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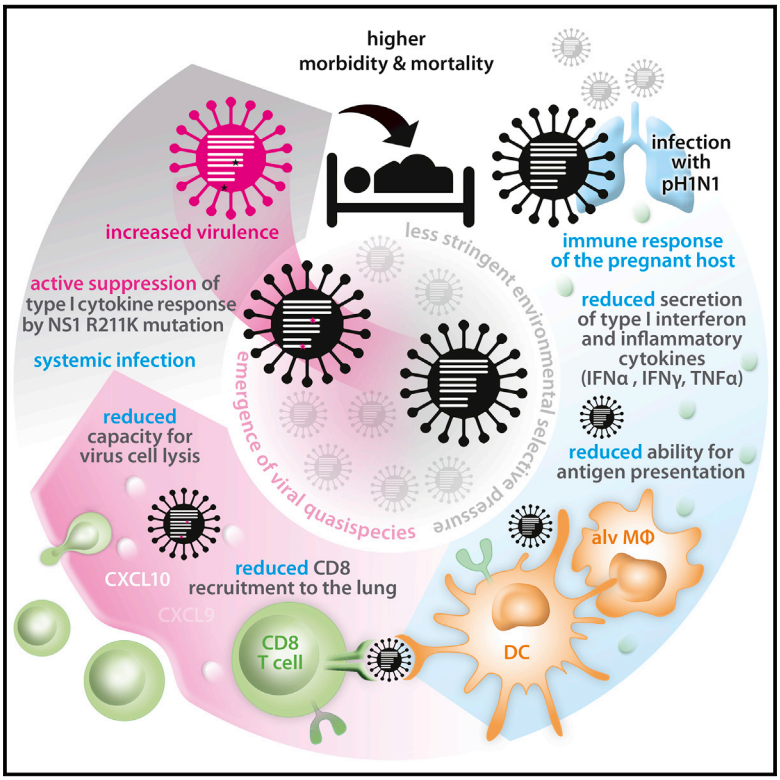
Pregnancy-Related Immune Adaptation Promotes the Emergence of Highly Virulent H1N1 Influenza Virus Strains in Allogenically Pregnant Mice

Graphical Abstract



Highlights

- Pregnancy-associated influenza susceptibility can be mimicked in pregnant mice
- Allogenically pregnant mice show a more severe infection than do syngenically mated mice
- $CD8^+$ T cell migration into the lung is impaired in allogenically pregnant infected mice
- H1N1 virus variants emerge in pregnant mice that are more virulent in non-pregnant mice

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In Brief

Pregnant women are at highest risk during influenza pandemics. Engels and colleagues present influenza infection models in mice and show that the immune response, which is tailored to accommodate the semiallogenic fetus, restricts the anti-viral immune response during gestation. Under these conditions, highly pathogenic virus variants can emerge.

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Pregnancy-Related Immune Adaptation Promotes the Emergence of Highly Virulent H1N1 Influenza Virus Strains in Allogenically Pregnant Mice

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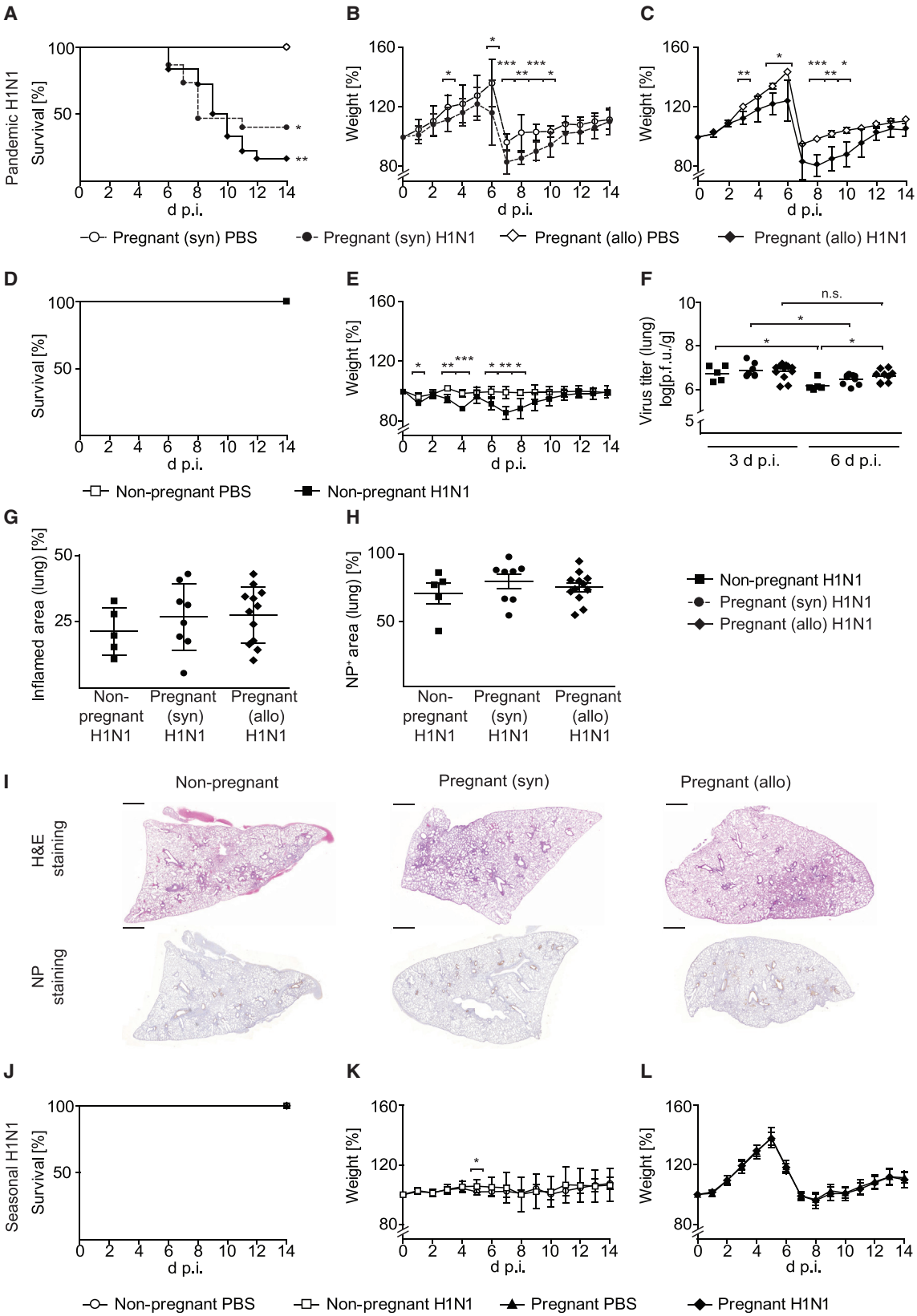
SUMMARY

Pregnant women are at high risk for severe influenza disease outcomes, yet insights into the underlying mechanisms are limited. Here, we present models of H1N1 infection in syngenic and allogenic pregnant mice; infection in the latter mirrors the severe course of 2009 pandemic influenza in pregnant women. We found that the anti-viral immune response in the pregnant host was significantly restricted as compared to the non-pregnant host. This included a reduced type I interferon response as well as impaired migration of $CD8^+$ T cells into the lung. The multi-faceted failure to mount an anti-viral response in allogenic pregnant mice resulted in a less stringent selective environment that promoted the emergence of 2009 H1N1 virus variants that specifically counteract type I interferon response and mediate increased viral pathogenicity. These insights underscore the importance of influenza vaccination compliance in pregnant women and may open novel therapeutic avenues.

INTRODUCTION

Influenza severely affects human populations through seasonal epidemics and random pandemics. While influenza epidemics occur yearly during autumn and winter (Jamieson et al., 2009), influenza pandemics strike irregularly, but recurrently. The most recent influenza pandemic occurred in the year 2009, when a new H1N1 influenza virus strain emerged (Dawood et al., 2012; Garten et al., 2009). One common denominator of all influenza pandemics recorded over the last century is the proportionally high morbidity and mortality rate among pregnant women (Freeman and Barno, 1959; Jamieson et al., 2009).

