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Influence of influenza A virus infections on the maternal immune system during pregnancy

Dissertation

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Table of contents

Table of c	ontents	.111
1 Introd	uction	1
1.1 Inf	luenza A virus (IAV) infection	1
1.1.1	Epidemiology	2
1.1.2	Influenza A virus infection during pregnancy	3
1.1.3	IAV vaccination during pregnancy	4
1.2 lm	mune response against IAV	5
1.2.1	Innate immune response to IAV	5
1.2.2	Adaptive immune response to IAV	7
1.3 Th	e immune system during pregnancy	8
1.3.1	Innate immune adaptation during pregnancy	8
1.3.2	Adaptive immune adaptation during pregnancy	9
1.3.3	Endocrine modulation of the immune system during pregnancy	9
1.4 Im	mune response against IAV during pregnancy	12
1.5 Ai	m of the thesis	14
2 Materi	al and Methods	15
2.1 Ma	iterial	15
2.1.1	Chemicals	15
2.1.2	Plastic material	17
2.1.3	Kits	17
2.1.4	Buffers and Solutions	18
2.1.5	Viral stocks	18
2.1.6	Instruments	19
2.1.7	Antibodies	19
2.1.8	Software	21
2.1.9	Primers	21
2.1.10	Statistics	23
2.2 Me	ethods	24
2.2.1	Virological assays	24
2.2.2	Animal experiments	26
2.2.3	Cell biology	30
2.2.4	Molecular biology	35
2.2.5	Histology	37

3	Re	esult	s39
	3.1	Es	tablishment of an allogenic pregnancy mouse model to study influenza A
	viru	is inf	ections
	3.2	Inv	restigation of the innate immune response in allogenically pregnant
	influ	uenz	a infected mice41
	3.	.2.1	Pregnant mice exhibit reduced pro-inflammatory cytokine production upon
	in	fluen	za virus infection41
	3.	.2.2	Impaired activation of antigen-presenting cells in pregnant IAV infected mice42
	3.3	Inv	restigation of the adaptive immune response in allogenically pregnant
	influ	uenz	a infected mice45
	3.	.3.1	Impaired migration of $CD8^+$ T cells to the lung in pregnant IAV infected mice45
	3.	.3.2	Comparable T cell proliferation in pregnant and non-pregnant IAV infected mice
	3.	.3.3	Increased frequencies of CD8 ⁺ T cells expressing pro-inflammatory cytokines in
	lu	ngs	of pregnant infected dams50
	3.	.3.4	Increased frequencies of T cells expressing the anti-inflammatory cytokine IL-10
	in	preg	nant infected dams
	3.	.3.5	Preexisting acquired immunity rescues pregnant mice from lethal IAV infection.54
	3.4	Inv	restigation of the effect of progesterone on the immune response towards
	IAV		
	3.	.4.1	Administration of a progesterone derivative to non-pregnant mice increased IAV-
	re	lated	l lethality in mice
	3.	.4.2	Progesterone receptor deficiency in CD11c ⁺ cells partly rescues pregnant mice
	fro	om l/	AV-related lethality
	3.5	Inf	luence of progesterone on T cells <i>in vitro</i> 61
	3.	.5.1	Absent effect of progesterone on antigen-dependent T cell activation61
	3.	5.2	Progesterone and glucocorticoids induce cell death in T cells isolated from
	pr	regna	ant and non-pregnant mice63
	3.	.5.3	Progesterone induces T cell death via binding to the glucocorticoid receptor67
	3.	.5.4	Comparable effect of progesterone on female and male T cells69
4	Di	scus	ssion71
	4.1	Sy	ngenic versus allogenic pregnancy mouse models73
	4.2	Th	e immune system between adaptation and virus elimination74
	4.3	Re	levance for pregnant women76
	4.4	Inf	luence of progesterone on IAV-related mortality77
	4.5	Inf	luence of progesterone on the immune system78

	4.6	Scientific significance and outlook	82
5	Sur	mmary	85
6	Zus	sammenfassung	86
7	Abl	breviations	vı
8	Bib	bliography	x
9	Lis	t of figures and tables	XXI
	9.1	List of figures	XXI
	9.2	List of Tables	XXII
10	A	cknowledgement	XXIII
11	C	urriculum Vitae	xxıv
12	A	ppendix	XXVIII
	12.1	Copyright	XXVIII
	12.2	Manuscript	xxxix
13	A	ffidavit	LXXIV

1 Introduction

Influenza A virus (IAV) infections lead to a highly contagious pulmonary disease that poses a major threat for public health all over the world. IAVs cause three to five million infections and 250,000 to 500,000 deaths annually worldwide. Especially, high risk groups like young children (< 5 years), elderly humans (> 65 years) and people with co-morbidities (asthma, chronic lung or heart disease) are at increased risk to develop severe illness after IAV infection (1). During the past years and especially during the IAV pandemic in 2009, it was demonstrated that also pregnant women suffer from a significantly increased risk to develop severe complications of IAV infections, like pneumonia or preterm delivery and even increased lethality (2). However, very little is known about the underlying molecular mechanisms leading to elevated risk in pregnant women. In this thesis, it is aimed to elucidate mechanisms of immunological adaptation during pregnancy that aggravate the course of IAV infection.

1.1 Influenza A virus (IAV) infection

Influenza viruses belong to the family of *Orthomyxoviridae* containing a negative-sense single-stranded segmented RNA genome (8 segments). They can be classified in influenza A, B, C and newly discovered D genera. Viruses of Type A and B are able to infect humans and cause worldwide disease outbreaks, whereas infections with Type C viruses are less common and so far locally restricted. Type A influenza viruses exhibit a great genetic diversity and can further be subdivided based on the expression of their surface glycoproteins hemagglutinin (HA) and neuraminidase (NA) (Figure 1). So far, 18 hemagglutinin and 11 neuraminidase subtypes are identified, whereas in humans, only HA subtypes H1-3 and NA subtypes N1-2 have caused worldwide epidemics and pandemics. Infections with avian IAV e.g. H5N1 and H7N9 have currently solely caused local outbreaks. HA mediates binding to the host cell via sialic acids. In human upper respiratory tract predominantly α 2,6-linked sialic acids are more common. Hence, most human adapted IAV HA bind preferentially to α 2,6-linked sialic acids. NA exhibits sialidase activity and after replication, facilitates virion release from cell membrane (3, 4).



Figure 1: Schematic model of an influenza A virus particle. Influenza A viruses consist of an 8-segmented viral genome that is associated with nucleoproteins (NP, red) and the viral polymerase (containing the acidic polymerase protein PA and the basic polymerase proteins PB1 and PB2, purple). The hemagglutinin (HA, blue) and neuraminidase (NA, orange) surface proteins are integrated in the virus membrane. Modified from (5).

1.1.1 Epidemiology

IAV is able to infect a variety of species. However, the natural reservoir of IAV are aquatic birds. The infection of other species like mammals is facilitated by a high mutation rate of the IAV. The RNA-dependent RNA polymerase lacks proofreading activity leading to an accumulation of point mutations. Point mutations mainly in the immunological dominant HA and NA proteins (antigenic drift) (6) can result in viral persistence in the population and reinfection of the host due to avoiding recognition by neutralizing antibodies (7). Moreover, the segmented viral genome allows reassortment of viral segments deriving from different viral classes when infecting the same host (antigenic shift). Pigs are thought to act as a so-called "mixing vessel" due to their ability to be infected with different avian and mammalian IAV strains. Reassortant viruses with substantial antigenic shift can cause pandemics with high lethality to an immunologically naïve population (8).

Epidemic IAV infection in humans is a seasonal occurring, acute respiratory infectious disease affecting people of all age groups. IAV circulates all around the world with peaks in wintertime for temperate climates and less determined peak seasons for tropical and subtropical climates. The highly contagious disease spreads rapidly from person to person by sneezing and coughing. IAV induces a sudden onset of typical symptoms such as cough, high fever, severe malaise (feeling unwell), headache, joint and muscle pain as well as pharyngitis and rhinitis. In severe cases IAV infection can lead to pneumonia and rarely to myocarditis. The incubation time is around 48 h. Most people recover within 1-2 weeks from the infection without seeking medical care. However, in severe cases IAV infection leads to

complications and even death. Especially among risk group patients that include young children, the elderly, immune-compromised people, patients with underlying chronic medical conditions and pregnant women IAV severity can be extremely increased. As IAV is continuously evolving and changing, infections of the same individual are possible multiple times by different IAV subclasses (1, 9).

Besides the annual occurring seasonal influenza epidemics, influenza pandemics may also occur irregularly. IAV pandemics are often mediated via antigenic shift leading to increased morbidity and mortality in humans (10, 11). In more recent times, four influenza A virus pandemics occurred:

In 1918/1919, the so-called Spanish flu caused by an H1N1 IAV; in 1957, the Asian influenza caused by an H2N2 IAV; and in 1968, the Hong Kong influenza caused by an H3N2 IAV (8). The first pandemic of the 21st century, caused by an H1N1 subtype, was initially recorded in Mexico in 2009. From there it spread within 6 months throughout the world leading to approximately 18,500 laboratory-confirmed deaths as reported by the WHO (12). Surprisingly, the mortality was highest in infected 20 – 40 year old patients. One explanation for this observation is the similarity of the 2009 H1N1 virus with the H1N1 virus from the pandemic in 1918/1919. Since the virus from 1918/1919 was circulating until the 60/70ies, older people still had some protective immunity. The pandemic 2009 IAV (2009 pH1N1) was a reassortant virus containing components of human, porcine and avian IAV (13). The 2009 pH1N1 has replaced the previously circulating H1N1 virus and is now, together with the H3N2 IAV subclass, the predominant circulating influenza virus (8, 9).

1.1.2 Influenza A virus infection during pregnancy

During the last decades, it was observed that pregnant women suffered from increased morbidity and mortality during IAV epidemics and pandemics (Figure 2A) (14). Already during the pandemic in 1918 (Spanish flu) (15) and more recently, during the pandemic in 2009, pregnant women were shown to be significantly vulnerable of influenza-related complications and death (Figure 2B) (16, 17). During the 2009 pandemic, pregnant women were more likely to develop severe disease, with a 4-fold increase in hospitalization and pneumonia development than age-matched non-pregnant women. Moreover, the risk of preterm delivery (3-fold increase) (17), stillbirth (2.4-fold increase) and low weight neonates (18) was severely increased in mothers infected with influenza. In the United States, pregnant women accounted for 5 % of all IAV-related deaths (17), whereas they represent only 1 % of the general population (16). Infection in the third trimester of pregnancy was associated with a more severe outcome compared with influenza during earlier gestation. Among pregnant

women who died during the first months of the IAV pandemic in 2009, 10 % were infected in the first, 30 % in the second and 60 % in the third trimester of pregnancy (17). Also in Germany, albeit fatality was very low, similar observations were made, with 27 % of infected pregnant women having been hospitalized and 2.6 % having developed pneumonia – in comparison to 4 % and 0.9 %, respectively, in the non-pregnant age-matched population (19).

The underlying molecular mechanisms leading to the increased disease susceptibility in pregnant women are largely unknown and will be the subject of this thesis.



Figure 2: Morbidity of pregnant women upon influenza A virus infection. (A) Estimated morbidity of pregnant women infected with the influenza A virus is shown in comparison to the general population at two pandemic years (1918, 1957) and two seasonal outbreaks (1978, 1983). (B) Estimated morbidity of pregnant women in different countries during the 2009 influenza pandemic (14).

1.1.3 IAV vaccination during pregnancy

Annual vaccination against IAV is the most effective prevention of IAV infection as medication is limited and often inefficient. Since 2012, the WHO recommends pregnant women at all trimesters to be vaccinated against influenza at first priority – before other high risk groups, including very young children, the elderly population and people with chronic cardiac or pulmonary diseases as well as immunosuppressed patients and health care workers (20). Inactivated trivalent vaccine – containing two influenza A and one influenza B virus strain – has been shown to be safe and effective during pregnancy in various studies (21-23). Moreover, maternal vaccination results in production of antibodies that are transferred via the placenta and breast milk to the baby, thereby protecting it against infections. Since no vaccine is currently licensed for infants < 6 months of age, maternal

vaccination is critical for the protection of the infant (24). Nevertheless, in Germany vaccination compliance among pregnant women is only 11 % (25). Pregnant women often consider the vaccination against IAV as not necessary and the vaccination as being a higher risk than the infection itself. Moreover, poor vaccination compliance of pregnant women in Germany can be ascribed to the low rates of physicians offering the vaccine to pregnant women (25).

1.2 Immune response against IAV

IAV infection initiates an immediate pro-inflammatory immune response in mice and humans. The host immune response to influenza viruses can be divided into the innate immunity being available within hours and the adaptive immunity evolving within days after infection.

1.2.1 Innate immune response to IAV

Upon virus entry in the host respiratory tract, IAV primarily infects respiratory epithelial cells. The alveolar epithelium is crucially involved in innate immunity against IAV (26). It produces granulocyte-macrophage colony stimulating factor (GM-CSF) in order to recruit dendritic cells (DCs) and macrophages (27). Moreover, epithelial cells secrete high amounts of type I (IFN- α and INF- β) and – especially – type III interferons (IFN- λ) which are involved in the initial immune response against IAV (28, 29). Recognition of viral RNA is mediated via at least three types of pattern recognition receptors (PRR), namely retinoic acid-inducible gene I (RIG-I), toll-like receptor (TLR) 3 and 7 and the nucleotide binding oligomerization domain (NOD)-like receptor (NLR) family pyrin domain containing 3 (NLRP3) protein (30). In respiratory epithelial cells, viral RNA is sensed predominantly by RIG-I and TLR 3, whereas TLR 7 is primarily expressed in plasmacytoid DC (31). Virus recognition results in activation of the nuclear factor kappa-light-chain-enhancer of activated B cells (NF-KB) and consequently, in the secretion of type I IFN and pro-inflammatory cytokines (32, 33). Type I IFN, produced by epithelial cells and immune cells, signal via the ubiguitously expressed IFN-α receptor (IFNAR). Thereby, type I IFN induce the expression of interferon-stimulated genes (ISGs) in neighboring - so far uninfected - cells and thus influence protein biosynthesis, cell growth and survival leading to inhibition of viral replication. Moreover, type I IFN directly induce DC maturation and activation (increased expression of costimulatory surface molecules and MHC) as well as lymphocyte and monocyte recruitment by the induction of cytokines and chemokines (32, 34). However, IAV has developed strategies to counteract the host immune system. Specifically, the non-structural protein 1 (NS1) from IAV

can inhibit type I IFN production by binding viral RNA and thus, preventing recognition by PRR (35, 36). Activation of the NLRP3 inflammasome in DCs and macrophages by IAV leads to pro-inflammatory IL-1 β and IL-18 production that contribute to leukocyte infiltration and activation in the lung (29, 37-39).

Innate immune cells, e.g. Natural Killer (NK) cells, macrophages and DCs, are crucial mediators of immediate defense against IAV. They induce killing of infected cells, secrete various pro-inflammatory cytokines and chemokines and act as professional antigenpresenting cells (APCs) to initiate adaptive immunity.

NK cells are able to kill virus-infected epithelial cells. Moreover, antibody-coated infected epithelial cells are targeted by NK cells and killed via antibody-dependent cellular cytotoxicity (ADCC) fulfilled by the release of perform and granzymes (8, 35, 40, 41).

Alveolar macrophages (alvMac) are resident in the lung and rapidly activated upon IAV infection of respiratory epithelial cells (29). They are characterized as $CD11c^{high}$ and $CD11b^{neg}$, $CD103^{neg}$ (42). alvMac phagocytose apoptotic IAV infected cells and thus, limit viral spread (43, 44). However, upon activation, alvMac produce high amounts of nitric oxide synthase 2 (NOS2) and tumor necrosis factor alpha (TNF- α). This contributes to the pro-inflammatory milieu in the lung and consequently, to virus elimination, but also to lung pathology (45-47). Moreover, by upregulation of costimulatory molecules like CD40, CD80 and CD86, alvMac are able to act as professional APCs and initiate adaptive T cell immunity (35).

After IAV infection, DCs secrete a wealth of chemokines causing recruitment of neutrophils and NK cells, but also activated and memory lymphocytes (48). During IAV infection two DC subpopulations in the lung are important - conventional DCs (cDC) and plasmacytoid DCs (pDC). cDCs are able to take up virions and apoptotic cells. This leads to the upregulation of costimulatory molecules (CD80, CD86) on cDCs and their migration from the lung to lungdraining lymph nodes where they prime naïve T cells (35). Phagocytosis of viral particles leads classically to activation of CD4⁺ T cells by presentation via MHC class II molecules. A mechanism called 'cross-presentation' enables the presentation of phagocytosed particles via MHC-I and thus, activation of CD8⁺ T cells (49) that are the most important lymphocyte population in response to IAV. Direct IAV infection of DCs is also possible, thereby activating CD8⁺ T cells by classical presentation of viral peptides via MHC-I molecules. On the other hand, plasmacytoid DCs (pDCs), characterized as CD11c^{dim}, CD11b^{neg} and CD45R⁺ (42), are major producers of IFN- α via TLR 7 activation during IAV infection (48, 50, 51). Although, they are also able to migrate to the lymph nodes, they are poor activators of naïve T cells due to low expression of costimulatory molecules (52). However, by the secretion of IFN-a pDCs activate and modulate adaptive immune responses in the lymph nodes (51, 53).

1.2.2 Adaptive immune response to IAV

The adaptive immune response builds the second line of defense against IAV and consists of the humoral and the cellular response.

The humoral immune response describes the specific antibody production against IAV surface proteins. Mostly, antibodies against HA are generated, which neutralize viral particles. They bind predominantly to the globular head domain of HA and inhibit virus attachment and entry to the cell. Moreover, via binding to Fc receptors on immune cells e.g. macrophages, these antibodies mediate phagocytosis of bound viral particles. Furthermore, they are able to initiate ADCC of infected cells by NK cells. As the globular head domain of HA, which varies substantially between IAV subclasses, is the target of most HA-specific antibodies, protection of the host via HA-specific antibodies is limited to the same IAV subclass (35, 54). However, a small proportion of HA-specific antibodies that are generated during an IAV infection are directed against the preserved stem region of HA that is highly conserved between IAV subclasses. Much effort is undertaken in current research to design vaccination strategies to generate broadly neutralizing antibodies directed against the conserved HA stem region of IAV (55).

The cellular adaptive immune response consists of CD4⁺ T helper cells (Th) and CD8⁺ cytotoxic T lymphocytes (CTL) that are activated by APCs in the lymph nodes. After activation, they subsequently migrate to the infected tissue – mostly the lung during IAV infection. CD4⁺ T helper cells are restricted to MHC class II molecules and can be divided into different subclasses. Th1 cells produce IFN- γ and IL-2 and thereby, activate various innate and adaptive immune cells. Other Th subpopulations, such as Th2 or Th17 cells, have been described but are less well studied in response to IAV. Moreover, during IAV infection regulatory CD4⁺ T cells (Treg) suppress T cell responses via multiple mechanisms, e.g. IL-10 production, and hence, reduce immune cell-mediated lung pathology and overshooting immune responses (35, 54).

CD8⁺ T cells, being restricted to MHC-I molecules, are essential in IAV clearance in the lung. They produce the pro-inflammatory cytokines IFN-γ and TNF-α that support antigen presentation by promoting upregulation of MHC molecules on APCs (35, 54). Moreover, they kill virus-infected cells via the release of perforin and granzymes (granzyme A and B) or the interaction of the Fas/FasL system (56). Additionally, CD8⁺ CTLs contribute to lung immunopathology during IAV infection (57-59). CD8⁺ T cells mainly recognize peptides derived from intracellular proteins e.g. the IAV nucleoprotein (NP). For C57BL/6J mice, CD8⁺ T cells preferentially target the peptide NP₃₆₆₋₃₇₄ in context with MHC-I (in C57BL/6J mice H-2D^b) (60). The IAV NP₃₆₆₋₃₇₄ peptide induces a strong CD8⁺ T cell response, therefore, being

described as immunodominant. After clearance of the IAV infection, long living CD8⁺ memory T cells are generated and mediate protection against recurrent infections. As CD8⁺ CTLs recognize intracellular proteins of IAV that are less frequently subject to mutation related change, memory CTLs can protect against IAV also from different subclasses (61).

1.3 The immune system during pregnancy

Pregnancy is a unique immunological situation where the maternal immune system has to tolerate the semi-allogenic fetus. As a histo-incompatible product – expressing antigens of maternal and paternal origin – the fetus is recognized as foreign by the maternal immune system, even though rejection is prevented (62). For a long time, it was believed that rejection prevention is mediated by global immune suppression. However, the maternal immune system during pregnancy is highly active and can react to invading pathogens and even fetal antigens. Then, a skewing of the immune system towards a Th2 phenotype was suspected (63, 64). By now, it is known that the immune regulation during pregnancy is highly complex and cannot be solely explained by mechanisms like global immune suppression (65) or a general Th2 skewing (66, 67). Nevertheless, women and mice during pregnancy are more susceptible to some pathogens and infection-related complications (68). While complex maternal immune adaptation to pregnancy is advantageous for fetal outcome, these circumstances can lead to severe disadvantages for the mother and the unborn child in case of infection.

1.3.1 Innate immune adaptation during pregnancy

Whereas, during early and late gestation pro-inflammatory responses are required for placentation and initiation of birth, respectively (65, 69), mid pregnancy is characterized by local (70) and peripheral maternal immune adaptation (71) to prevent fetal rejection. Destruction of the delicate balance of immune cells in the uterus during pregnancy can lead to severe pregnancy complications such as preeclampsia and preterm delivery (72-75). Especially, uterine NK cells are of major importance at the feto-maternal interface in human and mouse pregnancies. They are abundantly distributed in the uterus throughout pregnancy and are involved in tolerance induction towards the semi-allogenic fetus (76-78). Furthermore, DCs that are present at the feto-maternal interface are kept in an immune-tolerant/immature state (79). They are characterized by low expression of costimulatory molecules CD80 and CD86, low IL-12 production, enhanced IL-10 secretion and reduced capacity of antigen presentation (80, 81). These tolerogenic DCs are unable to migrate to

draining lymph nodes and are impaired in presenting fetal antigens to naïve T cells (82). Tolerogenic DCs mainly induce the differentiation of Tregs and thus, promote immune tolerance (83). Macrophages in the decidua are mainly polarized towards an alternatively activated, anti-inflammatory M2 phenotype and secrete large amounts of IL-10 and transforming growth factor beta (TGF- β) (83-86) (Figure 3).

1.3.2 Adaptive immune adaptation during pregnancy

During pregnancy, CD4⁺ and CD8⁺ T lymphocyte numbers increase substantially in the uterus. Moreover, shedding of fetal antigens during the second and third trimester leads to the generation of fetus-specific cytotoxic T cells that remain even after birth (82, 87). On the other hand, an immune reaction against the fetus is prevented, importantly, by elevated numbers of Tregs in the pregnant uterus. Due to their capacity for antigen-specific inhibition of T cell function by different mechanisms e.g. production of IL-10, Tregs are fundamental in tolerance induction at the feto-maternal interface (88). Furthermore, cytotoxic T cell priming is impaired in uterus-draining lymph nodes due to constrained migration of uterine DCs to the draining lymph nodes (82, 89). Also, the migration of cytotoxic T cells to the feto-maternal interface is restricted. C-X-C motif chemokine ligands CXCL9, CXCL10, CXCL11 and C-C motif chemokine ligand CCL5 are epigenetically silenced by repressive lysine 27 methylation of histone 3 (H3K27) in decidual cells, leading to a migration constrain for activated cytotoxic C-X-C motif receptor 3 (CXCR3) expressing T cells to the uterus (90) (Figure 3).

1.3.3 Endocrine modulation of the immune system during pregnancy

Hormonal changes during pregnancy are causative for most crucial immune adaptations (Figure 3). Not only pregnancy-related hormones like progesterone and estrogens rise throughout pregnancy but also immuno-suppressive glucocorticoids (GCs).

Progesterone is one of the most important sex hormones involved in the maintenance of pregnancy, as low progesterone levels are associated with miscarriage (91) and preterm labor (92). It is produced by the corpus luteum and rises up to 5-10-fold in serum and 10-100-fold in the uterus during human and mouse pregnancies (93). With primarily immunosuppressive activity, progesterone mediates immune adaptation during pregnancy by promoting an anti-inflammatory milieu. Consequently, high progesterone concentrations lead to suppression of the production of pro-inflammatory cytokines like IFN-γ and TNF-α in CD4⁺ and CD8⁺ T cells (94). Moreover, the capacity of T cells to secrete multiple cytokines (polyfunctional T cells) is reduced in the presence of progesterone (94), while anti-inflammatory Treg proliferation and IL-10 secretion are induced (93). Apart from induction of Pregnancy and influenza CD4⁺ Treg cells, progesterone increases the expansion of regulatory CD8⁺ CD122⁺ T cells that promote vascularization and therefore fetal growth (95).

As the nuclear progesterone receptor (PR) – that mediates the classical modulation of gene transcription – is thought to be absent in lymphocytes (94, 96), progesterone probably modulates these cells via binding to a membrane PR (97, 98) or the glucocorticoid receptor (GR) that is widely expressed in leukocytes (96).

GR activation - usually via endogenous cortisone (human) or corticosterone (rodents) exhibits strong immuno-suppressive effects. GCs are involved in physiological immunological processes also in non-pregnant hosts. Importantly, during pregnancy, GC concentration in maternal serum increases 2-3-fold in humans (99) and 5-10-fold in rodents (100, 101). Beside regulation of metabolic functions, at high concentrations, GCs act as global immunosuppressors, as they are able to reduce the expression of various pro-inflammatory cytokines (e.g. IL-1α, IL-1β, IL-2, IL-6, IFN-γ, TNF-α, GM-CSF) due to the direct interaction of the GR with transcription factors like NF- κ B or activator protein 1 (AP-1) (102). They suppress vasodilation and expression of chemokines and adhesion molecules (e.g. ICAM1, VCAM1, CCL2), thereby restricting leucocyte mobilization and recruitment (102). They inhibit DC maturation by inducing down regulation of MHC-II and costimulatory molecule (CD80, CD86) expression and reduced secretion of cytokines (IL-12, TNF- α), while they increase the secretion of anti-inflammatory IL-10 (103). Hence, they support the generation of tolerogenic DCs and limit the capacity of DCs to activate T cells (104). GCs interfere with lymphocyte differentiation by modulating the Th1/Th2 balance; while inhibiting pro-inflammatory Th1 differentiation and cytokine expression, they promote Th2 and Treg differentiation (105-107). To limit inflammation and prevent an overshooting immune response with elevated immunopathology, GCs mediate apoptosis of lymphocytes (108, 109), DCs (110) and eosinophils (102, 111). Due to strong immuno-suppressive effects, GCs presumably play a crucial role in shaping the immune system during pregnancy. The exact function and role of GCs during pregnancy, however, is still under investigation.

Estrogens, another group of crucial pregnancy related-hormones, rise up to 500-fold in maternal serum throughout pregnancy (112). Although, their contribution to maternal immune adaptation to pregnancy has not been extensively studied, estrogens seem to have a dose-dependent function on the immune response. At low concentrations, they primarily enhance immunity by promoting pro-inflammatory cytokine production by macrophages and monocytes and facilitate a Th1-type immune response (IFN- γ and TNF- α). At high concentrations – as during pregnancy – estrogens reduce pro-inflammatory cytokine production and promote a Th2-type and humoral immune response (IL-4, IL-10) (86).

While the immune adaptation to pregnancy mostly occurs at the feto-maternal interface, elevated systemic hormone levels contribute to altered development and progression of several diseases during pregnancy. This is beneficial in some autoimmune diseases e.g. rheumatoid arthritis (113, 114) and multiple sclerosis (115, 116). In a mouse model for multiple sclerosis, elevated systemic progesterone levels mediate effector T cell apoptosis resulting in elevated Treg frequencies and therefore, suppression of disease (96). Conversely, development and progression of certain infectious diseases like IAV infections are worsened in pregnant hosts (68).



Figure 3: Immunological adaptation to pregnancy. Local and systemic increase in hormone concentrations during pregnancy leads to immunological changes at the feto-maternal interface and systemically. Macrophages and dendritic cells (DC) are kept in a tolerogenic state and are prevented from migration to the draining lymph nodes. T cells produce less pro-inflammatory cytokines, while regulatory T cells (Tregs) are expanded that secrete high amounts of the anti-inflammatory cytokine IL-10. Furthermore, reduced expression of the chemokine ligands CXCL9, 10, 11 and CCR5 reduce the migration of pro-inflammatory cytotoxic CXCR3⁺ T cells (CTL) to the uterus. These immunological changes at the feto-maternal interface prevent fetal absorption and therefore, promote pregnancy maintenance. Modified from (117).

