primary infection in both the pulmonary and genital models in mice on a BALB/c background (20, 24).

Secondary infection with MoPn has been less well defined. Studies of secondary infection have demonstrated that athymic mice on a BALB/c background remain susceptible to rechallenge with the organism, while euthymic animals develop resistance (31), showing a prominent role for T cells in acquired resistance. Although data for a role for CD8⁺ T cells in immunity to reinfection exist (2, 21), in our pulmonary model of secondary infection with MoPn in which immune CD4⁺ or CD8⁺ T cells were adoptively transferred to athymic mice recipients which were subsequently given MoPn pneumonia, CD4⁺ T cells were more efficient than CD8⁺ T cells in conferring protection (27). Similar data were derived by adoptively transferring immunity to secondary infection by employing

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MoPn-specific CD4⁺ (Th1) and CD8⁺ BALB/c-derived T-cell clones in the genital infection model (9, 10). In these experiments, a higher degree of protection was afforded by the CD4⁺ clone. These data are further supported by the genital rechallenge studies of Morrison et al. with MoPn in major histocompatibility complex class I (MHC I)- or MHC II-deficient mice on a C57BL/6 background (with impaired CD8⁺ or CD4⁺ T-cell function, respectively [7, 12]) in which mice deficient in CD4⁺ T-cell function remained susceptible to rechallenge, while mice deficient in CD8⁺ T-cell function were as resistant as immunologically intact rechallenged control mice (17). Thus, CD4⁺ T cells appear critical in mediating protection in secondary infection with chlamydia as well as in primary infection. While the studies with T-cell clones described above show a role for Th1 T cells in rechallenge infection due to MoPn in mice on a BALB/c background, the relative roles and compensatory abilities of Th1 and Th2 CD4⁺ T cells, cellular versus humoral immunity, and the cytokines involved are still unclear in reinfection.

Although no direct data exist for an important role in host defense for a Th2 response in secondary infection with MoPn, in the pulmonary model, adoptively transferred polyclonal antibody (particularly when given locally) provided partial protection, which was more complete in immunologically intact than in T-cell-deficient mice (32). While this route of antibody administration is not physiologic, antibody isotype was not determined, and the relative roles of cellular and humoral immunity in this model are not clear, these observations and data showing a marginal effect of immunoglobulin A (IgA) or IgG monoclonal antibodies specific for the major outer membrane of *C. trachomatis* (when delivered by a backpack hybridoma tumor system) in preventing MoPn colonization of the genital tract (with a larger effect on ascending infection [4]) indicate antibody may play a role in secondary infection. On the other hand, compelling evidence against an indispensable role for antibody in secondary host defense against infection is provided by the lower genital tract model of reinfection due to MoPn in the mouse rendered B-cell deficient by treatment with anti- μ antibody. These mice lack humoral immunity but have intact cellular immunity (19). In these mice, significant resistance to reinfection was present despite the lack of antibody, implying a dominant role for cell-mediated immunity in resistance to reinfection.

Studies with knockout mice have the inherent problem that while positive data are easy to interpret, compensatory mechanisms make it impossible to attribute negative results concerning the difference in host defense as ruling out a role for the deleted host defense mechanism. However, the models are very useful if host defense is not severely impaired by the absence of an immunological function (shown in other models to be potentially important in host defense) because of the implication that effective compensatory mechanisms exist that are relatively effective. This is potentially even more useful if the potential compensatory mechanisms are more effective than those in the immunologically intact control. This information can be very important in vaccine design, where it may not be possible or desirable to stimulate all mechanisms which might play a role in natural resistance to reinfection with a pathogen. For this reason, we have undertaken a series of experiments with immunologically deficient mice to examine the effect of the deficiency on host resistance to reinfection with MoPn and the possible presence of effective compensatory mechanisms for the immunologic deficiencies.

MATERIALS AND METHODS

MoPn. The MoPn biovar of *C. trachomatis* was maintained in HeLa cell culture and was Renografin density purified (15). The HeLa cell material without MoPn contained less than 0.1 ng of endotoxin per ml by Limulus assay. MoPn was harvested and frozen at -70°C until used. Mice were infected intranasally following sodium pentobarbital anesthesia with 1×10^2 to 5×10^5 inclusion-forming units (IFU) of MoPn diluted in McCoy's modified 5A medium in a volume of 0.05 ml. Control mice were given the same volume of McCoy's modified 5A medium containing no MoPn. Purified elementary bodies (EBs) were prepared with a Renografin density gradient as in our prior studies (15). Quantitative culture of infected lung was performed with McCoy cell monolayers and reported as IFU per lung as in our previous studies (15). MoPn antigen levels were determined by enzyme-linked immunosorbent assay (ELISA) detecting chlamydial lipopolysaccharide LPS (Ortho, Raritan, N.J.) as in our previous publications (15, 28). The correlation of antigen levels with quantitative culture was at the 0.8 level.