Pregnancy creates a unique immunological situation in allogenic matings, since the placenta—which is in direct contact with the maternal immune system—expresses antigens derived from the father. To tolerate this "foreign" allogenic tissue, the maternal immune system in mice and humans mounts intricate processes of adaptation, which ensure that rejection of the fetus is suppressed (Arck and Hecher, 2013). These adaptational processes include the arrest of dendritic cells (DCs) in a tolerogenic state, mirrored by a reduced expression of costimulatory surface markers (Blois et al., 2007; Segerer et al., 2008). Moreover, effector T cells are constrained in their migration to the feto-maternal interface due to the epigenetic silencing of the C-X-C motif chemokine (CXCL) 10, a chemoattractant involved in recruiting $CXCR3^+$ leukocytes, into the uterus (Nancy et al., 2012; Chaturvedi et al., 2015). Tolerance toward the fetus and pregnancy maintenance is further sustained by the generation of $CD4^+$ and $CD8^+$ regulatory T (Treg), and Natural Killer (NK) cells that reside at the feto-maternal interface (Arck and Hecher, 2013; Solano et al., 2015; Moffett and Colucci, 2014). Also, B cells are increasingly recognized to promote fetal tolerance (Muzzio et al., 2014). These intricate pathways collectively create a tolerogenic, anti-inflammatory environment in which placental development and fetal growth can occur.



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In contrast to this tolerogenic immune response during pregnancy, influenza virus infection generally leads to an immediate activation of the immune response to mount anti-viral immunity and clear the infection (Gabriel and Arck, 2014; Miller et al., 2015). Here, the production of anti-viral, inflammatory cytokines such as interferon (IFN)-α/β by epithelial and immune cells is a critical first line of defense. Moreover, DCs become antigen presenting cells and induce the generation of virus-specific effector T cells (Gabriel and Arck, 2014; Unkel et al., 2012; Lambrecht and Hammad, 2012). Facilitated by chemokines, virus-specific effector T (T_{eff}) cells subsequently migrate into the lung to clear the infection (Lambrecht and Hammad, 2012).

These differential, if not opposing immune responses mounted during pregnancy or upon influenza virus infection strongly indicate that the pregnant host may face a contradictory demand to sustain immune tolerance required for fetal survival versus mounting an inflammatory response to eliminate the influenza virus (Gabriel and Arck, 2014; Krishnan et al., 2013; Pazos et al., 2012a). However, insights into the underlying pathways of this presumed predicament of the pregnant host's immune response are still sparse. Mouse models, which are pivotal tools to understand the immune response to pregnancy as well as to influenza virus infection, may help to identify such pathways. Indeed, some evidence is available to support an altered influenza-related morbidity during pregnancy (Chan et al., 2010; Marcelin et al., 2011; Kim et al., 2012). However, these studies were carried out in syngenically mated mice, which limits the translational relevance of the obtained findings for human pathologies. Thus, a tailored assessment of the unique features and facets of maternal immune responses mounted against an allogenic fetus during pregnancy was urgently needed, which prompted us to perform the present study.

RESULTS

Increased pH1N1 Influenza Morbidity and Mortality in Allogenic Pregnant Mice

Infection of BALB/c-mated allogenic pregnant C57BL/6 females at mid-pregnancy with a dose of 10^3 plaque forming units (PFU) of the 2009 pandemic (pH1N1) influenza virus resulted in an increased mortality in allogenic pregnant mice, compared to pH1N1 infected, C57BL/6-mated, syngenic pregnant C57BL/6 females (Figure 1A). Moreover, while surviving syngenic pregnant mice fully recovered within 14 days post infection (d.p.i.), as determined by a restored body weight as a proxy of morbidity during influenza infection (Figure 1B) (Gabriel et al., 2005), allogenic pregnant mice showed an increased and prolonged weight loss upon infection (Figure 1C). In non-pregnant, pH1N1 infected C57BL/6 females, mortality rates were not increased upon infection, and full recovery from influenza-related morbidity was reached at 14 d.p.i. (Figures 1D and 1E). Virus titers were higher on day 6 p.i. in the lungs of 2009 pH1N1 infected allogenic compared to syngenic pregnant mice (Figure 1F). Consistently, lungs of infected syngenic and allogenic pregnant mice presented increased numbers of infiltrated mononuclear cells defined as inflamed areas and higher frequencies of viral antigen (NP) positive areas compared to infected non-pregnant mice (Figures 1G–1I).

To test if the pregnancy-related mortality and morbidity is specific for the pH1N1 virus, we also infected pregnant and non-pregnant mice with 10^3 PFU of a 2006 seasonal H1N1 (sH1N1) strain, where fewer influenza-related complications were reported among pregnant women (Rasmussen and Jamieson, 2012). Here, the infection did not cause a significant weight loss or mortality in pregnant or non-pregnant mice (Figures 1J–1L). Similarly, increasing the sH1N1 virus concentration by 100× (10^5 PFU), all infected pregnant and non-pregnant mice survived. When reducing the pH1N1 virus infection dose 10× (10^2 PFU), the infection became non-lethal also in allogenic pregnant mice but still mediated a significant weight loss. When using a 10× higher pH1N1 virus dose (10^4 PFU), mortality and weight loss further increased in pregnant mice, and survival rate also decreased in non-pregnant mice (Figure S1).

We further observed that more animals presented systemic viral titers—assessed in the gut (Otte et al., 2011)—in the allogenic pregnant pH1N1-infected groups. However, NP^+ cells or viral titers were not detectable in placental tissue or viral titers in concepti. Also, no significant alterations of the course and outcome of pregnancy were present in infected syn- or allogenic pregnant mice (Figure S2).

Overall, these findings mirror the clinical observation of pH1N1-specific increase in morbidity and mortality during pregnancy, which is particularly profound in allogenic murine matings. Considering that allogenic matings in mice are also more comparable to human pregnancies, we performed all subsequent experiments exclusively in allogenic matings.

Figure 1. Pathogenicity of Pandemic H1N1 and Seasonal H1N1 in Syn- and Allogenic Pregnant and Non-pregnant Mice

BALB/c-mated, allogenic pregnant C57BL/6 (n = 18) and C57BL/6-mated, syngenic pregnant C57BL/6 females (n = 15) were infected with 10^3 PFU of pH1N1 virus. Syngenic and allogenic pregnant control groups received PBS only.

(A–C) Survival (A) and weight loss in syngenic (B) and allogenic pregnant mice (C) were monitored for 14 d.p.i. Note: the weight loss occurring around 6 d.p.i. and in PBS controls in (B) and (C) is associated with birth.

(D and E) Survival (D) and weight loss (E) in non-pregnant C57BL/6 females (n = 5) infected with 10^3 PFU of pH1N1 virus, compared to non-infected non-pregnant animals.

(F) pH1N1-virus titers were determined in lungs of infected mice at 3 and 6 d.p.i. (non-pregnant, n = 5; syngenic pregnant, n = 8; allogenic pregnant, n = 9).

(G and H) Inflamed areas (percentage of inflamed area over total lung area) (G) and percentage of viral antigen (NP^+ bronchioles over total number of bronchioles) (H) were assessed.

(I) Lung tissues were stained with H&E to detect inflamed areas and immunohistochemically (IHC) against the viral antigen nucleoprotein (NP) (non-pregnant, n = 5; syngenic pregnant, n = 8; allogenic pregnant, n = 12). Scale bar, 100 μm in H&E and NP staining.

(J–L) Mice were infected with 10^3 PFU of sH1N1 influenza virus (non-pregnant, n = 9; allogenic pregnant, n = 7); controls received PBS only. Survival (J) and weight loss in non-pregnant (K) and allogenic pregnant (L) infected mice were monitored for 14 d.p.i.

Data in (B), (C), (E)–(H), (K), and (L) are presented as mean ± SD. Statistical significance was calculated by Gehan-Brelow-Wilcoxon and Student's t test (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$).