1.4 Immune response against IAV during pregnancy

In pregnant women the physiological adaptation to pregnancy increases susceptibility to severe IAV. Anatomical adaptations i.e. elevation of the diaphragm, increased respiratory rate and intra-abdominal pressure, decreased chest compliance and increased risk for aspiration can drive respiratory failure during IAV infection (118-122). It has been postulated that maternal immune adaptation to pregnancy is crucial for rendering pregnant woman susceptible to severe infections. There is strong indication that elevated systemic concentrations of estrogens, progesterone and GCs modulate the immune response to IAV resulting in enhanced morbidity and mortality during pregnancy (123). Especially, progesterone and GCs have strong immuno-suppressive functions (76, 102). However, the exact mechanistic link between the immune modulation during pregnancy and the increased morbidity and mortality upon IAV infection still remains elusive. Insights from animal studies provide first hints explaining the complex regulation of the immune response against IAV during pregnancy.

Studies available so far, analyzing the immune response during pregnancy towards IAV, work with artificially elevated hormone levels to induce a pregnancy-like status or use syngenically pregnant mice (genetically identical father and mother). Elevation of estrogen levels in ovariectomised mice leads to improved survival and enhanced immune cell recruitment to the lung and elevated CCL2 and IL-6 mRNA expression upon IAV infection, whereas elevated progesterone levels are associated with increased lethality and enhanced expression of IL-10 in infected mice (124). On the contrary, low concentrations of progesterone rescue mice from lethal IAV infection due to elevated expression of TGF- β , IL-6 and amphiregulin that is involved in tissue repair (125). Despite proposed protection of low dose progesterone from primary infection (125, 126), it is discussed that reduced antibody and CD8⁺ memory T cell responses cause exacerbated outcome in secondary heterologous IAV infection with excessive lung immunopathology in progesterone-treated mice (126).

In 1977, Williams and Mackenzie established a mouse model by infecting syngenically pregnant C3H inbred mice with a sublethal dose of an H0N1 virus and observed high maternal and fetal mortality upon infection during the 3rd week of gestation. Interestingly, the infected mice did not display viremia, transplacental virus transmission or congenital malformations (127). Various studies using syngenically mated BALB/c females demonstrated increased lethality of pregnant mice infected with mouse-adapted or human-isolated H1N1 IAV strains (128-130). In literature, it is frequently proposed that increased

pro-inflammatory cytokine production resulting in a so-called 'cytokine storm' (128, 130) and severe lung pathology mediated by enhanced recruitment of neutrophils and macrophages (130) are the leading causes of death in pregnant IAV infected mice. A lately published study by Littauer and colleagues demonstrated that syngenically mated BALB/c females infected with seasonal H1N1 IAV have a significantly increased risk for preterm labor, stillbirth and small for gestational age (SGA) neonates. Moreover, viral load was increased in these infected pregnant mice compared to non-pregnant infected littermates, whereas pro-inflammatory cytokines (IL-1 β , IL-6) were decreased and anti-inflammatory cytokines (IL-10) increased in serum at 4 days post infection (d p.i.) (98). Also, in a study conducted with ferrets, which are often used in influenza research, infection of pregnant ferrets with a low dose of H1N1 IAV at early gestation resulted in impaired fetal outcome. Moreover, increased pro-inflammatory cytokine expression, elevated virus titers and enhanced alveolar damage but absence of intrauterine virus transmission was observed (131).

Although these studies already show increased severity of IAV infection during pregnancy, these observations are all based on syngenic mating combinations that, due to the common genetic identity of mother and father, can mirror only partially the complexity of human/allogenic pregnancies.

13

1.5 Aim of the thesis

The aim of this thesis was to elucidate the immune response of pregnant mice towards the influenza A virus. So far, relevant studies use either the induction of a pregnancy-like status by hormone administration or syngenically mated pregnant mice, and therefore fail to reproduce the complexity of human pregnancies. In order to mirror the human condition, we established an allogenic mating mouse model where C57BL/6J females were mated with BALB/c males and infected with IAV at mid-gestation (132). This study intends to analyze the innate and adaptive immune response during pregnancy towards IAV using this improved allogenic mouse model. It is hypothesized that pregnancy hormones, especially progesterone and glucocorticoids – that rise substantially during pregnancy – suppress the innate and adaptive immune response towards IAV leading to poor viral clearance and high maternal lethality.

To test this hypothesis, it was aimed to:

- I. Investigate the innate immune response in allogenically mated pregnant IAV infected mice in comparison to non-pregnant littermates by analyzing the cytokine secretion in the lung as well as DC and macrophage maturation.
- II. Determine the adaptive immune response towards IAV during pregnancy by characterizing the migration of CD8⁺ T cells to the lung and the frequency of cytokine expressing CD4⁺ and CD8⁺ T cells.
- III. Elucidate the role of progesterone in the development of an immune response towards IAV during pregnancy by injecting a progesterone derivative into nonpregnant mice and using mice lacking the progesterone receptor in DCs and macrophages.
- IV. Analyze the impact of progesterone and glucocorticoids on immune cell function *in vitro*

These results will improve our understanding on the function of the immune system during pregnancy and on the contribution of pregnancy-related hormones on the altered immune response against the influenza virus.

2 Material and Methods

2.1 Material

2.1.1 Chemicals

All standard chemicals used in puffers, solutions and media were purchased from Sigma-Aldrich Chemie GmbH (Munich, Germany), Merck Millipore (Darmstadt, Germany) and Gibco/ThermoFisher Scientific (Waltham, MA). Chemicals for special applications are listed in Table 1 and Table 2.

Chemical	Company	
Avicel (microcrystalline cellulose)	FMC BioPolymer, Philad	delphia, PA
Benzyl benzoate	Sigma-Aldrich Chem	ie GmbH, Munich,
	Germany	
Bovine serum albumin (BSA)	Sigma-Aldrich Chem	ie GmbH, Munich,
	Germany	
Brefeldin A	Sigma-Aldrich Chem	ie GmbH, Munich,
	Germany	
Castor oil	Sigma-Aldrich Chem	ie GmbH, Munich,
	Germany	
Cell Proliferation Dye eFluor 670	eBioscience, San Diego	, CA
Chicken erythrocytes with citrate	Lohmann Tierzucht, Cu	xhaven, Germany
Chicken ovalbumin ₂₅₇₋₂₆₄ (SIINFEKL)	Peprotech, London, UK	
Citrate buffer	DCS, Hamburg, German	ny
Collagenase D	Hoffmann-La Roche, Ba	asel, Switzerland
CompBeads (anti-rat/anti-hamster/anti-	BD Bioscience, Heidelb	erg, Germany
mouse Ig κ and negative control		
compensation particles)		
Corticosterone	Sigma-Aldrich Chem	ie GmbH, Munich,
	Germany	
Dexamethasone	Sigma-Aldrich Chem	ie GmbH, Munich,
	Germany	
Dextramer H2-Db ASNENVETM	Immudex, Copenhagen	, Denmark

Table 1: Chemicals

DNase I	Sigma-Aldrich Chemie GmbH, Munich,
	Germany
Dydrogesterone	Abbott Laboratories, Chicago, IL
Fetal bovine serum (FBS)	Gibco/ThermoFisher Scientific, Waltham, MA
Formaldehyde solution	Sigma-Aldrich Chemie GmbH, Munich, Germany
Hematoxylin	Ventana, Hoffmann-La Roche, Basel, Switzerland
Influenza peptide NP ₃₆₆₋₃₇₄ (ASNENVETM)	JPT, Berlin, Germany
Ionomycin	Sigma-Aldrich Chemie GmbH, Munich, Germany
L-Glutamine 200 mM (100x)	Gibco/ThermoFisher Scientific, Waltham, MA
Maxima/SYBR Green/Rox qPCR Master Mix	ThermoFisher Scientific, Waltham, MA
Normal rat serum (NRS)	Jachson ImmunoResearch Laboratories, West Grove, PA
Pacific Orange [™] Succinimidyl Ester (PacO)	ThermoFisher Scientific, Waltham, MA
Paraffin	DCS, Hamburg, Germany
Paraformaldehyde (PFA)	Biochemica, Billingham, UK
Penicillin (10.000 units)/Streptomycin	Sigma-Aldrich Chemie GmbH, Munich,
(10 mg/ml)	Germany
Phorbol-12-myristat-13-acetat (PMA)	Sigma-Aldrich Chemie GmbH, Munich, Germany
Progesterone	Sigma-Aldrich Chemie GmbH, Munich,
	Germany
Proteinase K	Germany Hoffmann-La Roche, Basel, Switzerland
Proteinase K Qiazol	Germany Hoffmann-La Roche, Basel, Switzerland Qiagen, Venlo, Netherlands
Proteinase K Qiazol Red blood cell (RBC) lysis buffer	Germany Hoffmann-La Roche, Basel, Switzerland Qiagen, Venlo, Netherlands eBioscience, San Diego, CA
Proteinase K Qiazol Red blood cell (RBC) lysis buffer RNAlater	Germany Hoffmann-La Roche, Basel, Switzerland Qiagen, Venlo, Netherlands eBioscience, San Diego, CA Qiagen, Venlo, Netherlands
Proteinase K Qiazol Red blood cell (RBC) lysis buffer RNAlater SuperBlock T20 (TBS)	Germany Hoffmann-La Roche, Basel, Switzerland Qiagen, Venlo, Netherlands eBioscience, San Diego, CA Qiagen, Venlo, Netherlands ThermoFisher Scientific, Waltham, MA
Proteinase K Qiazol Red blood cell (RBC) lysis buffer RNAlater SuperBlock T20 (TBS) TaqMan Universal PCR Master Mix	Germany Hoffmann-La Roche, Basel, Switzerland Qiagen, Venlo, Netherlands eBioscience, San Diego, CA Qiagen, Venlo, Netherlands ThermoFisher Scientific, Waltham, MA Life Technology, Carlsbad, CA
Proteinase KQiazolRed blood cell (RBC) lysis bufferRNAlaterSuperBlock T20 (TBS)TaqMan Universal PCR Master MixTriton-X-100	Germany Hoffmann-La Roche, Basel, Switzerland Qiagen, Venlo, Netherlands eBioscience, San Diego, CA Qiagen, Venlo, Netherlands ThermoFisher Scientific, Waltham, MA Life Technology, Carlsbad, CA Merck Millipore, Darmstadt, Germany
Proteinase K Qiazol Red blood cell (RBC) lysis buffer RNAlater SuperBlock T20 (TBS) TaqMan Universal PCR Master Mix Triton-X-100 TrueBlue [™] Peroxidase Substrate	Germany Hoffmann-La Roche, Basel, Switzerland Qiagen, Venlo, Netherlands eBioscience, San Diego, CA Qiagen, Venlo, Netherlands ThermoFisher Scientific, Waltham, MA Life Technology, Carlsbad, CA Merck Millipore, Darmstadt, Germany KPL Sera Care, Milford, MA
Proteinase K Qiazol Red blood cell (RBC) lysis buffer RNAlater SuperBlock T20 (TBS) TaqMan Universal PCR Master Mix Triton-X-100 TrueBlue [™] Peroxidase Substrate Trypan Blue Stain (0.4 %)	GermanyHoffmann-La Roche, Basel, SwitzerlandQiagen, Venlo, NetherlandseBioscience, San Diego, CAQiagen, Venlo, NetherlandsThermoFisher Scientific, Waltham, MALife Technology, Carlsbad, CAMerck Millipore, Darmstadt, GermanyKPL Sera Care, Milford, MAGibco/ThermoFisher Scientific, Waltham, MA

		Ohamia	Omb	Muniala
Trypsin-EDTA (TX)	Sigma-Aldrich	Chemie	GmbH,	wunich,
	Germany			
Tween-20	Serva, Heidelber	g, Germany	/	
Weihert's ion hematoxylin	Waldeck, Münste	er, Germany	/	
Xylene	Greyer, Renning	en, Germar	ıy	
β-Mercaptoethanol	Gibco/ThermoFis	sher Scienti	fic, Walthar	n, MA

Table 2: Anaesthetics, analgetics and additives

Name	Company
Forene (isofluran 100 %)	Abbvie, North Chicago, IL
Ketamine (100 mg/ml)	WDT, Garbsen, Germany
Sedaxylan	WDT, Garbsen, Germany
(xylazin-hydrochloride, 20 mg/ml)	
NaCl (0.9 %)	B. Braun Melsungen AG, Melsungen, Germany

2.1.2 Plastic material

The standard laboratory plastic materials were purchased from Sarstedt AG & Co. (Nümbrecht, Germany), Greiner Bio-One International GmbH (Kremsmünster, Austria), Nunc GmbH & Co. KG (Langenselbold, Germany), BD Bioscience (Heidelberg, Germany), GE Healthcare (Little Chalfont, UK) and Eppendorf AG (Hamburg, Germany).

2.1.3 Kits

If not stated explicitly in the methods all kits were used following manufacturers' instructions and listed in Table 3.

Table 3: Kits

Kits			Company
DNase elimination			Amgen, Thousand Oaks, CA
FITC BrdU Flow Kit			BD Pharmingen, Franklin Lakes, NJ
Fixation/Permeabilisation			eBioscience, San Diego, CA
FoxP3/Transcription	Factor	Staining	eBioscience, San Diego, CA
Buffer Set			
Pan T cell Isolation Kit			Miltenyi Biotec, Bergisch Gladbach, Germany

Pregnancy and influenza

ProcartaPlex [®]	Multiplex	Immunoassay	Affimetrix/eBioscience, San Diego, CA
Mix&Match Mou	ise 4-plex		
RNeasy Plus Ur	niversal Min	i Kit	Qiagen, Venlo, Netherlands
SensiMix SYBR	Hi-ROX		Bioline, London, UK
Superscript			Invitrogen, Carlsbad, CA
Zytochem-Plus	HRP Kit		Zytomed, Bargteheide, Germany

2.1.4 Buffers and Solutions

FACS buffer: 500 ml PBS with 0.5 % BSA

MACS buffer: 500 ml PBS with 0.5 % FBS, 2 mM EDTA

IMDM complete medium for immune cells: 500 ml IMDM media with 10 % FBS, 2 mM L-Glutamine, 1 % Penicillin/Streptomycin, 50 μM β-Mercaptoethanol

<u>Overlay medium:</u> 2x MEM media with 0.4 % BSA, 2 % L-Glutamin, 2 % Penicillin/Streptomycin

Avicel-Overlay medium: 50 % Overlay media, 50 % Avicel solution (2.5 % Avicel in PBS)

<u>MEM complete media for MDCK cells:</u> 500 ml MEM media with 10 % FBS, 1 % L-Glutamin, 1 % Penicillin/Streptomycin

Infection media for MDCK cells: 500 ml MEM media, 0.2 % BSA, 1 % L-Glutamin, 1 % Penicillin/Streptomycin

2.1.5 Viral stocks

Viral stocks were obtained from or generated in cooperation with the Heinrich Pette Institute, Hamburg, Germany and listed in Table 4. For mouse infection the human pandemic H1N1 virus A/Hamburg/NY1580/09 was used that was isolated from a pharyngeal swab of a 29year-old male patient before oseltamivir treatment at the University Medical Center Hamburg-Eppendorf in June 2009 and was already described before (133). For hemagglutination inhibition assays (HAI, see 2.2.1.5) the human pandemic H1N1 virus A/Sachsen-Anhalt/101/09 was used that was isolated during the pandemic in 2009 and also described in (133).

Table 4: Viral stocks

Name	Origin
A/Hamburg/NY1580/09 (pH1N1)	Sigrid Baumgarte, Institut für Hygiene und
\rightarrow for mouse infection	Umwelt, Hamburg, Germany (133, 134)
A/Sachsen-Anhalt/101/09 (pH1N1)	Dr. Brunhilde Schweiger, Robert-Koch-Institut,
\rightarrow for HAI assay	Berlin, Germany (133)

2.1.6 Instruments

All experiments were performed using standard laboratory equipment. Specialised instruments are listed in Table 5.

Table 5: Special instruments used

Instruments	Company
Bioruptor	Diagenode, Seraing, Belgium
CFX96 Real-Time System	Biorad, Hercules, CA
FACS LSR/Fortessa	BD Bioscience, Heidelberg, Germany
Infinite 2000 PRO NanoQuant reader	Tecan, Männedorf, Switzerland
Luminex 200	Biorad, Hercules, CA
Microtome SM2010R	Leica, Wetzlar, Germany
Mixer Mill MM400	Retsch Technology, Haan, Germany
Precellys 24 tissue homogenizer	PeQlab, VWR, Erlangen, Germany
Rotor-Gene Q-plex	Qiagen, Venlo, Netherlands
StepOne Plus [™] Real-Time PCR System	Applied Biosystems, Foster City, CA

2.1.7 Antibodies

Antibodies used in FACS assays (Table 6), plaque assays and immunohistochemistry (Table 7) are listed below.

Antigen	Conjugated fluorochrome	Clone	Dilution	Company
CD103	PerCP-Cy5.5	2E7	1:100	BioLegend, San Diego, CA
CD11b	BV711	M1/70	1:200	BioLegend, San Diego, CA

Table 6: Antibodies for FACS

Pregnancy and influenza

CD11c	AF700	N418	1:200	BioLegend, San Diego, CA
CD3	PerCP-Cy5.5	145-2C11	1:100	eBioscience, San Diego, CA
CD3	FITC	145-2C11	1:100	BD Bioscience, Heidelberg, Germany
CD3	eF610	145-2C11	1:100	eBioscience, San Diego, CA
CD4	APC-eF780	RM4-5	1:400	eBioscience, San Diego, CA
CD40	PE-Cy7	3/23	1:100	BioLegend, San Diego, CA
CD44	PE-Cy7	IM7	1:100	BioLegend, San Diego, CA
CD45	AF700	30-F11	2.5 µl/test	eBioscience, San Diego, CA
CD45R	APC	RA3-6B2	1:200	BD Bioscience, Heidelberg,
ODCOL	D) /744		4.000	Bish spend Can Diago CA
CD62L	BV711	MEL-14	1:200	BioLegend, San Diego, CA
CD8a	BV650	53.7.7	1:100	BioLegend, San Diego, CA
CD80	PE	B7-1	1:200	BD Pharmingen, Franklin Lakes, NJ
CD86	BV605	GL-1	1:200	BioLegend, San Diego, CA
CD90.1	V450	HIS51	1:200	eBioscience, San Diego, CA
CD90.2	PerCP	53-2.1	1:200	BioLegend, San Diego, CA
CXCR3	APC	CXCR3-173	1:100	eBioscience, San Diego, CA
Foxp3	FITC	FJK-16s	1:100	eBioscience, San Diego, CA
Gr-1	BV421	RB6-8C5	1:200	BD Bioscience, Heidelberg, Germany
Granzyme B	APC	GB12	1:50	Molecular Probes Inc., Eugene, USA
IFN-γ	PE-CF594	XMG1.2	1:200	BD Bioscience, Heidelberg, Germany
IL-10	PE	JES5-16E3	1:100	BD Bioscience, Heidelberg, Germany
MHC-II	AF700	M5/114.15.2	1:100	BioLegend, San Diego, CA
TNF-α	BV421	MP6-XT22	1:200	BioLegend, San Diego, USA

Antibody	Origin	Dilution	Company
Anti-NP (nucleoprotein)	Mouse	1:1000	Abcam, Cambridge, UK
	monoclonal		
Anti-Mouse IgG-horseradish Rabbit		1:2000	Southern Biotech, Birmingham,
peroxidase (HRP) polyclonal			UK
Anti-FPV (fowl plaque virus)	Rabbit	1:2000	Kindly provided by HD. Klenk,
	polyclonal serum		Marburg, Germany
	raised against		
	A/FPV/Rostock/		
	34 (H7N1)		
Anti-Rabbit-Biotin	Donkey	1:200	Jackson ImmunoResearch,
	polyclonal		Cambridgeshire, UK

Table 7: Antibodies for plaque assay and immunohistochemistry

2.1.8 Software

Special software that was used for data acquisition and analysis is listed in Table 8.

Table 8: Software used for data	a acquisition and analysis
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Software	Company
GraphPad Prism Version 7	GraphPad Software, La Jolla, CA
FlowJo Version 9.9.5 for Mac	TreeStar, Ashland, OR
FACSDiva [™] Software V.8.0.1	BD Bioscience, Heidelberg, Germany
Pannoramic Viewer	3DHISTECH, Budapest, Hungary

2.1.9 Primers

All primers for SYBR Green quantitative real-time PCR (qRT-PCR) were purchased at Eurofins (Ebersberg, Germany) and used at a final concentration of 100 nM (Table 9). The expression assays for TagMan® analysis were purchased at Applied Biosystems (Foster City, CA) (Table 10). Primers for Chip assay were listed in Table 11.

	Table 9:	Primer	for SYBR	Green aPCR
--	----------	--------	----------	------------

Primer	Sequence (5' > 3')
Gapdh fw	GGA-TGCAGGGATGATGTTCT

Gapdh rev	AACTTTGGCATTGTGGAAGG
Ccx/9 fw	CCCAAGCCCCAATTGCA
Cxcl9 rev	GCAGGTTTGATCTCCGTTC
Cxcl11 fw	GAGAAAGCTTCTGTAATTTACCCGAGTA
Cxcl11 rev	GTCCAGGCACCTTTGTCGTTTA

Table 10: Gene expression assays for TaqMan qPCR

Gene expression assay	Assay ID
Gapdh	Mm99999915_g1
Cxcl10	Mm99999072_m1

Table 11: Primer for Chip assay

Primer	Sequence (5' > 3')
Gapdh fw	CCCAGCCAAGGCTGTGTTAGACT
Gapdh rev	GCTGTGCATAGAGCCTCGGTAG
Cxc/9 fw	TGTTTTGAAAGGGTGGTTGTTGGCTTT
<i>Cxcl</i> 9 rev	CAATTTGCCTTCTGGGACAGGATG
Cxc/10 fw	GCATTCAGAACAGAAGCCGGAAGT
<i>Cxcl</i> 10 rev	CATCCTTGACCCTGTAACCACAC
<i>Ccl5</i> fw	CATATGGCTCGGACACCACT
<i>Ccl5</i> rev	ACACACTTGGCGGTTCCTTC
Cd8 fw	AAAATGCATGAAGAGTTTTACCACAA
Cd8 rev	TCCTCATCACGTCTATACATGCG
<i>Msi1</i> fw	TCCGAGACACCGACTTCTCT
Msi1 rev	CCCTTTCGGACTGTCTGGAG
Ache fw	CCGGTCAGGGGAATTAGCTC
Ache rev	GCTGAGAAGCCAGACCACAT
Atp8b5 fw	TACTTAGTCATGGCCCCTCT
Atp8b5 rev	AGGAACAAAGGCTGGGGTTTAT
Hoxc13 fw	TCGGGACGAAGTCTGCATTT
Hoxc13 rev	CTCCGACCTCAGAGGCTTTC

2.1.10 Statistics

All statistical analyses were performed using the Prism Graphpad Version 7. Gehan-Brelow-Wilcoxon test was used for assessment of statistical significance of survival rates. Where normal distribution of data was presumed the Student's *t* test was used, otherwise the Mann Whitney *U* test was used in order to assess the significance of two sample collections. Multiple group analysis was done using two-way ANOVA and Bonferroni's multiple comparison post-test as described in the figure legend. Only results reaching a level of difference were presented as significant with * < 0.05, ** < 0.01 or *** < 0.001. Nonsignificant differences were not explicitly stated.

2.2 Methods

Working steps with infectious material were conducted in Biosafety Level 2 facilities following the regulations and risk assessment of the *Gentechnikbehörde* of the City of Hamburg.

2.2.1 Virological assays

2.2.1.1 Cell culture

The influenza A virus (IAV) was propagated in MDCK cells. MDCK cells were incubated at 37 °C, 5 % CO₂ and 95 % humidity and split upon 80-90 % confluence. For detachment of cells, they were washed with PBS and incubated with Trypsin-EDTA for 20 min at 37 °C. Addition of cell culture media containing FBS inactivated the Trypsin-EDTA and cells were cultured as needed.

2.2.1.2 Virus generation

Viral stocks were passaged not more than twice to limit spontaneous occurring mutations due to the lack of proofreading activity of the viral polymerase (135).

For virus generation MDCK cells were seeded in 75 cm³ flasks. When 80-90 % confluence was reached, cells were washed with PBS and 1.5 µl virus dilution was added. After an incubation for 30 min at 37 °C remaining virus inoculum was removed and infection media containing 1 µg/ml TPCK-Trypsin was added. TPCK-Trypsin is needed to split the HA into subunits and therefore, provide virus entry into cells. Infected MDCK cells were cultured at 37 °C and regularly checked for presence of cytopathic effects (CPE). Upon occurrence of a CPE of greater than 50 % (after approximately 24-36 h) or positive hemagglutination assay (see 2.2.1.3) the supernatant was harvested. After centrifugation to remove remaining cells, the supernatant was collected and stored in aliquots at -80 °C. The protocol was described before (136).

2.2.1.3 Hemagglutination assay

In order to test the viral concentration, a hemagglutination assay (HA assay) was performed as previously described (136). IAV binds to $\alpha 2,3$ -linked and/or $\alpha 2,6$ -linked sialic acids expressed on erythrocytes. Therefore, virus containing cell culture supernatant (2.2.1.2) was added into the first row of a 96 well V-bottom plate. A twofold serial dilution of the virus was performed in PBS. Subsequently, 1 % chicken erythrocyte suspension (diluted in 0.9 % NaCl) was added to the samples and incubated for 30 min at 4 °C. After the incubation, agglutination of erythrocytes could be observed. Erythrocytes normally clot and gather at the bottom of the plate. In the presence of virus, the erythrocytes are bound and clotting is prevented. This leads to a cloudy appearance in the well (137). For analysis the reciprocal value of the highest dilution showing agglutination was described as hemagglutination units (HAU).

2.2.1.4 Plaque assay

The concentration of infectious viral particles in unknown tissue homogenates (2.2.3.2) or cell culture media (2.2.1.2) was determined using the plaque assay as described before (136) (modified from (138)). MDCK cells were seeded into a 6 well plate. Upon 80-90 % confluence, cells were washed with PBS and virus dilution was added. A tenfold serial dilution was prepared out of virus containing samples. Incubation of the MDCK cells with the virus dilutions took place at 37 °C for 30 min including occasional tilting. Following the incubation, avicel-overlay media containing TPCK-Trypsin was added for 72 h at 37 °C. Subsequently, overlay-media was discarded and cells were fixed with 4 % PFA for at least 30 min at 4 °C. This was followed by the addition of 0.3 % Triton-X for 30 min at a tumbling table at room temperature (RT) in order to permeabilize the cells and facilitate visualization of viral plaques. Visualization of plaques was reached by immunostaining. Therefore, 500 µl of the primary antibody, an anti-NP antibody against the IAV nucleoprotein (NP), was added for 1 h, following the staining with a secondary anti-mouse IgG-HRP (horseradish peroxidase) conjugated antibody for another hour. Visualization of the stained plaques was performed using the TrueBlue[™] Peroxidase Substrate. According to the number of plagues in the cell culture layer the viral concentration was calculated as plaque forming units per milliliter (p.f.u./ml) for each condition.

2.2.1.5 Hemagglutinin inhibition assay

Binding of influenza virus HA to sialic acids on erythrocytes can be prevented by IAV specific antibodies e.g. in sera of humans or animals. In order to determine the level of neutralizing antibodies in mouse sera, sera was heat inactivated (56 °C, 30 min) to remove complement activity. Then, 1:10 serum dilution was added into the first well of a V-bottom shaped 96-well plate and twofold serial diluted in PBS. Next, virus solution (A/Sachsen-Anhalt/101/09, diluted to HAU of 4) was added for 30 min at RT and finally, 1 % chicken erythrocytes were added for 30 min at 4 °C. Hemagglutination was observed in wells with absent neutralizing antibodies. Upon antibody presence hemagglutination inhibition was detected and thus, erythrocytes sediment to the ground, forming dots. The dilution factor of the highest dilution inhibiting hemagglutination is defined as the HI titer. If in wells with serum dilutions of 1:40

(HAI titer 40) or higher sedimentation of erythrocytes is observed, the tested serum is considered to contain sufficient neutralizing antibody concentrations to protect from recurrent infection with the same IAV subtype (139). The procedure was described before in (136).