Mice. Beta₂m^{-/-} mice (β_2 -microglobulin deficient, MHC I deficient) lacking CD8 $\alpha\beta$ ⁺ T-cell function (12) and class II^{-/-} mice (I-A-deficient mice functionally deficient in CD4⁺ T-cell function) (7) both on a C57BL/6J background and C57BL/6J control mice were purchased from GenPharm, Inc. (Sunnyvale, Calif.). B-cell-deficient C57BL/10J-Igh^{tm1Cgn} mice (Igh^{-/-}) with a targeted disruption of the membrane exon of the Ig μ -chain gene (11) and C57BL/10J controls were purchased from Jackson Laboratories (Bar Harbor, Maine), as were interleukin-4 (IL-4)-deficient C57BL/6J^{tm1Cgn129} (IL4^{-/-}) mice (13) and IFN- γ -deficient C57BL/6-Irf^{tm1} mice (Irf^{-/-}) with disrupted IFN- γ genes (5) and C57BL/6J controls. These mice were free of pathogenic bacteria and viruses as determined by culture and serology and were maintained under barrier conditions. They were used at between 6 and 12 weeks of age in these experiments.

Antibodies. Antibodies used to deplete cells or cytokines in vivo were given intraperitoneally on days 0 and 2 of infection except when stated otherwise. The following antibodies were employed: TIB-210 (anti-CD8), 1 mg per dose (a dose shown by us in preliminary experiments to effectively deplete CD8⁺ T cells in this model); XMG 1.2 (anti-mouse IFN- γ [anti-mIFN- γ]), 0.5 mg per dose; 22E9 (anti-mouse granulocyte-macrophage colony-stimulating factor [anti-mGM-CSF]; Endogen, Woburn, Mass.), 250 μg per dose; anti-asialo GM-1 (anti-NK cell; Wako Pure Chemicals, Richmond, Va.), 500 μg per dose (a dose known to deplete NK cells in this model [29]); and neutralizing polyclonal rabbit anti-mouse tumor necrosis factor alpha (TNF- α) (Genzyme, Cambridge, Mass.), 50 μg per dose. Control animals were given an equivalent amount of normal Ig from the appropriate species.

Cytokine assays. ELISAs of minced and filtered whole lung material for IFN- γ were performed with the anti-murine monoclonal antibodies XMG 1.2 and R4-6A2 as in our previous studies (15). ELISAs for TNF- α , GM-CSF, and IL-4 (Genzyme) were performed with the same material with commercial ELISAs according to the manufacturer's instructions.

Antibody assay. Plasma samples were collected at the times specified below and determined by ELISA as previously described (10).

Statistical analysis. Comparison of groups was performed by Mann-Whitney U test and Student's *t* test with correction for unequal variance. Significance is reported as $P < 0.05$ (two tailed unless otherwise stated).

RESULTS

Initial experiments were designed to examine the relative roles of CD4⁺ and CD8⁺ T cells in secondary infection in the pulmonary model of MoPn infection. Lungs of both Beta₂m^{-/-} mice (deficient in CD8⁺ T-cell function) and class II^{-/-} mice (deficient in CD4⁺ T-cell function) were culture negative, with low MoPn antigen levels by day 50 of primary infection by the intranasal route (data not shown). These mice were used for secondary infection at day 50 post primary infection in the studies reported here. In the experiment shown in Fig. 1A, MoPn antigen levels in C57BL/6, Beta₂m^{-/-}, and class II^{-/-} mice at day 3 of secondary infection were determined and were compared with levels at day 3 of primary infection in the same mouse groups. Mice were studied in groups of three to four mice each. Immunizing infection was with 10^2 IFU of MoPn. The primary and secondary infections shown in the figure were with 10^5 IFU of MoPn. On day 3 of rechallenge, MoPn antigen levels were significantly elevated in class II^{-/-} mice compared with those in either C57BL/6J or Beta₂m^{-/-} mice ($P < 0.05$), suggesting a major role for CD4⁺ T cells in resistance to reinfection. These data are generally consistent with the observations of Morrison et al. of genital infection with MoPn (17). A repeat experiment employing