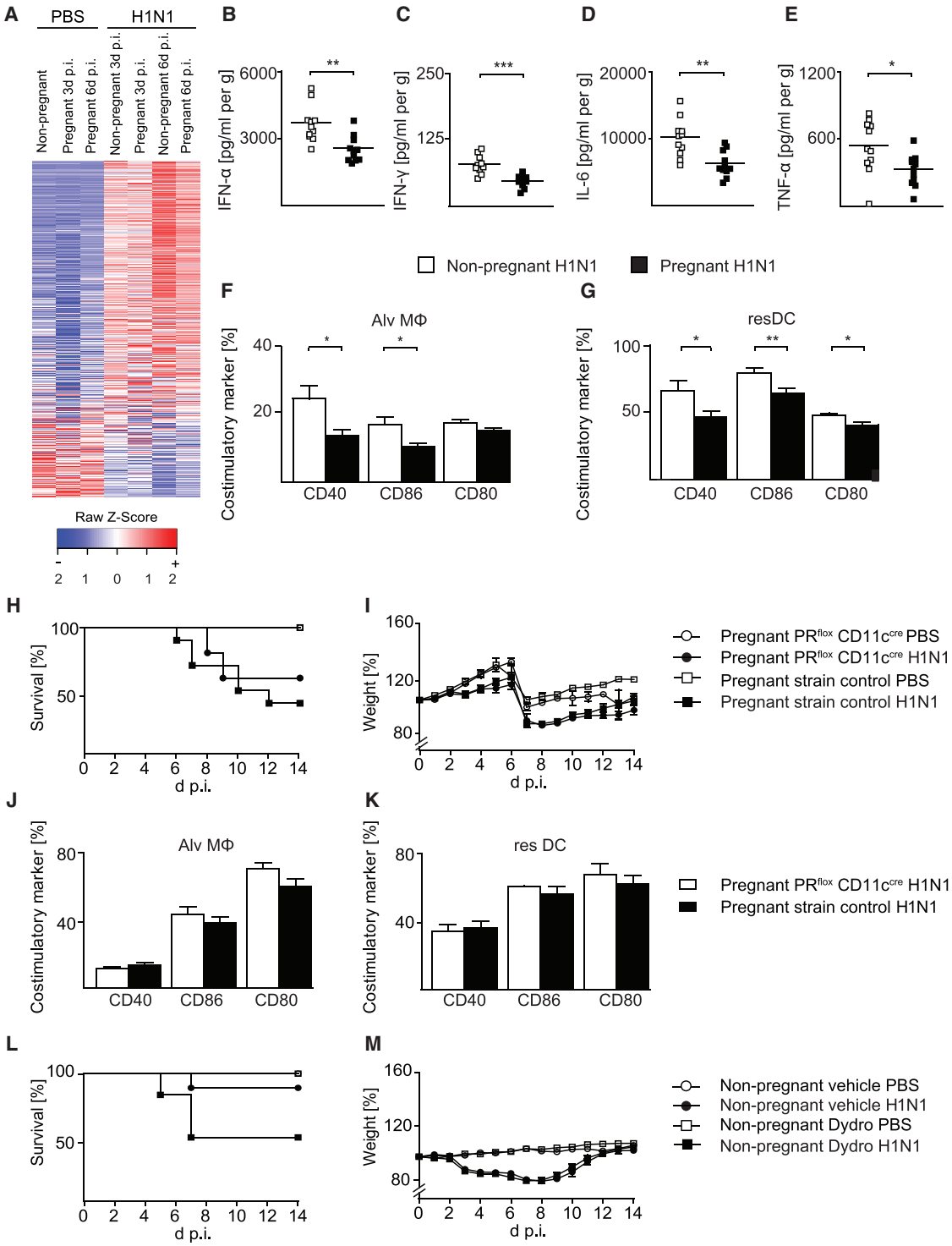

Figure 2. Reduced ISG Expression, Secretion of Type I Interferons and Inflammatory Cytokines, and Upregulation of Costimulatory Markers on APCs in Pregnant Infected Mice

(A) Differential expression of murine ISGs was analyzed by RNA-seq. The color code symbolizes the Z-score of normalized read counts according to the legend shown at the bottom. Full names of genes are provided in the Supplemental Information. PBS (3 d.p.i. or 6 d.p.i., respectively) reflects the PBS treated controls obtained on day 3 p.i. or 6 p.i. along with the infected animals.

(B–E) Cytokines (IFN-α [B], IFN-γ [C], IL-6 [D], and TNF-α [E]) determined by luminex assay in lungs of non-pregnant (open squares, n = 11) or pregnant mice (black squares, n = 12), respectively, infected with 10^3 PFU of pH1N1 and harvested on day 3 p.i. Values are normalized to the organ weight. If fewer measurement points than the indicated n are visible in the graphs, the ones not shown were out of range (i.e., below the range of the standard curve).

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Reduced ISG Expression, Type I IFN Response, and Inflammatory Cytokine Secretion in Pregnant Infected Mice

The host innate response, particularly type I IFNs, present the first line of defense against viral pathogens (Schneider et al., 2014). Thus, we investigate the expression pattern of key innate response genes during pregnancy by performing an mRNA expression analysis using next-generation sequencing (RNA-seq) of infected and uninfected lungs in pregnant versus non-pregnant mice. The mRNA transcripts were further analyzed using an innate immune response database, which covers greater than 196,000 experimentally validated molecular interactions within the inflammatory host response (Breuer et al., 2013). We found numerous genes, which were differentially regulated in infected pregnant versus non-pregnant lungs (Table S1). Most importantly, a clear pattern of downregulated innate response genes was present in 2009 pH1N1 infected pregnant compared to non-pregnant mice on day 6 p.i. (Figure 2A). We then re-assessed a number of differentially downregulated innate factors, such as IFN-γ, interleukin (IL)-6, and tumor necrosis factor (TNF)-α, in the lungs of infected pregnant and non-pregnant mice on a protein level and could independently confirm significantly reduced IFN-α, IFN-γ, IL-6, and TNF-α levels in lungs of pregnant infected dams on day 3 p.i. compared to non-pregnant infected mice (Figures 2B–2E). Thus, key cytokines crucial in suppressing viral spread (Durbin et al., 2000) are downregulated during pregnancy in 2009 pH1N1 infected dams.

Reduced Upregulation of Costimulatory Markers on Antigen-Presenting Cells

Next, we assessed the frequencies of leukocyte subsets in the lungs of infected mice at different days p.i. The overall frequencies of granulocytes, macrophages (MΦ), and DCs were generally higher in the lungs of pregnant infected mice, compared to infected, non-pregnant mice (data not shown). However, we observed a significantly reduced frequency of alveolar MΦ (alvMΦ) and distinct DC subsets expressing the costimulatory markers CD40, CD86, and CD80 in pH1N1 infected pregnant compared to infected non-pregnant mice (Figures 2F and 2G).

The maternal immune adaptation to pregnancy is significantly modulated by the pregnancy hormone progesterone (Arck and Hecher, 2013). Hence, in order to assess whether the observed innate cell defects are associated with pregnancy hormones and have functional consequences for influenza survival and morbidity during pregnancy, we utilized mice that lack the progesterone receptor (*Pgr*) specifically on CD11c$^+$ DCs (*Pgr*^{flox}*CD11c*^{cre} mice). We observed a—albeit marginal—higher survival during pH1N1 infection in allogenic pregnant *Pgr*^{flox}*CD11c*^{cre} mice, compared to infected, pregnant mice of the control strain (Figures 2H and 2I). Also, pregnant, infected *Pgr*^{flox}*CD11c*^{cre} mice revealed a slightly higher expression of the costimulatory marker CD80 and CD86 on alvMΦ and resDC in the lung (Figures 2J and 2K), similar to the expression on CD11c cells in non-pregnant infected wild-type mice (Figures 2F and 2G). The expression of CD40 was unaffected between mutant and control mice. The observation in pregnant, infected *Pgr*^{flox}*CD11c*^{cre} mice suggests that the high levels of progesterone during pregnancy account—at least in part—for the impaired innate immune response seen in infected, pregnant mice. We could independently show that injection of infected, non-pregnant mice with a progesterone derivative (dydrogesterone) in order to mirror pregnancy levels showed an increased mortality compared to sham-treated, infected mice (Figures 2L and 2M), suggesting that progesterone is involved in dampening the host's response against pH1N1.

Altered B Cell Response and Anti-pH1N1 Antibody Production in Pregnant Infected Mice

We also tested whether humoral immunity is affected in pregnant infected mice and observed a diminished humoral/B cell response during pregnancy, mirrored by a lower frequency of total splenic B lymphocytes, lower virus-specific hemagglutination inhibition (HI) titers, and a reduced co-expression of B cell-activation markers (Figure S2).

Impaired Leukocyte Recruitment to the Lung in Infected Pregnant Mice

When assessing the total number of leukocytes in the lung, we observed a reduction in infected pregnant mice at 3 and 4 d.p.i. (Figures 3A and 3B). This reduction also affected the frequency of CD8$^+$ T cells at 3 d.p.i. (Figures 3C and 3D). This led us to assess the expression of the chemoattractants for activated T cells, *Cxcl9* and *Cxcl10* (Loetscher et al., 1996; Bonecchi et al., 1998). We detected a significantly reduced expression of *Cxcl10* in lungs of pregnant, infected mice (Figure 3E), confirming our observations from the transcriptome analyses (Table S1). Similarly, the expression of *Cxcl9* was also reduced (Figure 3F). Based on these findings, we next tested whether the homing of CD8$^+$ T cells into the bona fide tissue effector site for CD8$^+$ T cells during influenza infection, the lung, is reduced (Figure 3G). We observed a significantly reduced frequency of migrated CD8$^+$ T cells bearing the cognate Cxcl10 receptor, CXCR3, to the lung (Figures 3H and 3I). In order to test if the ligands for CXCR3, *Cxcl9* and *Cxcl10*, are epigenetically silenced in the lung—similar to the observations made in the pregnant uterus (Nancy et al., 2012)—we analyzed the repressive histone H3 trimethyl lysine 27 (H3K27me3) enrichment at promoter regions of *Cxcl9* and *Cxcl10*. No significant difference of H3K27me3 enrichment was detected between the lungs of

(F and G) Expression of costimulatory markers (CD40, CD86, and CD80) on alveolar macrophages (alvMΦ) (F) and resident dendritic cells (resDC) (G) in the lungs of non-pregnant (n = 6) and pregnant mice (n = 6) upon infection with 10^3 PFU of pH1N1 as measured by flow cytometry at 3 d.p.i.