2.2.2 Animal experiments

All mice were purchased from Charles River, Envigo (Harlan) or bred in the animal facility of the University Medical Center Hamburg-Eppendorf. Mice were kept in individually ventilated cages (IVC) at a 12 hour light-dark rhythm with dry food and autoclaved drinking water *ad libitum* at the animal facility of the University Medical Center Hamburg-Eppendorf or the Heinrich Pette Institute, Hamburg. All animal studies were approved by the animal protection law and the relevant German authorities (Behörde für Gesundheit und Verbraucherschutz Hamburg; approval numbers G124/12, G53/16) and conducted at the animal facility of the Heinrich Pette Institute Hamburg and the University Medical Center Hamburg-Eppendorf. Mouse lines are listed in Table 12.

Table 12. Mouse lines

Marra II.	Ontata
Mouse line	Origin
C57BL/6J	Charles River, Sulzfeld, Germany
C57BL/6JRccHsd	Envigo (Harlan), Huntingdon, UK
BALB/c	Charles River, Sulzfeld, Germany
B6-THY1aPL (<i>B6.PL-Thy1a/CyJ</i>)	University Medical Center Hamburg-
	Eppendorf, Germany (originally Jackson, Bar
	Harbor, ME)
B6-OT1tg	University Medical Center Hamburg-
(C57BL/6-Tg(TcraTcrb)1100Mjb/J)	Eppendorf, Germany (140) (originally
	Jackson, Bar Harbor, ME)
Fir/Tiger (C57BL/6-Foxp3 ^{tm1Flv} /J; B6.129S6-	University Medical Center Hamburg-
IL10 ^{tm1flv} /J)	Eppendorf, Germany (141, 142) (originally
	Jackson, Bar Harbor, ME)
PR ^{flox} (<i>Pgr^{flox/flox}</i>)	University Medical Center Hamburg-
	Eppendorf, Germany (143)
CD11c ^{cre} (B6.Cg-Tg ^(Itgax-cre)1-1Reiz/J))	Provided by Prof. Dr. Manuel Friese,
	University Medical Center Hamburg-
	Eppendorf, Germany (144)

PR ^{flox} CD11c ^{cre} (<i>Pgt^{flox/flox}</i> ; B6.Cg-Tg ^{(ltgax-cre)1-}	Generated by crossing PR ^{flox} with CD11c ^{cre}
^{1Reiz/J)})	mice, University Medical Center Hamburg-
	Eppendorf, Germany (143, 144)
Lck ^{cre} (<i>Tg^{(Lck-cre)1Cwi}</i>)	Provided by Prof. Dr. Manuel Friese,
	University Medical Center Hamburg-
	Eppendorf, Germany (145, 146)
PR ^{flox} Lck ^{cre} (<i>Pgr^{flox/flox}; Tg^{(Lck-cre)1Cwi}</i>)	Generated by crossing PR ^{flox} with Lck ^{cre}
	mice, University Medical Center Hamburg-
	Eppendorf, Germany (143, 145, 146)
GR ^{flox} (<i>Nr3c1</i> ^{tm2GSc})	Provided by Prof. Dr. Manuel Friese
	University Medical Center Hamburg-
	Eppendorf, Germany (147)
GR ^{flox} Lck ^{cre} (<i>Nr3c1</i> ^{tm2GSc} ; <i>Tg</i> ^{(Lck-cre)1Cwi})	Provided by Prof. Dr. Manuel Friese
	University Medical Center Hamburg-
	Eppendorf, Germany (96, 145-147)

2.2.2.1 Timed pregnancy

After 1-2 weeks of adaptation, 8 week old female C57BL/6J mice or female mutant mice on C57BL/6J background (Table 12) were mated to BALB/c males for allogenic matings or to C57BL/6J males for syngenic matings as previously described (136). We considered the presence of a vaginal plug in the morning as gestational day (gd) 0.5. To control pregnancy, animals weight was taken at gd 8.5 and 10.5. Pregnancy was confirmed by a body weight increase of 1.5-2 g relative to the weight at gd 0.5. Virgin, age-matched littermates served as respective controls.

2.2.2.2 Virus infection

Prior to infection, mice were anesthetized. Therefore, mice were placed in a chamber inflated by an isoflurane/ O_2 mixture followed by intraperitoneal (i.p.) injection of 100 mg/kg ketamine and 10 mg/kg xylazine (added to 200 µl 0.9 % NaCl). Mice were infected with 10^3 p.f.u. of 2009 pH1N1 virus strain A/HamburgNY1580/09 or mock infected with PBS by pipetting 50 µl of the viral dilution or PBS slowly intranasally in both nostrils. Pregnant mice were infected at gd 12.5. For the following 14 days post infection (d p.i.) mice were monitored closely every day according to the criteria listed in Table 13. The infection protocol was described before (132, 136).

Table 13. Scoring criteria

Observation	Scoring points
Body weight [*]	
 Reduction of ≥ 15 % 	10
• Reduction of \geq 25 %	20
General constitution	
Ruffled fur, hazy eyes	5
 Abnormal posture, high muscle tonus, dehydration 	10
Seizures, paralysis, respiratory sounds, hypothermia	20
Spontaneous activities	
Abnormal attitude, restricted motor function	5
Isolation, pain, apathy	10
Automutilation	20

≥ 10 points: close monitoring more than once a day; ≥ 20 points: euthanasia of the animal (points are added). * Reduction in body weight for non-pregnant mice was calculated in comparison to the body weight of the same mouse at the day of infection (0 d p.i.). For pregnant mice as body weight increases due to pregnancy the weight reduction was calculated in comparison to the mean body weight of pregnant PBS treated mice at the respective day of gestation.

2.2.2.3 Reinfection

As IAV vaccination was not established in our laboratory, a reinfection experiment was performed to generate preexisting immunity in mice. Therefore, non-pregnant mice were infected as described in 2.2.2.2 or treated with PBS as control. After viral clearance (at 28 d p.i.) blood was taken to determine neutralizing antibody titers in infected mice (2.2.1.5). Subsequently, mice were mated to BALB/c males (2.2.2.1) and infected for a second time at gd 12.5. Control dams were treated with PBS. Mice were scored for the subsequent 14 days and survival and weight loss was determined. Another group of animals was sacrificed 3 days post second infection (44 days post first infection) and cytokine expression as well as costimulatory molecule expression on macrophages and dendritic cells was analyzed in lung tissue by Luminex (2.2.3.2) and flow cytometry (2.2.3.5), respectively.

2.2.2.4 Dydrogesterone administration

In order to generate a pregnancy-like status, 1.25 mg dydrogesterone were administered subcutaneously into virgin female C57BL/6J mice every second day as described previously (132). Dydrogesterone is a progesterone derivate with high affinity and selectivity to the progesterone receptor. Dydrogesterone was solved in castor oil containing 20 % benzyl benzoate. As a control, littermates were injected with castor oil vehicle only. 12 days after

start of dydrogesterone application, mice were infected with 10^3 p.f.u. 2009 pH1N1 as described in 2.2.2.2. Mice were scored for the subsequent 14 days (Table 13) or sacrificed at 3 d p.i. for organ harvest (2.2.3.1).

2.2.2.5 Bromodeoxyuridine administration

The *in vivo* proliferation of cells was measured by application of bromodeoxyuridine (BrdU) using the FITC BrdU Flow Kit and following the manufacturers' instructions. 1 mg BrdU solved in PBS was injected i.p. into infected pregnant and non-pregnant mice (for infection see 2.2.2.2) at 7 d p.i. and 18 h before organ harvest (see 2.2.3.1). BrdU gets incorporated into the DNA during proliferation. Subsequently, BrdU can be stained with specific antibodies and therefore, proliferating cells are detectable via flow cytometry. Therefore, isolated cells were stained with surface antibodies and subsequently, fixed and permeabilized using the Fixation/Permeabilisation kit (2.2.3.5). Next, cells were treated with DNase to expose BrdU and then, stained with 5 µl fluorochrome-conjugated anti-BrdU antibody.

2.2.2.6 Adoptive T cell transfer

To analyze T cell migration to the lung during influenza virus infection, an adoptive T cell transfer was performed as previously described (132). C57BL/6J mice expressing the CD90.1 epitope of the lymphocyte molecule CD90 (B6-THY1aPL) were infected with influenza. Eight days post infection T cells were isolated from whole spleen cells. Therefore, the Magnetic Activated Cell Sorting (MACS) kit Pan T cell Isolation was used according to company's instructions. In short, a single cell suspension was produced out of the isolated spleen as described in 2.2.3.3. Subsequently, the calculated volume of MACS buffer and an antibody cocktail were added that was targeting all leukocytes except T cells. After an incubation for 5 min at 8 °C, MACS buffer was added anew and the calculated volume of anti-streptavidine microbeads was added. During the incubation of 10 min at 8 °C, a LS-MACS column was prepared by placing the column into the MACS magnet and rinsing it with MACS buffer. Following the incubation, the cells were added to the column and washed three times with MACS buffer. The isolated T cells were present in the flow through. 2×10⁶ T cells from infected mice were injected intravenously (i.v.) into the tail vain of pregnant and non-pregnant infected mice at 3 d p.i..18 h after the T cell transfer (4 d p.i.), mice were sacrificed and organs were harvested as described in 2.2.3.1.

2.2.2.7 Administration of an anti-CD45 antibody

Mice were anesthetized with isofluran and subsequently, 2.5 µg of an anti-CD45 antibody was administered retrobulbary (r.b.). Three minutes after antibody administration, mice were euthanized for organ harvest (see 2.2.3.1). The injection of the anti-CD45 antibody leads to the staining of leukocytes in the vasculature. However, due to the short incubation time leukocytes inside the parenchyma are not stained with the injected antibody. This allows the differentiation of leukocytes in blood vessels from those in the parenchyma (Figure 4).



Figure 4: Identification of tissue resident leukocytes by anti-CD45 antibody administration. A fluorochrome labeled anti-CD45 antibody was injected retrobulbary 3 minutes before sacrificing the mice. Shown are representative dot plots from lung tissue of one uninfected and one IAV infected mouse (8 days post infection). CD45 unstained cells are localized inside the lung tissue, whereas CD45 stained cells are localized in blood vasculature.

2.2.3 Cell biology

2.2.3.1 Organ harvest

For the harvest of organs, mice were first anesthetized with isofluran and subsequently euthanized using cervical dislocation. Lung, spleen and lung-draining lymph nodes were stored in RNAlater at -80 °C for subsequent PCR analysis, in 4 % PFA for histology or in ice-cold PBS for immediate flow cytometric analysis. For Luminex assays tissue was stored without any additions at -80 °C. Blood was withdrawn from the *vena facialis* using lancets into EDTA-coated tubes. After centrifugation serum was stored at -80 °C.

2.2.3.2 Luminex assay

For detection of cytokines in the lung, tissue (0.04-0.15 g) was weighed into 2 ml-O-Ring tubes, filled with sterilized glass beads and first stored at -80 °C. For measurement, 1 ml PBS was added to thawed samples and homogenized in the Mixer Mill MM400 at 20 Hz, 4 °C for 10 min. Subsequently, samples were centrifuged (6000 g, 4 °C, 10 min) and supernatant (800 µl) was transferred into a new tube. After another centrifugation step (10000 g, 4 °C, 10 min), samples were pipetted into the Luminex plate. The cytokines IFN- α ,

IFN-γ, TNF-α and IL-6 were then detected by the ProcartaPlex[®] Multiplex Immunoassay Mix&Match Mouse 4-plex with magnetic beads following the company's instructions. Lung homogenate samples were run in duplicates and measured at the Luminex 200 machine. The procedure was described earlier (132).

2.2.3.3 Single cell isolation of mouse organs

For analysis with flow cytometry, single cell suspensions had to be generated out of lung, spleen and lung-draining lymph nodes.

Spleen and lymph node tissue was passed through a 40 μ m cell strainer using the plunger of a syringe and rinsed with PBS. Lung tissue was cut into small pieces and digested using 10 μ l collagenase D (200 mg/ml) and 3 μ l DNase (10 U/ μ l) for 30 min at 37 °C. Subsequently, the digested lung tissue was passed through a 40 μ m cell strainer using a plunger and rinsed with PBS. Lysis of erythrocytes was performed using 1xRBC lysis buffer for 5 min at RT. After completing another washing step with PBS, cell numbers were obtained using a hemocytometer and cells were used for flow cytometric analysis.

2.2.3.4 *In vitro* stimulation

For *in vitro* stimulation 2×10^6 spleen and lung cells were incubated in 1 ml IMDM complete medium. For antigen specific stimulation 10^{-6} M of the influenza nucleoprotein NP₃₆₆₋₃₇₄ (ASNENVETM) and for unspecific polyclonal stimulation 50 ng/ml phorbol-12-myristate-13-acetate (PMA) and 1 µM ionomycin were used for 4 hours at 37 °C. After 30 min, 10 µg/ml brefeldin A was added to block cytokine secretion.

2.2.3.5 Extracellular and intracellular staining for flow cytometry

1×10⁶ cells were used for staining. To reduce unspecific FcγRII/III binding of the fluorescence antibodies, cells were first incubated in 50 µI FACS buffer with 0.25 µg TruStain fcX (anti-CD16/32) and 1 % normal rat serum (NRS) for 15 min at 4 °C. Subsequently, the corresponding antibody staining mix for surface staining of cells was added and incubated for another 30 min at 4 °C in the dark. The optimal concentration of each antibody was determined before by titration. For elimination of dead cells, the fixable dead/life stain Pacific Orange[™] (1:1000) was added together with the antibody mix. After incubation, cells were washed with FACS buffer (450 g, 5 min, 4 °C) to remove unbound antibodies. Cells could be used directly for flow cytometry or stained intracellularly.

For intracellular staining of cytokines the Fixation/Permeabilisation kit was used. First, cells had to be fixed with 2 % PFA (IC Fixation) for 20 min at RT in the dark. All infected cell
samples were always fixated before measurement. Cells were washed twice with 1x permeabilization buffer (diluted 1:10 in distilled water) in order to permeabilize the cells and expose intracellular antigens. Subsequently, the corresponding antibody mix for cytokine staining was added in 50 μ l 1× permeabilization buffer and incubated for 30 min at RT in the dark. After the incubation, cells were washed again with 1× permeabilization buffer and resuspended in 200-300 μ l FACS buffer.

The staining of the transcription factor Foxp3 was performed using the kit FoxP3/Transcription Factor Staining Buffer Set following the company's instructions. Cells were measured with the LSR/Fortessa flow cytometer.

2.2.3.6 Analysis of flow cytometric data

With flow cytometry it is possible to characterize single cells according to their size, shape and expression of surface markers as well as intracellular cytokines and transcription factors. First, cell populations were distinguished by their size using the forward scatter (FSC) and their granularity using the side scatter (SSC). Then, by the exclusion of dead cells with help of the dead/life stain Pacific OrangeTM, living cells could be detected. The staining with the injected anti-CD45 antibody excluded cells in the vasculature and allowed the analysis of CD45 unstained cells in the parenchyma of specific tissues (Figure 4). Effector CD8⁺ T cells were further identified by the expression of CD8 and CD44 and the absence of CD62L. Effector CD4 helper cells were characterized as CD4⁺ CD44⁺ CD62L^{neg} and regulatory T cells (Tregs) as CD4⁺ Foxp3⁺ (Figure 5). Moreover, dendritic cells (DC) were identified as CD3⁻ CD11c⁺ MHC-II⁺.



Figure 5: Gating strategy for CD4⁺ and CD8⁺ T cells. To identify CD4⁺ T cells, regulatory T cells (Tregs) and CD8⁺ T cells, lung cells were isolated from mice, stained with fluorochrome-labeled antibodies and analyzed via flow cytometry. Via the size (forward scatter, FSC) and granularity (side scatter, SSC) of the cells, debris and dirt could be excluded. Single cells were identified using FSC-H (H=height) and FSC-A (A=aria) or SSC-H and SSC-A as these parameters show a linear correlation for single cells. Living cells were identified by exclusion of dead cells via Pacific OrangeTM dye and auto-fluorescence. Next, CD4⁺ T cells were identified by CD3 and CD4 expression and further, regulatory T cells (Treg) via expression of the transcription factor Foxp3. CD8⁺ T cells were identified by the expression of CD3 and CD8. Activated CD4⁺ and CD8⁺ T cells were determined by expression of CD44 and absence of CD62L. *Foxp3=Forkhead box protein 3*

Influenza virus-specific CD8⁺ T cells were identified using a Dextramer construct from Immudex. The Dextramer consists of a dextran backbone linked to fluorochromes and MHC-I (H-2D^b) molecules. The attached MHC-I molecules contain the IAV nucleoprotein NP₃₆₆₋₃₇₄ and thus, the MHC – NP₃₆₆₋₃₇₄ construct binds to the T cell receptor of influenza-specific CD8⁺ T cells. The linked fluorochromes enable the detection of these virus-specific cells with flow cytometry (Figure 6).



Figure 6: Detection of influenza A virus specific CD8⁺ T cells by flow cytometry. To identify IAV-specific CD8⁺ T cells, lung cells were stained with a Detramer construct consisting of MHC-I molecules binding the IAV nucleoprotein NP₃₆₆₋₃₇₄ and a fluorochrome. Shown are representative dot plots of CD8⁺ T cells from lungs of one uninfected and one IAV infected mouse (8 d p.i.).

2.2.3.7 Compensation of spectral overlay

Prior to sample acquisition, compensation of spectral overlays of the respective fluorochromes used in each panel was performed as described previously (148). Thus, antibodies were coupled to anti-mouse/rat/hamster Ig Kappa (κ) beads diluted in 100 µl FACS buffer depending on the respective host in which the antibody was produced. Antibody concentrations were empirically determined and were between 0.1 and 1.25 µl/sample. Single antibody staining of beads was performed for 20 min at RT in the dark and followed by washing with FACS buffer and centrifugation (450 g, 5 min, 4 °C). For staining with Pacific Orange[™], spleen cells were used and half of the cells were killed at 70 °C for 5 min before staining. In addition unstained beads and cells were used to determine auto-fluorescence. Samples were measured at the LSR/Fortessa in the Compensation Setup mode of FACSDiva software and automated calculation of compensation values was done. For post measurement compensation, 'fluorescence minus one' (FMO) samples were used. FMOs describe the staining of cells with all fluorescent antibodies used in a respective panel minus one antibody.

2.2.3.8 DC - T cell co-culture

To determine the effect of progesterone and glucocorticoids on the DC – T cell cross talk, an in vitro co-culture system was established. On the one hand, spleen cells were isolated from non-pregnant or allogenically pregnant (gd 12.5) mice and incubated with 10⁻⁶ M chicken ovalbumine₂₅₇₋₂₆₄ peptide (OVA, SIINFEKL) for 1 h at 37 °C. Subsequently, cells were washed three times with 50 ml PBS to remove unbound peptide. On the other hand, T cells were isolated from spleens of B6-OT1tg mice using the Pan T cell isolation MACS kit (2.2.2.6). In these mice, CD8⁺ T cells carry a T cell receptor (TCR) specific for OVA. Isolated T cells were stained with the Cell Proliferation Dye eFluor670 to detect proliferation. Thus, cells were resuspended in a 2 µM solution of the proliferation dye and incubated for 10 min at 37 °C. Next, cells were washed and cell number was calculated using a hemocytometer. Peptide pulsed spleen cells (1×10⁵ cells) containing antigen-presenting cells (APCs) and isolated T cells (4×10⁵ cells) were then cultured in a 24 well plate in 1 ml IMDM complete media. Progesterone (10⁻⁶ M), dydrogesterone (10⁻⁶ M) or dexamethasone (10⁻⁶ M) were added, diluted in DMSO. As a control DMSO alone was added for 72 h at 37 °C, 5 % CO₂. Subsequently, cells were stained for flow cytometric analysis with anti-CD8, anti-CD44, anti-CD62L and Pacific OrangeTM to determine dead, activated and proliferated CD8⁺ T cells. Each condition was measured in duplicates.

2.2.3.9 In vitro T cell assay

The effect of hormones on T cells *in vitro* was determined as described previously (149). Thus, spleen cells were isolated from non-pregnant and BALB/c-mated C57BL/6J mice at gd 7.5 and 18.5 as described in 2.2.3.1. After determining the cell number via a hemocytometer, 1×10^6 cells were cultured in each well of a 24 well plate in 1 ml IMDM culture media. Progesterone (10^{-6} M), dydrogesterone (10^{-6} M), corticosterone (10^{-7} M) and dexamethasone (10^{-8} M) diluted in DMSO were added and cells were cultured at 37 °C and 5 % CO₂ for 48 h. As a control DMSO was added alone. Subsequently, cells were stained for flow cytometric analysis with anti-CD4, anti-CD8, anti-CD44, anti-CD62L, anti-Foxp3 and Pacific OrangeTM to detect dead T cells and the frequencies of T cell subsets. Each condition was measured in duplicates.

2.2.4 Molecular biology

2.2.4.1 RNA isolation

In order to analyze the mRNA expression of the C-X-C motive chemokines *Cxcl9*, *Cxcl10* and *Cxcl11*, quantitative RT-PCR was performed. First, RNA was isolated out of lung homogenates of IAV infected allogenically pregnant and non-pregnant mice at 3 and 4 d p.i. as well as uninfected control animals using the RNeasy Plus Universal Mini Kit. Prior to RNA extraction, lung tissue preserved in RNAlater was transferred into micro packaging vials with ceramic beads (1.4 mm) and 700 µl qiazol and homogenized using the Precellys 24 tissue homogenizer. To eliminate DNA, a DNase treatment was performed by addition of 100 µl gDNA Eliminator Solution. Subsequently, chloroform was added and after a centrifugation step at 13.000 rpm for 15 min at 4 °C, the upper clear phase was transferred into a new tube containing 70 % ethanol. The mixed suspension was pipetted onto a column and centrifuged (8000 g, 30 sec). The flow-through was discarded and the column was washed by addition of washing buffers. Next, the column was placed into a new tube and RNA was eluted in 40 µl of RNase free water by a centrifugation at 8000 g for 1 min. To increase the yield, the water was pipetted onto the column once more and centrifuged. RNA concentration was measured at the Infinite 200 PRO NanoQuant reader and RNA was stored at -20 °C.

2.2.4.2 cDNA transcription

DNase treatment of the RNA was performed to increase RNA purity. $5 \mu g$ RNA were incubated with 10×DNase I Buffer, rDNase I, RNase Inhibitor (RNase OUT) at 30 °C for 30 min. Subsequently, DNase Inactivation Reagent was added and incubated for 2 min at RT. After a centrifugation step at 10,000 g for 1.5 min, 30 μ I of the supernatant were

transferred into a new tube. For reverse transcription 1 μ g RNA was mixed with dNTPs and primers (0.25 μ g/ μ I) and incubated for 5 min at 65 °C, then chilled on ice shortly. Subsequently, 8 μ I of the master mix including 5×First-Strand buffer, 0.1 M DTT, RNase OUT (40 U/ μ I) and Superscript II RT (200 U/ μ I) were added and incubated at 25 °C for 10 min, at 42 °C for 50 min and finally at 70 °C for 15 min. The cDNA concentration was measured at the Infinite 200 PRO NanoQuant reader.

2.2.4.3 Quantitative reverse-transcription polymerase chain reaction (qRT-PCR)

Quantification of *Cxcl10* gene expression levels was performed using the StepOnePlus Real-Time PCR System and corresponding software. For the reaction, 100 ng cDNA was used together with the 2×TagMan Universal PCR Master Mix in a final volume of 20 µl following the manufacturers' instructions. Standard cycling conditions were used as demonstrated below.

50 °C 2 min 95 °C 10 min 95 °C 15 sec 60 °C 60 sec 40 cycles

Expression levels of *Cxcl9* and *Cxcl11* genes were quantified using CFX96 Real-Time System and corresponding software. For the amplification, 5 ng cDNA and the Maxima/SYBR Green/Rox qPCR Master Mix were used following the cycling conditions shown below.

95 °C 10 min 95 °C 15 sec 60 °C 60 sec 72 °C 20 sec 40 cycles

All quantifications were run in triplicates and normalized to the expression of the housekeeping gene G*apdh*. For *Cxcl11* only a signal at cycle 38 was obtained using the given concentration. For calculation of relative transcript levels the equation described in (150) was used. The relative gene expression level of the target gene in non-pregnant mice at 3 d p.i. was set as 1.

2.2.4.4 Chromatin immunoprecipitation (Chip) assay

In order to investigate the H3K27 3 methylation (H3K27me3) of *Cxcl9*, *Cxcl10*, *Ccl5* and *Cd8* in lung tissue of pregnant and non-pregnant infected mice at 3 d p.i., a Chip RT-PCR was

used as described previously (132, 151). Whole lung homogenates were first treated with 2 mg collagenase D for 30 min at 37 °C and then cross-linked with 1 % formaldehyde for 15 min at RT. Next, cell lysis and nuclei isolation was achieved by centrifugation. Subsequently, chromatin extraction and fragmentation was performed by sonication to an average length of 100-500 bp using a Bioruptor. A small part of the fragmented chromatin was put aside for preparation of the input controls by phenol-chloroform treatment. For immunoprecipitation, 100 µl of chromatin were first pre-cleared with BSA blocked protein G sepharose beads. Subsequently, chromatin was incubated with 2 µl anti-H3K27me3 antibody for 16 h at 4 °C. As a control chromatin was incubated with normal rabbit IgG. Chromatinimmunocomplexes were further precipitated using unblocked protein G sepharose beads and washed with increasing salt concentrations. Subsequently, chromatin was eluted and decrosslinked for 16 h at 65 °C. gRT-PCR was performed using Rotor-Gene Q-plex and corresponding software. Prior to gRT-PCR analysis of precipitated chromatin, DNA was purified by phenol-chloroform and eluted in ethanol. Gene-specific primers for qRT-PCR were selected according to (90) (Table 11). Positive (Msi1, Ache, Hoxc13) and negative (Gapdh, Atp8b5) controls for H3K27me3 were assigned with the help of USCS Genome Browser. Positive and negative controls showed increased or reduced H3K27me3, respectively within 1000 bp upstream of the transcriptional start site. 1.5 µl of input DNA or precipitated chromatin were used together with SensiMix SYBR Hi-ROX kit and 3 pmol genespecific primers (Table 11) in a reaction volume of 10 μ l at the following cycling conditions.

95 °C 10 min 95 °C 10 sec 57 °C 25 sec 72 °C 20 sec 40 cycles

2.2.5 Histology

2.2.5.1 Preparation of histological slides

Lung tissue samples were fixed in 4 % PFA over night before being embedded in paraffin. Therefore, samples were dehydrated by increasing concentrations of ethanol, then cleared from ethanol by xylene and subsequently infiltrated by paraffin what resulted in embedding in a paraffin block. Subsequently, paraffin-embedded tissue was cut in 4 µm tissue sections using a microtome.

2.2.5.2 Hematoxylin and Eosin (H&E) staining

H&E staining was used to visualize infiltrating immune cells into lungs of infected animals. The Hematoxylin dye is staining basophilic nuclei blue/violet and the Eosin dye is staining eosinophilic cytoplasm red.

Procedure:

Hematoxylin solution	4 min
ddH ₂ O	10 sec
Tap water washing	4 min
Eosin G-solution 1 %	30 sec
3× ddH ₂ O	15 sec
2× ethanol 70 %	15 sec
2× ethanol 90 %	15 sec
2× ethanol 100 %	15 sec
3× xylene	5 min

Quantification of inflamed area in H&E-stained lung tissue slides from infected mice was performed using the Panoramic Viewer. Inflamed area, characterized by infiltrated leukocytes, proteins and erythrocytes was determined in relation to total lung area.

2.2.5.3 NP immunohistochemistry staining

Detection of virus infected cells was facilitated by a polyclonal rabbit anti-FPV serum generated against the chicken H7N1 virus A/FPV(fowl plaque virus)/Rostock/34. After treatment of paraffin-embedded tissue sections with 0.1 M citrate buffer (pH 6.0), the primary rabbit serum was added that was detected by a biotin-conjugated anti-rabbit antibody. Finally, using the Zytochem-Plus HPR kit enabled viral detection. For histopathological analysis, tissues were counterstained with hematoxylin. The number of infected pulmonary bronchioles was determined and calculated in relation to the total number of bronchioles.

3 Results

The results of 3.1 to 3.4 including Figures 7-14, 19-22 were published in (132) with shared first authorship between Dr. Géraldine Engels, Alexandra Maximiliane Hierweger, Dr. Julia Hoffmann and Dr. René Thieme. The manuscript is attached in the Appendix. In the present thesis, the published results that were predominantly generated by myself are presented, along with yet unpublished data that I generated.