FIG. 1. (A) Lung MoPn antigen levels in C57BL/6J, class II^{-/-}, and Beta₂m^{-/-} (B2m^{-/-}) mice on day 3 of primary or rechallenge infection with 10⁵ IFU of MoPn intranasally. Rechallenged mice had received a primary intranasal infection with 10⁵ IFU of MoPn 50 days earlier. MoPn antigen levels had returned to the baseline prior to reinfection. Class II^{-/-} mice remained susceptible to rechallenge infection, while the other groups were significantly resistant. (B) Lung MoPn levels in C57BL/6J, class II^{-/-}, Ifg^{-/-}, C57BL/10J, and Igh^{-/-} mice on day 3 of rechallenge infection with MoPn. Rechallenge was performed intranasally at day 50 after low-dose primary infection (at which time MoPn levels in all mouse groups had returned to baseline). Primary infection in C57BL/6J mice on day 3 at the same MoPn dose as secondary infection is included for comparison. Compared to rechallenged C57BL/6J controls, MoPn levels were significantly elevated in class II^{-/-} mice and significantly decreased in Ifg^{-/-} mice ($P < 0.0002$ and $P < 0.005$, respectively [two tailed]). Compared to rechallenged C57BL/10J controls, levels in rechallenged Igh^{-/-} mice were significantly increased ($P < 0.05$). There were 8 to 15 animals in each group, except for the primary infection group, which had 3 mice. *n*, number of mice per group.

class II^{-/-} and C57BL/6 mice and a slightly higher MoPn dose (5×10^5 IFU) to determine that the results were representative at other dosage levels showed a similar increase in MoPn antigen in mice functionally deficient in CD4⁺ T cells ($P < 0.05$) (data not shown). Quantitative culture data from the same experiment confirmed the antigen data, with $2,600 \pm 853$ IFU in the C57BL/6 mice and $163,000 \pm 72,473$ IFU in the class II^{-/-} mice, respectively ($P < 0.05$). On day 3 post rechallenge, geometric mean levels of antibody specific for MoPn were measured (Table 1). Serum geometric mean IgG levels in

TABLE 1. Serum and lung antibody titers on day 3 after rechallenge

Mice	Geometric mean titer ^a		
	Serum IgG	Serum IgA	Lung IgA
C57BL/6J	758	1,549	28
Beta ₂ m ^{-/-}	269	ND ^b	95
Class II ^{-/-}	<10	<10	<10
C57BL/10J	10,240	6,457	320
Igh ^{-/-}	<10	ND	<10

^a Standard error is <2 for all values shown.

^b ND, not done.

the C57BL/6J, Beta₂m^{-/-}, and class II^{-/-} mouse groups were 758, 269, and 9 (standard error of <2 for each), respectively, showing an apparent correlation of susceptibility to MoPn with a lack of specific antibody production in class II^{-/-} mice. Lung geometric mean IgA levels (Table 1) in the same groups again showed a lack of significant antibody production in the susceptible class II^{-/-} mice (standard error of <2 [$P < 0.05$ compared to both other groups]). When serum isotype determinations of the MoPn-specific antibody responses were made at day 3 of rechallenge (Table 2), geometric mean IgG2a titers were higher than IgG1 titers in C57BL/6 mice ($P < 0.05$, while IgG1 titers dominated in Ifg^{-/-} mice and IgG2a titers dominated in IL4^{-/-} mice (the latter are discussed below). As expected, titers of both IgG1- and IgG2a-specific antibody were <12 in class II^{-/-} mice (data not shown in Table 2). These data show production of both Th1- and Th2-dependent antibody in C57BL/6J mice and a lack of each in class II^{-/-} mice. Geometric mean serum IgA levels were 1,549 and <10, respectively (Table 1). Geometric mean serum IgG antibody levels just prior to rechallenge were 8,128 and 16 in the C57BL/6 and class II^{-/-} mice, respectively, and geometric mean serum IgA levels were 1,584 and <10 in the two groups, respectively, thus confirming the apparent correlation of susceptibility with lack of significant antibody production.