(H and I) Pregnant *Pgr*^{flox}*CD11c*^{cre} mice (n = 5) and pregnant mice of the control strain (n = 10) were infected with 10^3 PFU of pH1N1 virus. Control groups of both strains received PBS only. Survival (H) and weight loss (I) were determined within 14 d.p.i.

(J and K) Expression of costimulatory markers (CD40, CD86, and CD80) on alvMΦ (J) and resDC (K) in the lungs of pregnant *Pgr*^{flox}*CD11c*^{cre} (n = 5) and pregnant mice of the control strain (n = 7) were measured by flow cytometry at 3 d.p.i.

(L and M) Non-pregnant mice were injected with Dydrogesterone (Dydro) (n = 12) or vehicle (n = 9) every second day 12 days before and 14 days after infection with 10^3 PFU pH1N1. Control groups treated with Dydro or vehicle received PBS only. Survival (L) and weight loss (M) were determined within 14 d.p.i.

All data are presented as mean ± SEM, except (A), (H), and (L). The statistical significance was calculated by Student's t test (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$).

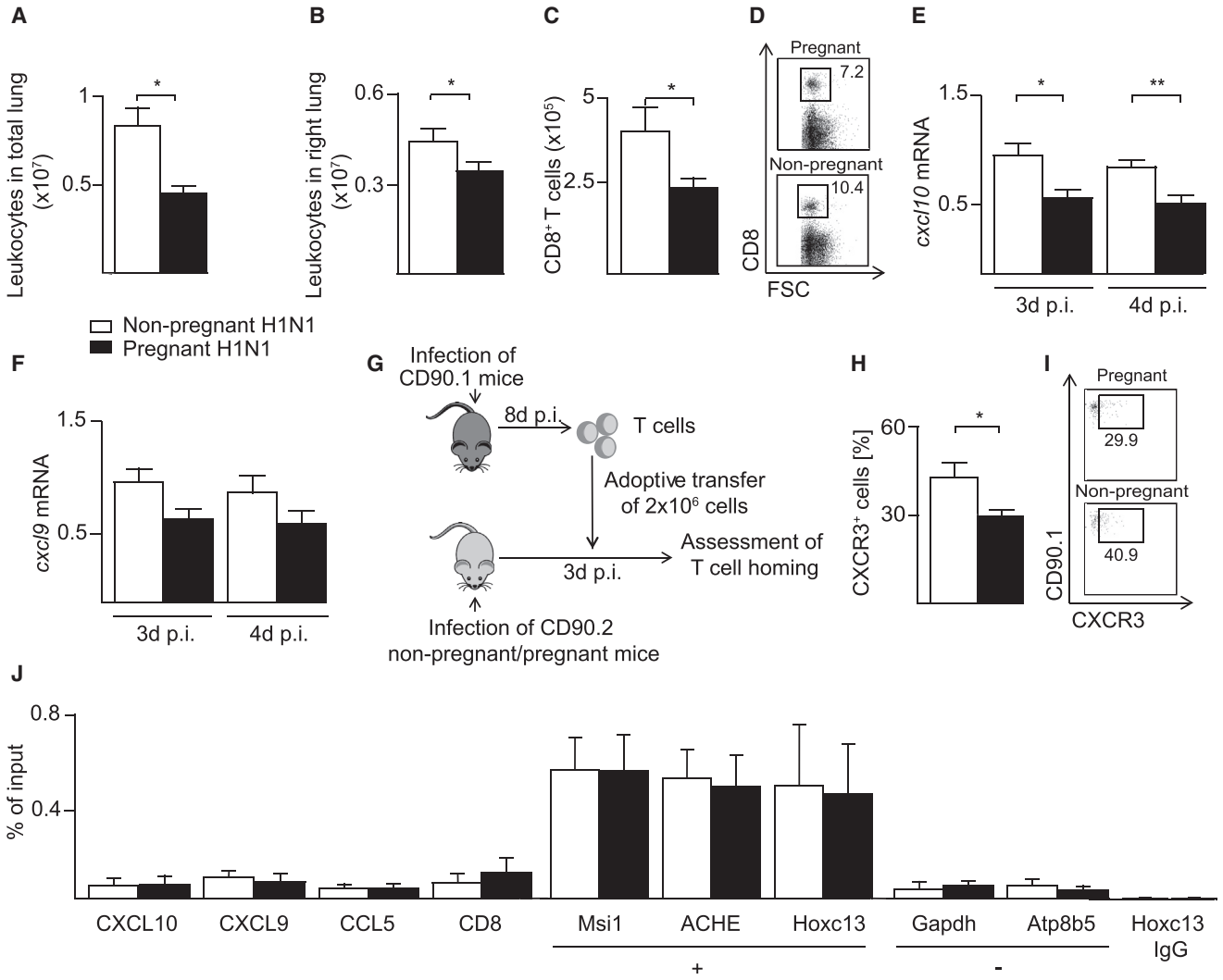


Figure 3. Recruitment of Leukocytes to the Lung in Infected Mice

(A and B) Absolute leukocyte numbers of non-pregnant (n = 6) and pregnant (n = 6) mice, present in lungs at 3 d.p.i. (A) and 4 d.p.i. (B).

(C) Frequency of $CD8^+$ T cells in the lung of non-pregnant (n = 12) and pregnant (n = 19) infected mice, as assessed by flow cytometry at 4 d.p.i.

(D) Representative dot plots of (C).

(E) Relative mRNA expression of Cxcl10 at 3 and 4 d.p.i. in lungs of non-pregnant (n = 25 at 3 d.p.i., n = 35 at 4 d.p.i.) and pregnant (n = 17, n = 25) infected mice, quantified by qRT-PCR.

(F) Relative mRNA expression of Cxcl9 in lungs of non-pregnant (n = 15 at 3 d.p.i., n = 36 at 4 d.p.i.) and pregnant (n = 9, n = 25) infected mice.

(G) Experimental setup of adoptive transfer assay of $CD90.1^+$ $CD8^+$ T cells from infected non-pregnant donor mice into non-pregnant (n = 12) and pregnant (n = 19) infected $CD90.2^+$ recipient mice.

(H and I) Frequency of $CD90.1^+$ $CXCR3^+$ T cells among $CD90.1^+$ $CD8^+$ T cell subsets (H), as assessed by flow cytometry 18 hr post transfer (I).

(J) Assessment of H3K27me3 histone modification in lung tissue of pregnant (n = 5) and non-pregnant (n = 5) infected mice. In vivo ChIP RT-PCR was performed in mouse lung cells 3 days post-infection. y axis represents the percentage of recovered material relative to the starting material. *Msi1*, *ACHE*, and *Hoxc13* were used as positive controls and *Gapdh* and *Atp8b5* as negative controls.

For (A)–(C), (E), (F), (H), and (J), mean values ± SEM are shown, and the statistical significance was calculated using Mann-Whitney U test (*$p < 0.05$, **$p < 0.01$).

pregnant and non-pregnant infected mice (Figure 3J), nor in $CD45^{neg}$ lung stroma cells (Figure S2).