3.1 Establishment of an allogenic pregnancy mouse model to study influenza A virus infections

To study influenza A virus (IAV) infections during pregnancy, we established a new allogenic pregnancy mouse model that mimics observations in pregnant women with severe course of IAV infection. Therefore, C57BL/6J females were mated to BALB/c males. At gestational day (gd) 12.5, mice were infected intranasally with 10^3 plaque forming units (p.f.u.) of a human H1N1 virus from the pandemic in 2009 (A/Hamburg/NY1580/09, 2009 pH1N1). The mouse lethal dose for 2009 pH1N1, when 50 % of mice died upon infection, (MLD₅₀) was $10^{3.83}$ p.f.u.. As a mouse pregnancy lasts 18 - 20 days, this resembles the end of the second/beginning of the third trimester of pregnancy in mice. To compare the allogenic mating model with a syngenic mating strategy, we included C57BL/6J females mated to C57BL/6J males that were also infected with 10^3 p.f.u. 2009 pH1N1 on gd 12.5 (Figure 7).



Figure 7: Pregnancy mouse model to study influenza A virus infections. C57BL/6J females were either mated allogenically to BALB/c males or syngenically to C57BL/6J males. On gestational day (gd) 12.5, pregnant mice were infected with 10³ plaque forming units (p.f.u.) of 2009 pH1N1. As controls, non-pregnant mice were infected with 10³ p.f.u. of 2009 pH1N1. In addition pregnant and non-pregnant mice were treated with PBS. For the subsequent 14 days post infection (d p.i.), survival and weight loss were monitored.

Using this infection mouse model, infected allogenically pregnant mice demonstrated increased lethality (80 %) (Figure 8A) compared to non-pregnant (0 % lethality) (Figure 8B)

and even to syngenically mated pregnant mice (60 % lethality) (Figure 8A). Whereas surviving syngenically pregnant and non-pregnant mice fully recovered from infection within 14 days post infection (d p.i.) (Figure 8C,E), allogenically pregnant mice showed prolonged weight loss – a measurement for morbidity (Figure 8D). The weight loss occurring around 6 d p.i. in pregnant infected and PBS treated mice was due to parturition. Increased disease severity was indicated by elevated virus titers and lung pathology in allogenically pregnant infected mice. At 3 d p.i., all infected mice contained high virus titers in the lung. Whereas at 6 d p.i., non-pregnant and syngenically pregnant mice were able to clear the virus significantly, allogenically pregnant mice still exhibited high virus titers in the lung (Figure 9A). In the cohort of allogenically pregnant IAV infected mice, more animals presented systemic virus titers - assessed in the gut, whereas, no virus titers were detected in the placenta or concepti (Figure 9B). Allogenically and syngenically pregnant infected mice exhibited increased inflamed areas and virus infected bronchioles in the lungs at 3 d p.i. compared to non-pregnant infected littermates as assessed by H&E and NP (polyclonal staining of IAV) staining, respectively (Figure 9C-E). As allogenic pregnancies show a more severe course of infection and in general display more closely the complexity of a human pregnancy, we conducted all subsequent experiments exclusively within allogenically mated mice. Using this mouse model mirroring the observations seen in the clinic, mechanistic links between maternal immune adaptation to pregnancy and enhanced lethality upon IAV infection could be further clarified.



Figure 8: Influenza A virus infection of non-pregnant, syngenically pregnant and allogenically pregnant mice. Non-pregnant (n=5), syngenically pregnant (n=15) and allogenically pregnant (n=18) mice were infected with 10^3 plaque forming units (p.f.u.) of 2009 pH1N1; pregnant mice on gestational day (gd) 12.5. Survival of allogenically pregnant, syngenically pregnant (A) and non-pregnant mice (B) was determined for the subsequent 14 days post infection (d p.i.). Weight loss of syngenically pregnant (C), allogenically pregnant (D) and non-pregnant mice (E) was assessed within 14 d p.i.. Shown are means ± SEM. Statistical significance was calculated using the Gehan-Brelow-Wilcoxon or the Student's *t* test with p < 0.05, p < 0.01, p < 0.001. Data were generated in cooperation with Dr. Géraldine Engels, Dr. René Thieme and Dr. Julia Hoffmann. Modified as published in (132).



Figure 9: Influenza A virus induced pathogenicity in non-pregnant, syngenically pregnant and allogenically pregnant mice. Non-pregnant (n=5), syngenically pregnant (n=15) and allogenically pregnant (n=18) mice were infected with 10^3 p.f.u. of 2009 pH1N1; pregnant mice on gd 12.5. Virus titers were determined in the lung (A), gut, placenta and concepti (B) at 3 and 6 d p.i.. Histological assessment of inflamed areas (C) and NP⁺, infected bronchioles (polyclonal staining against IAV) (D) in lung tissue isolated from infected mice at 3 d p.i.. (E) Representative images of infected lung tissue stained with H&E and NP. (A-D) Shown are means ± SEM. Statistical significance was calculated using the Student's *t* test with p < 0.05. Data were generated in cooperation with Dr. Géraldine Engels, Dr. René Thieme and Dr. Julia Hoffmann. Modified as published in (132).

3.2 Investigation of the innate immune response in allogenically pregnant influenza infected mice

3.2.1 Pregnant mice exhibit reduced pro-inflammatory cytokine production upon influenza virus infection

In this thesis, the immune response of allogenically mated pregnant females towards IAV was investigated. Infection with the IAV normally leads to a strong immediate innate immune response in mice. To test if the initiation of the innate immunity is different in pregnant mice, we used the allogenic mating model. Previous RNA sequencing (RNAseq) data from our

laboratory had indicated a general down regulation of various innate response genes in pregnant compared to non-pregnant IAV infected mice at 3 and 6 d p.i. (132). To validate the RNAseq data, C57BL/6J female mice were mated with BALB/c males and infected with 10^3 p.f.u. of the 2009 pH1N1 virus on gd 12.5 (Figure 10A). As controls, we used non-pregnant, virgin mice that were also infected with IAV or pregnant and non-pregnant mice treated with PBS. At 3 d p.i., mice were sacrificed, lung tissue was isolated and the expression of pro-inflammatory cytokines was measured in the lung. Pro-inflammatory cytokine expression was overall increased upon infection (data not shown). The expression of IFN- α , IFN- γ , TNF- α and IL-6 was reduced in the lungs of pregnant infected mice compared to lungs of non-pregnant infected littermates (Figure 10B-E).



Figure 10: Cytokine production in lungs of infected mice. (A) Allogenically mated (n=12) and virgin (n=11) C57BL/6J mice were infected with 10^3 p.f.u. of 2009 pH1N1 (pregnant mice on gd 12.5) and lungs were harvested at 3 d p.i.. (B) Lung homogenates were analyzed by Luminex for the expression of pro-inflammatory cytokines IFN- α , IFN- γ , TNF- α and IL-6. Values were normalized to the organ weight. If the number of measurement points depicted in the graph does not correspond to the indicated n, the ones not shown were below the range of the standard curve and thus, considered as out of range. (B) Shown are means ± SEM. Statistical significance was calculated with Student's *t* test with p < 0.05, p < 0.01, p < 0.001. Data were generated in cooperation with Dr. Swantje Thiele and published in (132).

3.2.2 Impaired activation of antigen-presenting cells in pregnant IAV infected mice

Pro-inflammatory cytokines are produced early after infection by infected epithelial cells and innate immune cells like macrophages, monocytes, dendritic cells (DCs) and Natural Killer cells (NK cells) and provide the first part of the anti-viral innate immune response by, for example inducing the activation of antigen presenting cells (APCs). Hence, we investigated the activation of different macrophage and DC subsets in pregnant mice at 3 d p.i. (Figure Pregnancy and influenza 42

11A), as these cells can act as professional APCs and initiate the adaptive immune response. The predominant macrophage population in the lung are alveolar macrophages (alvMac) being characterized as CD11c⁺, CD11b^{neg}, CD103^{neg}. On the other hand, there are several distinct DC subsets present in the lung e.g. tissue-resident DCs (CD11c⁺, CD11b⁺, CD103^{neg}, Gr-1^{neg}) and migratory DCs (CD11c⁺, CD11b^{neg}, CD103⁺), the latter migrating to the lung-draining lymph nodes upon infection. Plasmacytoid DCs (pDC), which are characterized as CD11c^{dim} CD45R⁺, are major producers of IFN-α but they are poor in antigen presentation. Monocyte-derived inflammatory DCs (CD11c⁺, CD11b⁺, Gr-1⁺, CD103^{neg}) are recruited into the lung upon infection (42). To assess the activation of these DC subsets and macrophages during IAV infection in pregnant mice, we analyzed the frequency of cells expressing the costimulatory molecules CD40, CD80 and CD86 that are crucial for the cross-talk with and priming of T cells. The gating strategy is shown in Figure 11B and Figure 11C. The frequency of DCs and macrophages expressing costimulatory molecules was generally increased upon infection (Figure 12A, data not shown). Reduced frequencies of alvMac expressing CD40 and CD86 were observed in lungs of pregnant compared to lungs of non-pregnant infected mice (Figure 12B). Strikingly, significantly reduced frequencies of resident DCs expressing all three costimulatory molecules (CD40, CD80, CD86) tested could be isolated from lungs of pregnant infected mice 3 d p.i in comparison to lungs of non-pregnant infected littermates (Figure 12A, C). In pregnant infected mice slightly less pDCs expressed CD40 (Figure 12D). Almost 100 % of inflammatory DCs and migratory DCs expressed CD40, CD80 and CD86 upon infection in pregnant and non-pregnant mice, without detectable differences (Figure 12E, F).

The reduced production of pro-inflammatory cytokines and the reduced frequency of activated DCs and macrophages in lungs of pregnant infected mice suggest that the innate, first line of defense is impaired in pregnant IAV infected mice and thus, an adequate activation of innate immune cells could be compromised early after infection.



Figure 11: Gating strategy to detect macrophage and dendritic cell (DC) populations in the lung of infected animals. (A) BALB/c-mated C57BL/6J females were infected with 10³ p.f.u. of 2009 pH1N1 on gd 12.5 and lungs were harvested at 3 d p.i.. Control animals were treated with PBS. (B) Alveolar macrophages (alvMac; CD11c^{high}, CD11b^{neg}, CD103^{neg}), migratory DCs (migrDC; CD11c^{high}, CD11b^{neg}, CD103⁺), resident DCs (resDC; CD11c^{high}, CD11b⁺, CD103^{neg}, Gr-1^{neg}), inflammatory DCs (inflDC; CD11c^{high}, CD11b⁺, CD103^{neg}, Gr-1⁺) and plasmacytoid DCs (pDC; CD11c^{dim}, CD45R⁺) were distinguished by flow cytometry based on the characterization published in (42). (C) Representative dot plots of lung cells from one uninfected and one infected mouse.



Figure 12: Expression of costimulatory molecules on macrophage and dendritic cell subsets in the lung of infected mice. Allogenically pregnant (n=10) and non-pregnant (n=8) C57BL/6J mice were infected with 10^3 p.f.u. of 2009 pH1N1 (pregnant mice on gd 12.5). Lungs were harvested at 3 d p.i. and the expression of the costimulatory molecules CD40, CD86 and CD80 was analyzed by flow cytometry – representative histograms (A) – on alveolar macrophages (alvMac) (B), resident DCs (resDC) (C), plasmacytoid DCs (pDC) (D), migratory DCs (migrDC) (E) and inflammatory DCs (inflDC) (F). (B-F) Shown are means ± SEM. Statistical significance was calculated using Mann-Whitney *U* test with p < 0.05, p < 0.01. Results were published in (132)

3.3 Investigation of the adaptive immune response in allogenically pregnant influenza infected mice

3.3.1 Impaired migration of CD8⁺ T cells to the lung in pregnant IAV infected mice

We hypothesized that the impaired innate immune response in pregnant IAV infected mice could lead to incomplete priming of the T cell response. Indeed, we observed decreased numbers of total leukocytes and CD8⁺ T cells in lungs of pregnant infected mice at 4 d p.i. (Figure 13A-D). Nancy and colleagues showed that the expression of C-X-C motif chemokine ligands, namely *Cxcl9*, *Cxcl10* and *Cxcl11* was epigenetically repressed in the decidua during pregnancy. This downregulation limited the recruitment of activated T cells expressing

their cognate receptor CXCR3 to the decidua (90). As progesterone was claimed to be causative for chemokine silencing in the decidua and progesterone concentrations rise also in the periphery during pregnancy, we hypothesized that chemokine ligand expression could also be altered in lungs of pregnant mice. Therefore, we analyzed the expression of Cxcl9 and Cxcl10 in lungs of allogenically pregnant and non-pregnant IAV infected mice at 3 and 4 d p.i. (Figure 13A). The expression of Cxcl11 was not investigated because, in C57BL/6J mice, Cxcl11 is a pseudogene and therefore, it is not translated (152). mRNA expression of Cxcl9 and Cxcl10 was enhanced upon IAV infection (data not shown). Indeed, we could demonstrate reduced mRNA expression of Cxcl9 and Cxcl10 in lungs of pregnant IAV infected dams at 3 and 4 d p.i. compared to non-pregnant infected mice (Figure 13E, F). Based on the study by Nancy et al. showing an epigenetic modification of Cxcl9, Cxcl10 and Ccl5 chemokine ligand promoter regions by repressive H3K27 methylation (H3K27me3) in the decidua, we tested the methylation of Cxcl9, Cxcl10 and Ccl5 promoters in the lungs of our mice. However, no pregnancy-induced alteration in H3K27 methylation at Cxcl9, Cxcl10 and Ccl5 promoter regions were found in our infected mice (Figure 13G). At Cd8 promoter region H3K27 methylation was comparable in tissue of pregnant and non pregnant mice isolated from the uterus (90) or the lung. Msi1, Ache and Hoxc13 were used as positive controls and Gapdh and Atp8b5 were used as negative controls as these genes contain a known H3K27me3 enrichment or absence, respectively. For pulldown controls with IgG only, *Hoxc13* IgG is shown as a representative example (Figure 13G).



Figure 13: Expression of *Cxcl9* **and** *Cxcl10* **in lungs of infected mice.** (A) BALB/c-mated pregnant C57BL/6J and non-pregnant mice were infected with 10^3 p.f.u. of 2009 pH1N1; pregnant mice on gd 12.5. At 4 d p.i., lung tissue was analyzed for total leukocyte (B) and CD8⁺ T cell numbers (C) in pregnant (n=19) and non-pregnant (n=12) mice as shown in the representative dot plots (D). *Cxcl10* (E) and *Cxcl9* (F) relative mRNA expression was determined at 3 and 4 d p.i. in pregnant (n=9-17 at 3 d p.i. and n=25 at 4 d p.i.) and non-pregnant (n=15-25 at 3 d p.i. and n=35 at 4 d p.i.) mice by qRT-PCR. (G) Assessment of H3K27me3 at the promoter regions of *Cxcl9*, *Cxcl10*, *Ccl5* and *Cd8. Ex vivo* CHIP qRT-PCR was performed on total lung homogenates of non-pregnant (n=5) and pregnant (n=5) mice at 3 d p.i.. *Msi1*, *Ache* and *Hoxc13* were used as positive controls, *Gapdh* and *Atp8b5* as negative controls. *Hoxc13* IgG is representatively shown for pulldown with IgG antibody only. Shown is the percentage of recovered material in relation to the starting material. Shown are means ± SEM. Statistical significance was calculated using Mann-Whitney U test with p < 0.05, p < 0.01. (G) Data was generated in cooperation with Svenja Siebels. Data was published in (132).

Based on the observation of reduced expression of chemokine ligands *Cxcl9* and *Cxcl10*, we next aimed to investigate the migration of T cells to the lung upon infection in pregnant and non-pregnant mice. First, we infected congenic C57BL/6J non-pregnant mice expressing CD90.1 (B6-THY1aPL). At 8 d p.i., spleen and lung-draining lymph nodes were harvested and T cells were isolated. Subsequently, the isolated T cells were adoptively transferred into

allogenically pregnant and non-pregnant IAV infected mice expressing CD90.2 at 3 d p.i. (Figure 14A). 18 h after the transfer (4 d p.i.), significantly less CXCR3⁺CD8⁺ donor derived (CD90.1⁺) T cells had migrated into the lungs of pregnant compared to non-pregnant infected mice (Figure 14B, C).

Thus, reduced chemokine ligand expression in lung of pregnant infected mice resulted in reduced migration of activated CD8⁺ T cells to the target site of IAV infection – the lung.



Figure 14: T cell migration during influenza virus infection. (A) Donor CD90.1⁺ mice were infected with 10^3 p.f.u. of 2009 pH1N1 and T cells were isolated out of spleen and lung-draining lymph nodes at 8 d p.i.. Isolated T cells were adoptively transferred into CD90.2⁺ recipient non-pregnant and allogenically pregnant mice at 3 d p.i.. (B) 18 h post transfer CXCR3⁺ CD8⁺ donor derived (CD90.1⁺) T cells were analyzed in the lungs of non-pregnant (n=12) and pregnant (n=19) mice. (C) Representative dot plots are shown gated on donor derived (CD90.1⁺) CD8⁺ T cells. (B) Shown are means ± SEM. Statistical significance was calculated using Mann-Whitney *U* test with p < 0.05. Data was published in (132).

3.3.2 Comparable T cell proliferation in pregnant and non-pregnant IAV infected mice

At 4 d p.i., reduced numbers of CD8⁺ T cells were present in lungs of IAV infected pregnant mice compared to non-pregnant littermates. However, previous results from our laboratory

indicate that pregnant mice exhibit increased numbers of activated and virus-specific CD8⁺ T cells in the lungs at 6 and 8 d p.i. (132). Since we did not observe increased migration of T cells in pregnant infected mice, we investigated whether the increase of virus-specific T cells at a later time point of infection is mediated by enhanced proliferation of these cells in pregnant infected hosts. We first infected BALB/c mated pregnant C57BL/6J females with 2009 pH1N1 on gd 12.5. At 7 d p.i., we administered bromodeoxyuridin (BrdU) to infected pregnant and non-pregnant mice. BrdU is a uridine analogue that is incorporated into newly synthesized DNA upon replication and can be stained for flow cytometry. 18 hours after BrdU administration, the mice were sacrificed and CD8⁺ T cells in the lung were analyzed for BrdU incorporation that correlates with proliferation (Figure 15A). Shortly before sacrificing the mice, we injected a fluorochrome-labeled anti-CD45 antibody retrobulbary (r.b.) into the mice. The administered anti-CD45 antibody binds to leukocytes in the blood vasculature. Due to the short incubation time (3 min), leukocytes in the parenchyma are not stained. Therefore, this approach enabled us to distinguish the immune cells inside the lung parenchyma from cells in blood vessels of the lung (Figure 15B). We observed an increase in proliferation of CD8⁺ T cells in the lung parenchyma of infected compared to uninfected mice (Figure 15C, D). However, similar BrdU accumulation and therefore, similar proliferation rates of CD8⁺ T cells inside the lung parenchyma (CD45 unstained) were detected in pregnant and non-pregnant infected mice (Figure 15C, D).

Consequently, the increased numbers of virus-specific $CD8^+$ T cells in lungs of pregnant infected mice at 6 and 8 d p.i. cannot be attributed to an enhanced proliferation of these cells between 7 and 8 d p.i.

49



Figure 15: Proliferation of CD8⁺ T cells in lungs of infected mice. (A) Allogenically pregnant (n=9) and nonpregnant (n=9) C57BL/6J females were infected with 10^3 p.f.u. of 2009 pH1N1 – pregnant mice on gd 12.5. Control mice were treated with PBS (n=5). At 7 d p.i., bromodeoxyruridine (BrdU) was administered intraperitoneally. 18 h after BrdU injection (8 d p.i.) mice were sacrificed. (B) Three minutes before sacrifice, a fluorochrome-labeled anti-CD45 antibody was applied retrobulbary to distinguish cells in lung parenchyma from circulating leukocytes by flow cytometry as depicted in the respective dot plots. (C) Subsequently, lung tissue was harvested and the frequency of proliferated CD8⁺ T cells was analyzed, measured by BrdU incorporation. (D) Shown are representative histograms of CD45 unstained CD8⁺ T cells. (C) Shown are means \pm SEM.

3.3.3 Increased frequencies of CD8⁺ T cells expressing pro-inflammatory cytokines in lungs of pregnant infected dams

Pregnant mice succumb to the IAV infection around 6 - 8 d p.i. – the time point when the T cell response is established and contributes crucially to virus defense. Moreover, we observed that the innate immune response is impaired in pregnant infected mice. Hence, we hypothesized that this leads to an altered priming of the T cell response and consequently, to high lethality in pregnant infected mice. To this end, we investigated the frequency of CD4⁺ and CD8⁺ T cells expressing pro-inflammatory cytokines in pregnant and non-pregnant mice 6 and 8 days post IAV infection (Figure 16A). For distinguishing between cells in the lung parenchyma and those circulating in the vasculature, a fluorochrome-labeled anti-CD45 antibody was injected 3 min before sacrificing the mice. As only few T cells express cytokines ex *vivo*, we stimulated CD4⁺ T cells with PMA/ionomycin for 4 h. The PMA/ionomycin stimulation enabled us, to investigate T cells exhibiting effector functions that were recruited to the lung upon IAV infection. We observed that the frequency of CD4⁺ T cells in the lung parenchyma (CD45 unstained) expressing the pro-inflammatory cytokines



IFN- γ and TNF- α was comparable in pregnant and non-pregnant mice at 6 and 8 d p.i. after ex vivo polyclonal stimulation with PMA/ionomycin for 4 hours (Figure 16B-D).



Figure 16: Frequency of CD4⁺ T cells expressing pro-inflammatory cytokines after influenza infection. (A) BALB/c-mated C57BL/6J pregnant (n=14-16) and non-pregnant (n=12-13) mice were infected with 10³ p.f.u. of 2009 pH1N1; infection of pregnant mice took place on gd 12.5. At 6 and 8 d p.i., mice were sacrificed and the lungs were harvested. Three minutes before sacrifice, mice were injected with a fluorochrome-labeled anti-CD45 antibody to identify leukocytes inside the lung parenchyma (CD45 unstained). (B) Representative dot plots for cytokine staining of CD45 unstained CD4⁺ T cells after ex vivo PMA/ionomycin stimulation are shown isolated at 6 d p.i.. The expression of IFN- γ and TNF- α was analyzed at 6 (C) and 8 (D) d p.i. in CD45 unstained CD4⁺ T cells after 4 h ex vivo PMA/ionomycin stimulation. For (C,D) means ± SEM are shown.

Regarding CD8⁺ T cells, a tendency towards an increased frequency of IFN-y expressing cells was observed after 4 h stimulation with PMA and ionomycin at 6 and 8 d p.i. (Figure 17A-C). Further, ex vivo stimulation with the immunodominant IAV NP₃₆₆₋₃₇₄ peptide facilitates the detection of NP-specific CD8⁺ effector T cells. Importantly, increased frequencies of CD8⁺ T cells expressing IFN-y and TNF- α or IFN-y alone in pregnant infected dams could be observed most strikingly at 6 d p.i. after 4 h stimulation with the NP₃₆₆₋₃₇₄ peptide (Figure 17A, D). At 8 d p.i., the frequency of CD8⁺ T cells expressing IFN- γ and TNF- α was overall declined and differences between pregnant and non-pregnant mice were smaller (Figure 17E). In addition, CD8⁺ T cells expressing granzyme B – an enzyme involved in clearance of infected cells - were significantly enriched in lungs of pregnant mice at 6 and 8 d p.i. (Figure 17A, F, G). This suggests that, during pregnancy, the adaptive immune response is not generally suppressed upon IAV infection and even increased, thereby potentially aggravating lung immunopathology in pregnant infected mice.



Figure 17: Frequency of CD8⁺ T cells expressing pro-inflammatory cytokines after influenza infection. Mice were infected with 10³ p.f.u. of 2009 pH1N1; infection of pregnant mice took place on gd 12.5. Six and eight d p.i., allogenically pregnant (n=14-16) and non-pregnant (n=12-13) mice were sacrificed and the lungs were harvested. Three minutes before sacrifice, mice were injected with a fluorochrome-labeled anti-CD45 antibody to identify leukocytes inside the lung parenchyma. (A) Representative dot plots for cytokine and granzyme B staining of CD45 unstained CD8⁺ T cells are shown isolated at 6 d p.i.; upper panels after 4 h PMA/ionomycin stimulation, middle panels after 4 h NP₃₆₆₋₃₇₄ stimulation and lower panels without *ex vivo* stimulation. The expression of INF- γ and TNF- α was analyzed in CD45 unstained CD8⁺ T cells after 4 h stimulation with either PMA/ionomycin (B,C) or an immunodominant IAV peptide (NP₃₆₆₋₃₇₄) (D,E) at 6 and 8 d p.i.. The expression of granzyme B was determined in CD8 T⁺ cells at 6 (F) and 8 (G) d p.i. without *ex vivo* stimulation. For (B-G) means ± SEM are shown. Statistical significance was calculated using the Mann-Whitney U test (p < 0.05, p < 0.01, p < 0.001).

3.3.4 Increased frequencies of T cells expressing the anti-inflammatory cytokine IL-10 in pregnant infected dams

Upon excessive and prolonged activation, CD4⁺ and CD8⁺ T cells start producing the antiinflammatory cytokine IL-10 (153, 154). IL-10 dampens the immune response and prevents overshooting immunity with excessive immunopathology. IL-10 is predominantly expressed by regulatory T cells (Tregs) that are characterized by the expression of the transcription factor Forkhead box protein 3 (Foxp3). We analyzed the production of IL-10 by CD4⁺,CD8⁺ T cells and CD4⁺Tregs. To this end, we used mice containing reporters for Foxp3 (FOXP3-IRES-mRFP, Fir mice (141)) and IL-10 (GFP, Tiger mice (142)) that allow a direct detection of Tregs and IL-10 producing cells. The C57BL/6J Fir/Tiger mice were allogenically mated and infected with 10³ p.f.u. of 2009 pH1N1 on gd 12.5 (Figure 18A). At 8 d p.i., CD8⁺, CD4⁺ T cells and CD4⁺ Tregs from the lung were analyzed for their IL-10 production. Detection of cells inside the lung parenchyma was facilitated by anti-CD45 antibody application to mice shortly before sacrifice. Virus-specific CD8⁺ T cells were detected via staining with a dextramer. The dextramer construct consists of a dextran backbone binding H-2D^b MHC-I molecules that harbor the immunodominant NP₃₆₆₋₃₇₄ peptide of IAV. MHC - NP₃₆₆₋₃₇₄ complexes are recognized and bound by NP₃₆₆₋₃₇₄-specific CD8⁺ T cells. Since the dextran backbone is in addition coupled to a fluorochrome, NP₃₆₆₋₃₇₄ reactive CD8⁺ T cells can be specifically visualized by flow cytometry (Figure 18B). Significantly increased frequencies of conventional (Foxp3^{neg}) CD4⁺ T cells (Tcon) expressing IL-10 could be detected in pregnant infected dams at 8 d p.i. compared to non-pregnant infected littermates (Figure 18C, D). Further, we could show a tendency towards more virus-specific (detramer⁺) activated (CD44⁺ CD62L^{neg}) CD8⁺ T cells producing IL-10 in pregnant infected mice (Figure 18C, E). Although total Treg (Foxp3⁺) frequencies were comparable in infected pregnant and non-pregnant mice, the frequency of IL-10 expressing Tregs was slightly increased in lungs of pregnant infected mice (Figure 18C, F, G).

Taken together, our findings indicate that, at 8 d p.i. pregnant mice exhibit increased frequencies of T cells expressing pro-inflammatory (IFN- γ , TNF- α) cytokines but also anti-inflammatory (IL-10) cytokine in the lung than non-pregnant littermates.



Figure 18: Frequency of IL-10 producing CD4⁺ and CD8⁺ T cells after influenza infection. (A) Allogenically pregnant (n=5) and non-pregnant (n=4) Fir/Tiger mice harboring reporters for Foxp3 and IL-10 were infected with 10^3 p.f.u. of 2009 pH1N1. Pregnant mice were infected on gd 12.5. At 8 d p.i., mice were sacrificed, three minutes after administration of an anti-CD45 antibody. (B) Representative dot plots to detect virus-specific CD8⁺ T cells using dextramer staining. The expression of IL-10 was determined as shown in representative dot plots (C) in CD45 unstained conventional CD4⁺ (Foxp3^{neg}) T cells (Tcon) (D) and CD45 unstained virus-specific (dextramer⁺) activated CD8⁺ (CD44⁺ CD62L^{neg}) T cells (E). Moreover, the frequency of Foxp3⁺ regulatory T cells (Tregs) (F) and the IL-10 production by these cells (G) was analyzed. (D-G) Shown are means ± SEM. The Mann-Whitney *U* test was used to calculate statistical significance (p < 0.05).