Figure 1B demonstrates MoPn antigen levels in the lungs during rechallenge infection in C57BL/6J, class II^{-/-}, Ifg^{-/-}, C57BL/10J, and Igh^{-/-} mice. The mice were rechallenged with 10⁴ IFU of MoPn on day 50 after primary infection with 10² IFU of MoPn, at which time MoPn levels had returned to the baseline in the lungs in two mice sampled from each group. Figures 1B and 2 are the results of duplicate experiments with a final total of 8 to 16 mice per group of rechallenged mice as indicated on the figures. MoPn levels were determined on day 3 of rechallenge in all mice except the group labeled "primary infection." A small number of C57BL/6J mice on day 3 of primary infection were included for comparison. Levels in Igh^{-/-} mice were modestly but significantly elevated compared to those in rechallenged C57BL/10J mice ($P < 0.05$ [two tailed]). The Igh^{-/-} mice have an arrest in B-cell development at the pre-B-cell level and have an absence of mature B cells,

TABLE 2. Serum IgG antibody isotype on day 3 after rechallenge

Mice	Geometric mean titers ^a	
	IgG1	IgG2a
C57BL/6J	190	640
Ifg ^{-/-}	1,288	<10
IL4 ^{-/-}	<10	710

^a Standard error is <2 for all values shown.

as well as no cells expressing IgM, IgD, IgG, or IgA (11), so that the defect would involve both the ability of B cells to present antigen and to produce antibody. While three randomly selected rechallenged C57BL/10 mice in these experiments had serum geometric mean IgG and IgA titers of antibody to MoPn of at least 6,457, all values in the *Igh*^{-/-} mice were <1:10, consistent with their B-cell deficiency (Table 1). Lung IgA levels followed a similar pattern (Table 1). MoPn levels in class II^{-/-} mice were significantly elevated compared to those in C57BL/6J control rechallenged mice ($P < 0.0002$ [two tailed]). Of perhaps the greatest interest, MoPn levels in *Igf*^{-/-} mice were paradoxically lower than those in rechallenged C57BL/6J controls ($P < 0.005$) (Fig. 1B). Levels in four *Igf*^{-/-} mice sampled on day 6 of rechallenge were even lower at 882 ± 174 ng of protein per ml and significantly lower than those in C57BL/6J controls ($12,845 \pm 1,644$ ng/ml) on the same day ($P < 0.005$), confirming that this was not an observation confined to a single day. A repeat experiment was performed with a higher rechallenge dose of MoPn on day 3 (5×10^4 IFU) (three mice per group) to determine that this paradoxical effect was not strictly dose dependent. A similar albeit slightly less dramatic effect was seen with $24,980 \pm 2,808$ ng of MoPn per ml in the C57BL/6J mice and $13,971 \pm 1,900$ ng in *Igf*^{-/-} mice ($P < 0.03$). The ability to effectively immunize *Igf*^{-/-} mice has also been observed in rechallenge experiments with *Listeria monocytogenes* (8) and thus appears to be a more generalizable phenomenon. Serum antibody measured in the *Igf*^{-/-} mice disclosed a pattern of IgG1 to IgG2a geometric mean antibody titers on day 3 of rechallenge consistent with a lack of Th1-dependent antibody production in these mice (Table 2).

Figure 2A shows IFN- γ levels in lung in the same groups. IFN- γ levels were at the lower limits of detection of the assay in *Igf*^{-/-} mouse lung ($P < 0.0002$ compared to those in C57BL/6J-rechallenged controls). Levels of IFN- γ were modestly but significantly elevated in *Igh*^{-/-} lungs compared to those in C57BL/10J-rechallenged controls at 152 ± 10 versus 95 ± 10 ng/ml ($P < 0.0006$). It is of interest that the levels in *Igf*^{-/-} lungs were consistently very low but not at the absolute baseline, and the very low values were reduced by half by incubating the samples with antibody to IFN- γ (XMG 1.2) prior to measurement. It is possible that this represents a truncated IFN- γ protein. IFN- γ levels in uninfected mice were all <5 ng/ml in the lungs. There was also significant IFN- γ production in class II^{-/-} mice (but not a significant elevation) despite the lack of CD4⁺ T-cell function. Our prior studies have suggested that early IFN- γ production, such as that at day 3 of primary infection, is at least in part NK dependent (14), while later IFN- γ production can be either CD4 or CD8 dependent (15). Treatment of the class II^{-/-} mice in a separate experiment with antibody to NK cells in vivo significantly reduced lung IFN- γ from 223 ± 21 ng/ml to 160 ± 10 ng/ml on day 3 of rechallenge ($P < 0.05$), while treatment with antibody to CD8⁺ T cells reduced it from the same baseline to 144 ± 24 ng/ml ($P < 0.05$), suggesting a role for both NK and CD8⁺ T cells in its production. To determine if IFN- γ had any activity in this model, class II^{-/-} mice were treated with neutralizing antibody to IFN- γ on days -2 and 0 of rechallenge. This treatment reduced IFN- γ lung levels to a third of that in class II^{-/-} mice given an equal amount of normal rat Ig and increased lung MoPn levels from $48,790 \pm 8,488$ ng/ml to $72,039 \pm 6,376$ ng/ml ($P < 0.07$ [two tailed]), thus showing some possible borderline efficacy of IFN- γ in these animals in the absence of CD4⁺ T-cell function. IFN- γ levels in the rechallenged Beta₂m^{-/-} mice were not significantly different

FIG. 2. (A) Lung IFN- γ levels at day 3 of primary infection in C57BL/6J mice and day 3 of rechallenge infection in C57BL/6J, class II^{-/-}, *Igf*^{-/-}, C57BL/10J, and *Igh*^{-/-} mice. Levels were significantly decreased in *Igf*^{-/-} mice ($P < 0.0002$) compared to those in C57BL/6J controls after rechallenge and were significantly elevated in *Igh*^{-/-} mice compared to those in C57BL/10J rechallenged controls ($P < 0.0006$ [two tailed]). There were 9 to 16 mice in all groups, except for the primary infection group, which had 5 mice. (B) Lung TNF- α levels on the same days and in the same groups. Levels were significantly elevated in *Igf*^{-/-} and *Igh*^{-/-} mice compared to those in rechallenged C57BL/6J and C57BL/10J controls ($P < 0.0002$). (C) Lung GM-CSF levels on the same days and in the same groups. Levels were significantly elevated in *Igf*^{-/-} mice compared to those in C57BL/6J rechallenged controls ($P < 0.003$ [two tailed]) and were significantly reduced in class II^{-/-} mice ($P < 0.02$ [two tailed]). n, number of mice per group.

from those in the rechallenged C57BL/6J mice (data not shown).

Figure 2B demonstrates a significant elevation of lung TNF- α in both *Igf*^{-/-} and *Igh*^{-/-} lungs compared to appropriate C57BL/6J and C57BL/10J rechallenged controls (each $P < 0.0002$). This is of interest particularly because our prior

studies have shown that TNF- α plays a significant role in host defense against MoPn in the mouse (28), and therefore the elevation observed might be a compensatory mechanism. Baseline TNF- α levels in uninfected lungs were <50 pg/ml. A repeat experiment employing the higher MoPn rechallenge dose (5×10^4 IFU) (which generated higher levels of TNF- α) in class II $^{-/-}$ and Ifg $^{-/-}$ mice showed a significant elevation of TNF- α levels in both of these groups compared to those in C57BL/6J controls ($P < 0.03$ and $P < 0.02$, respectively). Thus, this phenomenon is not dose specific. To determine if TNF- α were playing a role in host defense in Ifg $^{-/-}$ mice in secondary infection, mice were rechallenged with 10^5 IFU of MoPn while receiving 50 μ g of rabbit polyclonal anti-mouse TNF- α -neutralizing antibody or control antibody intraperitoneally on days 0 and +2 of rechallenge in two separate experiments, each with groups of four mice. This treatment reduced lung TNF- α levels by 57% ($1,397 \pm 82$ to 602 ± 49 pg/ml) ($P < 0.008$). When data from the two experiments were combined (leading to a total of eight mice per group), treatment of Ifg $^{-/-}$ mice with anti-TNF- α antibody increased lung MoPn levels at day 3 of rechallenge from $37,081 \pm 8,402$ ng/ml to $58,689 \pm 8,382$ ng/ml ($P < 0.03$). Thus TNF- α appears to play a role in host defense in secondary infection in these mice. The exact extent of the role will require complete neutralization of TNF- α to determine. Figure 2C demonstrates a significant elevation of GM-CSF levels in Ifg $^{-/-}$ mice compared to those in C57BL/6J controls ($P < 0.003$). Levels in class II $^{-/-}$ mice were significantly decreased compared to those in controls ($P < 0.02$). To determine if this cytokine were playing a role in host defense in this setting, Ifg $^{-/-}$ mice were given 250 μ g of neutralizing antibody to GM-CSF intraperitoneally on days 0 and +2 of infection with 5×10^4 IFU of MoPn. This had no significant effect on lung levels of MoPn compared to those in controls given normal rat Ig ($9,800 \pm 519$ versus $13,971 \pm 1,900$ ng/ml, respectively [$P < 0.15$]). In this experiment, pulmonary GM-CSF was again significantly higher in Ifg $^{-/-}$ mice than in C57BL/6J mice ($P < 0.003$), but the antibody treatment reduced lung GM-CSF by only 31% (681 ± 20 pg/ml to 473 ± 95 pg/ml), so the results must be regarded as preliminary. Thus, no evidence was seen for a role in host defense under these conditions, but further study with increased amounts of antibody is warranted.