Enhanced Frequency of pH1N1 Virus-Specific and Effector $CD8^+$ T Cells, but Reduced Capacity for Virus Cell Lysis in Pregnant Infected Mice

We next tested the local frequency of $CD8^+$ T cells in the lung upon infection and observed significant increased frequencies of $CD44^+CD62L^{neg}$ $CD8^+$ T_{eff} cells at 6 d.p.i. (Figures 4A and 4B) and virus-specific $CD8^+$ T cells (identified by MHC class I dextramer staining) in lungs of pregnant mice at 6 and 8 d.p.i. (Figures 4C and 4D). We then assessed the effectiveness of the T cell response in an in vivo killing assay by adoptively transferring virus-peptide loaded $CFSE^{high}$ and control $CSFE^{low}$ splenocytes into infected non-pregnant and pregnant mice and respective controls

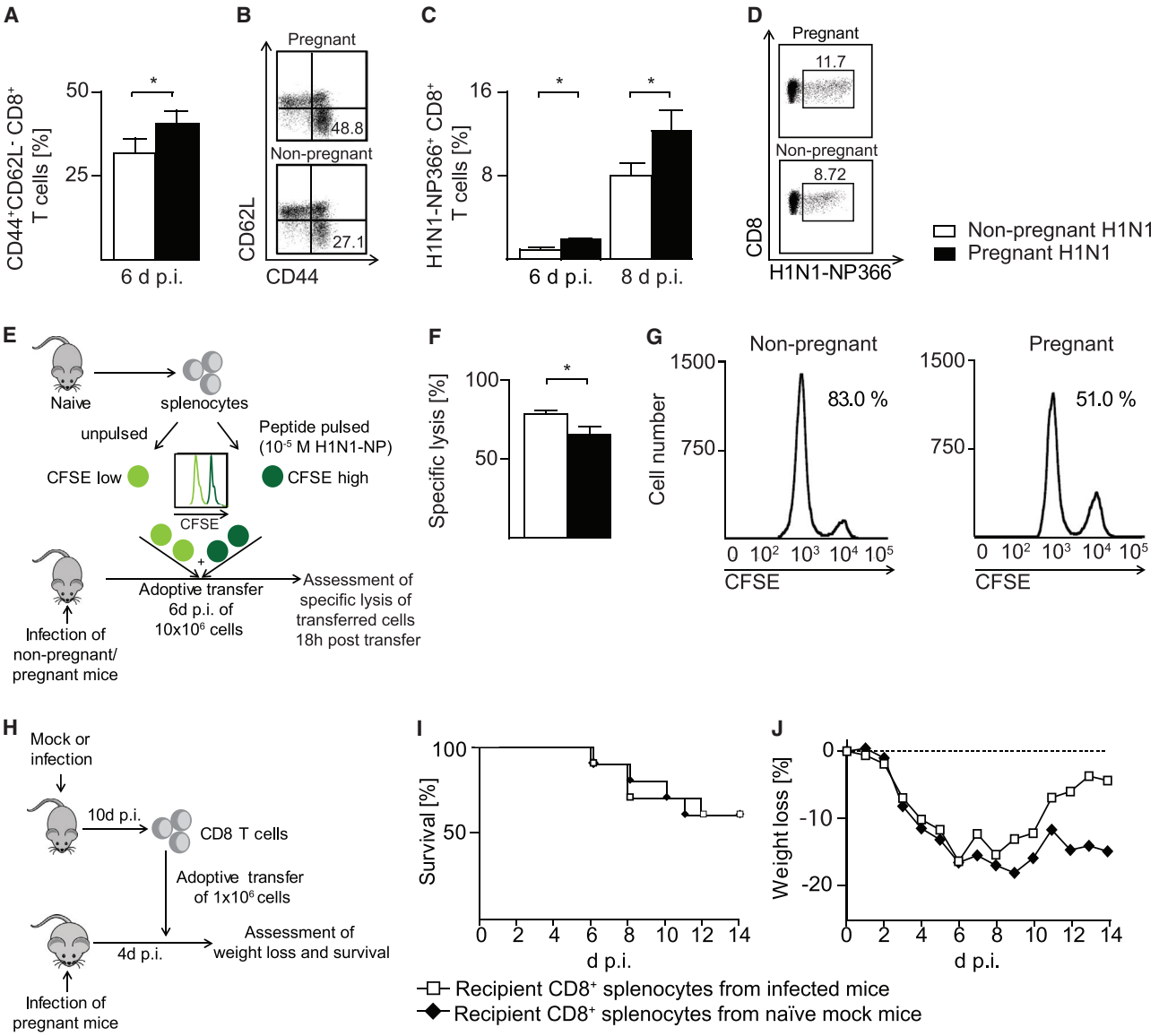


Figure 4. Enhanced Frequency of pH1N1 Virus-Specific and Effector CD8$^+$ T Cells, but Reduced Capacity for Virus Cell Lysis in Pregnant Infected Mice

(A) Frequency of CD44$^+$CD62L$^-$ cells among CD8$^+$ T cells at 6 d.p.i. in lungs of non-pregnant and pregnant infected mice.

(B) Representative dot plots of CD44$^+$CD62L$^-$ cells among CD8$^+$ T cells.

(C) Frequency of MHC dextramer$^+$ (H1N1-NP366$^+$) among CD8$^+$ T cells in lungs of non-pregnant and pregnant infected mice at 6 and 8 d.p.i.

(D) Representative dot plots of MHC dextramer$^+$ cells and CD8$^+$ T cells in lungs of non-pregnant (n = 9) and pregnant (n = 8) mice infected with 10^3 PFU of pH1N1.

(E) In vivo killing assay of virus peptide loaded cells was performed in order to determine the specific lysis of virus-peptide-loaded transferred cells. Control splenocytes were CFSE^{low} (left peak) and virus-peptide-loaded splenocytes were CFSE^{high} (right peak). Specific lysis was determined by the presence of CFSE^{high} cells in the spleen of infected mice 18 hr post transfer of CFSE^{low} and CFSE^{high} cells into the respective hosts.

(F) Specific lysis of CFSE^{high} cells in infected non-pregnant (n = 6) and pregnant (n = 8) mice.

(G) Representative histograms of data in (F).

(H) CD8$^+$ T cells harvested at 10 d.p.i. from the spleens of naive or infected non-pregnant mice were adoptively transferred (1×10^6) into pregnant mice 4 d.p.i. with 10^3 PFU of pH1N1 (n = 10).

(I and J) Survival (I) and weight loss (J) were monitored for 14 d.p.i.

Data shown in (A), (C), and (F) are presented as mean ± SEM. The statistical significance was calculated by Student's t test (*$p < 0.05$).

(Figure 4E). We observed a significantly lower specific lysis of virus-peptide loaded CSFE^{high} cells in pregnant infected animals compared to non-pregnant infected mice (Figures 4F and 4G).

Adoptive Transfer of CD8$^+$ T Cells to Infected, Pregnant Mice Improves Not Survival, but Recovery

We also tested whether adoptive transfer of virus-specific CD8$^+$ T cells from infected non-pregnant donors would improve the

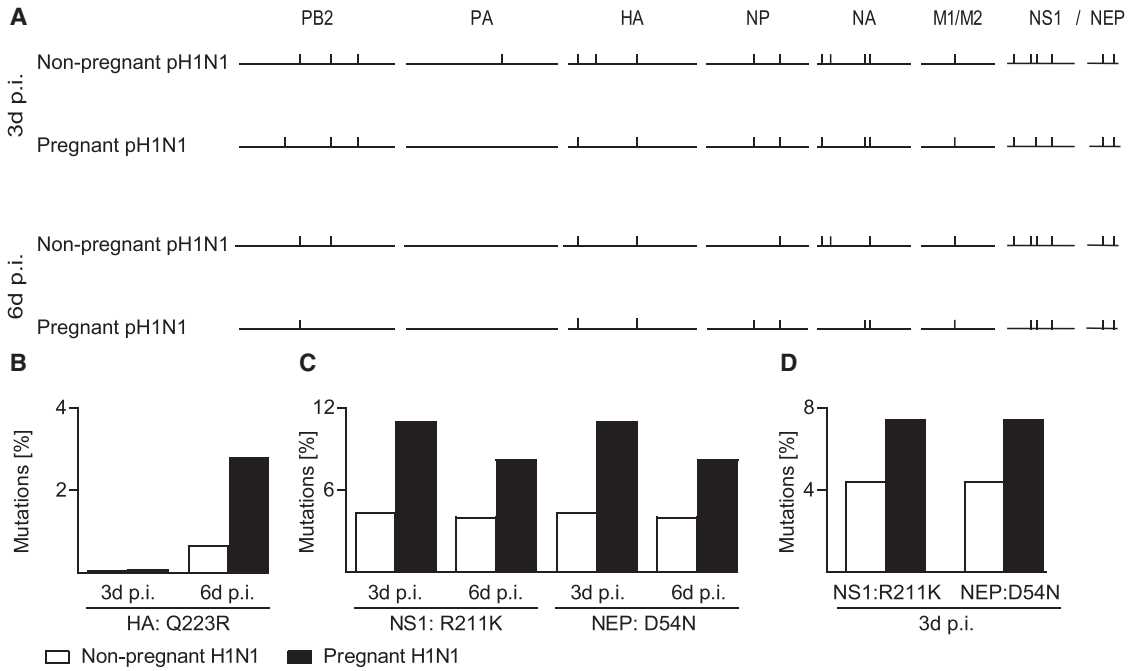


Figure 5. pH1N1 Viral Mutation Frequencies during Pregnancy

(A) Viral RNA in the lung homogenates was compared to the parental strain by high-throughput sequencing (RNA-seq). Non-synonymous mutations of pH1N1 virus occurring in the eight RNA segments are represented by vertical lines.

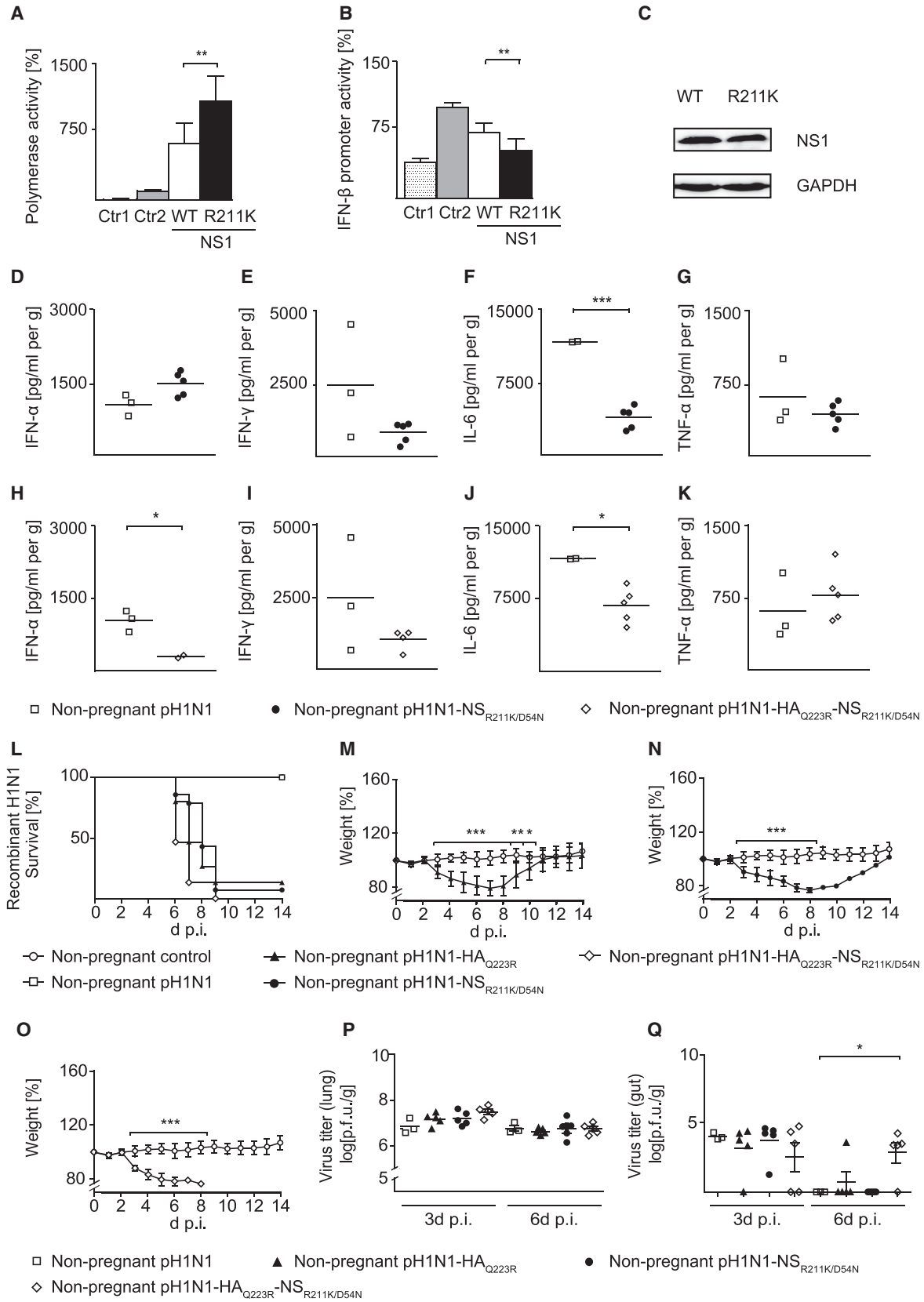
(B and C) Non-synonymous mutations primarily affected the HA (B) and the two distinct NS proteins (NS1, NEP) (C) of the virus, reflected by high frequencies.

(D) Frequency of the non-synonymous mutation, which occurred in the viral NS proteins (NS1, NEP) in an independent 2009 pH1N1 infection experiment in pregnant and non-pregnant mice.

survival of pH1N1 infected pregnant animals (Figure 4H). Here, survival rates did not differ between pregnant infected dams receiving virus-specific $CD8^+$ T cells or naive (mock) $CD8^+$ T cells (Figure 4I). However, the recovery from influenza virus infection improved upon virus-specific $CD8^+$ T cell transfer in the non-moribund pregnant mice (Figure 4J).

The Less Stringent Selective Pressure in the Pregnant Host Facilitates the Emergence of Viral Quasi Species that Mediate Reduced Cytokine Response and Increased Virulence

The results described so far strongly support the concept that a series of events involved in the maternal immune response fail to mount immunity and clear pH1N1 influenza virus infection during pregnancy. In order to assess whether this failure might give rise to the emergence of novel virus variants since the influenza virus RNA-dependent RNA polymerase (RdRp) lacks proofreading activity (Gabriel and Fodor, 2014), we sequenced the entire viral RNA genome obtained from infected lungs of pregnant and non-pregnant mice and could identify various synonymous and non-synonymous mutations in each of the eight viral RNA segments (Figure 5A, data not shown). In pregnant mice, the sequences for hemagglutinin (HA) and the non-structural (NS) gene-encoded proteins, NS1 and nuclear export protein (NEP), were affected (Figures 5B and 5C). In order to assess whether the detected high-frequency mutations in the NS and HA genes represent a one-time event or whether they might have biological relevance, we repeated this experiment and sequenced again the entire virus genome obtained from lungs of infected pregnant and non-pregnant mice. Repeatedly, we could detect the NS mutation as the most frequent mutation in pregnant compared to non-pregnant mice (Figure 5D). The HA mutation was not detected in the biological replicate experiment, particularly highlighting the importance of the NS mutation during pregnancy. Convergent evolution, namely independent evolution of similar features, is a strong parameter regarding the biological relevance particularly of the NS mutation in pregnancy. Therefore, we focused on the role of the NS mutation on protein function, since NS1 is a key viral determinant of type I IFN antagonism and pathogenicity (Ayllon and García-Sastre, 2015). The NS1 R211K mutation resulted in increased viral polymerase activity (Figure 6A) and repressed IFN-β induction in human cells (Figures 6B and 6C). Consistently, cytokine expression was generally reduced in the lungs of mice infected with NS recombinant virus harboring the NS1 R211K mutation except for IFN-α (Figures 6D–6G). Most significantly, IL-6 levels were reduced at 6 d.p.i., compared to mice infected with the parental WT strain (Figure 6F). Combination of the NS mutation with HA Q223R further revealed reduced cytokine expression at 6 d.p.i. (Figures 6H–6K), particularly of IFN-α and IL-6 (Figures 6H and 6J). Next, we analyzed the biological function of the HA Q223R mutation and could show that it mediates increased binding to α2,3-linked sialic acids, which act as attachment sites for influenza viruses predominantly expressed in the lower respiratory tract (Table S2A). Then, we assessed whether these high-frequency mutations that occurred during pregnancy alter viral pathogenicity. Therefore, we infected mice with recombinant influenza viruses harboring either the HA or NS mutation or both combined.



(legend on next page)

Strikingly, we observed an increased mortality and morbidity of these high-frequency pregnancy mediated mutant viruses even in non-pregnant mice compared to the parental pH1N1 virus, which was highest when both mutations encoded by HA and NS genes were combined (Figures 6L–6O and S3, and Table S2B). The increased pathogenicity observed upon combination of NS and HA mutations did not result in altered virus titers in the lungs of infected mice, but in extra-pulmonary organs (Figures 6P and 6Q), such as the gut, often observed in pathogenic 2009 pH1N1 infections in mice (Otte and Gabriel, 2011; Otte et al., 2011). However, the single NS mutation was sufficient to mediate 100% lethality in pregnant mice (Figure S3). Finally, we could confirm that the NS1 R211K mutation, which evolved independently to elevated frequencies during pregnancy, was not present in influenza virus strains, which circulated before the pandemic occurrence in the human population (Table S2C).

DISCUSSION

We provide here strong evidence for the multi-faceted failure of the immune system during pregnancy to mount an innate and adaptive immune response against 2009 pH1N1 influenza virus infection. Compared to the sub-lethality in non-pregnant mice, pH1N1-infected pregnant mice showed an increased mortality and morbidity. Moreover, we identified that syngenic pregnant C57BL/6 females are less affected by influenza than allogenic pregnant mice. Thus, we propose that our model using allogenic pregnant C57BL/6 mice is the most suitable translational model available to date to understand how the increased pathogenicity of influenza-related mortality and morbidity is operational.

Our findings significantly amend existing models in which lethal doses of pH1N1 were used in syngenic pregnant BALB/c females (Chan et al., 2010; Marcelin et al., 2011; Kim et al., 2012) or in C57BL/6 mice where pregnancy-like elevated estrogen was experimentally induced (Pazos et al., 2012b). Partly contradictory to our findings, increased levels of inflammatory cytokines have been described in some of these studies (Marcelin et al., 2011). This ambiguity may be explained by the different experimental approaches, e.g., the sub-lethal viral doses we used opposed to lethal doses used by the other groups, the different mating combinations (syn- versus allogenic pregnancies), as well as use of different genetic backgrounds (C57BL/6 versus BALB/c) (Chan et al., 2010; Marcelin et al., 2011; Kim et al., 2012), all of which considerably influence 2009 pH1N1 disease severity and related immune response (Otte and Gabriel, 2011).

We also show that in infected pregnant mice, the production of anti-viral and inflammatory cytokines such as type I IFNs is reduced, accompanied by the failure to activate the innate immune response, e.g., via upregulation of costimulatory marker on alvMΦ and DCs. The latter is partly restored in pregnant $Pgr^{flox}CD11c^{cre}$ mice, suggesting that progesterone is involved in suppressing the activation of DCs during pregnancy. Taken together, the inadequate first line of defense against influenza during pregnancy may—at least in part—account for an increased viral load and the failure to present antigens to $CD4^+$ and to $CD8^+$ T cells during the early phase of infection (Legge and Braciale, 2003).

Antigen presentation, e.g., by activated DC, initiates the generation of virus-specific $CD8^+$ T cells (Belz et al., 2001), which then resolve the infection by clearing the virus, along with viral-specific antibodies (Yap et al., 1978; Lambrecht and Hammad, 2012). In the present study, we were surprised to detect increased frequencies of virus-specific and $CD8^+$ T_{eff} cells at 6 d.p.i., while leukocyte and $CD8^+$ T cell numbers were significantly lower in pregnant mice at 3 d.p.i. Subsequent functional assays unveiled a reduced ability of $CD8^+$ T cells to lyse virus peptide-presenting cells. Considering the reduced function of $CD8^+$ T cells present in pregnant mice to lyse virus-loaded cells, one might argue that the host's immune system was continuously aiming to generate an effective $CD8^+$ T cell response against the steadily replicating virus without any success, which would explain the increased $CD8^+$ T cell frequencies. Very similar observations have been made in chronic viral infections such as HIV, where infection results in expansion of dysfunctional T cells (Trautmann et al., 2006).

Further, we detected a reduced expression of the chemokine *Cxcl10* in the lung of pregnant infected mice compared to non-pregnant infected mice. An epigenetic silencing of genes coding for CXCR3 ligands, including *Cxcl9 and Cxcl10*, has recently been shown to promote fetal tolerance by restricting the migration of anti-fetal effector T cells into the uterus (Nancy et al., 2012). Since we could not confirm increased H3K27me3 histone methylation of *Cxcl9* and *Cxcl10* in the lungs of pregnant mice, we

Figure 6. In Vitro and In Vivo Characterization of the Viral High-Frequency Variants that Evolved during Pregnancy

(A) The polymerase activity of pH1N1 was analyzed in H1299 cells co-expressing NS1 WT or NS1 R211K (each 2 μg). Activity of viral ribonucleoproteins in cells transfected with pcDNA3.1 empty vector as positive control (Ctr2) was set to 100%. As a second control, cells were transfected with vRNPs omitting the PB2 subunit (Ctr1). Data shown represent mean ± SD of three independent experiments performed in triplicate.

(B) The IFN-β promoter activity in HEK293T transfected with NS1 WT or NS1 R211K (each 2 μg) and infected with A/WSN/33 virus (MOI 1). Activity of IFN-β promoter in cells transfected with reporter constructs and empty vector as positive control (Ctr2) was set to 100%. As a negative control, cells transfected with reporter constructs were mock infected (Ctr1). Data shown represent mean ± SD of three independent experiments performed in triplicate.

(C) Confirmation of equal NS1expression in H1299 cells by western blot analysis. GAPDH was used as a loading control.

(D–G) Cytokines (IFN-α [D], IFN-γ [E], IL-6 [F], and TNF-α [G]) determined by Luminex assay in lungs of non-pregnant mice infected with 10^3 PFU of pH1N1 WT (open square, n = 11) or pH1N1-$NS_{R211K/D54N}$ mutant (black circle, n = 11) virus, respectively, and harvested on day 6 p.i.

(H–K) Cytokines (IFN-α [H], IFN-γ [I] IL-6 [J], and TNF-α [K]) determined by luminex assay in lungs of non-pregnant mice infected with 10^3 PFU of pH1N1 WT (open square, n = 3) or pH1N1-HA_{Q223R}-$NS_{R211K/D54N}$ mutant (open diamond, n = 5) virus, respectively, and harvested on day 6 p.i. If fewer measurement points than the indicated n are visible in the graphs, those not shown were out of range (i.e., below the range of the standard curve).

(L–O) Survival (L) and weight loss (M–O) in non-pregnant mice infected with 10^3 PFU of recombinant pH1N1 quasi species (pH1N1-HA_{Q223R}, n = 15; pH1N1-$NS_{R211K/D54N}$, n = 14; pH1N1- HA_{Q223R}+$NS_{R211K/D54N}$, n = 15), or WT pH1N1 control virus (n = 14), monitored for 14 d.p.i. in infected animals and uninfected controls.

(P and Q) Virus titers of pH1N1 (n = 3) and recombinant viruses (pH1N1-HA_{Q223R}, n = 5; pH1N1-$NS_{R211K/D54N}$, n = 5; pH1N1- HA_{Q223R}+$NS_{R211K/D54N}$, n = 5) were determined in lung (P) and gut (Q) of non-pregnant mice at 3 and 6 d.p.i.

(D)–(K) and (M)–(Q) are presented as mean ± SD. Statistical significance was calculated by Student's t test (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$).

propose that the reduced *Cxcl9* and *Cxcl10* mRNA expression and reduced migration of adoptively transferred $CD8^+$ T cells to the lung in pregnant mice is —opposed to the observations in the pregnant uterus—not mediated via epigenetic modification of H3K27me3. Moreover, since we observed an improved recovery in pregnant, infected recipients of $CD8^+$ T cells harvested from infected mice compared to naive mice, one might argue that these adoptively transferred cells may have promoted viral clearance in the periphery, at least in the non-moribund mice.

We could also show that the presence of anti-viral antibodies prior to pregnancy significantly improved survival and morbidity of an influenza infection during a subsequent pregnancy, accompanied by low viral titers and the redundancy of mounting an anti-viral immune response (Figure S4). These observations underpin the importance of vaccination against influenza especially for women during their reproductive years. Alternative approaches to enhance or restore the defective immune responses during gestational influenza infection are rather limited due to the potential teratogenicity. However, therapeutic avenues pursued to treat influenza infected pregnant women during the 2009 pandemic include the use of neuraminidase inhibitors, which reduce virus load. Published evidence from Japan confirms that such intervention may indeed improve maternal survival, while teratogenic effects were not observed (Saito et al., 2013).

Influenza viruses possess an error-prone RdRp that can be affected in an environment where the selective pressure is less stringent, as shown here in the lung of pregnant mice. Indeed, we detected mutations especially in the HA and NS genes in pregnant compared to non-pregnant mice. The HA Q223R mutation, which increases pathogenicity in mice, mediates enhanced binding to α2,3-linked sialic acid receptors that are predominantly expressed in the human lower respiratory tract. In patients, infections with influenza viruses that prefer attachment to receptors of the lung are associated with pneumonia and death (van Riel et al., 2006; Shinya et al., 2006).

The NS1 R211K mutation reduces IFN-β promoter activity in vitro and in vivo in the murine lung where it leads particularly to low IL-6 levels, a known marker for disease severity in 2009 pH1N1 infected patients, further validating the clinical relevance of the murine pregnancy model used in this study (Paquette et al., 2012). Moreover, the reduction of the cytokine levels upon infection with NS and/or HA recombinant viruses on day 6 p.i., but not on day 3 p.i. (Figure S3), further highlights the findings of the innate response transcriptome analysis. There, a general reduction of innate gene transcription was particularly observed on day 6 p.i., 3 days after the occurrence of the viral escape variants in the lungs of infected pregnant mice. This most likely represents the consequence of increased replicative fitness of the escape variants due to suppression of key anti-viral cytokine responses. Consistently, the NS1 R211K mutation increases viral polymerase activity in vitro as well as in vivo, most significantly in combination with the HA Q223R mutation. Strikingly, introduction of these two NS and HA mutations into the pH1N1 virus backbone, either alone or in combination, resulted in 100% lethality in non-pregnant mice compared to the parental pH1N1 strain. Moreover, introduction of the NS mutation alone was sufficient to significantly increase lethality in pregnant mice.