3.3.5 Preexisting acquired immunity rescues pregnant mice from lethal IAV infection

Since pregnant women are highly vulnerable to IAV infection, the WHO recommends influenza vaccination to pregnant women as the first priority (20). Vaccination was shown to be effective and safe during pregnancy, protecting both the mother and child from severe IAV infections (21-23). As a next step, we aimed to investigate whether in our IAV mouse model

that resembles clinical observations, preexisting immunity against IAV can protect pregnant mice from lethal IAV infection. Since IAV vaccination was not established in our laboratory, we chose previous infection before pregnancy to generate immunity against IAV as shown in Figure 19A. Non-pregnant C57BL/6J females were infected with 10³ p.f.u. of 2009 pH1N1; controls were treated with PBS. After full recovery (28 days), mice were mated to BALB/c males and infected a second time with the same dose of 2009 pH1N1 (10³ p.f.u.) on gd 12.5. Interestingly, infected pregnant mice with preexisting immunity due to prior infection showed reduced lethality as all animals survived the second infection and demonstrated no weight loss. However, pregnant infected mice that had not been infected before showed a mortality rate of approximately 20 % and remarkable weight loss during infection (Figure 19B, C). Protective neutralizing antibodies against IAV (HAI titer \geq 40) were detected in all dams that were infected during the first and/or second infection time point at comparable levels (Figure 20A). Mice being infected for the first time during pregnancy contained high virus titers in the lung at 3 d p.i., whereas, mice with preexisting immunity had already cleared the virus at 3 d p.i., as demonstrated by absent virus titers in their lungs (Figure 20B). Additionally, we analyzed the activation of the innate immune system in these animals by measuring the expression of costimulatory molecules on macrophages and different subsets of DCs in the lung at 3 d p.i.. We observed reduced frequencies of CD40, CD80 and CD86 expressing cells among almost all macrophage and DC subsets tested in mice with preexisting immunity in comparison to mice without immunity against IAV (Figure 20C-G). Pro-inflammatory cytokine (IFN-a, TNF-a, IL-6) concentrations, except IFN-y concentration, were also reduced in the lungs of dams with preexisting immunity at 3 d p.i. (Figure 20H-K).

These results demonstrate that preexisting immunity can protect pregnant mice from lethal IAV infection with the same subtype during pregnancy as the virus is rapidly cleared and the innate immune response is already reduced at 3 d p.i..



Figure 19: Secondary IAV infection during pregnancy in mice. (A) Non-pregnant mice were infected with 10^3 p.f.u. of 2009 pH1N1 or treated with PBS. After viral clearance (day 28), mice were allogenically mated and infected for a second time on gd 12.5. Survival (B) and weight loss (C) were determined for 14 days post secondary infection (non-pregnant PBS – pregnant pH1N1 n=13, non-pregnant pH1N1 – pregnant pH1N1 n=10) compared to non infected controls (non-pregnant PBS – pregnant PBS n=5, non-pregnant pH1N1 – pregnant PBS n=5). (B-C) represents means ± SEM. Statistical significance was calculated using Student's t-test (p < 0.05, p < 0.01, p < 0.001). Results were generated in cooperation with Dr. Géraldine Engels and published in (132).



Figure 20: Pathogenicity of IAV infection after secondary infection during pregnancy. (A) Neutralizing antibody titers were determined in serum at day 28 and 69 after first and second IAV infection with 10³ p.f.u. of 2009 pH1N1 (d28 uninfected n=45, infected n=42; d69 uninfected controls n=5 each, non-pregnant PBS pregnant pH1N1 n=13, non-pregnant pH1N1 - pregnant pH1N1 n=10). Shown are geometric mean titers (GMT) (B) Virus titers were measured in the lung at day 44 in uninfected controls (n=5 each), non-pregnant PBS pregnant pH1N1 (n=11) and non-pregnant pH1N1 - pregnant pH1N1 (n=9). The expression of costimulatory molecules on alveolar macrophages (alvMac) (C), resident dendritic cell (resDC) (D), inflammatory DC (inflDC) (E), migratory DC (migrDC) (F) and plasmacytoid DC (pDC) (G) was assessed at day 44 in the lungs of nonpregnant PBS - pregnant pH1N1 (n=11) and non-pregnant pH1N1 - pregnant pH1N1 (n=9) mice. (H-K) Proinflammatory cytokine expression (IFN- α , IFN- γ , IL-6 and TNF- α) was analyzed at day 44 in lungs of nonpregnant PBS - pregnant pH1N1 (n=11) and non-pregnant pH1N1 - pregnant pH1N1 mice (n=9) by Luminex assay. Values were normalized to organ weight. If the number of measurement points depicted in the graph does not correspond to the indicated n, the ones not shown were below the range of the standard curve and thus, considered as out of range. Shown are means ± SEM. Statistical significance was calculated using Student's t test or Mann-Whitney U test (p < 0.05, p < 0.01, p < 0.001). Results were generated in cooperation with Dr. Géraldine Engels and Dr. Swantje Thiele and published in (132).

3.4 Investigation of the effect of progesterone on the immune response towards IAV

3.4.1 Administration of a progesterone derivative to non-pregnant mice increased IAV-related lethality in mice

Pregnant mice show elevated morbidity and mortality upon IAV infection. During pregnancy hormonal changes mediate physiological and immunological adaptation to pregnancy (76). Especially progesterone is known for its immune modulatory function and increases at the feto-maternal interface and in serum during pregnancy (93). Therefore, we hypothesized that high systemic progesterone levels during pregnancy could be involved in immune modulation and finally, in increased lethality of pregnant mice upon IAV infection. To test this hypothesis, we injected dydrogesterone, a progesterone derivative with high affinity and selectivity for the progesterone receptor (PR), into non-pregnant mice every second day for 12 days. Subsequently, mice were infected with 10³ p.f.u. of 2009 pH1N1 and dydrogesterone application was continued (Figure 21A). Mice receiving dydrogesterone showed increased mortality after IAV infection, as 50 % of the dams succumbed to the infection. Control animals treated with the vehicle substance only showed 10 % lethality after infection (Figure 21B). However, no difference in regard of the weight loss was observed between infected animals receiving dydrogesterone or controls (Figure 21C). Furthermore, we measured the frequency of macrophages and DCs expressing costimulatory molecules in the lung. No difference in the frequency of CD40, CD80 and CD86 expressing alvMac and resident DCs between dydrogesterone- and vehicle-treated infected littermates was detected (Figure 21D, E).



Figure 21: Influence of dydrogesterone injection on the pathogenicity of influenza infections. (A) Nonpregnant mice were injected with 1.25 mg dydrogesterone (Dydro) subcutaneously every second day for 12 days. Controls were treated with the vehicle substance only. After infecting the mice with 10^3 p.f.u. of 2009 pH1N1, survival (B) and weight loss (C) were determined in dydrogesterone-treated (n=12) and vehicle-treated (n=9) mice compared to uninfected controls (n=5 each). Expression of costimulatory molecules was determined in alveolar macrophages (alvMac) (D) and resident dendritic cells (resDC) (E) in lungs of dydrogesterone (n=12) and vehicle (n=9) treated mice at 3 d p.i.. (B-E) Shown are means \pm SEM. Part of the data was published in (132).

3.4.2 Progesterone receptor deficiency in CD11c⁺ cells partly rescues pregnant mice from IAV-related lethality

As artificial stimulation of the PR in non-pregnant mice by administration of dydrogesterone led to increased mortality upon IAV infection, we next analyzed how progesterone modulates the immune response during infection. Therefore, we utilized mice harboring a PR deficiency mainly restricted to CD11c⁺ DCs and alvMac to investigate if DCs and alvMac are the predominant target of progesterone-mediated immune modulation during IAV infection. Mice containing the PR gene flanked with loxP sites (PR^{flox}) were mated to animals expressing the cre recombinase under the control of the CD11c promoter (CD11c^{cre}) (155). These PR^{flox}CD11c^{cre} mice were mated allogenically and infected on gd 12.5 with 2009 pH1N1 (Figure 22A). Pregnant infected mice of the control strains (PR^{flox} (CD11c^{cre}-neg) or PR^{WT}CD11c^{cre}) exhibited 60 % lethality, whereas pregnant littermates lacking the PR on DCs (PR^{flox}CD11c^{cre}) showed reduced lethality of 40 % (Figure 22B). The weight loss of pregnant

mice from control strains and PR^{flox}CD11c^{cre} was comparable during infection (Figure 22C). Next, we assessed the activation of CD11c⁺ alvMac and resident DCs and demonstrated slightly elevated frequencies of alvMac and resident DCs expressing the activation molecules CD80 and CD86 when isolated from pregnant infected PR^{flox}CD11c^{cre} mice compared to those from infected animals of the control strains (Figure 22D, E). However, no difference in the frequencies of CD40 expressing alvMac and resident DCs were observed in lungs of PR^{flox}CD11c^{cre} mice and respective controls (Figure 22D, E).

Taken together, our results show that PR deficiency solely in CD11c⁺ DCs and alvMac results in a moderately increased survival and slightly elevated activation of DCs and alvMac upon IAV infection.



Figure 22: Influence of progesterone on the immune response upon influenza infection. (A) PR^{flox}CD11c^{cre} mice (n=5) and respective controls (n=10; PR^{flox} n=5, PR^{WT}CD11c^{cre} n=5) were allogenically mated and infected with 10³ p.f.u. of 2009 pH1N1 on gd 12.5. Survival (B) and weight loss (C) are shown in comparison to uninfected controls (n=3 each). At 3 d p.i. expression of costimulatory molecules was assessed on alveolar macrophages (alvMO) (D) and resident dendritic cells (resDCs) (E) in the lungs from infected PR^{flox}CD11c^{cre} mice (n=5) and respective controls (n=6; PR^{flox} n=2, PR^{WT}CD11c^{cre} n=4) by flow cytometry. (B-E) Shown are means ± SEM. Data was published in (132).

3.5 Influence of progesterone on T cells in vitro

3.5.1 Absent effect of progesterone on antigen-dependent T cell activation

Using *in vitro* co-culture models, the direct targets of progesterone- and glucocorticoid- (GC) induced immunomodulation were characterized. First, possible target cells for pregnancy related hormones were investigated within the DC – T cell crosstalk. As APCs we used spleen cells from non-pregnant or pregnant mice on gd 12.5. APCs were pulsed with chicken ovalbumin₂₅₇₋₂₆₄ peptide (OVA) and co-cultured with CD8⁺ T cells from OT-1 mice expressing a T cell receptor (TCR) specific for OVA (Figure 23A). After 72 h of incubation, proliferation and activation of CD8⁺ T cells were analyzed. Neither the addition of physiological pregnancy-associated concentrations (10^{-6} M) of progesterone, dydrogesterone or dexamethasone – a synthetic GC, nor the isolation of APCs from pregnant mice altered the frequency of proliferated or activated CD8⁺ T cells after priming by DCs *in vitro* (Figure 23B-E).



Figure 23: Influence of progesterone and glucocorticoids on T cell proliferation and activation *in vitro*. (A) Non-pregnant C57BL/6J (n=3) and BALB/c-mated C57BL/6J females (n=3) were sacrificed – pregnant mice on gd 12.5. As antigen presenting cells (APC) spleen cells were isolated, pulsed with 10^{-6} M chicken ovalbumine peptide (OVA) and cultured with OT-1 CD8⁺ T cells that were labeled with a proliferation dye. 10^{-6} M progesterone

(P4), 10^{-6} M dydrogesterone (Dydro) and 10^{-6} M dexamethasone (DEX) were added to the culture and after 72 h the frequency of proliferated OT-1 CD8⁺ T cells (B) as well as the frequency of activated CD8⁺ CD44⁺ CD62L^{neg} T cells (C) was assessed by flow cytometry. Representative dot plots depicting proliferation (assessed by the dilution of the proliferation dye) (D) and CD44/CD62L staining (E) of CD8⁺ T cells. Shown are representative results from one out of 3 independently performed experiments. (B,C) Shown are means ± SEM.

3.5.2 Progesterone and glucocorticoids induce cell death in T cells isolated from pregnant and non-pregnant mice

To analyze the direct effect of steroid hormones on T cells, spleen cells isolated from nonpregnant C57BL/6J or allogenically pregnant C57BL/6J females on gd 7.5 and 18.5 were cultured with progesterone, dydrogesterone, dexamethasone and corticosterone – the predominant GC in rodents – *in vitro* (Figure 24A) and after 48 h of incubation T cell populations were assessed as described in Figure 24B and Figure 24C. Addition of progesterone, dexamethasone and corticosterone increased death of CD4⁺ and CD8⁺ T cells. Dydrogesterone addition, however, did not induce T cell death *in vitro* (Figure 25A-C). Strikingly, CD4⁺ and CD8⁺ T cells isolated from pregnant mice at early (gd 7.5) and late (gd 18.5) gestation were similarly sensitive to progesterone- and GC-induced death than T cells from non-pregnant mice (Figure 25A-C). Since the baseline death in CD4⁺ and CD8⁺ T cells was decreased in cultures from pregnant mice at gd 18.5 in comparison to cultures from nonpregnant and pregnant mice at gd 7.5, cell death was normalized to the frequency of dead cells in the DMSO control. The hormone-induced relative increase in dead CD4⁺ and CD8⁺ T cells was most prominent in cells from pregnant mice at late gestation (Figure 25D, E).

Within surviving CD4⁺ T cells, Tregs were more resistant to hormone-induced death induction, resulting in a relative increase in Treg frequencies in progesterone- and GC-stimulated cultures from non-pregnant and pregnant mice (Figure 26A, E). This increase in Treg frequencies was not observed in cultures stimulated with dydrogesterone. Foxp3^{neg} conventional T cells (Tcon) showed overall high susceptibility to hormone-mediated death induction. However, naïve CD4⁺ T cells (CD62L⁺ CD44^{low}) were particularly sensitive, whereas activated effector CD4⁺ T cells (Teff) (CD62L^{neg} CD44⁺) were relatively refractory. This resulted in a decreased relative frequency of naïve CD4⁺ T cells and an increased relative frequency of CD4⁺ Teff cells, especially in GC-stimulated cultures (Figure 26B, C, E). As the different conditions exhibited variations in T cell death, we calculated the ratio between Tregs and Teff cells and observed an increase in the Treg/Teff ratio for progesterone- but not dexamethasone-stimulated cultures indicated by a ratio > 1. The Treg/Teff ratio was particularly increased in T cell cultures from pregnant mice at gd 18.5 (Figure 26D).



Figure 24: Steroid hormone stimulation of spleen cells *in vitro.* (A) Spleen cells were isolated from nonpregnant and BALB/c-mated pregnant C57BL/6J females on gestational day (gd) 7.5 and 18.5. Progesterone (10^{-6} M) , dydrogesterone (10^{-6} M) , dexamethasone (10^{-8} M) and corticosterone (10^{-7} M) were added and after 48 h of incubation at 37 °C, cells were analyzed by flow cytometry following the depicted strategy (B, C). Data was published in (149).



Figure 25: Progesterone and glucocorticoids induce T cell death *in vitro.* Spleen cells were isolated from non-pregnant (n=6) and BALB/c-mated pregnant C57BL/6J females on gd 7.5 (n=6) and 18.5 (n=6). Progesterone (P4, 10^{-6} M), dydrogesterone (Dydro, 10^{-6} M), dexamethasone (DEX, 10^{-8} M) and corticosterone (Cort, 10^{-7} M) were added and after 48 h of incubation, cells were analyzed by flow cytometry. Dead CD4⁺ (A) and CD8⁺ (B) T cells were analyzed by staining with Pacific OrangeTM. (C) Representative dot plots demonstrating dead CD4⁺ T cells. The relative increase in dead CD4⁺ (D) and CD8⁺ (E) T cells was calculated in comparison to the respective DMSO control. Therefore, the mean of dead T cell frequencies after DMSO addition was set as 1. Shown are representative results pooled from 2 independently performed experiments. (A,B,D,E) Shown are means \pm SEM. Statistical significance was calculated using two-way ANOVA and Bonferroni's multiple comparison post-test (*p < 0.05, **p < 0.01, ***p < 0.001). (A,B) Statistical significance was calculated for each condition (P4, Dydro, DEX, Cort) in relation to the DMSO control for cultures from non-pregnant mice, pregnant mice at gd 7.5 and gd 18.5 separately. (D,E) Statistical significance was calculated for each condition between cultures isolated from non-pregnant and pregnant mice either at gd 7.5 or gd 18.5. Data was published in (149).



Figure 26: Progesterone and glucocorticoids modulate T cell subset composition *in vitro.* Spleen cells were isolated from non-pregnant (n=6) and BALB/c-mated pregnant C57BL/6J females on gd 7.5 (n=6) and 18.5 (n=6). Progesterone (P4, 10^{-6} M), dydrogesterone (Dydro, 10^{-6} M), dexamethasone (DEX, 10^{-8} M) and corticosterone (Cort, 10^{-7} M) were added and after 48 h of incubation, cells were analyzed by flow cytometry. (A) Percentages of surviving Foxp3⁺ regulatory CD4⁺ T cells (Tregs) among all living CD4⁺ T cells are shown. Surviving naïve (CD62L⁺ CD44^{low}) (B) and effector (Teff, CD62L^{nég} CD44⁺) T cells (C) are given in relation to surviving Foxp3^{neg} conventional CD4⁺ T cells (Tcon). (D) The ratio of Treg/Teff cells was calculated. (E) Representative dot plots demonstrating Foxp3 as well as CD62L and CD44 staining of surviving CD4⁺ T cells. Shown are representative results pooled from 2 independently performed experiments. (A-D) Shown are means ± SEM. Statistical significance was calculated using two-way ANOVA and Bonferroni's multiple comparison posttest (*p < 0.05, **p < 0.01, ***p < 0.001). Statistical significance was calculated for each condition (P4, Dydro, DEX, Cort) in relation to the DMSO control for cultures from non-pregnant mice, pregnant mice at gd 7.5 and gd 18.5 separately. Data was published in (149).

3.5.3 Progesterone induces T cell death via binding to the glucocorticoid receptor

The PR is discussed to be absent in T cells (96). Our observations that dydrogesterone treatment – that specifically binds to the PR – did not induce T cell death *in vitro*, also suggests the binding of progesterone to other steroid receptors on T cells e.g. the glucocorticoid receptor (GR). To test this hypothesis, we isolated spleen cells from mice harboring a specific knock-out of either the PR or the GR on T cells (PR^{flox}Lck^{cre} and GR^{flox}Lck^{cre}, respectively). To generate these mice, we crossed PR^{flox} or GR^{flox} mice with mice expressing the cre recombinase under the promoter of the lymphocyte-specific protein tyrosine kinase (Lck) that is expressed in T cells. T cells lacking the PR showed death induction by progesterone and GCs – not dydrogesterone – stimulation comparably to wild type T cells. On the other hand, in T cells lacking the GR death induction by progesterone and GCs – not dydrogesterone – stimulation by progesterone and GCs – not dydrogesterone – stimulation by progesterone and GCs – not dydrogesterone – stimulation the progesterone and GCs was completely abrogated (Figure 27A, B, F). Moreover, Treg enrichment was comparable in PR^{flox}Lck^{cre} and wild type cells, but did not occur in GR^{flox}Lck^{cre} T cells (Figure 27C-E, G). This strongly indicates a direct effect of progesterone on the GR – rather than the PR – in T cells to induce T cell death.


Figure 27: Influence of progesterone and glucocorticoids on T cells isolated from PR^{flox}Lck^{cre} and GR^{flox}Lck^{cre} mice. Spleen cells were isolated from non-pregnant C57BL/6J wild type mice (n=6), mice lacking the progesterone receptor (PR) specifically in T cells (PR^{flox}Lck^{cre}, n=2) as well as mice lacking the glucocorticoid receptor (GR^{flox}Lck^{cre}, n=3) in T cells. Spleen cells were cultured with progesterone (P4, 10⁻⁶ M), dydrogesterone (Dydro, 10⁻⁶ M), dexamethasone (DEX, 10⁻⁸ M) and corticosterone (Cort, 10⁻⁷ M) and after 48 h of incubation, cells were analyzed by flow cytometry. Dead CD4⁺ (A) and CD8⁺ (B) T cells were identified by staining with Pacific OrangeTM. (C) Surviving Foxp3⁺ regulatory CD4⁺ T cells (Tregs) among all surviving CD4⁺ T cells are shown. Surviving naïve (CD62L⁺ CD44^{low}) (D) and effector (Teff, CD62L^{neg} CD44⁺) (E) T cells are given in relation of surviving Foxp3^{neg} conventional CD4⁺ T cells (Tcon). Representative dot plots demonstrating dead CD4⁺ T cells (F) and Foxp3 staining (G). Shown are results pooled from three independently conducted experiments. (A-E) Shown are means ± SEM. Statistical significance was calculated using two-way ANOVA and Bonferroni's multiple comparison post-test (*p < 0.05, **p < 0.01, ***p < 0.001). Statistical significance was calculated for each condition (P4, Dydro, DEX, Cort) in relation to the DMSO control for cultures from non-pregnant mice. Statistical analysis was not possible for cultures from PR^{flox}Lck^{cre} mice because of the small sample size. Cultures from GR^{flox}Lck^{cre} mice did not reach levels of significance. Data was published in (149).

To test if the GR is also crucial for T cell death induction during pregnancy, we used allogenically pregnant GR^{flox}Lck^{cre} mice at gd 7.5. As shown before, in T cells isolated from pregnant wild type mice progesterone and GCs – not dydrogesterone – induced T cell death (Figure 28A, B) and a change in T cell subset composition (Figure 28C-E). On the contrary, T cell death as well as Treg, Teff and naïve CD4⁺ T cell frequencies were unchanged in GR^{flox}Lck^{cre} T cells from pregnant mice after cultivation with hormones in comparison to the DMSO control (Figure 28A-E). Reproductive outcome of allogenically pregnant GR^{flox}Lck^{cre} mice was comparable to wild type C57BL/6J mice (data not shown) (96).



Figure 28. Abrogated effect of progesterone on T cells from pregnant $GR^{flox}Lck^{cre}$ mice. Spleen cells were isolated from pregnant C57BL/6J wild type (WT, n=4) and pregnant $GR^{flox}Lck^{cre}$ (n=3) mice at gd 7.5 and cultured for 48 h with progesterone (P4, 10⁻⁶ M), dydrogesterone (Dydro, 10⁻⁶ M), dexamethasone (DEX, 10⁻⁸ M) and corticosterone (Cort, 10⁻⁷ M). Dead CD4⁺ (A) and CD8⁺ (B) T cells were identified by staining with Pacific OrangeTM. (C) Surviving Foxp3⁺ regulatory CD4⁺ T cells (Tregs) are shown as percentage of surviving CD4⁺ T cells. Surviving naïve (CD62L⁺ CD44^{low}) (D) and effector (Teff, CD62L^{neg} CD44⁺) (E) T cells are given in relation to surviving Foxp3^{neg} conventional CD4⁺ T cells (Tcon). Shown are pooled results from three independently conducted experiments. Shown are means ± SEM. Statistical significance was calculated using two-way ANOVA and Bonferroni's multiple comparison post-test (*p < 0.05, **p < 0.01, ***p < 0.001). Statistical significance was calculated for each condition (P4, Dydro, DEX, Cort) in relation to the DMSO control for cultures from non-pregnant mice. Cultures from GR^{flox}Lck^{cre} mice did not reach levels of significance. Data was published in (149).

3.5.4 Comparable effect of progesterone on female and male T cells

Differential behavior of male and female cells to sex hormones has been proposed (156, 157) and sex-specific immune responses are well studied (158). To investigate if male T cells respond differentially to hormonal treatment, we isolated spleen cells from male and female C57BL/6J wild type mice and cultured them with hormones as described before. In male and female T cells similar T cell death induction by steroid hormones was observed (Figure 29A,



B). The effect of steroid-hormones on CD4⁺ T cell subsets was also not sex-specific (Figure 29C-E).

Figure 29: Progesterone and glucocorticoids induce death in female and male T cells. Spleen cells were isolated from non-pregnant female (n=4) and male mice (n=4). Progesterone (P4, 10^{-6} M), dydrogesterone (Dydro, 10^{-6} M), dexamethasone (DEX, 10^{-8} M) and corticosterone (Cort, 10^{-7} M) were added and after 48 h of incubation at 37°C, cells were identified by flow cytometry. Dead CD4⁺ (A) and CD8⁺ (B) T cells were analyzed by staining with Pacific OrangeTM. (C) Surviving Foxp3⁺ regulatory CD4⁺ T cells (Tregs) are shown as percentage of surviving CD4⁺ T cells. Surviving naïve (CD62L⁺ CD44^{low}) (D) and effector (Teff, CD62L^{neg} CD44⁺) (E) T cells are given in relation to surviving Foxp3^{neg} conventional CD4⁺ T cells (Tcon). Shown are pooled results from two independently conducted experiments. Shown are means ± SEM. Statistical significance was calculated for each condition (P4, Dydro, DEX, Cort) in relation to the DMSO control for cultures from male and female mice separately. Data was published in (149).

4 Discussion

This thesis demonstrates the multi-faceted failure of innate and adaptive immunity to mount an adequate response against the influenza A virus (IAV) in pregnant hosts and presents evidence of progesterone regulating the development of an anti-viral immune response.

We successfully established an influenza mouse model using allogenically pregnant mice. In this model, C57BL/6J females mated with BALB/c males were infected with a human IAV from the pandemic in 2009 (2009 pH1N1) on gestational day (gd) 12.5 - that resembles the end of the second/beginning of the third trimester in human pregnancies. Using this infection model, increased morbidity and mortality in allogenically pregnant mice compared to nonpregnant, virgin mice was observed. The increased lethality in allogenically pregnant mice after IAV infection can be partly explained by a reduced activation of the innate immune response. In this thesis, I demonstrated that activation of antigen-presenting cells (APC) and cytokine production were reduced in lungs of pregnant mice 3 days post infection (d p.i.). The impaired innate immune response is associated with alterations in the adaptive immunity against IAV. I observed reduced numbers of total leucocytes and of CD8⁺ T cells in the lung and reduced migration of CXCR3⁺ CD8⁺ T cells to the lung, the target organ of IAV infection early after infection. Unexpectedly, in our group elevated frequencies of activated CD8⁺ T cells including elevated frequencies of virus-specific CD8⁺ T cells were detected in lungs of pregnant mice at 6 and 8 d p.i. (132). The mechanism leading to elevated CD8⁺ T cell frequencies in the lungs of pregnant mice is currently unclear. It could be caused either by (i) increased proliferation, (ii) reduced cell death or (iii) reduced emigration of these cells from the lung in the periphery. Increased proliferation of CD8⁺ T cells could already be excluded as I showed that the proliferation rate of virus-specific CD8⁺ T cells was comparable in lungs of pregnant and non-pregnant mice at 8 d p.i.. However, albeit increased in numbers, data from our group demonstrated that CD8⁺ T cells from pregnant infected mice were impaired in specific killing of their target cells (132), indicating impaired T cell function. In this thesis, I showed increased frequencies of activated CD8⁺ T cells expressing pro-inflammatory cytokines at 6 and 8 d p.i that could suggest an ongoing immune activation in pregnant infected mice, probably due to insufficient viral clearance and thus, virus persistence in the lung. In addition, elevated frequencies of granzyme B expressing CD8⁺ T cells isolated from lungs of pregnant in comparison to non-pregnant mice at 6 and 8 d p.i. could contribute to the increased lung pathology observed in those pregnant mice. Taken together, the results obtained using this established allogenic mating model suggest that the activation of an innate immune response in pregnant infected mice is impaired, thereby resulting in altered

initiation of the T cell response, reduced viral clearance and enhanced lung pathology resulting in increased lethality (Figure 30).