As stated above, in primary infection with MoPn, both IFN- γ and TNF- α play a significant role in host defense, while antibody does not (25, 28). Thus, resistance to reinfection in these studies was significantly different from what we had observed in primary infection, in that B cells either through antibody production or other B-cell function, such as antigen presentation, played a modest role in host defense, while IFN- γ appeared to be unnecessary based on the data to date. Because other cytokines might be playing a compensatory role in host defense in the mice which are congenitally deficient in IFN- γ , we performed antibody depletion studies with rechallenged immunologically intact C57BL/6J mice similar to those we had performed for primary infection (24) to see if we could demonstrate any role for IFN- γ in rechallenge by using that model. C57BL/6J mice were given neutralizing antibody to IFN- γ gamma on days -2 and 0 of reinfection with MoPn. Antibody treatment reduced measured IFN- γ in lungs on day 3 of rechallenge from 180 ± 13 ng/ml to 40 ± 2 ng/ml ($P < 0.0001$). This treatment led to a significant increase in MoPn levels in lungs ($P < 0.002$) (Fig. 3), showing at least a partial role of this cytokine in resistance to rechallenge. A repeat experiment with a slightly higher MoPn dose showed a similar result. Antigen levels in the XMG 1.2-treated animals were $29,596 \pm 5,413$ ng/ml versus $6,812 \pm 1,200$ ng/ml in the controls

FIG. 3. Lung MoPn levels in C57BL/6J mice on day 3 of rechallenge. Mice were treated with either 0.5 mg of anti-IFN- γ antibody (XMG 1.2)-containing ascites or the same amount of normal rat Ig on days -2 and 0 prior to rechallenge on day 0. Depletion of IFN- γ led to a significant increase in MoPn levels in the lungs in these rechallenged mice ($P < 0.002$ [two tailed]). n, number of mice per group.

given normal rat Ig ($P < 0.02$) (data not shown). Thus, these data in toto do not prove that IFN- γ plays no role in resistance to reinfection, but they do imply that the role in C57BL/6J mice is not indispensable in that other factors can apparently compensate for its loss in mice congenitally deficient in the cytokine.

A final experiment examined resistance to rechallenge in IL4 $^{-/-}$ mice and C57BL/6J controls (eight mice per group in rechallenge studies). IL4 $^{-/-}$ mice, which have reduced Th2 responses, including delayed production of IgG1 and IgE and a shift towards a Th1 response (13, 23), were resistant to rechallenge with MoPn on day 3. MoPn levels in control and IL4 $^{-/-}$ mice were $20,611 \pm 3,510$ versus $22,466 \pm 4,954$ ng/ml (P , not significant). Two mice each from the IL4 $^{-/-}$ and control groups were given a primary infection with MoPn concurrently as a positive control. As expected, MoPn levels were much higher in these two groups, with means of 260,000 ng/ml in the C57BL/6 mice and 110,000 ng/ml in the IL4 $^{-/-}$ mice, but the small numbers prevented meaningful interpretation. Further studies will be required to determine if an IL-4 deficiency is beneficial in primary MoPn infection. MoPn-specific IgG1 levels were <10 in all eight rechallenged IL4 $^{-/-}$ mice with positive controls, but substantial IgG2a levels were present, perhaps contributing to successful host defense (Table 2). The results are consistent with the development of immunity in the absence of significant Th2 antibody in this model.

DISCUSSION

These studies suggest the following conclusions concerning rechallenge chlamydial pneumonia. (i) Resistance to reinfection is highly dependent on CD4 $^{+}$ T cells. (ii) In the absence of CD4 $^{+}$ T cells and their products (class II $^{-/-}$ mice), IFN- γ (apparently produced by NK and CD8 $^{+}$ T cells) plays only a marginal role in host defense against rechallenge infection. (iii) Despite the apparent correlation of susceptibility to rechallenge infection with deficient antibody production in class II $^{-/-}$ mice, the level of GM-CSF is also significantly reduced, showing an impairment in cellular immunity as well as humoral immunity in these mice. (iv) The studies with rechallenged Ifg $^{-/-}$ mice show only a modest increased susceptibility com-

pared to that of rechallenged controls, particularly when MoPn levels are compared to those in mice undergoing a primary infection. This suggests either a limited role of antibody or other B-cell function in resistance to rechallenge or the presence of effective compensatory mechanisms. *Igh*^{-/-} mice may be compensating for their B-cell deficiency by an increase in their cell-mediated immune responses (IFN- γ and TNF- γ), as has been previously described in other models of B-cell deficiency (22). It should be further noted that while *Igh*^{-/-} mice may have a decrease in absolute numbers of CD8⁺ T cells at some stages of primary viral infection (1), relative numbers of these and CD4⁺ T cells are comparable to those of controls, and CD8 T-cell-dependent memory (which would be of importance in rechallenge MoPn infection) is intact in these mice (1), suggesting that the modestly increased susceptibility observed is probably due to the B-cell defect and not the confounding variable of concomitant T-cell dysfunction. Furthermore, the fact that these mice are reasonably resistant to MoPn while totally lacking lung IgA is counter to the concept that this antibody is critical to host defense with MoPn, as had been suggested by others (17). Thus, while B cells apparently play a role in resistance to MoPn rechallenge, the role is not critical, and other mechanisms can provide successful host defense in their absence. (v) IL-4-deficient mice are not hypersusceptible to reinfection compared to controls, suggesting that intact Th2 CD4 T-cell function is also not a requirement for successful resistance to reinfection. These mice do produce Th1-dependent antibody (IgG2a), which might contribute to successful host defense. Other possible compensatory host defense mechanisms remain to be explored. (vi) The most important observation of these studies is that although IFN- γ plays some role in host resistance to reinfection in rechallenged C57BL/6J and class II^{-/-} mice (as demonstrated by in vivo depletion by antibody), IFN- γ is apparently dispensable in rechallenge infection. Immunized *Igf*^{-/-} mice are not more susceptible than controls to reinfection. Indeed, they are actually more resistant on both days 3 and 6 in studies employing two differing doses of MoPn (while having increased levels of cytokines other than IFN- γ , perhaps in compensation). This is remarkable, because extensive data exist that IFN- γ - and Th1-mediated immune responses are critical in host defense against MoPn (reviewed in reference 33 and see references 9, 10, 14–16, 20, 21, 24–26, and 28).

The studies reported here employing antibody-mediated TNF- α depletion in *Igf*^{-/-} mice (even though depletion was not complete) strongly suggest that this cytokine plays a role in host defense in this setting of secondary MoPn infection, as it did in our model of primary infection (28). The elevated TNF- α levels in these mice may therefore play a compensatory role in host defense in the absence of IFN- γ . Similarly, in *Igh*^{-/-} and class II^{-/-} mice (at a higher dose of MoPn), there is an elevation of cytokines compared to the level in controls, suggesting that cell-mediated mechanisms could play a compensatory role for the congenital defect that each mouse exhibits as well. The fact that mice with a congenital defect in IFN- γ are actually significantly more resistant to MoPn than rechallenged controls suggests that these putative cell-mediated compensatory resistance mechanisms may indeed be highly effective and important to vaccine development and need to be further evaluated. While we could not show in the studies reported here that GM-CSF by itself was an additional potential compensatory mechanism in MoPn infection in *Igf*^{-/-} mice, the studies were preliminary and not definitive because of inadequate neutralization of GM-CSF. The role of GM-CSF alone and in combination with TNF- α clearly deserves further study in this model.

It could be maintained additionally that *Igf*^{-/-} mice are more resistant than controls to reinfection because of a switch to a Th2 antibody response (which would be putatively more effective than the mixed antibody response in control animals). The fact that IL4^{-/-} mice which lack a Th2 antibody response are not more susceptible than controls to reinfection argues against this hypothesis, however. Indeed, the ability of IL4^{-/-} mice which have a defect in Th2 CD4 T-cell activity, including IL-5 and IL-6 production, as well as IL-4 production (13, 23), to control infection is most consistent with a dominant role for Th1 CD4 T-cell-mediated mechanisms (possibly including antibody) in host resistance to MoPn reinfection.

A similar phenomenon of increased resistance to rechallenge infection in *Igf*^{-/-} mice has been observed in *L. monocytogenes* reinfection (8). In these studies, CD8⁺ T cells played a major role in the resistance to *Listeria* and could be a possible candidate with MoPn as well (particularly in view of the fact that *Igf*^{-/-} mice have an increase in cytotoxic cell activity [5]). However, data by Starnbach et al. that chlamydia-specific CD8⁺ T cells function much less well in *Igf*^{-/-} mice than in controls (21) and our data that functional CD8 deficiency does not prevent the development of acquired immunity argue against this view. We have performed one preliminary experiment with in vivo CD8 depletion in *Igf*^{-/-} mice infected with MoPn with no exacerbation of infection (23a). Further studies in this area are necessary however.

It may also be important that these experiments were conducted with *Igf*^{-/-} mice on a C57BL/6 background. This background of mouse is more resistant to MoPn by the pulmonary route than the BALB/c background mice which we have used in most of our prior studies with this model and has a greater propensity towards a Th1-mediated response (33). The C57BL/6 mouse also produces more TNF- α in response to genital infection than C3H mice (19a). There might thus also be greater redundancy in the protective cell-mediated responses in this strain than in more susceptible strains such as C3H and BALB/c. Therefore, differing data may be obtained with *Igf*^{-/-} mice on a BALB/c background. This area also requires further study.

As discussed above, these studies confirm those of Morrison et al. performed with the mouse genital tract that MHC II-restricted mechanisms are critical in host defense against MoPn infection (17). An important difference, however, is that our studies suggest that cytokine-dependent mechanisms play an important role in host defense in secondary infection with MoPn.

It should also be emphasized that these studies of secondary infection in *Igf*^{-/-} mice differed significantly from those of primary infection, in which the *Igf*^{-/-} mice were significantly more susceptible than were controls (23a).

While a summary of all of these data is difficult (because compensatory immunologic mechanisms in many of these mice mean that negative data do not rule out a role for a cytokine or cell in host defense in rechallenge infection), the major findings of these studies are that host defense against rechallenge infection with MoPn is flexible and multifactorial and is not dependent on a single immunologic modality, with the apparent exception of CD4⁺ T cells. CD4⁺ T cells are critical in host defense, and IFN- γ (although produced by several cell types) plays a role as well. In addition, these studies indicate that potential compensatory mechanisms for a lack of CD4⁺ T cells (including IFN- γ production by NK and CD8⁺ T cells) are not very effective. In contrast, those for a lack of local IgA and particularly of IFN- γ (which include TNF- α) are potentially quite effective and will be the subject of further study.

Our planned studies include examining protective functions

(in addition to control of antibody and production of IFN- γ) not yet described in this setting for CD4⁺ T cells (since IFN- γ is only marginally effective in the absence of CD4⁺ T cells and their products [class II^{-/-} mice]) and antibody is by itself dispensable in reasonably successful host defense (Igh^{-/-} and IL-4^{-/-} mice). Other Th1-dependent cytokines, such as IL-2 and GM-CSF, as well as the possibility for a role for CD4⁺ cytotoxic T cells in resistance to reinfection with MoPn, will be examined. The additive effect of differing cytokine mechanisms such as TNF- α and GM-CSF is also a major candidate for further study. In addition, the interaction of cellular and humoral immunity as in antibody-dependent cellular cytotoxicity is of interest. Of particular importance in this endeavor is the further definition of cytokine production (both direct and indirect) and function of our protective CD4⁺ MoPn-specific T-cell clones (10).

The relationship of these findings to mucosal immunity also deserves comment. While IgA has classically been considered a major factor in mucosal host defense (reviewed in reference 18), cytokines such as IFN- γ are generated by T cells at mucosal sites, and memory T cells involved in cytokine production may recirculate between mucosal and nonmucosal compartments (6). The studies in this paper suggest that such cell-mediated immune mechanisms play a significant role in rechallenged mucosal infection with MoPn.

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