Neonatal antibody responses are attenuated by interferon gamma produced by NK and T cells during RSV infection

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Abstract

Respiratory syncytial virus (RSV) infects most children in the first year of life and is a major single cause of hospitalization in infants and young children. There is no effective vaccine, and antibody generated by primary neonatal infection is poorly protective against re-infection even with antigenically homologous viral strains. Studying the immunological basis of these observations in neonatal mice, we found that antibody responses to infection were low and unaffected by CD4 depletion, in contrast with adult mice, which had stronger CD4 dependent antibody responses. NK cell depletion or co-depletion of $CD4^+$ and $CD8^+$ cells during neonatal RSV infection caused a striking increase in anti-RSV antibody titer. These cells are major sources of the cytokine interferon gamma (IFN-γ), and blocking IFN-γ also enhanced RSV specific antibody responses in neonates. In addition, infection with a recombinant RSV engineered to produce IFN-γ reduced antibody titer, confirming that IFN-γ plays a pivotal role in inhibition of antibody responses after neonatal infection. These unexpected findings show that the induction of a strong cellular immune response may limit antibody responses in early life and that vaccines which induce IFN-γ secreting cells might, in some situations, be less protective than those that do not.

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Introduction

Antibody responses to vaccines are poor in infancy (1) and this can lead to increased infection severity and decreased vaccination efficacy. It is believed that the paucity of the infantile antibody response is caused by immaturity of the cells involved (2, 3). Neonatal B cells are immature, expressing lower levels of MHC-II (4) and when B cells are transferred from adult mice into the footpads of neonates the antibody response is restored to adult levels (5). In addition to poor B and T cell interactions, the development of follicular dendritic cells is delayed in both neonatal mice (6) and infants (7). Improved understanding about B cell responses is required to improve early life vaccine strategies, particularly in the control of respiratory infections, which are a major cause of morbidity and mortality in infancy.

Respiratory syncytial virus (RSV) is the principal cause of viral lung infection in infants (8). Hospitalization rates due to RSV bronchiolitis in the USA are 17 per 1,000 children below 6 months of age (9), with an estimated 33.8 million cases of RSV associated lower respiratory tract infection and 66,000-199,000 deaths globally per annum (10). Re-infection is common and does not necessarily depend on antigenic changes (11), 47% of children infected in the first year of life are re-infected in their second year of life (12). An effective RSV vaccine would be a major breakthrough in child health and since most children with RSV bronchiolitis are under 6 months of age, it would need to be effective in infancy. A core component of protection against RSV infection is antibody – high-titer, maternally-derived, RSV-neutralizing antibody is protective against hospitalization (13) and protection against re-infection in humans

is associated with high titers of antibody (14). Antibody responses seen in infants after both RSV infection (15, 16) and vaccination (17, 18) are weak, an effect usually attributed to reduced T cell help. Few CD4 T cell lymphocytes are found in the lungs of RSV infected infants (19) and the V gene repertoire for anti-RSV antibody is restricted in neonatal humans (20). We have developed a mouse model of neonatal RSV infection (21) where we observed that infection of neonatal mice led to a T cell response that caused disease on subsequent re-infection (22). Furthermore, we subsequently showed that this pathogenic cellular response is dependent upon the mouse MHC haplotype (23).

Antibody titers are lower following experimental neonatal mouse RSV infection compared to adult infection (24). Studying the basis of these observations, we wished to assess whether the cellular response to neonatal RSV affected the antibody titer. We found that $CD4^+$ cell depletion did not reduce neonatal antibody titers, but depleting natural killer (NK) cells or $CD4^+$ and $CD8^+$ cells together increased antibody titer. During neonatal RSV infection, NK and T cells produced the cytokine IFN-γ, which has been shown to inhibit the antibody response (25). When IFN-γ was depleted during neonatal RSV infection, the antibody titer significantly increased. This novel demonstration of the inhibition of antibody production by the cellular response during neonatal infection has important implications for the control of RSV in early life.

Results

Reduced antibody response in neonates compared to adults after RSV infection. BALB/c mice were infected with $4x10^4$ PFU/g body weight RSV at either 4 days (neonatal; 10^5 PFU) or 28 days (adult; 10^6 PFU) of life (schematic of experimental protocol in Figure 1A). Mice infected as neonates had significantly lower viral loads than adults on day 4 post primary infection (Fig 1B, $p<0.01$), as previously observed (21, 26). Serum was collected 8 weeks after primary infection to allow maturation of responses. Sera from mice that had primary RSV infection as adults had significantly more RSV-specific ELISA binding (Fig 1C, $p<0.001$) and neutralizing (Fig 1D, $p<0.001$) antibody than mice that had a primary infection as neonates. RSV-specific IgG subtypes were quantified by ELISA in sera. The neonatal RSV-IgG response was significantly skewed towards IgG1 (indicating a Th2 bias; $p<0.001$), whilst adult primary infection led to a balanced IgG1/IgG2a response (Fig S1A).

Mice were re-infected with 10^6 PFU of the same RSV strain 8 weeks after their initial infection (Fig 1A). Previous exposure to RSV, regardless of age, significantly reduced viral load during secondary RSV infection, compared to primary RSV infection (Fig 1B, $p<0.01$). But mice that were primed as adults had a significantly reduced viral load than mice primed as neonates ($p<0.05$). Whilst re-infection significantly boosted antibody titers in both neonatally and adult primed mice (Fig 1C, $p<0.001$), adult primed mice still had significantly greater antibody titers (Fig 1C, $p<0.001$) and more potent neutralizing responses (Fig 1D, $p<0.001$) than neonatally primed mice after re-infection. Adult re-infection of neonatally primed mice reversed the initial IgG1 bias, leading to a predominantly IgG2a response (Fig S1B). Neonatal mice generated weaker antibody responses to RSV infection, which may reduce protection against

subsequent viral re-exposure and contribute to the delayed sequelae of neonatal RSV infection (22).

T cells and NK cells inhibit the neonatal antibody response.

We have previously observed that depletion of T cells during neonatal RSV infection can have a protective effect during re-infection (22). To assess their effect on antibody responses, T cells were depleted during primary neonatal RSV infection and antibody responses measured before and after re-infection (schematic in Fig 1A). Cell depletion significantly reduced the targeted cell type (Fig S2). $CD4^+$ cell depletion had no effect on the weak anti-RSV antibody response in neonates after primary (Fig 2A) or secondary infection (Fig 2B). However, depletion of both $CD4^+$ and $CD8^+$ cells together significantly boosted IgG after primary ($p<0.001$) and secondary ($p<0.05$) RSV infection. Depletion of $CD4^+$ cells during primary adult RSV infection did not significantly change the antibody titer before re-infection (Fig 2C) but caused a significant reduction in anti-RSV IgG titer after re-infection (Fig 2D, $p<0.05$), as previously observed (27). CD4 and CD8 co-depletion during primary adult RSV infection had no effect on IgG titer (Fig 2D). To define the timing of the effect, T cells were depleted during secondary RSV infection of neonatally primed mice. When T cells were depleted during secondary RSV infection of neonatally primed mice, $CD8^+$ cell depletion made no effect on antibody titer, but $CD4^+$ cell depletion or depletion of both $CD4^+$ and $CD8^+$ cells together significantly reduced the titer (Fig 2E, $p<0.01$). In addition, cell depletion during primary neonatal infection increased levels of IgG1 compared to control neonatal infection (Fig S1C-D).

To test whether NK cells also affected the neonatal antibody response, we depleted NK cells with anti-asialo GM1 during primary neonatal RSV infection. NK cell depletion had no effect on antibody titer before re-infection (Fig 2F). However, NK cell depleted neonatal mice had significantly higher anti-RSV IgG responses than untreated mice after re-infection (Fig 2G, $p<0.05$). Suppression of antibody responses by NK cells has been previously observed (28) and was linked to inhibition of antigen presenting cells (29). This suppression may be caused by NK cell killing the antigen presenting cells using the FasL pathway. To test this, antibody responses to RSV infection were measured in $FasL^{-/-}$ deficient (gld) mice. The $FasL^{-/-}$ mice are on a C57BL/6 background so we confirmed that cell depletion affected antibody levels in C57BL/6 mice (Fig 2H). Neonatal $CD4^+$ cell depletion had no effect, but $CD8^+$ cell depletion significantly increased RSV-specific antibody titer ($p<0.05$). When levels were compared, there was no significant difference in antibody titer between the wild type and $FasL^{-/-}$ deficient mice (Fig 2I).

Interferon gamma inhibits the neonatal antibody response.

The lower viral load during primary neonatal infection may contribute to the reduced neonatal antibody responses. RSV viral titer was measured on day 4 after primary neonatal infection with and without cell depletion (Fig 3A). As previously demonstrated (30), NK depletion significantly increased viral load ($p<0.05$). However, T cell depletion had no effect on viral load, suggesting that increased viral load does not account for the increased antibody titer after T cell depletion. To further address the role of viral load on antibody response, neonatal mice were infected with different doses of virus ($1.2x10^4$ PFU, $5.5x10^4$ PFU and $2.6x10^5$ PFU). There was no apparent difference in antibody responses between groups after secondary infection

(Fig 3B). Likewise there was no difference in antibody titer when adult mice (Fig 3C) were infected with different primary doses of virus ($8.0x10^4$ PFU or $5.0x10^5$ PFU). These data suggest that the increased antibody titer seen after cell depletion is not caused by differences in viral load.

IFN-γ has been shown to have an inhibitory effect on antibody responses (25). In previous studies, we observed that neonatal mice infected with recombinant RSV expressing IFN-γ have reduced antibody responses (31). Whilst, there is a well described Th2 bias in neonatal life (3), we and others, have shown that there are RSV specific IFN-γ secreting CD8 cells following neonatal RSV infection (22, 32). Mice were infected at day 4 of life and cytokine levels measured in lung homogenate supernatants at various time points after infection. RSV infected mice had significantly more IFN-γ in the lungs at days 1 and 7 post infection than naïve littermate controls (Fig 3D, $p<0.01$). IFN-γ levels increased in the lungs with age in both naive and infected mice, which may reflect increased immunological maturity or the increased size of the lungs. To define which cells produced IFN-γ, cells were isolated during primary infection and stimulated with PMA/ionomycin. Infected mice had significantly more IFN-γ producing NK cells on day 1 post infection (day 5 of life) compared to age matched controls (Fig 3E, $p<0.05$). A significant increase in IFN-γ producing CD8 cells was observed on days 7 and 11 post infection (Fig 3F, $p<0.05$) and CD4 cells on day 11 post infection (Fig 3G, $p<0.001$). To determine whether cell depletion affected IFN-γ production, levels were measured in lung homogenates on day 4 after neonatal infection with and without cell depletion (Fig 3H). Reduced levels were seen in the anti-CD4 and CD8 double depletion group and the anti-NK group.

We next investigated whether IFN-γ might have an inhibitory effect on antibody responses. Neonatal mice were infected with RSV and IFN-γ was blocked using three doses of anti-IFN-γ (clone XMG1.2) i.p. on days -1, +2 and +5. Blocking IFN-γ during primary infection significantly boosted antibody prior to (Fig 4A, $p<0.05$) and after (Fig 4B, $p<0.001$) re-infection. IFN-γ blockade lead to a more balanced IgG1:IgG2a ratio than control treatment (Fig S1E, $p<0.05$). To confirm that the IFN-γ was affecting the antibody response we used a recombinant RSV that expressed IFN-γ (33). As we have observed previously (31), there was no effect on antibody titer prior to rechallenge (Fig 4C), but after rechallenge with wild type virus, the antibody response in the rRSV-IFN-γ primed animals was significantly lower than wild type RSV primed mice (Fig 4D, $p<0.001$). Infection with rRSV-IFN-γ significantly reduced the IgG1 response (Fig S1F, $p<0.05$). To support the argument that increases in antibody responses are independent of viral load, mice treated with anti-IFN-γ had significantly lower viral loads during primary neonatal infection than control treated mice but greater antibody responses (Fig 4E, $p<0.001$). As previously observed (31) the rRSV-IFN-γ infected mice had a significantly lower viral titer than control infected neonatal mice ($p<0.05$). We therefore conclude that neonatal anti-RSV antibody responses are inhibited by the cellular immune response, and that IFN-γ plays a vital role in this inhibition.

Discussion

The antibody response following neonatal RSV infection was lower than the adult response. Cellular depletion (CD4, CD8 or NK) during primary neonatal infection significantly increased the antibody response. These cells all produced IFN-γ during neonatal infection and if IFN-γ was blocked the antibody response was significantly enhanced. The absence of effect of neonatal $CD4^+$ cell depletion suggests that neonatal B cells have a reduced requirement for CD4 help and that possibly the early life antibody response to RSV is T cell independent, unlike the adult response. This would fit with the observation that the neonatal anti-RSV response has reduced somatic hypermutation (20) and that there are reduced numbers of T follicular helper cells (Tfh) in neonates (34). Levels of the TNF family receptors critical for the development and maintenance of B cells (BAFF-R, BCMA and TACI) have been demonstrated to be lower in cord blood (35) and neonatal mice (36). Whilst germinal centers are immature in early life (6), adjuvants that induce maturation of follicular dendritic cells can restore antibody levels (37). While we did not directly address the role of IFN-γ on antibody response in adult mice, we did observe that they have CD4 dependent antibody responses to RSV infection (Fig 2D). In addition we have previously shown that there is a significantly greater RSV specific IFN-γ response in adult mice than neonatal mice (22). This suggests that the absence of strong CD4: B cell interactions in neonates contributes to the suppression of the antibody response by IFN-γ.

Inhibition of antibody by IFN-γ has been observed in other systems, IFN-γ receptor deficient mice have enhanced antibody responses to *Bordetella pertussis* infection

(38) and STAT1 receptor deficient mice have enhanced antibody responses to RSV infection (39). This inhibitory role for IFN-γ in the neonatal immune response is surprising because of the reported Th2 skew of responses, but Th1 responses can be observed in human neonates for example to BCG (40) and in mice can be boosted by the addition of TLR ligands (41). There are several mechanisms by which IFN-γ might inhibit the antibody response. It is possible that the cell and cytokine manipulations increased the viral load, thereby increasing the antigen exposure. However, multiple strands of evidence suggest that antibody titer is not solely influenced by viral load: depleting neonatal T cells had no effect on viral load (Fig 3A), anti-IFN-γ treatment decreased viral load (Fig 4E), the rRSV-IFN-γ virus was significantly attenuated as previously observed (33, 42) and when neonatal or adult mice were infected with different amounts of virus, there was no effect on antibody response (Fig 3B, C). The decrease in viral load after neonatal anti-IFN-γ treatment was in contrast to adult mice, which either had unchanged (43) or increased (44) viral load after anti-IFN-γ treatment. The decreased viral titer after neonatal anti-IFN-γ treatment may suggest that removing the suppression of antibody response by IFN-γ also has an effect on the viral load. From our studies we cannot rule out the role of other effectors or cells in the inhibition of the neonatal antibody response, but believe that during neonatal RSV infection, NK and T cells produce IFN-γ, which acts on cells important for the generation of antibody inhibiting their function, for example B cells (25, 45, 46).

The cell depletion experiments, reported in the current study, do not distinguish between cells producing IFN-γ or cells responding to IFN-γ and the effect on antibody response. Possible targets for IFN-γ include NK cells, T cells and B cells and further

studies are required to identify which plays a role. Co-depletion of NK and T cells together did not further increase the antibody titer over single cell type depletion. Since NK cells have been described as limiting antibody by inhibiting APC (29), it may be that the T cells generate the IFN-γ in response to the viral infection, activating the NK cells leading to APC inhibition. However, we observed no difference in FasL$^{-/-}$ deficient mice, suggesting the inhibitory mechanism is independent of this pathway. It may be that other killing mechanisms or cell types are involved (47), for example Treg cells, which utilize granzyme B to resolve inflammation after RSV infection (48) or CD8 cells, which have been shown to kill LCMV infected B cells (49). CD8 T cells have been shown to downregulate the Th2 CD4 T cell response to RSV (50) and transferring RSV specific CD8 cells into adult mice has an inhibitory effect on antibody responses (51). However, this does not explain why CD4 and CD8 co-depletion enhanced antibody responses. Neonatal B cells have been shown to have reduced expression of IL-4 receptor, which may potentiate the inhibitory effects of IFN-γ (52). While IFN-γ has been shown to inhibit IL-4 and thereby reduce classical Th2 help for B cells, the effect of IFN-γ on other CD4 helper subsets which may play a role, such as T follicular help, is not well characterized, recently IL-21 depletion has been shown to reduce anti-RSV antibody (53).

The effect of IFN-γ on the anti-RSV antibody response in early life may go some way to explain why infants can be re-infected with the same virus in the same or consecutive seasons. We have not succeeded in our attempts to determine how general our observations are and so are unable to say whether this is an RSV specific effect. The inhibition of antibody responses by the cellular response may have an impact on the development of RSV vaccines for use in early life. If vaccines induce a strong

cellular response producing IFN-γ, it may reduce the anti-RSV antibody response. By understanding the effect of IFN-γ on antibody responses we can optimize pediatric vaccine formulations and schedules to minimize the inhibitory effects.

Methods

Mice and Virus. Time mated pregnant BALB/c or C57BL/6 mice (Harlan, Bevil's Hill, UK) were purchased at <14d gestation and pups were weaned at 3wk old. Mice were infected i.n. with $4x10^4$ PFU/gram body weight RSV A2 at 4 days (neonatal ~ 10^5 PFU in 20µl) or 4-6 weeks of age (adults ~ 5 x 10^5 PFU in 100µl) under isofluorane anesthesia. Mice were re-infected i.n. 8 weeks later, with 10^6 PFU RSV in 100µl. Recombinant RSV expressing IFN-γ (rRSV-IFN-γ) was obtained from Dr Peter Collins and was generated as described previously (33). Breeding pairs of Fas ligand ($FasL^{-/-}$) deficient mice (gld) were obtained as a kind gift from Professor Anthony Warrens (Imperial College London) and the litters from these were used in the same protocol as the wild type mice. RSV A2 strain was grown in HEp-2 cells and viral titer determined by immune-plaque assay.

RSV viral load was assessed by extracting RNA from lung tissue using STAT-60 or RNeasy mini kit (Qiagen) and then converting it into cDNA. Quantitative RT-PCR for the RSV L gene using primers and probes previously described, (21) was performed and the results normalized against 18s or GAPDH endogenous RNA levels. L gene copy number was determined using a RSV L gene standard and presented relative to µg lung RNA.

For cell depletion, mice were treated with 500µl (adults) or 100µl (neonates) of 1mg/ml antibody i.p. on d-1, d+2 and d+5 post infection. Cells were depleted using anti-CD4 (hybridomas YTA 191 and YTA 3), anti-CD8 (clone YTS 156) and control treatment used an irrelevant matched isotype control. All antibodies were IgG2b and a kind gift of S. Cobbold, Oxford University. IFN-γ was blocked in neonatal mice using

100µl of 1mg/ml antibody from clone XMG1.2 (eBioscience, UK) i.p. on d-1, d+2 and d+5 post infection. NK cells were depleted with 100µl anti-asialo GM1 (Wako, Japan) on d-1 and d+2 post infection. All work was approved and licensed by the UK Home Office.

RSV-specific antibody quantification. A quantitative assay was used to determine serum antibody levels adapted from Donelly et al (54). 96-well plates were coated with RSV antigens and blocked with 1% BSA. A dilution series of recombinant murine IgG was used on each plate as a standard to quantify the RSV specific antibodies. Sera were diluted 1:500, 1:5,000 and 1: 50,000 to ensure the absorbance reading measured fell within the linear range of the standard curve. Bound IgG was detected by incubation for 2 hr at 37 ºC with HRP-conjugated goat anti-mouse IgG (AbD Serotec). Plates were washed and developed with 50 µl TMB/E substrate and the reaction was terminated by the addition of 50 µl of 2M H_2SO_4 and read at A_{450}. For IgG subtype measurements, a similar protocol was performed using biotinylated anti-IgG1 or anti-IgG2a and detected using HRP-Streptavidin, compared to a standard curve of recombinant murine IgG1 or IgG2a. Viral neutralization by antibody was assessed as described before (55).

IFN-γ cytokine ELISA. Cytokine levels were assessed in lung homogenate supernatants by ELISA using a pair of capture and biotinylated detection antibodies (R&D systems) following the manufacturer's instructions. Mediator concentrations were quantified by comparison to recombinant cytokine standards.

Cell preparation and flow cytometry. After infection, animals were culled using i.p. pentobarbitone. Cells were harvested as described previously (56). Prior to staining cells were blocked with CD16/32 (BD). For surface staining antibodies against the surface markers CD3 (17A2, FITC), CD4 (RM4-5, APC), CD8 (53.6-7, PerCP) and CD49b (DX5, PE) were added in 1:100 dilution. For intracellular staining, cells were stimulated for 4h at 37°C in the presence of 10 μg/ml Brefeldin A, 100μg/ml PMA and 10μg/ml ionomycin. Cells were permeabilised with 0.5% saponin and stained with directly conjugated anti-IFN-γ (XMG2.1, FITC). Samples were run on an LSR (BD) and analyzed using Winlist (Verity).

Statistical analysis. Results are expressed as mean ± S.E.M.; statistical significance was calculated by ANOVA followed by Tukey tests or t tests using GraphPad Prism software, as indicated in figure legends.

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Author Contributions

JST: Designed Research, Performed Research, Analyzed Data, Interpreted results, Wrote the paper; BW, JM, YY, JA, MG: Performed Research; CJ: Wrote the paper, Analyzed data; AB: Contributed new reagents, Wrote the paper; PC: Contributed new reagents; PO: Designed Research, Interpreted results, Wrote the paper.

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Figure Legends

Figure 1. Antibody responses to RSV infection in early life are less than adult responses. BALB/c mice were infected with $4x10^4$ PFU RSV per gram body weight intranasally as neonates (age 4 days, ▪) or as immature adults (age 4-6 weeks, ●) (1°RSV). 8 weeks after the primary infection, mice were given a secondary infection of 10^6 PFU RSV (2° RSV). Schematic experimental protocol (A) including cellular depletion protocol (Ig indicates an antibody depletion time point). Lung viral load was measured by quantifying RSV L gene expression on day 4 of primary (1° RSV, closed symbols) or secondary (2° RSV, open symbols) infection (B). RSV-specific IgG (C) and *in vitro* RSV neutralization (D) measured in sera collected after primary and secondary infections. Points represent individual mice, lines represent mean of n≥4 mice per group +/- SEM (A and C show pooled data from 2 experiments, B is representative of 2 experiments), * $p<0.05$, ** $p<0.01$, *** $p<0.001$, calculated using t-test.

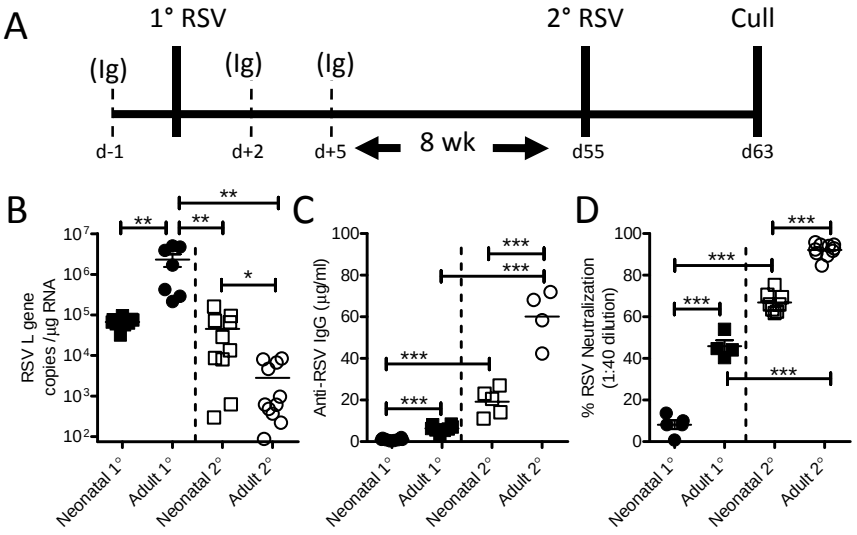
Figure 2. Cell depletion increases neonatal antibody response to RSV infection. CD4, CD8 or NK cells were depleted during primary RSV infection of neonatal (A, B, F, G), or adult (C, D) BALB/c mice or during secondary RSV infection of BALB/c mice initially infected as neonates (E). RSV-specific IgG was measured on day-1 (A, C, F) or day 7 (B, D, E, G) of RSV secondary infection. Wild type (wt) C57BL/6 were infected as neonates with RSV, with or without cell depletion (H) or compared to $FasL^{-/-}$ mice (I) and serum anti-RSV IgG measured on day 7 of RSV secondary infection. Points represent individual mice, lines represent mean of n≥3 mice per group +/- SEM, * $p<0.05$, ** $p<0.01$, *** $p<0.001$, calculated using ANOVA and

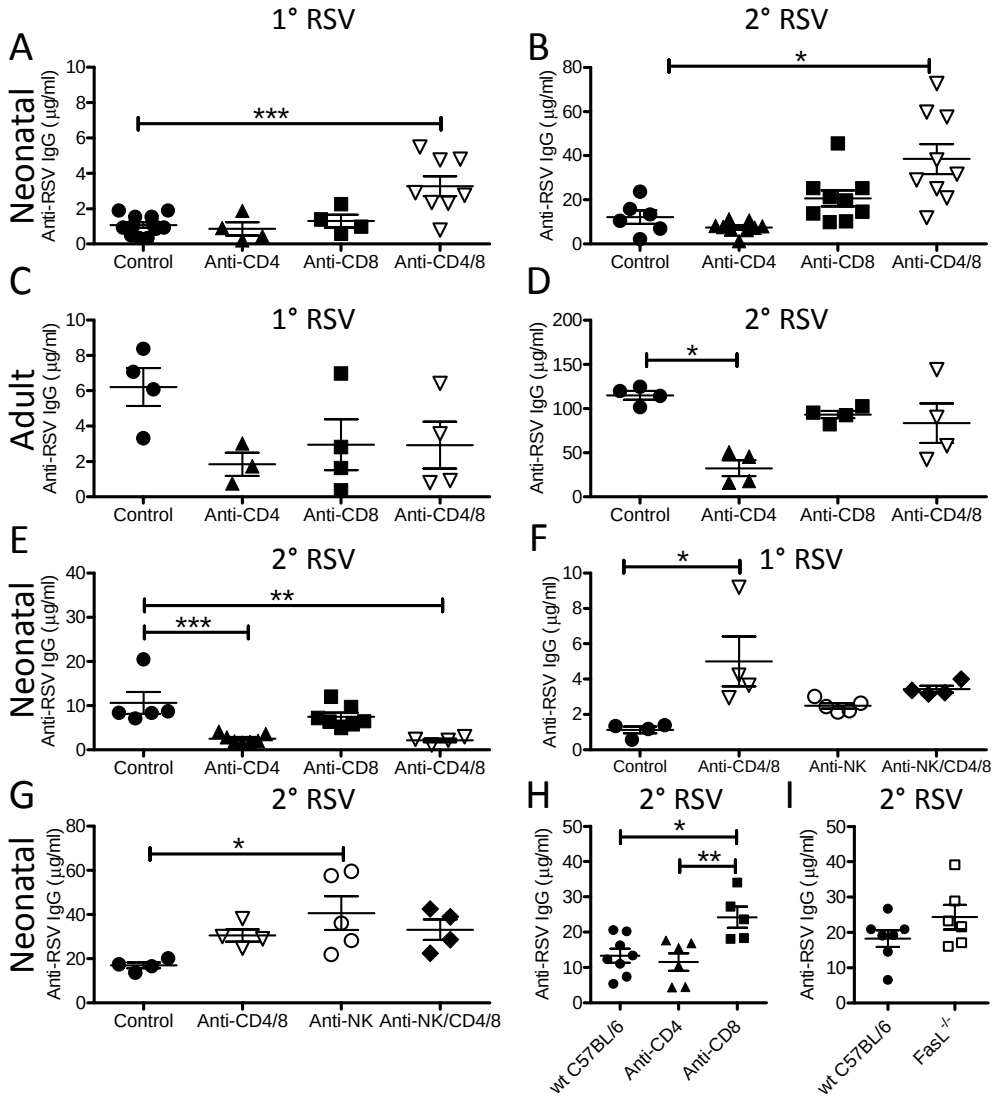
post test. Data presented are pooled from 2 experiments except panels C, D which is from a single experiment.

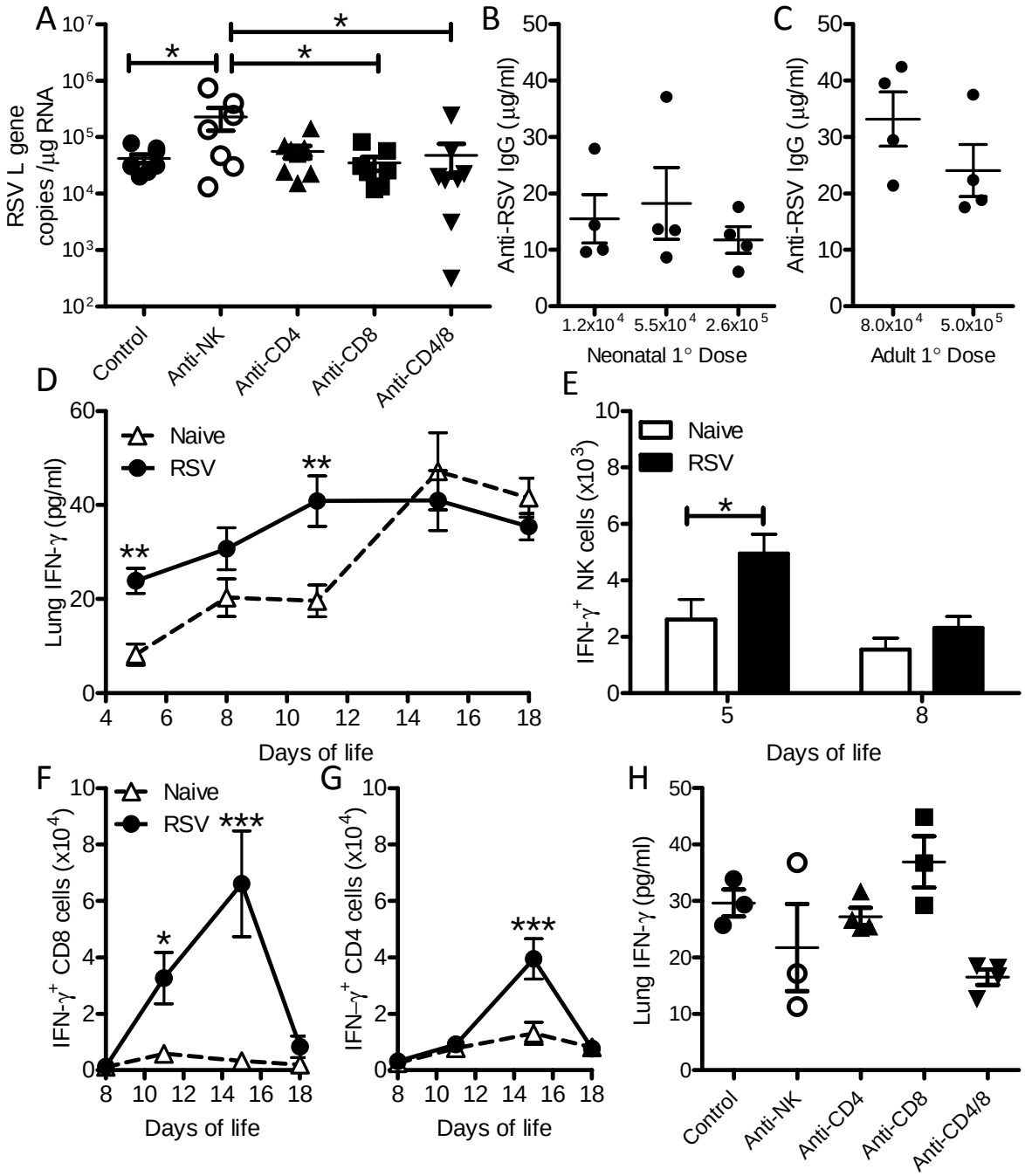
Figure 3. Interferon gamma is produced during neonatal RSV infection. Neonatal BALB/c mice were infected with 10^5 PFU RSV; CD4, CD8 or NK cells were depleted during RSV infection and lung viral load was measured by quantifying RSV L gene expression on day 4 post primary infection (A). Neonatal (B) and adult (C) mice were infected with different primary doses of RSV and RSV-specific IgG titer measured on day 7 after secondary RSV infection. 4 day old mice were infected with RSV (●) or left naïve (Δ), IFN-γ was measured in lung homogenate by ELISA (D) and IFN-γ expression measured in PMA/ionomycin stimulated lung NK ($DX5^+$) (E), CD8 (F) and CD4 (G) cells by flow cytometry. IFN-γ levels were measured in lung homogenate at day 4 post primary RSV infection, with and without cell depletion (H). Points represent individual mice, lines represent mean of n≥3 mice per group (A,B,C,H), bars/points represent mean of n≥4 mice per group ± SEM (D,E,F,G). Data shown is representative of one experimental repeat, * $p<0.05$, ** $p<0.01$, *** $p<0.001$, calculated using ANOVA and post test.

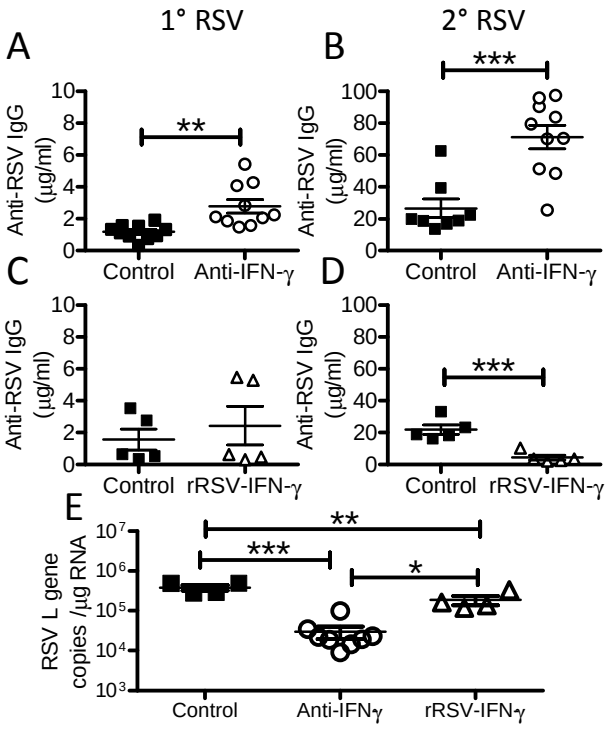
Figure 4. Interferon gamma inhibits antibody responses to neonatal RSV infection. During primary RSV infection, neonatal BALB/c mice were treated with anti-IFN-γ or left untreated, 8 weeks later, mice were re-infected with RSV. RSV-specific IgG was measured in sera on day-1 (A) and day 7 (B) of secondary infection. Neonatal mice were infected with recombinant RSV expressing IFN-γ (rRSV-IFN-γ) or wild type RSV A2, and 8 weeks later mice were re-infected with wild type RSV A2. RSV-specific IgG was measured in sera on day-1 (C) and day 7 (D) of secondary

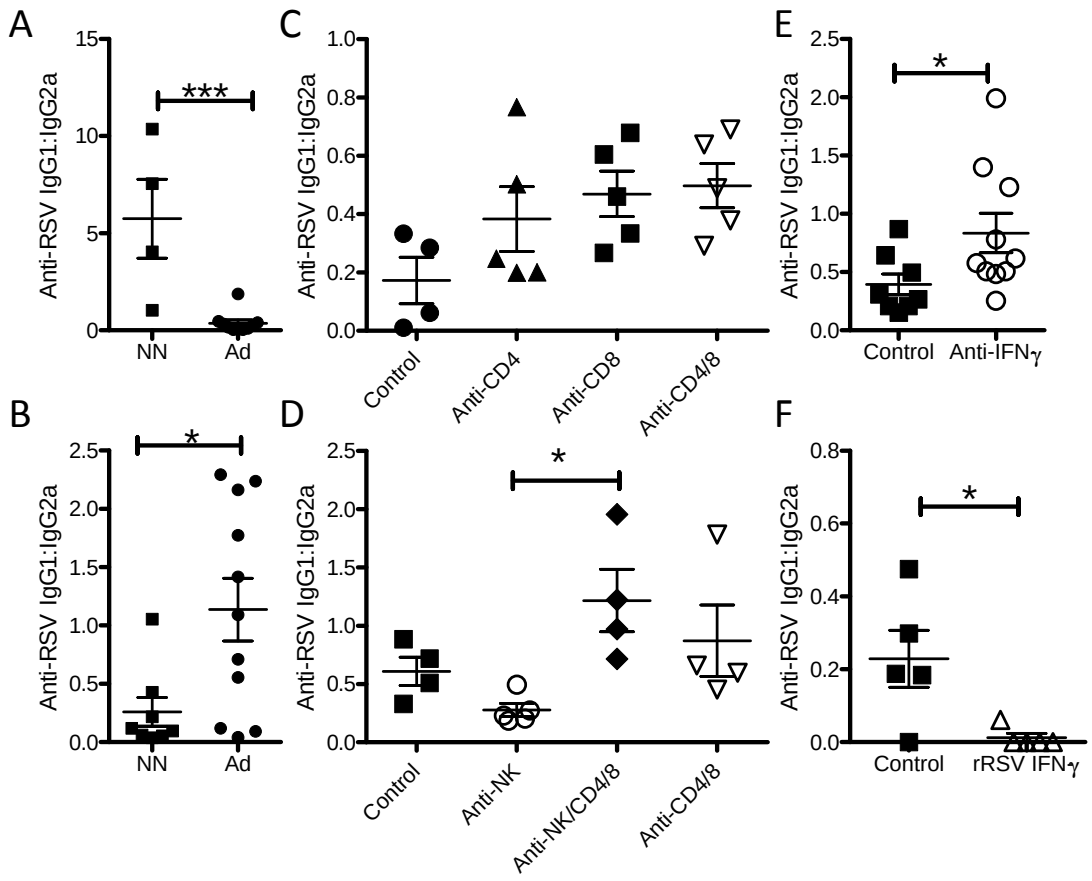
infection. Viral load (RSV L gene) was measured during primary neonatal infection by RT-PCR in lungs of control mice or mice infected with RSV and treated with anti-IFN-γ or infected with rRSV-IFN-γ (E). Points represent individual mice, lines represent mean of n≥4 mice per group. The data is pooled from 2 experimental repeats. * $p<0.05$, ** $p<0.01$, *** $p<0.001$, calculated using ANOVA and post test.

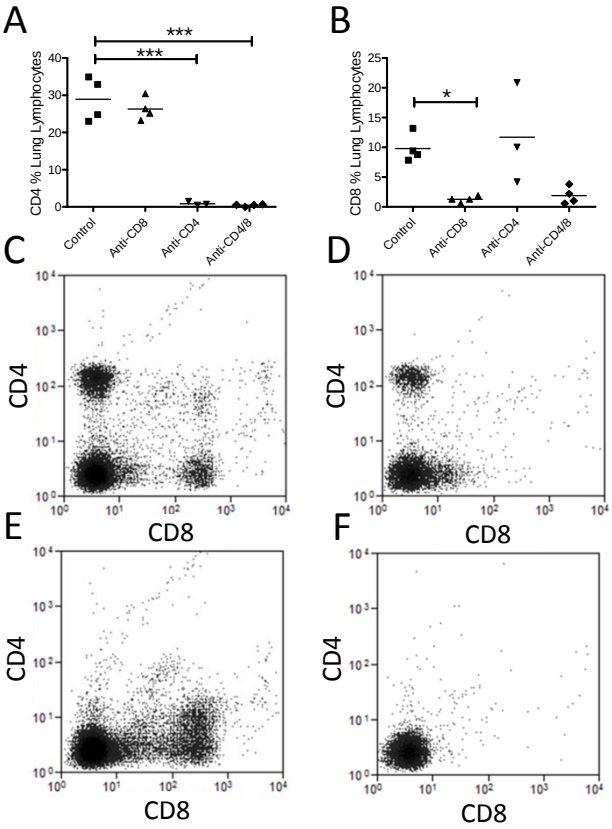

A
1° RSV
2° RSV
Cull
(Ig)
(Ig)
(Ig)
d-1
d+2
d+5
8 wk
d55
d63
B
RSV L gene copies /µg RNA
Neonatal 1°
Adult 1°
Neonatal 2°
Adult 2°
C
Anti-RSV IgG (µg/ml)
D
% RSV Neutralization (1:40 dilution)


A
1° RSV
Neonatal
Anti-RSV IgG (µg/ml)
***
Control
Anti-CD4
Anti-CD8
Anti-CD4/8
B
2° RSV
*
C
1° RSV
Adult
D
2° RSV
*
E
2° RSV
Neonatal
**
***
F
1° RSV
*
Anti-CD4/8
Anti-NK
Anti-NK/CD4/8
G
2° RSV
H
2° RSV
**
wt C57BL/6
I
2° RSV
FasL -/-


A
RSV L gene copies /µg RNA
Control
Anti-NK
Anti-CD4
Anti-CD8
Anti-CD4/8
B
Anti-RSV IgG (µg/ml)
1.2x10^4
5.5x10^4
2.6x10^5
Neonatal 1° Dose
C
Anti-RSV IgG (µg/ml)
8.0x10^4
5.0x10^5
Adult 1° Dose
D
Lung IFN-γ (pg/ml)
Naive
RSV
Days of life
E
IFN-γ+ NK cells (x10^3)
Naive
RSV
Days of life
F
IFN-γ+ CD8 cells (x10^4)
Naive
RSV
Days of life
G
IFN-γ+ CD4 cells (x10^4)
Days of life
H
Lung IFN-γ (pg/ml)
Control
Anti-NK
Anti-CD4
Anti-CD8
Anti-CD4/8


1° RSV
2° RSV
A
B
C
D
E
Anti-RSV IgG (µg/ml)
RSV L gene copies /µg RNA
Control
Anti-IFN-γ
rRSV-IFN-γ
**
***
*


A
Anti-RSV IgG1:IgG2a
15
10
5
0
***
NN
Ad
C
Anti-RSV IgG1:IgG2a
1.0
0.8
0.6
0.4
0.2
0.0
Control
Anti-CD4
Anti-CD8
Anti-CD4/8
E
Anti-RSV IgG1:IgG2a
2.5
2.0
1.5
1.0
0.5
0.0
*
Control
Anti-IFNγ
B
Anti-RSV IgG1:IgG2a
2.5
2.0
1.5
1.0
0.5
0.0
*
NN
Ad
D
Anti-RSV IgG1:IgG2a
2.5
2.0
1.5
1.0
0.5
0.0
*
Control
Anti-NK
Anti-NK/CD4/8
Anti-CD4/8
F
Anti-RSV IgG1:IgG2a
0.8
0.6
0.4
0.2
0.0
*
Control
rRSV IFNγ


A
***
***
CD4 % Lung Lymphocytes
40
30
20
10
0
Control
Anti-CD8
Anti-CD4
Anti-CD4/8
B
*
CD8 % Lung Lymphocytes
25
20
15
10
5
0
Control
Anti-CD8
Anti-CD4
Anti-CD4/8
C
D
CD4
CD8
E
F
CD4
CD8