In the context of this allogenic pregnancy mouse model, in our group we could show that the impaired immune response against IAV in pregnant mice led to a reduced immunological pressure and facilitated the evolution of viral quasi species. Enhanced viral replication in pregnant mice favored mutation of the viral genome as the viral RNA-dependent RNA polymerase lacks general proofreading capacity. We demonstrated the emergence of viral quasi species in pregnant mice containing (i) a Q223R mutation in the hemagglutinin that changes receptor binding specificity from α 2,6-linked towards α 2,3-linked sialic acids, the latter being expressed in the lower respiratory tract and thus, being associated with pneumonia and severe disease outcome (159, 160) and (ii) a R211K/D54N mutation in the non-structural (NS) gene-encoded proteins NS1 and NEP (nuclear export protein) that reduces IFN- β promoter activity. Both viral quasi species exhibited increased virulence even in non-pregnant mice (132). Hence, we may assume that infected pregnant women could infect family members with viral quasi species that may cause severe infection also in non-pregnant individuals. Of note, these results stress the importance of enhanced prevention and treatment strategies for pregnant women against IAV.



Figure 30: Graphical summary. Upon influenza A virus (IAV) infection, pregnant mice mount an altered innate and adaptive immune response. The secretion of pro-inflammatory cytokines and the activation of antigen-presenting cells (alveolar macrophages (alvMac) and dendritic cells (DC)) are impaired in lungs of pregnant IAV infected mice. This is associated with dysfunctional adaptive immunity, as (i) CD8⁺ T cell migration to the lung is impaired due to reduced *Cxcl9* and *Cxcl10* expression in the lung and (ii) the capacity of specific killing by CD8⁺ T cells is reduced. Consequently, the reduced immunological pressure facilitates the emergence of viral quasi species with increased virulence. Overall these events result in increased lethality in pregnant dams upon IAV infection (132).

4.1 Syngenic versus allogenic pregnancy mouse models

In literature, predominantly syngenically pregnant mice or artificial elevations of pregnancyassociated hormone levels were used in mouse models to study the increased IAV-related lethality during pregnancy. Here it was observed that allogenically mated mice showed increased lethality and impaired recovery from IAV infection compared to syngenically mated littermates (132). In allogenic mating combinations, the fetus expresses antigens from maternal and paternal origin and thus, is histo-incompatible to the maternal immune system. To prevent fetal rejection, the maternal immune system undergoes a variety of adaptations that were summarized in section 1.3. Although these immune adaptations are crucial for pregnancy maintenance, they can have detrimental consequences for the mother upon viral infections. In syngenic pregnancies, however, due to genetic identity of mother and father, immunological adaptation to pregnancy is less challenging (80). Therefore, we claim that allogenic mating combinations are better suited to explain the complex modulation of the immune system in pregnant IAV infected hosts. Although, results from syngenically pregnant mice identified increased pro-inflammatory cytokine production as the leading cause of death in IAV infected mice (128, 130), I could not observe a so-called 'cytokine storm' in allogenically pregnant IAV infected mice. Albeit, pregnant mice displayed increased frequencies of pro- and anti-inflammatory cytokine expressing CD8⁺ T cells at 6 and 8 d p.i., the overall cytokine production was not exorbitant. Littauer and colleagues claim that infection of syngenically pregnant mice with seasonal H1N1 leads to preterm labor, stillbirth and small for gestational age neonates (98). However, in our group we could not detect alterations in pregnancy outcome of infected allogenically pregnant mice compared to sham treated dams (132). Whereas in our model increased lung damage in pregnant IAV infected dams was observed, a recent study by Klein and colleagues, which was again conducted in syngenically pregnant mice, proposed preservation of lung function during IAV infection in pregnant mice (161).

The ambiguity to other pregnancy mouse models could be further explained by different mouse strains and IAV strains used. Whereas in other models often lethal IAV doses were used, in our model a sub-lethal dose of 2009 pH1N1 was used for mouse infection. It is known that different genetic backgrounds of mice can lead to alterations in severity of disease and in immune responses (134). Moreover, I experienced that even C57BL/6J mice purchased from different companies show different courses of disease

4.2 The immune system between adaptation and virus elimination

During IAV infection, the immediate recognition of viral RNA and the initiation of the innate immune response are crucial for successful virus control. During pregnancy, however, immune adaptation aims to prevent excessive immune activation to protect the allogenic fetus. Indeed, in this thesis I observed reduced activation of innate immune cells in IAV infected pregnant mice. This is in accordance with a previously conducted RNA sequencing analysis in our laboratory where various pro-inflammatory pathways were down regulated in pregnant infected mice at 3 and 6 d p.i. (132). In addition, PBMCs from pregnant women stimulated with p2009 H1N1 produced less IFN- α and IFN- λ compared to PBMCs from non-pregnant women (162). Further, in a pregnancy mouse model of syngenically mated C57BL/6, mice reduced IFN- γ expression in the lung was observed at 3 days post H3N2

infection (112). A recent study showed that rapid secretion of type III IFNs in the lung could protect from fulminant IAV-mediated lung pathology early after infection in the mouse (163). Hence, reduced type III IFN production in pregnant mice could also contribute to increased lethality upon IAV infection and therefore, it would be interesting to characterize type III IFN expression in our pregnancy infection mouse model.

The already mentioned RNA sequencing analysis revealed that *Cxcl10* (C-X-C motif ligand 10 chemokine) expression was also affected in pregnant mice during IAV infection. Consistently, *Cxcl10* and *Cxcl9* expression was significantly reduced in lungs of pregnant infected mice determined by quantitative RT-PCR (qRT-PCR), which was associated with reduced CXCR3⁺ CD8⁺ T cells migrating into the lung. Similar observations were made in the pregnant uterus where epigenetic repression of *Cxcl9,10,11* and *Ccl5* (C-C motif 5 chemokine) promoters resulted in a migration constrain for activated cytotoxic T cells and thus, prevention of fetal abortion (90). In contrast, in this thesis, epigenetic repression of *Cxcl9/10* (H3K27me3) was not detected in the lungs of IAV infected BALB/c mice increased lethality with fulminant lung pathology (164). In that study, CXCL10 was administered before IAV infection (164) thereby leading to excessive immediate innate immune cell recruitment and thus, increased immune pathology. In our study, T cell migration was analyzed at 4 d p.i. under physiological conditions without prior artificial elevation of CXCL10 concentration.

Albeit cytotoxic CD8⁺ T cells are important in IAV clearance, they might also contribute to excessive lung immunopathology (57-59). Data presented in this thesis suggest, that impaired innate immune activation during pregnancy upon IAV infection might be responsible for incomplete and dysregulated priming and function of the CD8⁺ T cell response, which then could lead to delayed virus clearance. In agreement to my results, dysregulated CD8⁺ T cell responses were also observed in a mouse pregnancy model upon H3N2 infection and were related to altered lung DC function and impaired IFN-y signaling (112). Excessive IFN-y expression is suspected to reduce cytotoxic CD8⁺ T cell numbers in order to prevent fulminant pathology (165, 166). In our mouse model, reduced IFN-y secretion in pregnant infected mice could therefore lead to uncontrolled CD8⁺ T cell expansion due to the absence of negative feedback. This would explain the observation from our group of increased frequencies of activated CD8⁺ T cells including virus-specific CD8⁺ T cells in the lungs of pregnant IAV infected mice at 6 and 8 d p.i.. Although these CD8⁺ T cells were impaired in specific killing (132), they could still contribute to lung pathology, since high frequencies of $CD8^+$ T cells expressing IFN-y and TNF- α in pregnant infected mice were demonstrated within this thesis.

Stimulating PBMCs from pregnant and non-pregnant women with 2009 pH1N1 resulted in rapid expression of exhaustion markers like programmed cell death protein 1 (PD-1) and

cytotoxic T lymphocyte-associated protein 4 (CTLA-4) on CD8⁺ T cells from pregnant women (167). Thus, it might be interesting to investigate exhaustion marker expression on T cells in our mouse model.

In addition to enhanced secretion of pro-inflammatory cytokines in T cells from pregnant infected mice, the frequency of CD4⁺, CD8⁺ T cells and regulatory T cells (Tregs) expressing the anti-inflammatory cytokine IL-10 was also increased at 8 d p.i.. Prolonged elevated IL-10 expression could facilitate secondary bacterial pneumonia (168), as observed for infections with the measles virus where a persisting immune suppressive milieu promotes secondary infections (169, 170).

4.3 Relevance for pregnant women

Our newly established allogenic pregnancy mouse model replicates clinical observations that pregnant women suffer from increased morbidity and mortality upon IAV infection (16). Functional analyses of immune responses in context with human reproduction are often limited. Therefore, mouse models are a valuable approach to analyze these processes. In both mouse and human pregnancies, a hemochorial placenta, which refers to the invasion of trophoblast cells into the uterus, allows the bidirectional contact and transfer of cells between mother and fetus. This makes the immunological tolerance during pregnancy extremely important. Hence, the use of allogenic mating combinations is necessary to mimic immunological processes occurring during human pregnancies. By using the human 2009 pH1N1 IAV for mouse infection rather than a mouse adapted H1N1 IAV, increases the value of these data for the human situation. Systemic IAV infection is rarely detected in humans, albeit, viremia can occur in severe infections and with some highly pathogenic avian IAVs (171). In contrast, during pregnancy viremia and thus infection of the placenta and amniotic fluid was observed in fatal (172, 173) and non-fatal cases (174). Since during the 2009 pandemic patients frequently presented with gastro-intestinal symptoms such as diarrhea, we assessed extra-pulmonary virus titers in the gut (16, 175). Extra-pulmonary virus titers in the gut were rarely detected in non-pregnant mice. However, pregnant infected dams presented more frequently with viremia (positive titers in the gut), although, no virus could be isolated from placenta or concepti. Similarly, during the 2009 IAV pandemic, virus was not detected in the placenta of pregnant women (176, 177). However, limited access to material from pregnant IAV infected patients in 2009 could have biased this finding.

Vaccination against IAV is the most effective prevention of severe IAV and is recommended by the WHO for pregnant women in all trimesters (20). In this thesis I aimed to test the efficiency of vaccination in our allogenic pregnancy mouse model. As vaccination was not established in our laboratory, I infected mice before pregnancy with 2009 pH1N1 to generate preexisting immunity and challenged them during pregnancy with the same IAV subtype. This model demonstrated protection from lethal IAV infection during pregnancy in mice with preexisting immunity. Again, these results mimic clinical findings suggesting that vaccination before and during pregnancy can protect the mother from lethal IAV infection (21-23). In accordance with my observations, various studies in syngenically pregnant mice demonstrated IAV vaccination against H1N1 and H3N2 (22-24) as well as H5N1 (178) as protective for mother and child. Albeit strong data on safety and effectiveness of IAV vaccination during pregnancy is available, IAV vaccination compliance among pregnant women differs extremely between countries ranging from 1.7 % to 88.4 % for seasonal IAV and from 6.2 % to 85.7 % for pandemic IAV (179). Whereas the United States account for highest vaccination reports, in Germany compliance is estimated at around 11 % (25). This was recently confirmed by a pregnancy cohort study in Hamburg where 12 % of enrolled pregnant women reported IAV vaccination during pregnancy (136). Pregnant women were often unaware of the risk of IAV infections and their complications for themselves and their unborn children. The poor vaccination compliance in European countries could probably be increased by specific educational programs for physicians (25, 179) or the distribution of information e.g. on social networking sites in the internet (180).

As this established allogenic pregnancy mouse model shares similar features as the human IAV infection in pregnant women, it can be further used for risk assessment of newly occurring IAV strains. This provides immediate data on virulence in pregnant individuals and implements consequences for prevention and treatment strategies.

4.4 Influence of progesterone on IAV-related mortality

In this thesis, I provide evidence that progesterone plays a role in increasing lethality from IAV infection in mice. The injection of dydrogesterone – a progesterone analogue with high selectivity for the progesterone receptor (PR) – in non-pregnant females increased mortality after IAV infection. Infecting pregnant mice lacking the PR on CD11c⁺ DCs and alveolar macrophages (PR^{flox}CD11c^{cre}) moderately ameliorated IAV-related mortality in comparison to wild type pregnant mice. PR^{flox}CD11c^{cre} mice have already been analyzed in our laboratory in the context of reproductive outcome. It was observed that the loss of the PR on DCs in PR^{flox}CD11c^{cre} mice resulted in increased expression of activation molecules (CD80 and CD86) on DCs and reduced frequencies of CD4⁺ and CD8⁺ regulatory T cells in the uterus

during pregnancy. Consequently, fetal weight was reduced at gd 13.5 (155). These data point out a partial loss of immunological tolerance in pregnant mice lacking the PR specifically on DCs. Nevertheless, abortion rate and implantations were normal in PR^{flox}CD11c^{cre} mice (155) indicating the existence of compensatory strategies. Hence, during infections additional effects of progesterone in the periphery, potentially not mediated via its binding to the PR in DCs, contribute to increased virus pathogenicity.

Since dydrogesterone selectively binds to the PR, GR-mediated effects of progesterone cannot be studied in this model. As PR expression in immune cells is highly discussed (96) and we and others could already provide evidence of progesterone binding to the GR *in vitro* (96, 149), effects mediated by progesterone binding to the GR in immune cells remain to be elucidated in case of IAV infection. Interestingly, Thomas and colleagues observed significantly increased viral replication and lung inflammation following glucocorticoid (GC) treatment of mice prior to IAV infection. Reduced mRNA and protein expression of type I and type III IFNs was observed in GC-treated infected mice (181). The similarity of my observations in pregnant mice with those by Thomas *et al.*, could suggest that GR activation, at least in part by progesterone, could play an important role in IAV induced mortality during pregnancy.

Further, the effects of progesterone on APCs which indirectly also affects T cell activation, should be distinguished from direct effects on T cells. To this end, it is planned to infect mice lacking either the PR or the GR in DCs or in T cells with IAV. Results of these studies will provide more information on the targets of progesterone during IAV infection. Moreover, analyzing the anti-IAV response in these mice will shed light on the molecular mechanism of progesterone-mediated modulation of T cell responses during pregnancy. A better understanding of the molecular mechanisms underlying hormone modulation of the immune system could help developing prevention and therapeutic strategies aiming to decrease maternal morbidity and mortality upon IAV infection.

4.5 Influence of progesterone on the immune system

To investigate the mechanism underlying the impact of steroid hormones on the immune system during IAV infection, an *in vitro* co-culture system of DCs and T cells was established. However, no effect of progesterone and GCs on antigen-induced T cell activation *in vitro* could be observed, as T cell proliferation and activation were comparable in cultures with and without addition of steroid hormones or when DCs were isolated from pregnant mice. However, the use of chicken ovalbumin₂₅₇₋₂₆₄ peptide (OVA) for DC loading

induces a strong signal for OT-1 CD8⁺ T cells that contain a T cell receptor specific for OVA. Recombinant variants of OVA peptides containing specific amino acid exchanges induce a weaker signal for OT-1 CD8⁺ T cells and therefore, moderate T cell activation. Hence, using OVA variants could probably increase sensitivity to detect hormone-mediated changes in T cell proliferation. In contrast to my observations, it has already been shown that GCs reduce the expression of MHC-II and of the costimulatory molecules CD40 and CD86 on DCs and render DCs unable to activate T cell (103, 104).

In the present study, physiological concentrations of progesterone were shown to induce death in T cells isolated from non-pregnant and pregnant mice. Conventional CD4⁺ T cells (Tcon) were highly sensitive to progesterone-mediated death induction, whereas Tregs were relatively robust. This resulted in a relative increase of CD4⁺ Treg over Tcon cell frequencies. Whereas GCs induce a significant reduction in naïve CD4⁺ T cells, progesterone did not (149). Progesterone is considered to be crucial for immunological tolerance towards the semi-allogenic fetus during pregnancy. Here a mechanism leading to immunological tolerance is shown. The selective elimination of CD4⁺ Tcon cells and the consequent increase in Treg frequencies could contribute to prevention of fetal rejection. The frequency of Treg cells is increased during pregnancy (182-184) and they inhibit anti-fetus immune reactions. In the study from Engler and colleagues, a similar change in the ratio of Tcon and Treg was observed in vitro (96), however, only non-pregnant mice were included in this study. Surprisingly, I could demonstrate that T cells isolated from pregnant mice at early and late gestation were similarly sensitive to steroid hormone-induced death and relative Treg enrichment as compared to T cells isolated from non-pregnant littermates. T cells isolated at gd 18.5, however, showed highest death induction upon hormone stimulation. Importantly, the Treg over Teff ratio was especially increased when T cells were isolated from pregnant mice at gd 18.5 (149). This ensures the sensitivity of T cells towards high progesterone concentrations throughout pregnancy.

The nuclear PR was shown to be absent in murine and human T lymphocytes (94, 96) which is in accordance with data from the Immunological Genome Project (185). Therefore, the effect of progesterone on T lymphocytes is proposed to be mediated via a membrane bound PR (94, 97, 186), the GR (96) or both. Kon and colleagues postulate apoptosis induction by progesterone via a membrane PR in Jurkat cells (97). However, in my study the addition of dydrogesterone, which selectively binds to the PR and the usage of mice lacking the PR in T cells (PR^{flox}Lck^{cre}) independently demonstrated T cell death induction by progesterone irrespective of the PR. In contrast, in T cells lacking the GR (GR^{flox}Lck^{cre}) T cell death

induction by progesterone was completely abrogated indicating the importance of the interaction between progesterone and the nuclear GR in modulating the fate of T cells (149).

Evidence of progesterone-mediated T cell apoptosis is sparse (96, 187). On the other hand, GC-mediated T cell apoptosis induction has been thoroughly described in various scenarios; foremost in the context of positive and negative selection in the thymus (109, 188). Similar to high sensitivity of immature thymocytes towards GC-mediated death, increased sensitivity of naïve, in comparison to activated CD4⁺T cells to GC- but not progesterone-mediated death was demonstrated within this thesis. I could also detect increased annexin V staining, which is a measurement for apoptosis, in CD4⁺ and CD8⁺ T cells upon progesterone and GC treatment (data not shown). This is in agreement with the results from Engler and colleagues (96). Together this implicates the involvement of the apoptosis pathway in hormone-induced T cell death induction.

Surprisingly, T cells from pregnant mice at early and late gestation were comparably sensitive to hormone induced cell death as T cells from non-pregnant mice (149). 10^{-6} M of progesterone was used for *in vitro* cultures, which represents approximately three times the serum concentration in pregnant mice at gd 16.5 – the peak of progesterone concentrations during pregnancy (189). However, 10^{-6} M is considered to be within the physiological range of progesterone concentrations found at the feto-maternal interface. Using 10^{-8} M of progesterone for *in vitro* cultures, no effect of progesterone on T cells was observed (data not shown). Lymphocytes express the enzyme 20α -hydroxysteroid dehydrogenase (20α -HSD) that metabolizes progesterone into inactive derivatives (190), possibly leading to resistance to progesterone-induced T cells up to a certain progesterone concentration. However, T cells might become sensitive to progesterone if concentrations exceed the enzyme's capacity. Hence, possible different expression levels of 20α -HSD in Treg and Tcon cells could account for their diverse response to progesterone and might also explain the lower level of apoptosis in T cells from pregnant mice at gd 18.5 under control conditions.

My results offer valuable insights into the mechanisms of progesterone-induced immunological tolerance during pregnancy. Albeit being beneficial at the feto-maternal interface, peripheral T cell apoptosis could result in detrimental consequences in the case of maternal infection. Reduced total leukocyte and CD8⁺ T cell accumulation in the lungs of pregnant mice after IAV infection could thus, be a result of hormone-mediated T cell apoptosis. However, increased Treg frequencies could not be detected in lungs of pregnant infected dams at 6 and 8 d p.i.. These findings may indicate that, at this time point, the

consequences of IAV infection might overwrite the effects of progesterone. Moreover, the progesterone concentration in the periphery is lower than at the feto-maternal interface, where tolerance induction is mostly relevant. Furthermore, an overall decrease in progesterone serum concentrations upon infection in pregnant mice was detected in our laboratory (132). In general, a better comprehension of the mechanisms of progesterone-mediated tolerance induction at the feto-maternal interface and systemically will open new therapeutic avenues to treat pregnancy complications, autoimmune diseases and importantly, viral and bacterial infections during pregnancy. I propose here a mechanism for progesterone to induce immunological tolerance at the feto-maternal interface and probably in the periphery, by selective killing of Tcon and the consequent increase of Treg frequencies (Figure 31).



Figure 31. Graphical summary. Mechanism of hormone-mediated T cell apoptosis. Progesterone (P4) and glucocorticoids (GC) enter the T cell and bind to the cytoplasmic glucocorticoid receptor (GR). Via direct effects of the GR on the mitochondria or localization to the nucleus and effects on gene expression, T cell apoptosis is induced. Whereas high concentrations of steroid hormones – as during pregnancy (right picture) – lead to death of conventional T cells (Tcon), regulatory T cells (Treg) are relatively refractory. This results in a relative Treg enrichment upon stimulation with high steroid concentrations in contrast to low steroid concentrations – as present in non-pregnant hosts (left picture) (149).

4.6 Scientific significance and outlook

Data presented in this thesis improve the understanding on why pregnant women suffer from increased morbidity and mortality during IAV pandemics. Maternal immune adaptation during pregnancy is advantageous for both mother and fetus, as it prevents fetal rejection and leads to amelioration of some autoimmune diseases. Nevertheless, maternal immune adaptation can also result in severe disadvantages for maternal health, as seen in the case of IAV infection. Understanding the molecular mechanisms of the altered immune system generally reacts to external challenges during pregnancy. This knowledge might provide a basis for investigating a variety of pregnancy complications and possibly approaching strategies to treat these conditions.

According to the data obtained within this thesis and additional results from our group, we still do not fully understand the exact cause of death of IAV infected pregnant mice. Thus, further investigations are needed. A cytokine storm - as seen in other IAV infection mouse models (128, 130) and in severe human IAV infection (191) – was not observed in our model. We consider viral persistence in the lung during pregnancy and the resulting continuous immune activation as well as altered T cell function as the cause of excessive lung pathology and death in pregnant IAV infected dams. Impairment in lung repair could contribute to this course of disease and especially account for the delayed recovery from IAV infection seen in pregnant mice. The early innate immune response is critical for successful immunity against IAV and, when uncontrolled, is associated with severe lung damage. Hence, it is important to further investigate the exact mode of action in early IAV response. In our group, increased frequencies of granulocytes and macrophages were detected in the lungs of pregnant dams at 3 and 6 d p.i.. Since they produce vast amounts of pro-inflammatory cytokines, they might cause fulminant lung pathology. In a next step, single innate immune cell populations (granulocytes, macrophages, NK cells, DCs, innate lymphocytes) should be thoroughly characterized based on their migration pattern, cytokine profile, activation status and local proliferation. Importantly, epithelial cells contribute to the first line of anti-IAV defense. Consequently, type I IFN production by epithelial cells in response to IAV will be analyzed in vitro. To investigate a possible effect of progesterone and GCs on epithelial cells, steroidhormones could be added to epithelial cell cultures and the type I IFN response of epithelial cells could be analyzed.

To investigate the effect of progesterone and GCs on the immune response to IAV *in vivo*, it is further planned to infect mice with specific deletion of the PR or GR on defined leukocyte

populations. This enables us to translate our *in vitro* observations on steroid hormonemediated effects on T cells to the situation of *in vivo* IAV infection.

Using the allogenic pregnancy mouse model, we have so far neither observed differences in reproductive outcome of IAV infected dams nor in the weight gain of the offspring from IAV infected and PBS treated mice within the first week after birth (132). However, consequences of maternal IAV infection during pregnancy on the offspring could still arise in later life. Thus, it is planned to further investigate susceptibility to infections (homologous and heterologous IAV strains, or bacterial pathogens such as *Staphylococcus aureus*), to autoimmune diseases (asthma, multiple sclerosis) and changes in behavior in offspring of infected mothers later in life. Especially, the protection of the newborn from homologous and heterologous IAV infection by the transfer of maternal antibodies and possibly memory T cells to the offspring *in utero* and during breast-feeding, could point out the benefit of maternal IAV vaccination on the protection of the newborn. This is particularly important as currently no vaccine is licensed for children less than 6 months of age.

The established allogenic pregnancy mouse model that is described in this thesis in the context of IAV infection is suited to investigate immunity during pregnancy in general. It has already been used to analyze autoimmunity during pregnancy, in particular in autoimmune encephalomyelitis a model for multiple sclerosis, where reduced disease severity during pregnancy could be demonstrated (96). Maternal infections with a variety of different bacteria and viruses can cause detrimental consequences for mother and child. For example maternal infection with *Listeria monocytogenes* often results in stillbirth, abortion or prematurity and is associated with increased viral load in the placenta and in the concepti (192). Probably the immune suppressive milieu in the placenta during pregnancy facilitates bacterial transmission to the fetus. Using the allogenic mating model in combination with mice lacking the PR or GR in leukocyte subsets could shed light into the mechanism of listeria-mediated pathology at the feto-maternal interface and listeria transmission to the fetus.

In conclusion, our established allogenic pregnancy mouse model mimics central clinical features observed in IAV infected pregnant women with severe courses of disease. Thus, we established a tool to investigate the immunological mechanisms leading to increased IAV-related lethality during pregnancy. Results presented in this thesis and additional data obtained within our group, already provide strong evidence suggesting failure of the innate and adaptive immune response against IAV during pregnancy. Based on this model, we could already show that the less stringent immunological pressure in pregnant mice also

affects the virus. It was demonstrated that in pregnant mice highly virulent IAV quasi species emerged. Finally, these observations are associated with the death of pregnant animals after IAV infection (Figure 30).

5 Summary

Influenza A virus (IAV) infections pose a large burden to public health in seasonal epidemics and irregular pandemics. During the pandemic in 2009, especially pregnant women suffered from severe and even fatal IAV infection. The underlying molecular mechanisms causing increased morbidity and mortality from IAV infection during pregnancy are still unknown. Evidence from mouse models confirm increased lethality of pregnant mice upon IAV infection (128-130). However, these studies were carried out in syngenically mated animals, not fully reflecting the complexity of the immunological adaptation in human allogenic pregnancies.

Within this thesis, an allogenic pregnancy mouse model was established. Specifically, C57BL/6J females were mated to BALB/c males and increased morbidity and mortality of allogenically pregnant mice was observed upon infection with the pandemic H1N1 IAV from 2009. In this model, reduced secretion of pro-inflammatory cytokines and reduced activation of antigen-presenting cells in the lungs of pregnant infected hosts was associated with impaired activation of adaptive immunity. There was reduced leukocyte accumulation at 4 days post infection and reduced CXCR3-dependent CD8⁺ T cell migration to the target organ of IAV infection – the lung. At later time points of infection, increased frequencies of CD8⁺ T cells expressing pro-inflammatory cytokines could contribute to severe lung pathology in pregnant infected mice. Since progesterone is considered to be important for immune adaptation to pregnancy, its influence on the immunity to influenza was assessed. Treatment of non-pregnant mice with a progesterone analogue to induce pregnancy-like hormone concentrations moderately increased IAV-related lethality, whereas the use of pregnant mice lacking the progesterone receptor in CD11c⁺ DCs and alveolar macrophages moderately decreased IAV-related lethality. The mechanism by which progesterone modulates the immune response against IAV remains elusive. In vitro data show that progesterone and alucocorticoids induce death in CD4⁺ and CD8⁺ T cells and induce a selective enrichment of regulatory T cells. This process is mediated via the glucocorticoid rather than the progesterone receptor. Further investigations are needed, e.g. by infecting mice lacking the glucocorticoid or progesterone receptor in specific leukocyte populations in order to elucidate the exact mechanism for progesterone-mediated alteration of the immune response towards IAV.

In conclusion, the results obtained within this thesis demonstrate the multifaceted failure of innate and adaptive immunity to IAV in pregnant mice. These findings have direct relevance for pregnant women and contribute to the understanding of how the immune response generally reacts to challenges during pregnancy.

6 Zusammenfassung

Influenza A Virus (IAV) Infektionen stellen eine für schwangere Frauen extreme Gesundheitsgefährdung dar, die leider immer noch häufig unterschätzt wird. Die zugrundeliegenden molekularen Mechanismen, die zur erhöhten Morbidität und Mortalität während der Schwangerschaft führen sind jedoch noch weitgehend unbekannt. In syngenen Verpaarungsmodellen konnte bereits eine erhöhte Letalität in trächtigen Mäusen nach IAV Infektion aufgezeigt werden (128-130). Diese spiegeln allerdings nur bedingt die Komplexität der immunologischen Anpassung an den semi-allogenen Fötus in schwangere Frauen wieder.