In order to confirm that these high-frequency mutations that occur during pregnancy might be of general relevance, we sequenced lungs of pregnant and non-pregnant mice in an independent 2009 pH1N1 infection experiment. There, the NS gene mutation could be identified independently in higher frequency in allogenic pregnant mice compared to non-pregnant mice, suggesting convergent evolution and thus biological relevance of the escape variant during pregnancy. The fact that the NS R211K position is a very low-frequency position in influenza virus strains circulating in the human population, but its occurrence increases in 2009 pH1N1 infected pregnant patients (Gíria et al., 2012), further supports the concept that the NS1 R211K mutation contributes to disease severity during pregnancy. Thus, the less stringent selective pressure in the pregnant host seems to facilitate the emergence of viral quasi species that mediate increased virulence particularly due to the acquisition of mutations in the NS1 gene that additionally antagonize interferon response and fuel viral replication, inflammation, and virulence during pregnancy.

In conclusion, we provide here evidence on how the allogenic pregnant host fails to mount an appropriate response against pH1N1 influenza virus infection particularly due to the emergence of more virulent pH1N1 virus variants. Future studies in humans should aim at confirming the translational and clinical relevance of our observations in mice. The enhanced pregnancy-related emergence of virulent virus variants in mice could point toward an increased risk for a severe infection of, e.g., family members if a pregnant woman is infected. Also, studies addressing the risk for virus transmission to other immunocompetent hosts will be required.

Moreover, we propose that the improved understanding on how the maternal immune system fails to mount an appropriate response to influenza virus infection will increase the communities' alertness and result in an improved vaccination compliance of women during their reproductive years. This is particularly important since influenza vaccination before or during pregnancy is of benefit to mother and child (Håberg et al., 2013; Rastogi et al., 2007). Thus, in the aftermath of the 2009 influenza pandemic, the World Health Organization Strategic Group of Experts recommends that pregnant women have highest priority in influenza vaccination programs (WHO, 2010). Yet, poor vaccination compliance among women during their reproductive years is an impending clinical problem (Blanchard-Rohner et al., 2012). Lastly, our mouse model presented here also offers the opportunity to be used as a screening system, which allows assessing the pathogenic potential of future circulating influenza A virus variants for pregnant individuals.

EXPERIMENTAL PROCEDURES

Animal Experiments

8- to 10-week-old female C57BL/6J or $Pgr^{flox}CD11c^{cre}$ mice were mated with male BALB/c or C57BL/6J mice. Using standardized protocols, non-pregnant and pregnant females, the latter on gestation day (gd) 12.5, were infected with 10^1–10^5 PFU of the 2009 pH1N1 virus strain A/Hamburg/NY1580/09, the 2006 sH1N1 virus strain A/*Solomon* Islands/3/2006-like, or the recombinant pH1N1 viruses on A/Hamburg/NY1580/09 background (pH1N1-HA_{Q223R}, pH1N1-$NS_{R211K/D54N}$, or pH1N1-HA_{Q223R}+$NS_{R211K/D54N}$) (more details in Supplemental Experimental Procedures). Mice receiving PBS were used as control group. Non-pregnant C57BL/6 mice were subcutaneously (s.c.) injected with 1.25 mg dydrogesterone every second day: 12 days before and 14 days after infection with 10^3 PFU of 2009 pH1N1. Some of these mice were monitored for weight loss and signs of disease until 14 d.p.i. Additional groups of mice were sacrificed on 3 and 6 d.p.i. for organ harvest. All animal experiments were

performed according to the guidelines of animal protection law and the approved protocols by the relevant German authority (Behörde für Gesundheit und Verbraucherschutz Hamburg, approval number G124/12 and G53/16).

Immunohistochemistry

Lungs were paraffin embedded and deparaffinized before H&E staining. Viral antigens were detected with an antibody against the viral nucleoprotein (NP) (more details in Supplemental Experimental Procedures).

Detection of Virus Titers and Cytokines

Supernatants of homogenized lungs from infected pregnant and non-pregnant mice were used to assess virus titers as described before (Otte et al., 2011) (more details in Supplemental Experimental Procedures) and cytokine measurements (IFN-α, IFN-γ, IL-6, and TNF-α) were performed using the ProcartaPlex Multiplex Immunoassay Mix&Match Mouse 4-plex (Affymetrix/eBioscience) with magnetic beads following the manufacturer's instructions (more details in Supplemental Experimental Procedures).

In Vitro Assays

Polymerase and IFN-β promoter activity of plasmids encoding for NS1 WT or R211K mutation were performed using the Dual-Luciferase Reporter Assay System (Promega) according to the manufacturer's instructions (Supplemental Experimental Procedures).

Western blot analysis was used to confirm the expression levels of HH15-NS1 (WT or R211K) in H1299 cells (more details in Supplemental Experimental Procedures).

Flow Cytometry Analyses

Mice were perfused via the right ventricle using PBS. Single-cell solutions were prepared of lung, spleen, and paraaortic lymph nodes followed by surface and intracellular staining using our standardized protocol (Solano et al., 2015) (more details in Supplemental Experimental Procedures).

In Vivo Killing Assay of Virus Peptide Loaded Cells

Splenocytes from naive mice were loaded with virus-specific peptide ASNENVETM (JPT) and subsequently labeled with 5 mM carboxyfluorescein succinimidyl ester (CFSE; LifeTechnologies) ($CFSE^{high}$); control cells were cultured without the peptide and labeled with 0.5 mM CFSE ($CFSE^{low}$). 10×10^6 $CFSE^{high}$ and $CFSE^{low}$ cells (1:1) were injected into the tail vein of pH1N1 infected non-pregnant and pregnant mice at 6 d.p.i. and in naive mice as controls. Specific killing of virus peptide loaded cells was determined at 18 hr post transfer in the spleen. More details are provided in the Supplemental Experimental Procedures.

Adoptive Transfer of Virus-Specific $CD8^+$ T Cells

$CD8^+$ T cells were purified from spleens of either naive or infected non-pregnant mice isolated at 10 d.p.i. using the Magnetic Cell Isolation and Cell Separation (MACS) $CD8a^+$ T cell kit (Miltenyi Biotec). Purity of isolated $CD8a^+$ T cells was controlled by flow cytometric analyses, and 1×10^6 $CD8^+$ T cells from infected or naive non-pregnant mice were adoptively transferred into the tail vein of pregnant infected mice at 4 d.p.i. Survival and weight loss were monitored for the subsequent 10 days.

Adoptive Transfer of $CD8^+$ T Cells from $CD90.1^+$ Donor Mice into $CD90.2^+$ Recipient Mice and Assessment of Leukocyte Homing

T cells were isolated from infected congenic $CD90.1^+$ donor mice at 8 d.p.i. from the spleen using the Pan T cell isolation kit II (Miltenyi Biotec) and MACS isolation. Purity of isolated T cells was confirmed by flow cytometric analysis. 2×10^6 purified T cells were administered into the tail vein of non-pregnant and pregnant infected wild-type $CD90.2^+$ recipient mice at 3 d.p.i. 18 hr after the adoptive transfer, mice were sacrificed and lymphocyte populations were analyzed in the lung using flow cytometric analysis.

ACCESSION NUMBERS

The accession number for the sequence data of all samples reported in this paper is European Nucleotide Archive (ENA): PRJEB12200.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, four figures, and two tables and can be found with this article online at http://dx.doi.org/10.1016/j.chom.2017.02.020.

AUTHOR CONTRIBUTIONS

Conceptualization, G.G. (virology) and P.C.A. (immunology); Methodology, G.G., P.C.A., K.K., H.-W.M.; Investigation, G.E., A.M.H., J.H., R.T., S.T., S.B., C.D., P.R.-I., H.J., D.I., S.S., V.S., D.M; Resources, K.T., N.F., and F.J.; Formal Analysis, M.A. and A.G.; Writing – Original Draft, G.G. and P.C.A.; Writing – Review & Editing, G.G. and P.C.A.; Funding Acquisition, G.G., H.-W.M., G.E., and P.C.A.; Joint Senior Supervision, G.G. and P.C.A.

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