In der vorliegenden Arbeit wurde deshalb ein allogenes Verpaarungsmodell etabliert, in welchem C57BL/6J Weibchen mit BALB/c Männchen verpaart wurden. In diesem Mausmodell konnte nach Infektion mit dem pandemischen H1N1 IAV von 2009 während der Trächtigkeit eine erhöhte Morbidität und Mortalität aufgezeigt werden. 3 Tage nach Infektion wurde eine verminderte Zytokinexpression und eine verminderte Akkumulation von aktivierten Antigen-präsentierenden Zellen in den Lungen von trächtigen Tieren beobachtet. Weiterhin konnte gezeigt werden, dass die Akkumulation von Leukozyten in der Lunge 4 Tage nach Infektion reduziert war und die CXCR3-abhängige Einwanderung von CD8⁺ T-Zellen in die Lunge vermindert war. Zu einem späteren Zeitpunkt, könnte eine erhöhte Frequenz von CD8⁺ T-Zellen, welche pro-inflammatorische Zytokine produzieren die beobachtete schwere Lungenschädigung in trächtigen Tieren nach IAV Infektion bedingen. Da Progesteron vermutlich eine wichtige Rolle in der Toleranzentwicklung des Immunsystems gegenüber dem Fötus spielt, wurde der Einfluss von Progesteron auf die Immunreaktion gegen IAV untersucht. Hierzu wurde zum einen ein Progesteronanalogon in nicht-trächtige Tiere injiziert um einen schwangerschaftsähnlichen Zustand zu generieren. Dies führte zu einer geringen Verbesserung der Letalität nach IAV Infektion. Zum anderen wurden Mäuse verwendet welchen der Progesteronrezeptor in CD11c⁺ Zellen fehlte. Dies hatte eine leichte Verschlechterung der Letalität nach Infektion zur folge. Der zugrundeliegende Mechanismus, wie Progesteron die Immunantwort gegen IAV moduliert, ist jedoch noch unbekannt. In vitro Daten zeigten, dass Progesteron in T-Zellen Zelltod induziert und eine spezifische Anreicherung von regulatorischen T-Zellen bewirkt. Dieser Prozess war unabhängig vom Progesteronrezeptor, aber abhängig von der Expression des Glucocorticoidrezeptors in T-Zellen. Weitere Experimente sind notwendig, beispielsweise die Infektion von Tieren, welchen entweder der Glucocorticoid- oder der Progesteronrezeptor in bestimmten Leukozytenpopulationen fehlt. Die Ergebnisse dieser Arbeit verbessern unser Verständnis über die veränderte Immunität während der Schwangerschaft.

7 Abbreviations

20α-hydroxysteroid dehydrogenase
Antibody-dependent cellular cytotoxicity
Alveolar macrophage
Activator protein 1
Allophycocyanin
antigen presenting cell
Base pairs
5-bromo-2-deoxyuridine
Bovine serum albumin
Brilliant Violet
C-C motif chemokine ligand
Cluster of differentiation
Center for Disease Control and Prevention
Conventional DC
Chromatin immunoprecipitation
Cytopathic effect
recombinase derived from P1 bacteriophage
Cycle threshold
Cytotoxic lymphocyte
Cytotoxic T lymphocyte-associated protein 4
C-X-C motif ligand
C-X-C motif receptor
Days post infection
Dendritic cell
Dimethyl sulfoxide
Deoxyribonucleic acid
Deoxynucleotide phosphate
Ethylene diamine tetraacetic acid
Fluorescence activated cell sorting
Fetal bovine serum
Fragment, crystallizable
Fluorescein isothiocyanate

Flox	Flanked with loxP sites
FMO	Fluorescence minus one
Foxp3	Forkhead box protein 3
FSC	Forward scatter
Gapdh	Glycerinaldehyd-3-phosphat-dehydrogenase
GC	Glucocorticoid
gd	Gestational day
GM-CSF	Granulocyte-macrophage colony stimulating factor
GR	Glucocorticoid receptor
H&E	Hematoxylin and Eosin
H3K27	Histone 3 lysin 27
H3K27	Trimethylated histone 3 lysin 27
НА	Hemagglutinin
HAU	Hemagglutination units
HRP	Horsradisch peroxidase
i.p.	Intra peritoneal
i.v.	Intra venous
IAV	Influenza A virus
ICAM	Intracellular adhesion molecule
IFN	Interferon
IFNAR	Interferon a receptor
lg	Immunoglobulin
IL	Interleukin
IMDM	Improved modified eagle medium
IMDM	Iscove's Modified Dulbecco's Medium
ISG	Interferon stimulated gene
IVC	Individually ventilated cage
JAK	Janus kinase
Lck	Lymphocyte-specific protein tyrosine kinase
MACS	Magnetic activated cell sorting
MDCK	Madin-Darby canine kidney cells
MEM	Minimal essential media
MEM	Minimum Essential Medium
MHC	Major histocompatibility complex
mRNA	Messenger RNA
NA	Neuraminidase

Pregnancy and influenza

NaCl	Sodium chloride
NEP	Nuclear export protein
NF-кВ	Nuclear factor kappa-light-chain-enhancer of activated B cells
NK cell	Natural killer cell
NLAP3	NLR family pyrin domain containing 3
NLR	NOD-like receptor
NOD	Nucleotide binding oligomerization domain
NOS	Nitric oxide synthase
NP	Nucleopeptide
NRS	Normal rat serum
NS1	Non-structural protein 1
OVA	Ovalbumin
p.f.u.	Plaque forming units
PacO	Pacific Orange
PBMC	Peripheral blood derived mononuclear cells
PBS	Phosphor buffered saline
PCR	Polymerase chain reaction
PD-1	Programmed cell death protein 1
pDC	Plasmacytoid DC
PE	Phycoerythrin
PerCP	Peridinin-Chlorophyll Protein
PFA	Paraformaldehyde
pH1N1	Pandemic H1N1
PMA	Phorbol-12-myristat-13-acetat
PR	Progesterone receptor
PRR	Pattern recognition receptor
r.b.	retrobulbar
RBC	Red blood cell
RIG-I	Retinoic acid-inducible gene 1
RNA	Ribonucleic acid
Rpm	Rounds per minute
RT	Room temperature
RT-PCR	Reverse transcription polymerase chain reaction
SEM	Standard error of the mean
SGA	Small for gestational age
SSC	Side scatter

Pregnancy and influenza

STAT	Signal transducer and activator of transcription
TCR	T cell receptor
TGF	Transforming growth factor
Th	T helper cell
TLR	Toll-like receptor
TNF	Tumor necrosis factor
Treg	Regulatory T cell
VCAM	Vascular cell adhesion molecule
WHO	World Health Organization
WT	Wild type

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9 List of figures and tables

9.1 List of figures

Figure 1: Schematic model of an influenza A virus particle2
Figure 2: Morbidity of pregnant women upon influenza A virus infection4
Figure 3: Immunological adaptation to pregnancy11
Figure 4: Identification of tissue resident leukocytes by anti-CD45 antibody administration30
Figure 5: Gating strategy for CD4 ⁺ and CD8 ⁺ T cells
Figure 6: Detection of influenza A virus specific CD8 ⁺ T cells by flow cytometry33
Figure 7: Pregnancy mouse model to study influenza A virus infections
Figure 8: Influenza A virus infection of non-pregnant, syngenically pregnant and allogenically
pregnant mice40
Figure 9: Influenza A virus induced pathogenicity in non-pregnant, syngenically pregnant and
allogenically pregnant mice41
Figure 10: Cytokine production in lungs of infected mice42
Figure 11: Gating strategy to detect macrophage and dendritic cell (DC) populations in the
lung of infected animals44
Figure 12: Expression of costimulatory molecules on macrophage and dendritic cell subsets
in the lung of infected mice45
Figure 13: Expression of <i>Cxcl9</i> and <i>Cxcl10</i> in lungs of infected mice47
Figure 14: T cell migration during influenza virus infection48
Figure 15: Proliferation of CD8 ⁺ T cells in lungs of infected mice50
Figure 16: Frequency of CD4 ⁺ T cells expressing pro-inflammatory cytokines after influenza
infection51
Figure 17: Frequency of CD8 ⁺ T cells expressing pro-inflammatory cytokines after influenza
infection52
Figure 18: Frequency of IL-10 producing CD4 ⁺ and CD8 ⁺ T cells after influenza infection54
Figure 19: Secondary IAV infection during pregnancy in mice56
Figure 20: Pathogenicity of IAV infection after secondary infection during pregnancy57
Figure 21: Influence of dydrogesterone injection on the pathogenicity of influenza infections.
Figure 22: Influence of progesterone on the immune response upon influenza infection60
Figure 23: Influence of progesterone and glucocorticoids on T cell proliferation and activation
in vitro62
Figure 24: Steroid hormone stimulation of spleen cells <i>in vitro</i> 64

Figure 25: Progesterone and glucocorticoids induce T cell death in vitro65	5
Figure 26: Progesterone and glucocorticoids modulate T cell subset composition in vitro60	6
Figure 27: Influence of progesterone and glucocorticoids on T cells isolated from PR ^{flox} Lck ^{ct}	е
and GR ^{flox} Lck ^{cre} mice68	3
Figure 28. Abrogated effect of progesterone on T cells from pregnant GR ^{flox} Lck ^{cre} mice69	9
Figure 29: Progesterone and glucocorticoids induce death in female and male T cells70	С
Figure 30: Graphical summary7	3
Figure 31. Graphical summary8	1

9.2 List of Tables

Table 1: Chemicals	15
Table 2: Anaesthetics, analgetics and additives	17
Table 3: Kits	17
Table 4: Viral stocks	19
Table 5: Special instruments used	19
Table 6: Antibodies for FACS	19
Table 7: Antibodies for plaque assay and immunohistochemistry	21
Table 8: Software used for data acquisition and analysis	21
Table 9: Primer for SYBR Green qPCR	21
Table 10: Gene expression assays for TaqMan qPCR	22
Table 11: Primer for Chip assay	22
Table 12. Mouse lines	26
Table 13. Scoring criteria	28
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11 Curriculum Vitae

12 Appendix

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A.M.H. performed various experiments analyzing the immune response during influenza infection and analyzed the data generating Figure 2H-M, 3B-J, S4F-J. Moreover, A.M.H. contributed to Figures 1A-I, 2B-G, 6D-K, S2G,P, S3G-P, S4A-E and K-N.

<u>Alexandra Maximiliane Hierweger</u>, Jan Broder Engler, Manuel A. Friese, Holger M. Reichardt, John Lydon, Francesco DeMayo, Hans-Willi Mittrücker[#], Petra Clara Arck[#], *Progesterone modulates the T cell response via glucocorticoid receptor-dependent pathways*. American Journal of Reproductive Immunology (2019) # Co-senior authors

Article

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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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Graphical Abstract

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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.

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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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 fetomaternal 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.

Cell Host & Microbe 21, 321-333, March 8, 2017 © 2017 Elsevier Inc. 321

<u>Cell</u>Press



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322 Cell Host & Microbe 21, 321–333, March 8, 2017

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 influenzarelated 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 10³ 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–1).

To test if the pregnancy-related mortality and morbidity is specific for the pH1N1 virus, we also infected pregnant and nonpregnant 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 $100 \times (10^5$ PFU), all infected pregnant and non-pregnant mice survived. When reducing the pH1N1 virus infection dose 10x $(10^2$ PFU), the infection became non-lethal also in allogenic pregnant mice but still mediated a significant weight loss. When using a 10x 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 10³ 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 10³ PFU of pH1N1 virus, compared to non-infected non-pregnant

(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) For assesses, (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; syndenic pregnant, n = 8; allogenic pregnant, n = 12). Scale bar, 100 um in H&E and NP staining.

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 10⁵ 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,

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).

Cell Host & Microbe 21, 321–333, March 8, 2017 323



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 (FN-α [B], IFN-γ [O], IL-6 [D], and TNF-α [E]) determined by luminex assay in lungs of non-pregnant (open squares, n = 11) or pregnant mice (black

(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 10³ 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).

324 Cell Host & Microbe 21, 321–333, March 8, 2017

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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 (RNAseq) of infected and uninfected lungs in pregnant versus nonpregnant 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-y, 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-a, IFN-y, IL-6, and TNF- $\!\alpha$ 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 (M Φ), 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 M Φ (alvM Φ) 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*^{10x}CD11c^{cre} mice). We observed a–albeit marginal–higher survival during pH1N1 infection in allogenic pregnant *Pgr*^{10x}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 $alvM\Phi$ 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. Cxc/9 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 3l). In order to test if the ligands for CXCR3, Cxc/9 and Cxc/10, 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 Cxc/9 and Cxc/10. 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 (alvM Φ) (F) and resident dendritic cells (resDC) (G) in the lungs of non-pregnant (n = 6) and pregnant mice (n = 6) upon infection with 10³ PFU of pH1N1 as measured by flow cytometry at 3 d.p.i. (H and I) Pregnant *Pgr^{lick}CD11c*^{cre} mice (n = 5) and pregnant mice of the control strain (n = 10) were infected with 10³ PFU of pH1N1 virus. Control groups of both

(H and I) Pregnant Pgr^{ace}CD11c^{ere} mice (n = 5) and pregnant mice of the control strain (n = 10) were infected with 10° 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 alvM Φ (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 10³ 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).

Cell Host & Microbe 21, 321–333, March 8, 2017 325

CellPress



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 (C).

(c) 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.

(G) Experimental setup of adoptive transfer assay of CD90.1⁺ CD8⁺ T cells from infected non-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 $^{\rm neg}$ 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

326 Cell Host & Microbe 21, 321-333, March 8, 2017

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 10³ PFU of pH1N1. (b) Representative dot plots of MHC dextramer Cells and CDE 1 Cells in lungs of non-pregnant (n = 9) and pregnant (n = 6) mice intected with 10⁻PFO of PH N1.
(c) 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^{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^{high} cells into the respective hosts.
(c) 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 × 10⁶) into pregnant mice 4 d.p.i. with 10³ 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

Cell Host & Microbe 21, 321-333, March 8, 2017 327



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

328 Cell Host & Microbe 21, 321-333, March 8, 2017

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 α 2,3-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.





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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 alvM Φ 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 nonpregnant 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



⁽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 NS1 expression 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 10³ 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 10³ PFU of pH1N1 WT (open square, n = 3) or pH1N1-HA₂₀₂₂₈, NS_{R211KDE94}, 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 10³ PFU of recombinant pH1N1 quasi species (pH1N1-HA₂₀₂₂₈, n = 15; pH1N1-HA₂₀₂₂₈, n = 15; pH1N1-PH₂₀₂₂₈, n = 15; pH1N1-PH₂₀₂₂₈,

(L–O) Survival (L) and weight loss (M–O) in non-pregnant mice infected with 10° 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).

330 Cell Host & Microbe 21, 321-333, March 8, 2017

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 α 2,3-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 n 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 nonpregnant 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 (Giria 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^{100x}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 10⁻¹⁰0⁵ PEU 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_{0223R}, pH1N1-NS_{R211K/D54N}, or pH1N1-HA_{0223R}+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 10⁰ 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

Cell Host & Microbe 21, 321–333, March 8, 2017 331



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).

Immununohistochemistry 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 Immunoassav 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 (Supple mental 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 Exp dures).

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 Pentide 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 Supp tal Experimental Procedures

doptive Transfer of Virus-Specific CD8⁺ T Cells

CD8⁺ T cells were purified from spleens of either naive or infected nonpregnant 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 trans-ferred 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 × 10⁶ purified T cells were administered into the tail vein of nonpregnant 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.

332 Cell Host & Microbe 21, 321-333, March 8, 2017

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.; - Review & Editing, G.G. and P.C.A.; Funding Acquisition, G.G., Writing H.-W.M., G.E., and P.C.A.; Joint Senior Supervision, G.G. and P.C.A.

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Cell Host & Microbe 21, 321–333, March 8, 2017 333

Cell Host & Microbe, Volume 21

Supplemental Information

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Virus Strains in Allogenically Pregnant Mice

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Supplemental online material

Pregnancy-related immune adaptation promotes

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strains in allogenically pregnant mice

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1

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Supplemental methods

Animal experiments

Eight to ten weeks old female C57BL/6JRccHsd mice (Harlan, Netherlands), C57BL/6J (Charles River, Germany) or PR^{flox}CD11c^{cre} mice (institutional breeding colony) were mated with male BALB/c or C57BL/6J mice (Charles River, Germany). All infection experiments were performed under BSL-2 conditions. Viruses were propagated in MDCK cells as described previously (Otte et al., 2011). Non-pregnant and pregnant females, the latter on gestation day (gd) 12.5, were anaesthetised with 100 mg/kg ketamine and 10 mg/kg xylazin and intranasally inoculated with 101-105 p.f.u. of the 2009 pH1N1 virus strain A/Hamburg/NY1580/09 (50% mouse lethal dose (MLD₅₀)=10^{3.83} p.f.u.), the 2006 sH1N1 virus strain A/Solomon Islands/3/2006-like (MLD₅₀>10⁶ p.f.u.) or the recombinant pH1N1 on A/Hamburg/NY1580/09 background (pH1N1-HA_{Q223R}, pH1N1-NS_{R211K/D54N} or pH1N1-NS_{R21K/D54N} or pH1N1-NS_{R21K/D54N} or pH1N1-NS_{R21K/D54N} or pH1N1-NS_{R21K/D54N} or pH1N1-NS_{R21K/D54N} or pH1N1-NS_{R21K/D54N} or p HAQ223R+NSR211K/D54N) diluted in 50 µL PBS. A p.f.u. indicates the number of virus particles capable of forming plaques per unit volume. This assay allows the functional assessment of the virus, as defective viral particles or particles that fail to infect a target cell will not produce a plaque. Mice receiving PBS were used as control group. Non-pregnant C57BL/6JRccHsd mice were s.c. injected with 1.25 mg dydrogesterone (progesterone derivate, provided by Abbott, USA) in 0.2 ml vehicle (20% benzyl benzoate, 80% castor oil) every second day - 12 days before and 14 days after infection with 10³ p.f.u. of the 2009 pH1N1 virus strain A/Hamburg/NY1580/09. Some of these mice were monitored for weight loss and signs of disease until 14 d p.i. and euthanized upon >25 % weight loss, 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). Additional groups of mice were sacrificed on 3 and 6 d p.i. and organs removed for detection of virus titers, cellular, molecular and histopathological examinations (Hoffmann et al., 2014, Tarnow et al., 2014).

Immununohistochemistry

Lungs of infected animals were processed for pathological examination and immunohistochemistry (IHC) according to our established protocols. In brief, deparaffinised slides were treated with 0.1 M citrate buffer (pH 6.0) and a rabbit anti-H7N1 serum. The primary antibody was detected by a biotin-conjugated anti-rabbit antibody (Jackson ImmunoResearch, USA), as described before (Tarnow et al., 2014, Hoffmann et al., 2014), followed by the application of the Zytochem-Plus HPR kit (Zytomed, Germany) and the AEC-substrate (Dako, Germany). Tissues were counterstained with hematoxylin for pathological analysis.

Placental tissue was dissected into histological sections at the midsagittal plane. Masson-Goldner trichrome staining of placental paraffin sections was performed following our established protocols. Image acquisition was performed using a slide scanner (Mirax Midi, Zeiss) and processed using the Panoramic Viewer software (3DHISTECH Ltd., Hungary).

Progesterone detection

Progesterone levels were detected in serum of pregnant mice PBS treated or infected with 10^3 p.f.u. of pH1N1 on 3 (gd 15.5) and 6 d p.i. (gd 18.6) using a progesterone ELISA kit (Cayman Chemical, Germany) according to the manufacturer's instructions. Progesterone concentration was calculated based on a standard curve run simultaneously.

High throughput sequencing

RNA extraction: RNA was isolated from lungs of non-pregnant and pregnant mice infected with 10^3 p.f.u. of the pH1N1 virus at 3 and 6 d p.i. using the innuprep RNA Mini Kit (Analytik Jena, Germany) according to the manufacturer's protocol.

RNA-seq library construction, sequencing and quality control: Three to four replicates per group were pooled in one sample and 1µg total RNA was used for generating libraries with the Illumina TruSeq RNA Sample Preparation Kit v2 as recommended by the manufacturer. Size and quality of the libraries were assessed using a BioAnalyzer High Sensitivity Chip. Diluted libraries (2 nM) were multiplex-sequenced on the Illumina HiSeq 2500 instrument (2x 100 bp paired end run) with 40-50 million reads per sample.

Expression quantification of ISGs: Reads were aligned to the murine reference transcriptome (UCSC mm10) using Bowtie2 (v2.2.2)(Langmead and Salzberg, 2012). DESeq(Anders and Huber, 2010) was employed to assess differential expression. Full names of genes in alphabetical order: *Cxcl10:* C-X-C motif ligand 10; *Ddx58:* DEAD (Asp-Glu-Ala-Asp) box polypeptide 58; *Eif2ak2:* eukaryotic translation initiation factor 2-alpha kinase 2; *Ifih1:* interferon induced with helicase C domain 1; *Ifit3:*interferon-induced protein with tetratricopeptide repeats 3; *Isg15:* ISG15 ubiquitin-like modifier; *Mx1:* myxovirus (influenza virus) resistance 1, interferon-inducible protein p78 (mouse); *Oas1a:* 2'-5' oligoadenylate synthetase 1B; *Oas1c:* 2'-5'

oligoadenylate synthetase 1C; *Oas1g:* 2'-5' oligoadenylate synthetase 1G; *Socs1:* suppressor of cytokine signaling 1; *Stat1:* signal transducer and activator of transcription 1; *Usp18:* ubiquitin specific peptidase 18.

Variant calling to identify mutations of pH1N1 virus: Viral RNA in the lung homogenates was compared to the parental pH1N1 strain. Alignment to the reference sequences (accession no.: GU480807.1 (PB2), HQ104924.1 (PB1), HQ104925.1 (PA), HQ104926.1 (HA), HM598305.1 (NP), HQ104927.1 (NA), HQ104928.1 (M), HQ104929.1 (NS)) was performed with the Burrows Wheeler Aligner (v0.7.5a)(Li and Durbin, 2009). The program was parameterized to trim reads from the 3'-end using a quality threshold of 15. Putative PCR duplicates were removed using SAMtools (v0.1.19)(Li et al., 2009). Consecutive calling of variants was conducted with SAMtools and VarScan (v2.3.6)(Koboldt et al., 2012). Bases with Phred quality scores below 30 were not considered for calling variants.

Data Availability: Sequence data for all samples have been submitted to the European Nucleotide Archive (ENA) and are publicly available at http://www.ebi.ac.uk/ena/data/view/PRJEB12200.

Cells

Cell lines of human embryonic kidney cells (HEK293T) and human lung epithelial cells (H1299) were grown in Dulbecco's modified Eagle's medium (DMEM, Sigma, Germany) supplemented with 10 % fetal bovine serum (FBS; Biochrom, Germany), 1 % penicillin/streptomycin (P/S, Sigma, Germany), and 1 % L-Glutamine (Sigma, Germany) and were cultivated at 5 % CO_2 , 96 % rH and 37 °C.

Transfection and vectors

Transfections were performed using Lipofectamine 2000 (Invitrogen, Germany) according to the manufacturer's instructions. The wt and R211K mutant NS coding sequence from pHW2000-HH15-NS-wt or -NS-R211K was subcloned into pcDNA3.1 between EcoRI and NotI restriction sites by using gene specific primers (forward: 5'-GCGAATTC ACCATGGACTCCAACACCATGTCAAGC-3', reverse: 5'-GCGCGGCCGCTCATTTCTGCTCTGGAGGTAGTG-3'). To generate a construct only expressing NS1, a mutation at the splice acceptor site and a silent SmaI restriction site were introduced into pcDNA3.1-HH15-NS-wt or -NS-R211K by site-directed mutagenesis. The NS sequence 502-CCAG \downarrow GA-507 (the arrow shows the intron/exon junction) was changed to

502-CCCGGG-507 (forward: 5'- CCTTCTCTCCCGGGCATACTTATGAGG-3', reverse: 5'- CCTCATAAGTATGCCCGGGAAGAGAGAGG-3') without changing any amino acids, creating the expression constructs pcDNA3.1-HH15-NS1-wt-SAM and -NS1-R211K-SAM. Vector constructs encoding pPol-I-NP-Luc-human, pRL-TK (Promega, Germany) and p125-Luc were described before (Gabriel et al., 2005).

Polymerase activity assay

H1299 cells were transfected in 12-well plates $(2.0 \times 10^5 \text{ cells per well})$ with pHW2000 vector constructs (0.5 µg each, NP 1.0 µg) encoding for A/Hamburg/NY1580/2009 (HH15) PB1, PB2, PA, and NP together with the reporter constructs (0.5 µg each) pPol-I-NP-Luc (encoding for the vRNA ((-)ssRNA) of the firefly luciferase) and the pRL-TK Renilla luciferase construct (Promega, Germany) for normalization. In addition, pcDNA3.1 expression constructs for HH15-NS1-SAM (WT or R211K; 2µg each) or empty vector as negative control were transfected. Luciferase activity was measured 24 h post vRNP reconstitution using the Dual-Luciferase® Reporter Assay System (Promega, Germany) according to the manufacturer's instructions.

Western blot

Expression levels of HH15-NS1 (WT or R211K) in H1299 cells were confirmed by Western blot analysis. Whole cell lysates obtained in the vRNP reconstitution assay were mixed with 6x Laemmli sample buffer and boiled for 5 min at 95 °C. Subsequently, the lysates were subjected to SDS-PAGE and blotted onto nitrocellulose membranes. For the detection of NS1, a rabbit anti-NS1 antibody (kindly provided from Thorsten Wolff, Robert Koch Institute, Berlin, Germany) was used, followed by incubation with a horseradish peroxidase (HRP)-conjugated anti-rabbit secondary antibody (Sigma, Germany), respectively. As loading control, expression of GAPDH was detected using a rabbit anti-GAPDH antibody (Cell signaling). Protein signals were visualized using the SuperSignal West Femto Maximum Sensitivity Substrate Kit (Life Technologies, Germany) and the ImageQuant LAS 4000 (GE Healthcare, Germany).

IFN-β promoter assay

HEK293T cells were transfected in 12-well plates (2.0 x 10^5 cells per well) with pcDNA3.1 expression constructs for HH15-NS1-SAM (WT or R211K; 2µg each) or empty vector as negative control. In addition, the reporter constructs (0.5 µg each) p125-Luc (firefly luciferase gene under the control of the IFN- β promoter) and the pRL-TK Renilla luciferase construct (Promega, Germany) for normalization. At 16 h post transfection, cells were infected with influenza virus A/WSN/33 at an MOI of 1 and incubated for 24 h at 37°C. Luciferase activity was measured 24 h post infection using the Dual-Luciferase® Reporter Assay System (Promega, Germany) according to the manufacturer's instructions.

Cytokine detection

Cytokines were determined in supernatants of homogenized lungs and/or collected sera from infected pregnant and non-pregnant mice as well as uninfected control groups at 3 d p.i. Therefore, lung tissue of mice (~0.04-0.15 g) was weighed into 2 ml-O-Ring tubes filled with sterilized glass beads (~300 μ l) on the day of organ excision and stored at -80 °C until measurement. On the day of measurement, 1 ml sterile 1x PBS (Sigma, Germany) was added to the thawed sample and homogenization was performed in a mixer mill (Retsch, Germany) at 20 Hz and 4 °C for 10 min. After centrifugation at 6000 xg and 4 °C for 10 min, the supernatants (~800 μ l) were transferred to a new tube and centrifuged again at 10000 xg and 4 °C for 10 min directly before addition to the Luminex plate. Cytokines (IFN- α , IFN- γ , IL-6, and TNF- α) were then determined using the ProcartaPlex® Multiplex Immunoassay
Mix&Match Mouse 4-plex (Affymetrix/eBioscience, Germany) with magnetic beads following the manufacturer's instructions. Lung homogenate supernatant samples were run in duplicate. Measurement of cytokine levels was performed using the BioRad Luminex 200 machine. Supernatants of homogenized lungs were measured using FlowCytomix (eBiosciences, Germany) for IFN- α and a mouse inflammation cytometric bead array (BD, Germany) for IFN- γ , IL-6 and TNF- α according to the manufacturer's protocols. Serum cytokines were captured using the ProcartaPlex Mouse Cytokine & Chemokine Panel 1A (Affymetrix eBiosciences, Germany) multiplex immunoassay with magnetic beads following the manufacturer's instructions. Serum samples were run in duplicate. Measurement of cytokine levels was performed using the BioRad Luminex 200 machine.

Plaque Assay

Virus titers in lung homogenates of infected mice were detected by plaque assays on MDCK cell as described before (Otte et al., 2011).

Tissue processing and preparation of single cell solutions

Generation of single cell solution from lung and lymph nodes: tissue was collected by opening the thoracic cavity of mice and perfusing the right cardial ventrical with cold PBS. The lung, paraaortic lymph nodes or spleen was harvested at 3, 6 or 8 d p.i. respectively, dissected and digested with 30 IU DNase I (Sigmal, Germany) and 2 mg Collagenase D (Roche, Germany) at 37°C for 30 min. The resulting single cell solution was passed through a 40μ M strainer, washed with a minimum of 20 mL 2 mM EDTA/PBS and centrifuged at 300 x g for 8 min at 4 °C. Red blood cells were removed from single cell solution obtained from lung samples using RBC lyses buffer (eBiosciences, Germany), according to the manufacturer's protocols.

Flow cytometry analyses

Single cell solutions were analysed for cell surface markers by flow cytometry. Here, unspecific antibody binding was reduced by CD16/CD32 block (TruStain fcX[™] BioLegend, USA) and normal rat serum (Jackson Immuno Research, USA). Monoclonal antibodies specific to CD8 (clone 53-6.7), CD4 (RM4-5), CD11b (M1/70), Gr-1 (RB6-8C5), CD11c (N418), CD3e (145-2C11), F4/80 (CI:A3-1), CD45.2 (104), CD103 (2E7), CD40 (3/23), CD80 (B7-1 or 16-10A1), CD86 (GL-1), CD25 (PC61), CD44 (IM7), CD62L (MEL-14), CD19 (1D3), CD45R/B220 (RA3-6B2), CD69 (H1.2F3), CD90.1 (HIS51), CD90.2 (53-2.1), CD138 (281-2) and CXCR3 (R660) purchased from BioLegend, eBiosciences or BD via their distribution centers for Germany. Alexa Fluor 594, Alexa Flour 750 carboxylic acid succinimidyl ester or Pacific Orange (LifeTechnologies, Germany) was used to stain and exclude dead cells before fixation with 1% PFA for 15 minutes. Intracellular staining was done using fixation and permeabilization buffer according to the manufacturer's protocols (eBiosciences, Germany). Monoclonal antibodies specific for FoxP3 (FJK-16s) (eBiosciences, Germany) and nucleoprotein (NP; 104, Abcam, UK) were used for intracellular staining. Detection of virus specific cytotoxic CD8⁺ T cells was performed using a specific MHC dextramer containing a MHC I peptide of the viral nucleoprotein (NP) of the pH1N1 influenza A virus (H1N1-NP366, sequence ASNENVETM) (Immudex, Denmark) 10 minutes before cell surface staining. Cells were reconstituted in 0.5% BSA / 2 mM EDTA / PBS before acquisition. Multicolour acquisition was performed using the LSR II flow cytometer (BD, Germany), data were analyzed using FlowJo software (Tree Star, USA).

Hemagglutination inhibition assay

Antibody responses in non-pregnant and pregnant mice infected with 10^3 p.f.u. of pH1N1 28 d post challenge were detected by use of the hemagglutination inhibition assay. Serum

samples were heat-inactivated in order to remove residual complement activity (56°C, 30 min.) and a two-fold serial dilution was performed starting at a dilution of 1:10. Antibody titers were detected by adding 1 % chicken red blood cells according to standard protocols (Hackenberg et al., 2013).

In vivo killing assay of virus peptide loaded cells

Splenocytes from naïve mice were loaded with virus specific peptide ASNENVETM (JPT, Germany) by co-incubating both in RPMI 1640 (LifeTechnologies, Germany) at 37°C for 1 hour; control cells were cultured without the peptide. Virus peptide loaded cells were subsequently labelled with 5 mM carboxyfluorescein succinimidyl ester (CFSE; LifeTechnologies, Germany) at 37 °C for 10 min to generate CFSE^{high} cells, whereas control cells were labelled with 0.5mM CFSE and defined as CFSE^{low} cells. Virus peptide loaded CFSE^{high} and control CFSE^{low} cells were mixed at a ratio of 1:1 a total of $10x10^6$ cells were and injected into the tail vein of pH1N1 infected non-pregnant and pregnant mice at 6 d p.i. and in naïve mice as controls. Specific killing of virus peptide loaded cells was determined by assessment of the frequency of CFSE^{high} cells in the spleen of infected mice 18h post transfer, the percentage of target cells killing was calculated using the following formula: $100 - ((\% CFSE^{high} peptide loaded cells in infected mice/% CFSE^{low} peptide-loaded cells in control mice)) × 100 (Wong and Pamer, 2003).$

Adoptive transfer of virus-specific CD8⁺ T cells

Splenocytes from either naïve or infected non-pregnant mice were isolated at 10 d p.i. and used to purify $CD8^+$ T cells from a single cell suspension using the Magnetic Cell Isolation and Cell Separation (MACS) $CD8a^+$ T cell kit (Miltenyi Biotec, Germany). We chose to harvest $CD8a^+$ T cell at 10 d p.i., since our pilot experiments revealed a high frequencies of virus-specific $CD8^+$ T cells at this day, compared to e.g. at 6 or 8 d p.i.. Purity of isolated $CD8a^+$ T cells was controlled by flow cytometric analyses and either 1 x 10^6 $CD8^+$ T cells from infected, non-pregnant mice or from naïve non-pregnant mice were adoptively transferred into the tail vein of pregnant infected mice at 4 d p.i.. Survival and weight loss was monitored for the subsequent ten days.

Adoptive transfer of CD8⁺ T cells from CD90.1⁺ donor mice into CD90.2⁺ recipient mice and assessment of leukocyte homing

Congenic CD90.1⁺ donor C57BL/6 mice were infected with 10^3 p.f.u. pH1N1. T cells were purified at 8 d p.i from the spleen and lung draining lymph nodes using the Pan T cell isolation kit II (Miltenyi Biotec, Germany) and MACS isolation. Purity of isolated T cells was confirmed by flow cytometric analysis. Two x 10^6 purified T cells were administered intravenously (i.v.) into non-pregnant (n=12) and pregnant (n=19) infected wild type CD90.2⁺ recipient C57BL/6 mice at 3 d p.i. (equals gestation day 15.5). Eightteen hours after the adoptive transfer, mice were sacrificed, lungs were harvested and single cell solutions prepared. Lymphocyte populations were analysed using flow cytometric analysis.

Quantification of *Cxcl9, Cxcl10 and Cxcl11* expression in lung by Real-Timequantitative Polymerase Chain Reaction (RT-qPCR)

RNA isolation from lungs of non-pregnant and pregnant mice infected with 10³ p.f.u. of pandemic H1N1 at 3 and 4 d p.i. was performed using the RNeasy Plus Universal Mini Kit (Qiagen, Germany) or innuprep RNA Minikit (Analytik Jena, Germany) following the manufacturer's protocol. Prior to RNA isolation, lung tissue preserved in RNAlater (Qiagen, Germany) was homogenized using micro packaging vials with ceramic beads (1.4 mm diameter) in the Precellys 24 tissue homogenizer (PeQlab, Germany). Following RNA

isolation, DNAse I digestion was performed in order to minimise the amount of DNA in the sample using the rDNAse I Kit (LifeTechnologies, USA). In a next step, total RNA was reverse transcribed using SuperScript II Reverse Transcriptase (Invitrogen, Life Technologies) and cDNA concentrations were quantified by using the Infinite 200 PRO NanoQuant (TECAN, Switzerland). The gene expression analysis of Cxcl10 was performed using the StepOnePlus Real-Time PCR System and corresponding software (LifeTechnologie, Germany), using 100ng of cDNA. The following TaqMan Gene Expression Assays were purchased: Cxcl10 (Assay ID Mm99999072_m1) and Gapdh (Assay ID Mm99999915_g1 VIC). The amplifications were performed using 2×TaqMan Universal PCR Master Mix (LifeTechnologie, Germany). The RT-qPCR was conducted with cDNA as template in 20 µl reactions. Reactions were performed in 40 cycles using a standard two-step RT-qPCR: initial 50 °C for 2 min and 95 °C for 10 min, 15 s denaturation at 95 °C and 60 s annealing and extension at 60 °C. The gene expression analysis of Cxcl9 and Cxcl11 was conducted by the CFX96 Real-Time System (Biorad, Germany) and corresponding software. The following primer pairs were purchased: Cxcl9 (5'-CCCAAGCCCCAATTGCA-3' and 5'-(5'-GCAGGTTTGATCTCCGTTC-3'), Cxcl11 GAGAAAGCTTCTGTAATTTACCCGAGTA-3' and 5'- GTCCAGGCACCTTTGTCGT-TTA-3'), and Gapdh (5'-GGA-TGCAGGGATGATGTTCT-3' and 5'-AACTTTGGCATT-GTGGAAGG-3'). The amplifications were performed using Maxima SYBR Green/ROX qPCR Master Mix (Thermo Scientific by LifeTechnologies GmbH, Germany), using 5ng of cDNA. The RT-qPCR was conducted with cDNA as template in 12 µl reactions. Reactions were performed in 40 cycles using the following protocol: initial 95°C for 10 min, 15 s denaturation at 95°C and 60 s annealing at 60°C and extension at 72°C. All experiments were performed in technical triplicates. The expression of the genes of interest were normalized to the expression of *Gapdh*. Note, for *Cxcl11* at the cDNA concentration used, a signal was only obtained after 38 cycles. Relative transcript levels were calculated applying the equation described in (Pfaffl, 2001). The relative mRNA level of the target gene from non-pregnant animals at day 3 p.i. was set 1.

ChIP RT-PCR

ChIP assays were performed on whole lung tissue of pregnant and non-pregnant mice 3 days post infection as previously described by (Gunther et al., 2016) with the following minor modifications. Mouse lung tissue (half of the lung) was treated with 2 mg Collagenase D (Roche, Germany) for 30 min. at 37°C. Subsequently, cell suspension was cross-linked for 15 min in 1% formaldehyde. Cells were lysed and nuclei were isolated by centrifugation. Chromatin was extracted and fragmented by sonication using a Bioruptor (Diagenode) to an average length of 100-500 bp. A fraction of chromatin was set aside for the preparation of the input controls. For each IP 100µL chromatin was pre-cleared with BSA blocked protein-G sepharose beads (GE Healthcare, Germany) and incubated for 16 h at 4°C with 2 μ L α -H3K27me3 antibody (Millipore, Germany) or 2 µL normal rabbit IgG (Merck Chemicals, Germany). Chromatin-immunocomplexes were precipitated by the addition of protein-G sepharose beads, washed with increasing salt concentrations, eluted and de-crosslinked for 16 h at 65°C. DNA was purified by phenol-chloroform extraction and ethanol precipitation. Quantitative real-time PCR was performed using specific primers. Primer sequences for cxcl9, cxcl10, ccl5, cd8 and Gapdh have been published earlier (Nancy et al., 2012). In addition, positive and negative controls for H3K27me3 enrichment were selected using the USCS Genome Browser, data sets wgEncodeEM002637, wgEncodeEM002711, wgEncodeEM002641, wgEncodeEM002722, wgEncodeEM002724, wgEncodeEM002725,

showing increased or reduced enrichment of H3K27me3, respectively within 1000 bp upstream of the transcriptional start site: *mMsil fw* 5'-TCCGAGACACCGACTTCTCT-3'. mMsi1 5'-CCCTTTCGGACTGTCTGGAG-3', 5'rev mACHE fw CCGGTCAGGGGAATTAGCTC-3', mACHE rev 5'-GCTGAGAAGCCAGACCACAT-3', mHoxc13 fw 5'-TCGGGACGAAGTCTGCATTT-3', mHoxc13 rev 5'-CTCCGACCTCAGAGGCTTTC-3', mAtp8b5 fw 5'-TACTTAGTCATGGCCCCTCT-3', mAtp8b5 rev 5'-AGGAACAAAGGCTGGGGTTTAT-3'. 1.5µL of diluted input samples and ChIP samples was used in a 10µL reaction volume using SensiMix[™] SYBR[®] Hi-ROX Kit (Bioline) and 3 pmol gene-specific primers. Cycling was performed using a Rotor-Gene Qplex (Qiagen) at the following conditions: 10 min at 95°C, 50 cycles of 10 s at 95°C, 25 s at 57°C, and 20 s at 72°C. Melting curve analysis was performed using Rotor-Gene software. The percentage of recovered material relative to the input was calculated. Chip assays using 3x10⁵ stromal cells isolated from lung tissue of pregnant and non-pregnant mice 3 d p.i. were performed using ChromaFlash High-Sensitivity ChIP kit (Epigentek, Farmingdale, NY, USA), according to the manufacturer's protocol with minor modifications. CD45^{neg} Stromal cells were isolated of whole lung homogenates using the Magnetic Cell Isolation and Cell Separation (MACS) and processed as written above. 2,5 ul of the extracted ChIP-DNA was used for ChIP-qPCR with primer pairs spanning promoter sites (mGapdh fw 5'-CCCAGCCAAGGCTGTGTTAGACT-3', mGapdh rev 5'-GCTGTGCATAGAGCCTCGGTAG-3', mCxcl95'fw 5'-TGTTTTGAAAGGGTGGTTGTTGGCTTT-3', mCxcl9 rev CAATTTGCCTTCTGGGACAGGATG-3', mCxcl10 5'fw 5'-GCATTCAGAACAGAAGCCGGAAGT-3', mCxcl10 rev CATCCTTGACCCTGTAACCACAC-3'). The expression level of the target DNA sequence

is represented as fold enrichment percentage compared to non-immune IgG.

Receptor binding specificity upon de- and resialylation of turkey erythrocytes For the determination of receptor binding preferences of the viral hemagglutinin, a hemagglutination (HA) assay with modified turkey red blood cells (TRBCs) was conducted as described before (Paulson and Rogers, 1987, Glaser et al., 2006). Briefly, sialic acids (SAs) were removed from TRBCs by incubation with 50 mU neuraminidase from Vibrio cholerae (Roche) at 37°C for 1 h. Resialylation was performed by incubation with 6 mU of a2,3sialyltransferase from Pasteurella multocida (Sigma-Aldrich) or 38 mU α2,6-sialyltransferase from Photobacterium damsela (Sigma-Aldrich, Germany), respectively and cytidine-5'monophospho-N-acetylneuraminic acid sodium salt (CMP, Sigma Aldrich, Germany) at 37°C for 2 h. After washing, the RBC were resuspended in PBS containing 1% bovineserum albumin to a final concentration of 0.5% RBC. The HA assay with $\alpha 2,3$ -resiallyted and $\alpha 2,6$ resialylated TRBCs, respectively is performed in 96 well v-bottom plates. 100 µl of the virus pH1N1 (A/Hamburg/NY1580/09), pH1N1-HA_{Q223R}, pH1N1-NS_{R211K/D54N} or pH1N1-HA_{0223R}+ NS_{R211K/D54N} (2⁶ hemagglutination units (HAU)) are incubated with 50 µl 0.5 % TRBCs (non-treated or modified) for 30 min at 4 °C, and HAUs were recorded. As control viruses for the resialylation of the desialylated TRBCs A/Netherlands/213/03 (H3N2) was used as a control for a2,6-linked SA binding and a H5N1 reassortant virus with 7 genes of A/PR/8/34 (H1N1) and the HA segment of A/Vietnam/11/94 (H5N1) with a monobasic cleavage site as a control for $\alpha 2,3$ -linked SA binding.

Generation of viral recombinant pH1N1 quasispecies mutations and assessment of pathogenicity

In order to generate recombinant 2009 pH1N1 viruses by reverse genetics the pHW2000 based 8-plasmid system was used as previously described (Hoffmann et al., 2000). In brief, the single or multiple point mutations (pH1N1-HA_{Q223R}, pH1N1-_{NSR211K/D54N} or pH1N1-HA_{Q223R}+ NS_{R211K/D54N}) were introduced into the 2009 pH1N1 virus (A/Hamburg/NY1580/09) by site directed mutagenesis. The recombinant viruses were then sequenced to confirm introduced amino acid substitutions and to exclude additional mutations.

Statistics

For the statistical analyses of survival rates, the Gehan-Brelow-Wilcoxon test was used. For the experimental data, mean \pm SEM or mean \pm SD and p-values were calculated. Levels of significance between groups were tested using Student's t-test for normally distributed data and Mann-Whitney test if normal distribution was not present, as stated in each figure legend. Level of statistical significance was defined as p<0.05 (*equals p<0.05; ** equals p<0.01; *** equals p<0.001).

С A в 100 160 160 Seasonal H1N1 Weight [%] Survival [%] Weight [%] 10⁵ p.f.u. ** 120 120 50 Ħ 80 80 6 8 10 12 14 d p.i. 6 8 10 12 14 d p.i. 6 8 d p.i. 10 12 14 2 ò 2 ō 2 4 Ò 4 4 D Е F 160 160 100 10² p.f.u. Survival [%] Weight [%] Weight [%] 120 120 50 80 80 Pandemic H1N1 6 8 10 12 14 d p.i. 6 8 10 12 14 d p.i. 8 10 12 14 ό ż 4 ċ Ż 4 6 8 d p.i. ż 4 G н I 100 160 160 Weight [%] Weight [%] Survival [%] 104 p.f.u. 120 120 50 H * \$ \$ \$ \$ 80 80 6 8 10 12 14 d p.i. 10 12 14 6 8 d p.i. 10 12 14 ó 6 8 Ò Ż à 4 Ż 4 d p.i. -o- Non-pregnant PBS Pregnant PBS -D- Non-pregnant H1N1 Pregnant H1N1

Supplemental results

Figure S1: Pathogenicity of sH1N1 and pH1N1 in non-pregnant and pregnant mice upon infection with different virus doses, related to Figure 1. Non-pregnant or pregnant mice were infected with 10^5 p.f.u. of sH1N1 (non-pregnant: n=5; pregnant: n=9), 10^2 p.f.u. or 10^4 p.f.u. of pH1N1 (non-pregnant: n=5, pregnant: n=9) influenza virus. Non-pregnant and pregnant control groups received PBS. Survival (A, D, G) and weight loss (B,C,E,F and H,I) were observed for 14 d p.i.. The statistical significance in the experiments was calculated by Student's t-test (*p<0.05; **p<0.001, ***p<0.001).



Figure S2: Pregnancy parameter and humoral immunity in mice infected with pH1N1 influenza virus, related to Figure 1 and 3. Pregnant mice were infected with 10³ p.f.u. of pH1N1 influenza virus, while control groups were inoculated with PBS only. Placentae were stained with Masson-Goldner trichrome staining for histomorphological assessments and by immunohistochemistry for detection of viral antigen (NP staining) (A). Panoramic Viewer software (3DHISTECH Ltd., Hungary) was used to prepare the photomicrographs. Total number of implants (B), abortion ratio (C, D shows a gravid uterus harvested on gd 16.5, an

abortion is marked as '*'), fetal weight (E) and maternal serum progesterone levels (F) in pregnant pH1N1 (n=8-12) and pregnant control (n=8-9) dams, assessed at 3 and 6 d p.i.. (G) Virus titers were determined in non-pregnant (n=11) and allogenic pregnant (n=12) infected mice in the gut at 3 and 6 d p.i.. Virus titers were assessed in pregnant animals in the placenta at 3 d p.i. (n=4) and 6 d p.i. (n=3) and in concepti at 3 d p.i. (n=3). Gestational length (H) and number of living offspring (I) in non-infected pregnant (syngenic n=8, allogenic n= 3) and infected dams (syngenic n=12, allogenic n=15). Note that the data shown for H1N1 dams in (H,I) could only be assessed in surviving dams. (J) Frequency of B cells $(B220^+)$ in the spleen of pregnant infected mice (n=5), compared to non-pregnant infected animals (n=9), as measured by flow cytometry at 3 d p.i.. (K) Virus-specific hemagglutination inhibition (HI) titers in sera depicted as geometric mean titer (GMT) of pregnant infected mice (n=9) at 28 d p.i., compared to non-pregnant infected animals (n=12). (L) Frequency of CD19⁺CD138^{hi} plasma cells in the spleen of pregnant infected mice, compared to non-pregnant infected animals at 3 d p.i. Frequency of B cells co-expressing CD69 (M), CD80 (N) and CD86 (O) in paraaortic lymph nodes of pregnant infected mice (n=5), compared to non-pregnant controls (n=9). (P) Recruitment of H3K27me3 histone modification in stromal cells of the lung of pregnant (n=3) and non-pregnant (n=2) mice infected with Influenza 3 d p.i.. Chromatin immunoprecipitation was performed followed by RT-PCR. y-axis represents the fold enrichment based on the IgG control. Exemplarily, the IgG control for CXCL10 is illustrated. Data shown in (B,C,E-P) are presented as mean and SEM or SD. The statistical significance in the experiments was calculated by Student's t-test or Mann-Whitney test (*p<0.05).



Figure S3: Pathogenicity of recombinant H1N1 at different virus doses and secretion of inflammatory cytokines in lungs of non-pregnant mice infected with wild type pH1N1 and recombinant H1N1, related to Figure 6. Non-pregnant mice were infected with 10^2 or 10^1 p.f.u. of parental or recombinant pH1N1 strains (pH1N1: n=10; pH1N1-HA_{Q223R}: n=10;

pH1N1-NS_{R211K/D54N}: n=10; pH1N1- HA_{Q223R+} NS_{R211K/D54N}; n=5-10). Survival (A, E) and weight loss (B-D,F) were monitored for 14 d p.i. in infected animals and uninfected controls (n=2 or 5). Pregnant mice were infected with 10³ p.f.u. of recombinant pH1N1-NS_{R211K/D54N} at gd 12.5. Survival (G) and weight loss (H) were determined 14 d p.i. in pH1N1-NS_{R211K/D54N} infected pregnant mice (n=7) in comparison to uninfected controls (n=3). (I-L) Cytokines (IFN-a, IFN-y, IL-6, and TNF-a) determined by Luminex assay in lungs of non-pregnant mice infected with 10³ p.f.u. of pH1N1 WT (open square, n=11) or pH1N1-NS_{R211K/D54N} mutant (black circle, n=11) virus, respectively, and harvested on day 3 p.i.. (M-P) Cytokines (IFN-a, IFN-y, IL-6, and TNF-a) determined by Luminex assay in lungs of non-pregnant mice infected with 10³ p.f.u. of pH1N1 WT (open square, n=11) or pH1N1-HA_{Q223R}- $NS_{R211K/D54N}$ mutant (open diamonds, n=5) virus, respectively, and harvested on day 3 p.i.. If less measurement points than the indicated n are visible in the graphs, the not shown ones were out of range (OOR), i.e. they were too low to be quantified as their values were below the range of the standard curve. Data are presented as mean and SD normalized to the organ weight. The statistical significance in the experiments was calculated by Student's t-test (p<0.05, p<0.01, p<0.001).



Figure S4: Assessment of infectious course in secondary infected mice, related to Figure 1 and 2. Non-pregnant mice were either treated with PBS or infected with 10^3 p.f.u. pH1N1. 28 d p.i. mice were mated to BALB/c males and infected for a second time with 10^3 p.f.u. pH1N1 at gd 12.5 (A). Survival (B) and weight loss (C) were monitored over 14 d post

second infection (non-pregnant PBS - pregnant pH1N1 n=13; non-pregnant pH1N1 pregnant pH1N1 n=10) compared to uninfected controls. (D) 28 and 69 d post first infection antibody titers were measured in serum (28 d p.i. uninfected n=45, infected n=42; 69 d p.i. uninfected controls n=5, non-pregnant PBS – pregnant pH1N1 n=13, non-pregnant pH1N1 – pregnant pH1N1 n=10). (E) Virus titers were assessed in non-pregnant PBS - pregnant pH1N1 (n=11) and non-pregnant pH1N1 - pregnant pH1N1 (n=9) mice in comparison to uninfected controls (n=5) at 44 d post first infection. Expression of costimulatory markers (CD40, CD86 and CD80) were determined on alveolar macrophages (alvM Φ) (F), resident dendritic cells (resDC) (G), inflammatory DCs (inflDC) (H), migratory DCs (migrDC) (I) and plasmacytoid DCs (pDC) (J) in non-pregnant PBS - pregnant pH1N1 (n=11) and nonpregnant pH1N1 – pregnant pH1N1 (n=9) mice at 44 d post first infection. (K-N) Cytokines (IFN- α , IFN- γ , IL-6, and TNF- α) were determined by Luminex assay in lungs of nonpregnant PBS – pregnant pH1N1 (open circles, n=11) and non-pregnant pH1N1 – pregnant pH1N1 (black circles, n=9) mice at 44 d post first infection. If less measurement points than the indicated n are visible in the graphs, the not shown ones were out of range (OOR), i.e. they were too low to be quantified as their values were below the range of the standard curve. Values are normalized to the organ weight. (C-N) are presented as mean and SEM or SD. The statistical significance in the experiments was calculated by Student's t-test or Mann-Whitney U-test (*p<0.05, **p<0.01, **p<0.001).

Table S1, related to Figure 2: Log2 fold change of known innate immunity genes between non-pregnant (NP) and pregnant (P)

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Table S2, related to Figure 6: recombinant pH1N1 viruses, (A) sialic acid binding properties of 2009 pH1N1, (B) MLD₅₀ of pH1N1 and recombinant H1N1 in non-pregnant mice and (C) occurrence of 2009 H1N1-specific NS1-R211K mutation among human influenza A virus isolates

A

HAU								
Virus	Untreated TRBCs	VCNA treated TRBCs [†]	α2,3 SA TRBCs [#]	α2,6 SA TRBCs [#]				
2009 pH1N1	64	0	4	64				
2009 pH1N1-HA _{Q223R}	64	0	32	0				
2009 pH1N1-HA _{Q223R} .NS _{R211K/D54N}	64	0	8	0				
H3N2 (a2,6) control	64	0	0	128				
H5N1-HA _{monobasic} (α 2,3) control	64	0	128	0				

†HA assays were performed with two fold dilutions of red blood cells (RBC) from turkey with pH1N1 and reassortant viruses (pH1N1-HA_{Q223R}, pH1N1-NS_{R211K/D54N} or pH1N1-HA_{Q223R}+ NS_{R211K/D54N}). As control viruses A/Netherlands/213/03 (H3N2) and a 7 + 1 reassortant virus of A/PR/8/34 (H1N1) and the HA of A/Vietnam/11/94 (H5N1) with a monobasic cleavage site (H5N1) were used. Results from one representative run are shown in hemagglutination units (HAU) for each tested virus. ‡Removal of sialic acids (SA) was controlled after *Vibrio cholerae* NA (VCNA) treatment. #Subsequently, RBC were either resialylated with α 2,3-(N) sialyltransferase or α 2,6-(N)-sialyltransferase, respectively.

В

Virus	Dose for infection [p.f.u.]	Survival [%]	MLD ₅₀ [p.f.u.]	
2009 pH1N1	10^4 10^3 10^2	60 100 100	10 ^{3.83}	
2009 pH1N1 – HA _{Q223R}	$\frac{10^3}{10^2}$	13 80	10 ^{2.46}	
2009 pH1N1 – NS _{R211K/D54N}	$\frac{10^3}{10^2}$	7 70	10 ^{2.34}	
2009 pH1N1 – HA _{Q223R} + NS _{R211K/D54N}	10^{3} 10^{2} 10^{1}	0 40 100	10 ^{1.83}	

Non-pregnant mice were infected with serial 10-fold virus dilutions (10^1 to 10^4 p.f.u.) of pandemic H1N1 or recombinant pH1N1 (pH1N1-HA_{Q223R}, pH1N1-NS_{R211K/D54N} or pH1N1-HA_{Q223R} + NS_{R211K/D54N}) and observed for 14 d p.i.. The MLD₅₀ was calculated as described by Reed and Muench (Reed LJ, 1938).

С

		R211K positions in NS1 during pandemic phase (year)									
Gene product	2009 H1N1- specific amino acid position	2006 2009	2009	2010	2011	2012	2013	2014	2015	2016	total
NS1	R211K	0/820	17/5207*	0/983	4/561*	0/198	0/393	0/383	0/1509	6/4020*	27/14074*

The analyzed data include amino acid sequences of the protein-encoding genes for all human pH1N1 strains with NS1-sequence data available from the GISAID database from 1/1/06 to 12/31/16.

*Only 2009 pH1N1 strains presented the NS1-R 211K position.

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13 Affidavit

Ich versichere ausdrücklich, dass ich die Arbeit selbständig und ohne fremde Hilfe verfasst, andere als die von mir angegebenen Quellen und Hilfsmittel nicht benutzt und die aus den benutzten Werken wörtlich oder inhaltlich entnommenen Stellen einzeln nach Ausgabe (Auflage und Jahr des Erscheinens), Band und Seite des benutzten Werkes kenntlich gemacht habe.

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Unterschrift: