Antenatal endogenous and exogenous glucocorticoids and their impact on immune ontogeny and long-term immunity

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Antenatal endogenous and exogenous glucocorticoids and their impact on immune ontogeny and long-term immunity

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Abstract

Endogenous levels of glucocorticoids rise during pregnancy to warrant development and maturation of the fetal organs close to birth. However, during most of the gestation, the fetus is protected from excessive biologically active endogenous glucocorticoids by placental and fetal expression of 11β-hydroxysteroid dehydrogenase 2 (11β-HSD2). Maternal stress, which may overwhelm placental 11β-HSD2 activity with high glucocorticoid levels, or administration of synthetic glucocorticoids to improve the survival chances of the premature newborn, are associated to postnatal increased risk for immune diseases. Fetal exposure to excessive glucocorticoids may underlie this altered postnatal immunity. Here, we revise the role that placental and fetal 11β-HSD2, fetal glucocorticoid exposure and programming of the offspring's the hypothalamic-pituitary-adrenal (HPA) axis play on concerted steps in immune fetal development. We could identify gaps in knowledge about glucocorticoid induced programming of immune diseases. Finally, based on current evidence about glucocorticoid and HPA axis mediated immune regulation, we hypothesize on mechanisms that could drive the enhanced risk for atopies, infections and type I diabetes in offspring that were prenatally exposed to glucocorticoids.

Introduction

In mammals, maternal physiological adaptations to pregnancy ensure appropriate fetal growth and development [1]. These multisystem adaptations include the modulation of endocrine signals, such as those derived from the hypothalamic-pituitary-adrenal (HPA) axis [2]. The gradual increase in maternal release of adrenal glucocorticoids during the second half of gestation accelerates as birth approaches[3]. Concurrently, the developing embryo is protected from maternal glucocorticoids throughout most of gestation by mechanisms that include feto-placental expression of the glucocorticoid-inactivating enzyme 11β-hydroxysteroid dehydrogenase type 2 (11β-HSD2). These mechanisms may be inadequate in some circumstances, for example when maternal glucocorticoid levels are chronically elevated by stress, or evaded by synthetic glucocorticoids. Under these circumstances, exposure of the fetus to inappropriately high glucocorticoid levels impairs fetal growth and elicits neuroendocrine changes that persist into adulthood (termed "programming") [4,5]. Given the higher incidence of atopic diseases in individuals who

were exposed to maternal stress prenatally and the potent immunomodulatory properties of glucocorticoids, concerns have been raised about potential long-lasting effects of excessive prenatal glucocorticoid exposure on the developing fetal immune system[6-8]. Here, we (i) review current knowledge of the factors that determine fetal glucocorticoid exposure, and (ii) review the evidence relating to mechanisms by which excessive prenatal glucocorticoid exposure may affect the immune system.

Glucocorticoids in pregnancy

Physiology of glucocorticoids during pregnancy

The secretion of glucocorticoids (Box 1) is tightly controlled by the HPA axis. The cyclic activation of the axis results in the hypothalamic secretion of corticotrophin releasing hormone (CRH)[2], which stimulates the release of adreno-corticotrophin hormone (ACTH) from the pituitary. In turn, ACTH activates the adrenal release of glucocorticoids: cortisol in humans and most other animals, corticosterone in rats and mice[3]. The activity of the maternal HPA axis changes dramatically in pregnancy and post-partum (reviewed in[9,10]). Particularly during the second half of pregnancy, circulating glucocorticoids stimulate placental CRH synthesis[11-14]. CRH further stimulates pituitary ACTH release, which results in adrenal glucocorticoid production [15] and in a physiological state of "hypercorticolism", with glucocorticoids rising dramatically towards parturition[16,17]. In late pregnancy, the HPA axis also becomes hyporesponsive to stress, probably due to a reduced forward drive from hypothalamic CRH and vasopressin, rather than to a change in negative feedback regulation[18]. As described below, high maternal glucocorticoids levels likely contribute to fetal organ maturation before birth. To some extent, the rise in maternal plasma levels of corticosteroid-binding globulin (CBG; Box 1) induced by high estrogen levels in late pregnancy offsets the high maternal glucocorticoid levels, by reducing the fraction of free, biologically available glucocorticoids[16]. Despite the high maternal glucocorticoid levels in the late human and rodent pregnancies, for most of gestation fetal plasma glucocorticoid levels are 5 to 10 times lower than in the mother, though levels rise markedly close to parturition to mature fetal organs[19-21]. However, intracellular glucocorticoid action is not solely dependent upon circulating glucocorticoid levels, but can be modulated by the activity of the 11β-HSD enzymes. The type 2 isozyme, 11β-HSD2, catalyses the inactivation of cortisol and corticosterone, generating intrinsically inert cortisone and 11-dehydrocorticosterone, respectively. In contrast, the type 1 isozyme, 11β-HSD1, catalyses the opposite reaction, regenerating active glucocorticoids. As reviewed below, these enzymes play distinct temporal and tissue-specific roles in the feto-placental unit during pregnancy.

Glucocorticoid excess during pregnancy

Endogenous glucocorticoids: Maternal stress

Despite the hypo-responsiveness of the maternal HPA axis to stress in late pregnancy, it is still activated by strong or chronic stressors[22,23], including inflammation and infection[24]. Moreover, expression of placental 11β-HSD2 can be suppressed by stress[25,2] and saturation of maternal CBG may lead to increased levels of free glucococorticoids in plasma[26]. All of these potentially lead to excessive fetal glucocorticoid exposure[23]. Intrauterine infection and placental inflammation are associated with fetal glucocorticoid excess, as evidenced by a reduction in thymic size, a biological marker of glucocorticoid action, in human fetuses exposed to these situations *in utero*[27,28]. This could be at least partly mediated by maternal glucocorticoids, as a consequence of activation of the maternal HPA axis[23]. As well as affecting

glucocorticoid levels, the pronounced maternal immune system responses normally elicited by infection and inflammation could, of course, directly affect development of the fetal immune system, a large topic, beyond the scope of this review. Here, we restrict our discussion to the effects of maternal stress perception as a source of excessive fetal glucocorticoid exposure.

Synthetic glucocorticoids

Synthetic glucocorticoids are prescribed during pregnancy for several clinical indications. If the subject of treatment is the expectant mother, for instance to treat asthma and autoimmune disease such as systemic lupus erythematosus, or to prevent recurrent miscarriage in the first trimester of pregnancy, prednisolone is most commonly used, with doses being up to 40mg/day. Prednisolone and its inactive 11-keto metabolite, prednisone, are substrates for the 11β-HSD enzymes (reviewed in[29,30]). However, high doses of prednisolone are likely to overwhelm the capacity of placental 11β-HSD2, and reach the fetus. Prednisolone administered in the first trimester of pregnancy (weeks 4 to 13) did not have a teratogenic effect, but caused a two-fold increase in pre-term birth rate and reduced birthweight in term babies[31].

Probably more relevant to fetal development are glucocorticoids administered to expectant mothers at risk of preterm delivery between 24 and 34 weeks of gestation[32], though benefit is seen even up to 36 weeks [33], in order to improve neonatal survival if the baby is born preterm. A single course of antenatal corticosteroids reduces morbidity due to acute respiratory distress in neonates and does not appear to be associated with overt short-term adverse effects on mother or fetus[32]. Importantly, however, a recent trial, conducted in low-resource settings, has shown no benefit of antenatal corticosteroid treatment in the mortality rates of very small infants, and a significant increase in neonatal mortality of infants born at later gestational ages[34]. Moreover, synthetic glucocorticoids suppress maternal and fetal HPA axis activity for at least one week after administration[35], and exert long-lasting effects upon the HPA-axis in children [36]. Despite the existing guidelines for prenatal steroid administration, more than half of the treated women do not deliver within the window of effect of the drug (less than 7 days), and repeated courses of steroids have proved detrimental rather than beneficial[37]. Betamethasone and dexamethasone are the steroids of choice to reach fetal organs if premature birth is imminent. Both have higher affinities for the glucocorticoid receptor (GR) than cortisol and neither is inactivated by 11β-HSD2 [38], a property that allows them to bypass feto-placental 11β-HSD2 but also ensures a longer half-life in the maternal circulation. Both bind poorly to CBG. Thus, these compounds cross the placenta readily, bypass 11β-HSD2 and are 25-fold more potent than endogenous cortisol (reviewed in[39]), causing a sizable peak of supraphysiological glucocorticoid bioactivity in the fetus, that only recedes three days after the second injection of betamethasone[40].

Dexamethasone (1.5mg/day) is also used in cases of congenital adrenal hyperplasia to prevent masculinization of the female fetus. In this case, therapy starts as soon as pregnancy is recognized, and is continued until the end. A comprehensive metaanalysis[41] of the few available data (four studies, n=325 pregnancies) reports a reduction in fetus virilization and no deleterious effects on stillbirths, spontaneous abortions or foetal malformations. Inconsistent results were reported on parent-reported behavioural and developmental outcomes later in life[41], and no data exist on metabolic, cardiovascular or immunological outcomes.

Regulation of fetal glucocorticoid exposure: Metabolism of glucocorticoids by 11β-HSDs during gestation and fetal glucocorticoid production.

~~Expression of 11β-HSD1 and 11β-HSD2 in the uterus~~

~~Both 11β-HSD1 and 2 are expressed in the uterus in the non-pregnant state, though they differ in their temporal and spatial distribution[42]. In rodents, 11β-HSD1 is expressed in epithelial cells in the uterine wall whereas 11β-HSD2 is present in the endometrial stroma and in the myometrium[42,43]. Expression of both enzymes is dependent upon estrogen[42]. In contrast, in human endometrium, 11β-HSD2 is present in the glandular epithelium with little expression of 11β-HSD1 until menstruation[44]. 11β-HSD1 is markedly up-regulated following decidualisation of human endometrial stromal cells[45], suggesting a possible role in embryo implantation. It has been suggested that glucocorticoid inactivation in the uterine wall contributes to the maintenance of the fetal allograft[46]. However, mice lacking 11β HSD2 are fertile and have normal sized litters[5], so any such role is moot. In pregnant rats, the marked up-regulation of 11β HSD1 in the myometrium shortly before parturition is dependent upon the feto-placental unit[47], suggesting a possible feed-forward regulation as glucocorticoid levels rise close to birth. However, 11β-HSD1-deficient mice do not show a marked parturition phenotype, suggesting any effect on parturition is subtle.~~

11β-HSD2 expression in the feto-placental unit

From as early as 5 weeks of human pregnancy, 11β-HSD2 is expressed in the syncytiotrophoblast layer of the labyrinth zone of the placenta[48]. This syncytiotrophoblasts form the interface between the maternal and fetal circulations where nutrients and other substances are exchanged. During a normal pregnancy, 11β-HSD2 inactivates most of the maternal glucocorticoid passing through the placenta to the fetus[49,50]. By the end of the first trimester, 11β-HSD2 becomes expressed in the cytotrophoblast and extravillous trophoblasts[48], potentially impacting placentation. Indeed, placentas of *Hsd11b2*$^{-/-}$ mice show altered structure and function, with reduced placental vascularization and altered nutrient transport[4]. The ontogeny of placental 11β-HSD2 expression differs between species. In humans, 11β-HSD2 expression remains high until parturition[51,52], maintained by human chorionic gonadotrophin activation of cAMP signaling[53], though 11β-HSD2 activity may reduce close to birth[46,54]. Placental 11β-HSD2 expression declines towards the end of gestation in the rat, and still earlier in the mouse (reviewed in[29]), with negligible levels of *Hsd11b2* mRNA from E13[55] and enzyme activity markedly decreased by E16[50]. This decline in 11β-HSD2 activity is likely to be due to[56], and contribute to, the late gestation rise in fetal glucocorticoid levels essential for maturation of fetal tissues and organs, which may differ in timing between species.

11β-HSD2 is also widely expressed in the fetus during early to mid-gestation, where it protects the developing tissues from inappropriate glucocorticoid exposure[55,57,58]. This protective role of 11β-HSD2 has been investigated in detail in the brain. Specific deletion of neuronal 11β-HSD2, expressed here only in very early life, affects offspring behavior once adult[59]. These studies are important as the first to demonstrate a protective role for 11β-HSD2 specifically in the fetus, distinct from the protective role of placental 11β-HSD2. In most tissues in the mouse (mineralocorticoid target tissues being the exception), 11β-HSD2 expression decreases markedly in late gestation[55]. The same is likely true in humans[46].

Reductions in placental 11β-HSD2 activity associate with reduced birth weight and are found in a variety of conditions including intra-uterine growth retardation, maternal asthma, maternal undernutrition (reviewed in[29]) and maternal vitamin D deficiency[60]. Glucocorticoids are raised in at least some of these conditions, if not all and down-regulate placental 11β-HSD2[56], though possibly only in late gestation[61]. The effect of exogenous corticosteroids on the regulation of placental 11β-HSD2 is still controversial: dexamethasone administered right before delivery increases levels of mRNA encoding 11β-HSD2 in sheep [61] and mice[62], while high levels of exogenous cortisol in late pregnancy decrease 11β-HSD2 enzyme activity in sheep [56].

In vitro, placental 11β-HSD2 activity can be down-regulated or inhibited by a variety of factors. As well as glucocorticoids, hypoxia, and pro-inflammatory cytokines (IL-1β or TNFα) reduce placental 11β-HSD2 expression[48,63-65], potentially offering a unifying glucocorticoid-mediated mechanism for the fetal programming effects of these different stressors [50]. Importantly, placental 11β-HSD2 activity is reduced in human pregnancies complicated by pre-eclampsia[48,66]. Whether this reflects cause or effect is important to establish, though it is noteworthy that *Hsd11b2*$^{-/-}$ mice model aspects of pre-eclampsia, including intra-uterine growth retardation[5,4,67]

11β-HSD1 expression in the feto-placental unit

Although mRNA encoding 11β-HSD1 is present in the rodent placenta[68], the corresponding activity is not[69]. Similarly, 11β-HSD1 is little expressed in the fetus during most of gestation[68]. It becomes expressed close to birth, notably in the liver and lung[68,70], where it contributes to the maturational effects of glucocorticoids that occur just prior to birth[71]. 11β-HSD1 is also expressed at term in the chorion and the amnion, the fetal membranes that together comprise the amniotic sac[52,46,72]. Glucocorticoids induce 11β-HSD1 expression in cultured chorionic trophoblasts as well as amnionic fibroblasts[73] suggesting a feed forward mechanism to amplify glucocorticoid levels in the fetal membranes and the amniotic fluid, as birth approaches[72]. Moreover, whilst the pro-inflammatory cytokines IL-1 β or TNFα alone have only a modest effect on 11β-HSD1 expression in human amnion fibroblasts, they potentiate its induction by glucocorticoids[74]

The fetal HPA axis

The fetal hypothalamic-pituitary-adrenal (HPA) axis becomes active in mid to late gestation, potentially contributing to the increase in fetal plasma glucocorticoid levels close to birth. Glucocorticoid synthesis initiates in the fetal adrenal gland around the 28th week of pregnancy in humans, and at embryonic day (E) 14.5 in mice (reviewed in[21]). In mice, plasma glucocorticoid levels increase rapidly from E15, though in humans, levels only increase substantially in the week before birth. Negative feedback regulation of the HPA axis is established around E16.5 in mice[75]. The time at which the human HPA axis becomes responsive to the normal regulation is unclear. However, evidence suggests that HPA axis suppression occurs with intrauterine exposure to synthetic glucocorticoids[35], suggesting negative feedback of the HPA axis operates in the human fetus from the time of, or shortly after, initiation of adrenal glucocorticoid synthesis. Maternal undernutrition increases HPA axis activation in mice[50], though whether it also causes premature initiation of adrenal corticosteroid synthesis is currently unknown. However, this is likely given the ability of

the fetal HPA axis to compensate for maternal glucocorticoid deficiency with maternal adrenalectomy[76,50].

Glucocorticoid sensitivity in the placenta and the fetus

The main determinants of glucocorticoid sensitivity are the receptors: the higher affinity type 1 glucocorticoid receptor (or mineralocorticoid receptor, MR) and the type 2 glucocorticoid receptor (GR). Expression of MR is negligible in the rodent placenta[69,55]. However, MR immunoreactivity, as well as evidence for the encoding mRNA, has been reported in human placenta [77]. Whether this reflects a true species difference requires confirmation. GR, by contrast, is expressed in the labyrinth zone of the placenta, and at higher levels in the basal zone, the main site of placental hormone synthesis and varies little through gestation[69,43,68].

Within the fetus, MR and GR show negligible expression in the first half of gestation, at least in the mouse[55,68,78]. In mice, GR first appears in the fetus around E10, with initial sites of expression being the developing heart and the 3rd branchial arch[78], the latter giving rise to the thymus, which strongly expresses GR from E12.5[68]. From E12.5, GR expression becomes much more widespread. Thus, it is likely that the capability to respond to glucocorticoids via GR precedes, by up to several days depending on the tissue, the initiation of adrenal glucocorticoid synthesis at E14.5. MR, by contrast, shows little expression before E13.5[55]. There is transient expression of MR in muscle and a few other tissues between E14.5 and E18.5, but by E18.5, the distribution of MR expression is similar to the adult pattern. Importantly, however, MR is expressed in the developing thymus at E18.5, persisting into neonatal life[55], though expression has gone here by adulthood [79]. This suggests a window of susceptibility during which the thymus may be extremely sensitive to the effects of glucocorticoid, mediated either via GR[80] or MR.

However, whilst essential, the presence of GR and/or MR by itself is not always sufficient for the response to glucocorticoids. For example, levels of GR do not associate with sensitivity to glucocorticoid-induced apoptosis in thymocyte populations[81]. Glucocorticoid resistance, despite expression of GR, is a well-known phenomenon in chronic disease states such as asthma, as well as in acute lymphoblastic leukemia. In the latter, resistance is associated with a Warburg type metabolism[82] and can be overcome by inhibition of glycolysis[83]. Acquisition of the ability to respond to glucocorticoids may depend on expression of "competence" factors[84], such as transcription factors, which act co-operatively with GR to effect glucocorticoid-mediated gene regulation. It is interesting to speculate that these may allow, or be facilitated by, a switch in metabolism as a direct or an indirect effect of glucocorticoid action[85]. ~~Thus, many factors potentially regulate glucocorticoid sensitivity – acting upstream, downstream or cooperatively with GR[86,87,85], though the relevance to developmental actions of glucocorticoids is, as yet, largely unexplored.~~ Further, glucocorticoid action in the fetus is likely to prime subsequent responses; the response of the tyrosine aminotransferase gene is more rapid following a second exposure to glucocorticoid, than following the first. This memory effect is mediated by glucocorticoid-induced gene demethylation at one site, required for glucocorticoid-dependent transcription factor recruitment to a second site[88]. We return to this topic of glucocorticoid sensitivity in the context of developmental programming, below.

Consequences of fetal exposure to glucocorticoids

Although glucocorticoids are essential for the maturation of fetal tissues and organs prior to birth[19,20],

excessive or possibly premature exposure to glucocorticoids during sensitive windows of development reduces tissue accretion and body weight, and elicits permanent effects on organs and tissues. These effects, which manifest in the offspring once adult, include hypertension, hyperglycemia, altered HPA axis activity and anxiety or depressive-like behaviours, increasing the risk of an individual for cardio-metabolic and psychiatric disease[89,21]. This phenomenon has been termed developmental "programming". Maternal stress, which may overwhelm placental 11β-HSD2 with high maternal glucocorticoid levels, programmes adult behavior and HPA axis responses[90-92] and increases allergic airway responses [93]. Early life programming of adult disease susceptibility also occurs with maternal under-nutrition and maternal infection[94]. Glucocorticoids are central to the programming that occurs with maternal under-nutrition[50], though whether they play a central role in programming by maternal infection is currently unknown and important to establish. Programming by glucocorticoids and/or stress has been described in humans, non-human primates, sheep, rats and mice, as well as other animals and has been previously reviewed[89,29,95-97].

As mentioned above, placental 11β-HSD2 plays an important role in controlling fetal glucocorticoid exposure. In humans, mutations in *HSD11B2* are associated with reduced birth weight[98]. In mice, maternal stress or the absence of 11β-HSD2 reduces placental vascularization, causes placental dysfunction and alters nutrient transfer to the fetus[99,4,67] causing intrauterine growth restriction. Similarly, chronic glucocorticoid over-exposure increases vascular resistance in the feto-placental circulation[100]. Together, these data suggest that placental dysfunction contributes to the programming effects of glucocorticoids. Recent evidence has shown that pravastatin administration, which increases placental vascular endothelial growth factor (VEGF)-α expression, to *Hsd11b2*$^{-/-}$ mice restores placental vascularization and rescues their IUGR phenotype[67], suggesting a possible therapy to overcome at least some of the adverse effects of fetal glucocorticoid excess.

Programming by glucocorticoids depends upon windows of sensitivity -critical periods in the growth, development and/or maturation of the particular tissue or organ that is affected. For example, although dexamethasone administration in the third week of pregnancy in rats programmes hyperglycemia in adult offspring, administration of dexamethasone in the first or second week of pregnancy has no effect on glucose or insulin homeostasis[101]. Similarly, the children of women exposed to extreme maternal stress in the third trimester of pregnancy have altered basal cortisol levels at 1 year of age, whereas those exposed in the first trimester have normal cortisol levels [102]. Glucocorticoid resistance may be widespread in the early to mid-gestation fetus, in addition to the protection afforded by fetal expression of 11β-HSD2. This requires further investigation, for example, to examine whether glucocorticoid resistance arises from the hypoxic environment or the dominance of glycolytic metabolism that predominates during early development or whether developing tissues need to express "competency factors" to acquire glucocorticoid sensitivity.

The developmental windows of sensitivity to glucocorticoid action may differ between tissues. In GR$^{-/-}$ fetuses, impaired lung maturation is apparent by E15.5[103], whereas the impairment in heart maturation is not apparent until E16.5-E17.5[78]. For other organs that mature later, sensitivity can occur well into the neonatal period[104]. ~~Thus, the programming effects of glucocorticoids, stress, poor nutrition or infection are not solely restricted to the prenatal period, but also impact during the neonatal period. For example, neonatal exposure to low doses of endotoxin programmes hyperactivity of the HPA axis and has long-lasting effects on immune regulation, including increased sensitivity of lymphocytes to stress-induced suppression of proliferation and protection from adjuvant-induced arthritis[104].~~ These windows of sensitivity for

particular organs are also likely to differ between species. For example, bone marrow hematopoiesis is largely established during the second trimester in humans, but only takes place shortly before birth in mice[105]. This suggests that the effects of maternal stress or other factors that determine fetal glucocorticoid exposure may be highly dependent on the developmental stage of the tissue or organ affected.

Glucocorticoid-mediated programming of the hypothalamic-pituitary-adrenal axis

Key to the mechanisms that underpin the long-term effects of maternal stress or glucocorticoid over-exposure, is likely to be their effects on the fetal and/or neonatal HPA axis, leading to life-long HPA axis hyper-responsiveness[89,106,94,97]. In the case of maternal post-traumatic stress disorder, hypo-activity is programmed[102], though the mechanism is currently unclear. HPA axis hyperactivity plausibly accounts for the associations with metabolic (insulin resistance), cardiovascular (hypertension, increased coronary heart disease) and affective disorders (anxiety, depression). Given the potent immuno-modulatory effects of glucocorticoids[107,108], permanent changes in HPA axis activity are also likely to underpin at least some aspects of glucocorticoid programming of the immune system, though others are direct and mediated by GR and/or MR in fetal and/or neonatal immune tissues. In rodent models of stress/glucocorticoid programming, the HPA axis hyperactivity is mostly driven by increased hypothalamic expression of CRH and AVP[109] as well as altered GR/MR balance in the hippocampus[110]. However, although altered HPA axis responses are involved in the exacerbated pro-inflammatory response to LPS programmed by neonatal over-feeding, they are not centrally mediated. Instead, the adrenal response to ACTH following LPS challenge does not resolve efficiently, prolonging corticosterone release[111].

Consequences of fetal glucocorticoid exposure on postnatal immunity

Accumulating evidence from both animal and clinical studies suggests a link between prenatal glucocorticoid excess and programming of immune traits in the offspring. Although animal studies have provided valuable information on potential mechanisms, the findings are highly heterogeneous, possibly reflecting the multiplicity of hypotheses tested, as well as the species, strains and models used, as recently and thoroughly reviewed[105]. Here, we focus on clinical studies, which, due to the better defined samples and parameters assessed, as well as to the large number of individuals in epidemiological cohorts, provide more clear outcomes. Findings from the most outstanding clinical studies since 1980 that have addressed the programming of immune traits in postnatal life by maternal stress perception or prenatal steroid treatment are summarised in Tables 1 and 2, according to the study design: focused on early postnatal immune outcomes (Table 1), or epidemiology (Table 2).

Table 1 provides clues on the short-term effects of glucocorticoid exposure on immunity. With few exceptions[112-115], fetal cord blood was employed to measure parameters such as cytokine levels, cell counts or leukocyte function, which we classified into innate or adaptive immunity (refer to Table 1 for references). Hampered by the considerable heterogeneity in immune parameters between individuals[116], differences in the selected readouts and methodologies employed, and the small size of these studies, the collective data are conflicting and inconclusive. For example, studies that have measured interleukin (IL)-6 in cord blood report decreased [117] or unchanged[118,119] IL-6 levels in response to the same prenatal dose of betamethasone. In another study in which second trimester maternal stress perception occurred, IL-6

levels increased[120]. The discrepancies between these studies could reflect the fact that cohorts exposed to antenatal steroids often include preterm neonates, in whom the immune system is still immature, in contrast to prenatally stressed neonates, largely born at term. After prenatal steroid treatment, studies broadly agree concerning alterations in cord blood lymphocytes, though differ in the individual cell populations affected. Total lymphocyte and $CD4^+$ T cell numbers were decreased in one study[121] whereas in another study, T and NK cell numbers were unchanged though T cell proliferation was reduced and NK cell activation was increased[119]. Another study, in infants treated with antenatal corticosteroid, reported an absence of radiographic thymic shadow 36 h after birth, suggesting a decrease in thymic cellularity, but this was not associated with abnormal cell counts in peripheral blood[115]. Decreased neutrophil function[122,117] and a bias to immaturity[123] were observed in neonates following antenatal corticosteroid treatment, potentially increasing risk of morbidity and mortality from bacterial infection, as reported for multiple courses of glucocorticoids[124]. This, plausibly, could be due to HPA axis suppression, as a result of the treatment. More consistent outcomes were observed following prenatal stress exposure. For example, pro-inflammatory cytokine profiles[125,120], showed increases in IL-8 and IL-4 in cord blood[120] or in ex-vivo stimulated cells[125,114], though effects on IFN-γ were less clear. Higher IgE levels have been reported in cord blood of prenatally stressed newborns[126,127]. However the relevance of these findings is somewhat questionable, as fetal cord blood IgE is often contaminated with Ig of maternal origin[128]. Taken with caution, the collective data suggest that antenatal steroid treatment is detrimental for neutrophil function and the T lymphocyte compartment, whereas prenatal stress biases the inflammatory cytokine profile at birth towards a Th2 response. This Th2 bias in the cytokine response could be long lasting, as it is also observed in adolescents[129] and adult women[130] who experienced prenatal stress. Future studies with a greater number of participants as well as a comprehensive characterization of immune outcomes at birth or in neonates are needed to provide conclusive information on the short term, as well as long term, effects of endogenous or exogenous glucocorticoid exposure.

In contrast, epidemiological studies (table 2) involving large cohorts and clear clinical outcomes have provided important insights into the mid and long-term consequences of prenatal endogenous and exogenous glucocorticoid exposure. Table 2 summarizes studies involving at least 100 participants, which we classified according to the nature of the immune disease and the age at evaluation of the symptoms. From 23 epidemiological studies, 14 addressed the incidence of atopic diseases, predominantly asthma but also atopic dermatitis (refer to Table 2 for references). These studies, which ranged from 279 to 3.2 million participants, provide strong evidence that children exposed to prenatal stress are at a higher risk of developing atopic disease. Similarly, increased risk for asthma was observed in children prenatally treated with synthetic glucocorticoids[131]. Atopies are multifactorial diseases, exhibiting intensified Th2 responses, which drive high levels of IgE, and involve innate lymphoid cells, eosinophils and mast cells in particular [132,125].

While classic genetic association studies can explain only 1-2% of variation in IgE levels, epigenetic associations account for more than 13% of IgE variation [133]. This suggests that environmental signals and developmental differentiation programs are influenced by epigenetic mechanisms that regulate sensitivity to asthma. The risk for atopy is increased from 1 until 14 years of age (refer to Table 2 for references), whereas beyond 14 years, the risk may be attenuated, though only one study addressed this [134]. The age at which these immune traits become apparent may be a measure of the endurance of effect of the prenatal insult. When the most affected ages are assessed within a cohort, variability suggests effects are most likely to manifest in early infancy[134,131] or during adolescence[8]. Similarly, the association of prenatal stress with

asthma was stronger either in girls[8] or boys[135] depending on the cohort analysed. These discrepancies could be driven by differences in the nature or timing of maternal stress during pregnancy, and highlight the requirement to report these and other maternal and offspring (such as gender and age) categories in the analysis of cohorts.

Other studies addressed diverse diseases/immune traits that we have classified under the umbrella of "risk for infection". Antenatal steroid treatment is associated with fewer systemic infections[32] in the immediate neonatal period. However, when multiple courses of prenatal glucocorticoids were given, the risk for perinatal infectious morbidity and neonatal death increased[124]. Further, in low and mid-income countries antenatal corticosteroid therapy was associated with greater overall infant mortality and an increase in suspected maternal infection [34], suggesting that glucocorticoids could negatively impact neonatal health by affecting maternal immunity. Increased antibiotic use[7] and hospitalization because of infectious diseases[136,137] in children aged 1 to 14 have been also reported, indicating that both antenatal stress and steroid therapy confer a greater susceptibility to infections or a weakened ability to resolve them. This could be related to multilevel dysfunction in the innate and adaptive immune responses, which might be primed by altered microbiome colonization, as discussed below.

Similarly, an increased risk for autoimmune type 1 diabetes is associated with prenatal exposure to stress[138] or glucocorticoid therapy[139] in large cohorts of over 0.5 and 1.5 million participants, respectively. To date, no studies have examined associations between prenatal glucocorticoid exposure and other autoimmune diseases, which is not surprising given their relatively low frequency (compared to atopy), and late age of onset (mostly in adulthood, thus implying many years of follow up study). In addition to genetic risk, autoimmunity relies autoreactive T cells escaping negative selection, a process sensitive to glucocorticoids[140], and defects in immune regulation. Moreover, since children undergoing prenatal glucocorticoid therapy also have a higher risk for type 2 diabetes[139], it is likely that mechanisms that determine resistance to insulin or maintenance of beta cells are also affected by glucocorticoid therapy[141].

Finally, prenatal stress is also associated with an increased risk for any cancer [142], including acute lymphoblastic leukemia and Hodgkin's disease[143]. Interestingly, the authors argue that these hematopoietic cancers may have an infectious etiology, being triggered by microbial agents or Epstein-Barr-Virus[143], suggesting a link between prenatally-programmed susceptibility to infections and cancer.

Thus, prenatal stress or corticosteroid treatment are associated with higher risk of atopy, infection, type I diabetes and cancer in later (postnatal) life. Despite the high heterogeneity in the experimental design among clinical studies (the prenatal steroid therapy or the proxy used for stress, the time of pregnancy evaluated, the postnatal time considered for assessing readouts, and the number and selection of participants), the mid/long-term clinical immune outcomes were surprisingly homogeneous. This, despite (at least in the case of stress, where the maternal immune and sympathetic nervous system are involved) the possibility of a variety of contributing mechanisms. This highlights the importance of glucocorticoids as key mediators of stress effects. Remarkably, just one study showed an association between increased evening cortisol and pregnancy-specific stress and both measures independently predicted the risk for infant illness[7]. The remaining clinical studies reviewed in Tables 1 and 2 did not measure glucocorticoids or failed to find their association with maternal stress[114], probably due to difficulties obtaining reliable glucocorticoid measures due to differences in time of day or stage of pregnancy. Moreover, as described above, maternal stress may decrease 11β-HSD2 expression/activity[25], resulting in fetal glucocorticoid

overexposure independently of maternal glucocorticoid changes. To close these gaps in knowledge, considerable efforts have been placed in improving experimental design and sample collection, which will undoubtedly provide more conclusive results in the near future. It is also possible that at least to an extent, the programming effects of glucocorticoids are direct upon the feto-placental unit, rather than secondary to effects upon maternal physiology[5,50]. Indeed, as mentioned above, there is a steep gradient between the high levels of glucocorticoid in the maternal compartment and low in the fetal. However, current evidence indicates significant "synchronization" between maternal and fetal immunity. Examples of this are apparent in the numbers of T regulatory[144] or Th2 cells[145]. This synchrony may result from the continuous exchange of hormones, immune messengers, antigens, and even cells[146,1], that takes place at the feto-maternal interface. Clearly, stress-induced changes in transplacental transfer of maternal IgG or other passive immunity could also affect offspring immunity. Taken together, programming of immune disease by antenatal stress/corticosteroid therapy is likely to involve indirect mechanisms - changes in the mother eliciting fetal immune programming-, as well as direct effects on the placenta, fetal HPA axis and fetal immune organs. This requires further examination. Moreover, the time windows by which endogenous or exogenous glucocorticoids exert their programming effects during pregnancy might differ. Antenatal corticosteroid therapy is applied between 24 and 34 weeks of gestation [32] whereas maternal stress could take place outside of that window. ~~We here referred to the existence of distinct windows of sensitivity, depending on the developmental immune process that takes place at each time and the sensitivity to glucocorticoids among tissues and stages of development, given by dynamic changes in the expression of GR, 11β-HSDs enzymes and competence factor.~~ Whilst the role of exogenous and endogenous insults with regards to the windows of sensitivity has been addressed extensively in animal research, to date, most clinical studies assessed stress only once in pregnancy without discrimination between timepoints (Tables 1 and 2). An exception to this are the reports from Cookson et al. and Hartwig et al. that identify a higher risk of asthma with stress between 18-32/34 weeks, compared to earlier in gestation[8,6]. Interestingly, this later time point partially overlaps the window in which antenatal corticosteroid treatment is administered, suggesting that in humans, this could be the greatest window of sensitivity to the programming effects of glucocorticoids.

In order to elaborate on potential mechanisms driving the increase in susceptibility to immune diseases following excessive prenatal glucocorticoid exposure, in the next section we will review the different stages of fetal immune development. While T cell responses are well known to be affected by glucocorticoids, we will additionally examine known or potential susceptibility to glucocorticoids by other components of innate and adaptive immune system, as they affect the risk for atopies, infections, and autoimmunity.

Mechanisms that may underly glucocorticoid induced programming of the immune system

Effect of glucocorticoids on the ontogeny of the fetal immune system: Hematopoietic stem cells and early hematopoietic niches

Placental blood circulation is established on E9 in mice[147] and from the first trimester in human gestation, facilitating the nutrient and gas exchange between the fetal and the maternal systems. Thereafter, greater fetal exposure, especially at highly vascularized hematopoietic sites, to factors transferred from maternal

blood could be expected[148]. It remains unclear whether glucocorticoids affect the prenatal establishment of the definitive hematopoietic stem cell (HSC) pool, which endures through the individual's life[149]. In the present section we review the development of the immune system aiming to pinpoint windows of sensitivity to glucocorticoids and possible immune or stromal cell targets. Though scarce, we here focus on evidence arising from fetal tissues, as distinct glucocorticoid responses in prenatal and posnatal tissues are possible, driven for example, by differential HSC DNA methylation patterns[150] and/or coexpression of transcription factors[151,152].

The fetal hematopoietic system develops in a stepwise manner that involves the formation, proliferation, migration and differentiation of HSC (reviewed in[153,154,149]). By E7 in mice and early first trimester in humans, hematopoiesis starts from transient precursors in the yolk sac [155]. These develop into erythrocytes and the first immune cells. From this hematopoietic wave, only tissue resident macrophages will endure until adult life. All other blood components will be gradually replaced by the definitive hematopoiesis [156]. Consequently, this early stage of hematopoiesis poses a low vulnerability for any long-lasting effects of glucocorticoids on the immune system, in agreement with the lack of association between stress in the first trimester of gestation and the risk for atopic diseases[6,8].

Definitive HSC are not found in the aorta-gonad-mesonephros region and in the placental labyrinth[157] until E10.5-11 in mice[158] and at 4-6 weeks of gestation in humans[159]. From 7 weeks of gestation in humans[160] and E12 in mice[158], definitive HSC migrate to the liver. Supported by the niche created by the arterial portal vascular tree[161], liver HSCs proliferate rapidly until E16.5 in mice[162], when HSC homing to the bone marrow starts. Simultaneously, differentiation of HSCs into myeloid cell lineages (erythrocytes, granulocytes, monocytes, megakaryocytes) as well as the development of the first lymphoid progenitors and then NK cells and B cells [163,164]. Some liver lymphoid precursors and HSC migrate to colonize the thymus and spleen and give rise to differentiated cells from the lymphoid and myeloid/erythroid lineages, respectively[165]. Although fetal growth and lung and cardiovascular development are rather refractory to glucocorticoids prior to E14.5[103,21,4], evidence concerning the *in vivo* effects of glucocorticoid exposure on fetal stages of hepatic hematopoiesis remains scarce. Early hematopoietic steps appear independent of glucocorticoids, as $GR^{-/-}$ fetuses show no distinct alterations in hepatic hematopoiesis at least until E14.5, when adrenal steroidogenesis initiates (unpublished observations). However, it is possible that GR can be activated by maternally-derived glucocorticoid before (or after) that. In mice, hepatic hematopoiesis coincides with a reduction in liver GR expression from E12. GR expression then rises significantly again at E18.5 when hematopoiesis has already been established in the bone marrow[68]. The decrease in GR expression on E14.5-18.5 together with high placental and hepatic 11β-HSD2 expression during early to mid gestation are likely to be mechanisms to protect hematopoiesis from inappropriate glucocorticoid exposure at might occur in the case of maternal stress or infection. However, low GR expression does not prevent dexamethasone (a poor substrate for 11β-HSD2) from promoting the differentiation of immature hematopoietic cells ($Lin^{-}Sca\text{-}1^{+}c\text{-}Kit^{+}$)[163] and $Lin^{-}c\text{-}Kit^{Lo}$ lymphocyte precursors isolated from E15 mouse liver[151] into myeloid cells while at the same time disrupting their ability to form B lymphocytes *in vitro*[151]. Similar results were observed in adult human bone marrow[151], suggesting that this is a highly conserved effect of glucocorticoids. Thus, throughout fetal development, glucocorticoids may direct otherwise undifferentiated stem cells towards a myeloid cell fate over a lymphoid cell fate. *In vivo* a glucocorticoid-mediated impairment in lymphocyte differentiation may alter the B cell compartment in the short-term. This might explain the impaired neonatal humoral responses to tetanus [113] and Hepatitis B[114] vaccination in babies that received antenatal corticosteroid.

However, antenatal corticosteroid therapy was also associated with increased responses to *Haemophilus influenzae type b* [112], underscoring the need for more investigation on the role of prenatal glucocorticoids on humoral immune responses.

The *in vivo* effects of glucocorticoids on immune cells are highly dependent on the type, dose, timing and duration of the treatment[166]. In human fetal (7-12 weeks of gestation) nucleated liver cells, *in vitro* betamethasone stimulation significantly inhibited the hematopoietic colony-forming capacity in a dose dependent fashion. This was evidenced by a reduction in the number of burst-forming units-erythroid cells (BFU-E) and colony-forming units for granulocytes, erythroid cells, macrophages and megakaryocytes (CFU-GM and CFU-GEMM)[167]. Together these give rise to the myeloid blood components and/or erythrocytes. In contrast, it is well established that modest levels of dexamethasone promote self-renewal of early erythroid progenitors (BFU-E) and increase the production of terminally differentiated erythroid cells by fetal mouse liver cells *in vitro* and *in vivo*[168,169]. This seems relevant for immune function, since increased erythropoiesis may occur at the expense of a reduction in leukocyte hematopoiesis, as observed for *in vitro* lymphopoiesis[167]. A further unexplored question is whether glucocorticoid enrichment of BFU-E and the consequent increase in BFU-E derived $CD71^+$ colony forming units erythroblast[168] might alter the suppressive $CD71^+$ erythroid immune cell compartment. This neonatal cell population plays an important regulatory role in early neonatal immunity[170] by protecting the immature newborn against aberrant immune cell activation in the intestine upon colonisation with parturition-associated commensal microorganisms.

Interestingly, while in human pregnancies the evidence for effects of prenatal stress on the immune system remains scarce[8,6], animal models pinpoint hepatic and bone marrow hematopoiesis as key susceptible sites, with corresponding developmental windows of sensitivity, for prenatal immune programming[171,172,105]. Effects specific to the different sites (or stages) are difficult to dissect, as very few studies limited the stress to just one of these stages[93]. Similarly, T cell development in the thymus takes place during an overlapping developmental window (see following section), at least in mice. Taken together, glucocorticoid over-exposure might simultaneously affect different processes of immune ontogeny.

By E16.5 in mice and 13-14 weeks gestation in humans, bone vascularization and the concurrent transition from cartilage to a calcified matrix permit the HSC to migrate into the developing bone marrow (BM)[148,160] decreasing their number in the liver[153]. In the BM, the first quiescent adult-like HSC develop, probably as a result of their interaction with mesenchyme-derived stromal osteoblasts[148]. BM HSCs give rise to multipotent progenitors (MPPs) before differentiating into common myeloid progenitors (CMPs) and common lymphoid progenitors (CLPs), which then undergo a series of maturation steps. MMPs can replenish virtually all components of the immune system throughout life. Fetal BM HSC homing, self-renewal and differentiation are highly dependent on the stromal niche[173]. Whilst glucocorticoids seem dispensable for fetal bone and cartilage formation[174], evidence suggests that glucocorticoid excess or deficiency affects the microstructure and function of the bone marrow. Bone resorption is enhanced in infants treated with at least four courses of antenatal steroids[175,176]. *In vitro* glucocorticoids promote proliferation of mouse perinatal osteoblasts [152] and promote maturation of human osteoblasts[166]. Importantly, newborn mice with chronically low glucocorticoid levels as a result of the transgenic deletion of the corticotropin releasing factor receptor 1 gene, show increased osteoblast-bound CXCL12[152], which enhances chemotaxis and quiescence in HSC. They also have more bone marrow and circulating hematopoietic stem and progenitor cells[152].

Concomitant with the sequential traffic of fetal HSCs through hematopoietic sites, immune cells circulate in growing numbers in the secondary lymphoid organs and vasculature[155], where they are susceptible to the effects of glucocorticoids. It seems plausible that glucocorticoid-induced programming of the immune system would be mediated by effects on HSCs or other persistent progenitors, rather than upon the continually replaced, short-lived fully differentiated cell populations. However, a direct glucocorticoid effect could explain short-term changes in infant immunity (for example, the increased susceptibility to infections). This could, in turn, affect immune responses in later life[143]. For example, methylprednisolone treatment of human umbilical cord blood $CD34^+$ hematopoietic cell precursors accelerated NK cell differentiation and induced cytolytic activity[177]. It also promoted a switch in myeloid precursors toward immature NK cells[177]. Such a switch could explain the enhanced NK activation found in human cord blood cells of infants who received antenatal corticosteroid therapy[119].

Thus, further experimental investigation is required to establish whether and how excessive prenatal glucocorticoid exposure impacts upon HSC homing and proliferation and to determine any effects on hematopoiesis, with short and long term consequences for postnatal immunity. Key to the mechanisms, we hypothesize that long-lasting disease risk is driven by epigenetic mechanisms in HSCs, as outlined below.

Effect of glucocorticoids in T cell differentiation and selection

~~Shortly before birth, adrenal glucocorticoid production is low and circulating maternal glucocorticoids have been blocked by the placental 11β-HSD2 barrier.~~ The thymic epithelium produces its own glucocorticoid to support the rapid development of the late gestation thymus[178]. Mouse thymic epithelial cells (mTEC) express the enzymatic machinery to convert cholesterol to corticosterone, suggesting that the level of glucocorticoids in thymus is enhanced by paracrine delivery. In mice, mTEC production of a glucocorticoid intermediate was highest at birth and subsided through adulthood[80,179]. Recently Taves et al. confirmed that corticosterone levels in the embryonic and neonatal thymic tissues are elevated above blood levels[180]. ~~Production of corticosterone has been observed by thymocytes from older mice[181], and it was proposed to underlie age-related thymic atrophy[178].~~ Immature thymocytes readily undergo apoptosis induced by glucocorticoids[182]. Since removal of glucocorticoids by adrenalectomy causes thymus hyperplasia, it was suspected that glucocorticoids play a role in thymocyte death by neglect. However, local production of glucocorticoids in the thymus and a normal thymus size in diverse GR-deficient models did not support this concept (reviewed in[183]). A recent report suggests that ACTH may act via its receptor, MCR2, to directly increase thymocyte numbers, independently of glucocorticoids [184], suggesting that perturbations of the HPA axis may regulate thymic homeostasis through ACTH as well as glucocorticoids. Glucocorticoids also counteract TCR-derived selection signals in thymocytes. By dampening the effect of TCR signals that would otherwise lead to negative selection, glucocorticoid signals allow TCRs with higher affinity for self-MHC to be positively selected. Consequently, transgenic mice with reduced GR signalling in immature thymocytes show a bias to a less autoreactive T-cell repertoire[185,186]. Enhancement of the T-cell repertoire by endogenous glucocorticoids has also been demonstrated by the reduced antigen responsiveness of mice with T cell-specific disruption of GR signaling[187]. Of note, the first reported “knock out” of GR, that generated a truncated GR with residual activity[188,189], previously led to the conclusion that glucocorticoids play no role in thymic development[190,191]. However, while the thymocyte number and subset distribution of these mice were normal, their T-cell repertoires were not examined[190-192].

Elevated levels of glucocorticoid prenatally would be expected to add to the effects of endogenous thymic glucocorticoid production and further raise the threshold for negative selection, promoting the development of higher affinity and potentially auto-reactive T cells. Indeed, thymocyte apoptosis in the fetal thymus is induced by prenatal treatment with betamethasone at doses that mimic therapeutic levels [193]. This results in an accelerated refill of the thymic niche with immature precursors that are subject to selection in the presence of high glucocorticoid levels. Since 11β-HSD2 is not expressed in the fetal thymus, similar mechanisms could apply to excessive glucocorticoid exposure upon prenatal stress. Thus, expansion of a cohort of auto-aggressive T cells could underlie the increased incidence of asthma or autoimmune disease reported in the offspring of stressed or betamethasone-treated mothers[131,194].

Mechanistically, the antagonism of TCR and glucocorticoid signaling involves the glucocorticoid-inducible leucine zipper (GILZ) protein. Overexpression of GILZ in T-cell hybridomas inhibited TCR-induced apoptosis[195], implicating GILZ in glucocorticoid-mediated repression of TCR-induced transcription factors such as AP-1 and NF-kB. These factors are themselves direct targets of suppression by the GR, so GILZ may serve to amplify the repressive effects of glucocorticoids[196,197]. GILZ has been implicated in glucocorticoid effects in other immune cell types, in the dendritic cell-mediated expansion of Tregs[198,199], control of B cell survival[200] and endotoxin tolerance of macrophages[201]. Another potential target of glucocorticoid signaling during thymocyte selection is Nur77 (Nr4a1), whose transcriptional activity is sensitive to glucocorticoids[202] and its expression is upregulated by TCR signalling[203]. Transgenic expression of a dominant-negative form of Nur77 resulted in inefficient negative selection of autoreactive thymocytes [204]. ~~In addition, thymocyte-specific deletion of all three Nr4a family members blocked development of regulatory T cells and caused fatal autoimmune disease similar to that of mice and humans lacking the Treg-specific transcription factor Foxp3[205].~~

The elimination of autoreactive and potentially dangerous T cells before they leave the thymus constitutes the basis of central tolerance. While it is readily understandaable how central tolerance of T cells reactive against ubiquitous and thymic antigens is achieved, tolerance against tissue specific antigens such as insulin or myelin basic protein requires their “ectopic” or “promiscuous” gene expression by mTEC. This ectopic expression is dependent on the transcriptions factors such as AIRE[206] and FEZF2[207]. The regulated activity of these transcription factors ensures a representation of ‘self proteins’ in the thymic medulla which is displayed to maturing thymocytes during negative selection. Interestingly, a putative risk allele for Crohn’s disease is associated with the downregulation of AIRE expression mediated by glucocorticoids[208]. mTECs are also susceptible to glucocorticoids: Injection of high-dose dexamethasone in adult mice drastically, albeit transiently, depleted mTEC [209], which only resolved one week later. It is conceivable that a spike of glucocorticoid signaling at a sensitive time for the development of the T cell repertoire may compromise the transcription of tissue-restricted antigens in the thymus, thus impairing negative selection and favoring the production of autoreactive T cells.

Molecular mechanisms that underpin glucocorticoid programming

The mechanisms that underlie the permanent or programmed effects of glucocorticoids upon developing fetal tissues and organs remain unclear. Some aspects of stress/glucocorticoid programming can even be transmitted to future generations, without further experimental manipulation[210], raising interesting and important questions about the mechanism. A detailed overview of this topic is beyond the scope of this review, and so we restrict our discussion chiefly to mechanisms that may apply to immune programming.

Epigenetic variation has been suggested as a key mediator of the programming effects of glucocorticoids, with methylation of CpG residues in the promoter regions of key genes being implicated in the long-term effects of early life stress or glucocorticoid exposure[211,212]. However, no cause and effect relationship between methylation and long-term effects on physiology has yet been established [213]. Moreover, the relevance of the small differences in CpG methylation observed in most studies, particularly where these lie in largely methylation free CpG islands, to the transcriptional regulation of the associated programmed gene remains unclear. Clearer is the role of DNA methylation in HSC function and differentiation.

Epigenetic mechanisms of fetal programming of immune cells

As methylation can be transmitted from a cell to its progeny, variations in the HSC or HSPC methylation patterns, induced for example, by prenatal stress or glucocorticoid exposure, could have long-term effects upon the individual's immunity. Epigenetic mechanisms have been implicated in regulation of fundamental stem cell functions, such as self-renewal and multilineage differentiation (reviewed in[214]). Plasticity in DNA methylation patterns is related to HSC multipotency, stage of ontogeny and aging[215,150] as well as to their degree of differentiation[216,217]. The few differences observed in the overall DNA methylation pattern between mouse fetal liver and young postnatal HSCs were suggestive of a developmental restriction process. In young HSCs, DNA methylation was gained on regions associated with non-hematopoietic lineages, and lost at genomic regions associated with blood cell production[150], seeming to favour an emerging transcription profile typical of leukocytes rather than fetal HSCs. De novo DNA methylation is required to maintain the self-renewal capacity of HSCs [218], whereas HSC differentiation is associated with changes in DNA methylation patterns. In this sense, myeloid lineage commitment involved less global DNA methylation than lymphoid commitment[216,217]. Interestingly, the methylation of genes involved in glucocorticoid receptor signalling pathways is altered when HSC commit to common myeloid and megakaryocyte-erythrocyte progenitors[219], providing further clues that glucocorticoid related pathways are involved in HSC differentiation. In other tissues, glucocorticoid exposure has been suggested to lead to glucocorticoid resistance by inducing methylation of the GR gene promoter and suppressing its expression [220,221]. However, the relevance of these differences in GR methylation for glucocorticoid sensitivity requires further examination.

During development, T cell precursors become committed at the same time that alternative lineages are excluded. Several recent reports pinpoint dynamic changes in gene expression profiles and epigenetic marking over the process of T cell differentiation [222,223]. These demonstrate, for instance, how inheritable specification in helper and cytotoxic T cells involves stage-specific DNA methylation and demethylation events at the *Cd4* locus[224]. Differentiation of Th2 cells is induced by activation of the T cell receptor and IL-4 receptors. Th2 phenotype is subsequently maintained by a positive feedback mechanism and by repressing histone modifications at Th1 loci[225]. With regard to the effect of stress/glucocorticoids on T cell epigenetic programming, a genome wide DNA methylation profile in T cells demonstrated that the methylation levels of 2872 CpGs differed significantly in adolescents whose mothers underwent stressful events during an ice storm in Canada, compared to controls[226]. Many of these differentially methylated CpGs occurred in genes and pathways related to immune function, suggesting that maternal stressors may affect postnatal immunity by long lasting and widespread effects on DNA methylation across the entire genome of their unborn children. A drawback of this study is that no sample was collected at birth, thus,

some of the changes could be related to postnatal stress events. In addition, no cause and effect relationships with DNA methylation have been established yet. This will be important to address in the future, as the epigenetic changes may occur secondarily to transcriptional programming.

A clear example of prenatal programming of the immune system has been shown upon induction of innate immunity in pregnant mice. Injection of TLR agonists during pregnancy increases innate and adaptive immune responses in the offspring, and results in earlier onset of clinical symptoms of experimental autoimmune encephalitis[227]. Even though the underlying mechanisms were not evaluated, these experiments indicate that fetal programming of the immune system persists into adulthood and has consequences for health.

Potential mechanisms of glucocorticoid induced postnatal immune disease

The effects of glucocorticoids on the immune system are amazingly broad as a consequence of the variety of target cell types, the diversity of pathways affected, the time of action and a seemingly dichotomous effect on the immune response: glucocorticoids tend to enhance a ramp up innate response to microbial products and damaged tissue, while repressing subsequent adaptive immune responses, to promote the resolution of inflammation and restore homeostasis[228,229]. It is not surprising that, when looking at the consequences of fetal exposure to glucocorticoids as a result of maternal stress or by pharmacological indication, we find apparently discordant effects, namely exacerbated responses in atopy or insufficient immunity to infection.

A converging point of published studies on prenatal stress is an increased risk of developing allergies. Atopy is characterized by dominant Th2 responses, with an overproduction of Th2 cytokines such as IL-4 and IL-13, and high IgE levels in serum, leading to enhanced mast cell degranulation. Th2 responses have long been considered the anti-inflammatory counterpart of Th1 and, by suppressing IL-12 and IFN-γ, glucocorticoids shift the Th1/Th2 balance to Th2[230]. This process may be mediated, at least in part, by GILZ[231]. Psychological stress enhanced Th2 responses in asthmatic patients, detectable even one year after the stressful event[232]. Moreover, production of Th2 cytokines was increased in 13 year old children and adult women born from mothers who faced stressful conditions during pregnancy[129,130]. Together, these studies indicate programming of enhanced Th2 responses by stress and/or glucocorticoids. Interestingly, epigenetic mechanisms had 10-fold greater influence on the levels of serum IgE in asthmatic patients than classical inheritance of genetic traits[133], underlining the importance of epigenetic transmission of Th2 responses.

Importantly, atopic diseases such as asthma may have a multifactorial etiology[233]. In humans, mid to severe subtypes of asthma show altered airway remodeling, resembling developmental branching morphogenesis of the lung. This results from disease-mediated epithelial metaplasia and damage and hypertrophy and hyperplasia of mesenchymal airway smooth muscle[234,235]. Similarly, in mice, conditional deletion of GR in lung mesenchyma results in an immature lung phenotype and deletion of GR in lung epithelium increases cellularity of epithelial and non-epithelial compartments [236]. Thus, it is tempting to hypothesize that precocious or excessive prenatal glucocorticoid exposure promotes structural changes in the immature lung which could synergize with glucocorticoid driven immune alterations to enhance the vulnerability to airway diseases.

In addition to direct effects upon the maturing lung and immune system, programmed HPA hyper-responsiveness as a result of prenatal stress or exogenous glucocorticoid exposure may play a role in hypersensitivity to antigens in atopy[237]. For example, hypothalamic CRH responses, increased upon prenatal stress[109], promote mast cell degranulation[238]. Other mechanisms previously reviewed elsewhere [239,237], that involve substance P, arginine vasopressin or ACTH, may also contribute to atopy by potentiation of systemic immune proinflammatory effects. Of note, while the association between prenatal glucocorticoid induced immune and HPA dysfunction has been frequently demonstrated in animal studies (as detailed above), direct evidence remains scarce in humans. None of the studies summarized in tables 1 and 2 assessed HPA responses in infants. Similarly, as described above, increased prenatal exposure to glucocorticoids decreases birth weight and is associated with placental insufficiency. Low birth weight is also an important predictor of enhanced risk of developing asthma[240,241] and atopic dermatitis[242]. Whether the reductions in birth weight, alterations in lung structure and function and Th2 bias are all mechanistically related through excessive prenatal glucocorticoid exposure is an important question for the future.

At physiological concentrations, glucocorticoids exert potent immuno-modulatory effects[107]. Glucocorticoids alter dendritic cell function, rendering them tolerogenic[198], plausibly mediated by GILZ, and they promote LPS tolerance in previously activated macrophages[201]. Thus, programmed postnatal HPA axis hyper-responsiveness and concurrent elevated basal cortisol levels could influence immune responses in the offspring. Moreover, there is growing evidence that development of the immune system in the offspring depends on the intestinal microbiota (reviewed in [243]). In this context, a recent study reported that infants of highly stressed mothers with high cortisol concentrations during pregnancy showed aberrant colonization with abundant Proteobacterial groups (containing pathogens related to *Escherichia, Serratia,* and *Enterobacter)*, and reduced commensal bacteria, such as *Lactobacillus*[244]. In addition to the reported higher incidence of gastrointestinal symptoms and allergic reactions in these infants, the abnormal microbiota could elicit long lasting changes in the composition of the immune system, resulting in, for example, an enhanced infection risk. Moreover, prenatal stress exposure as well as antenatal corticosteroid treatment or preterm birth can prime the risk for diseases in early postnatal life[7,245]. This, in turn, may exert long-lasting effects upon HPA responses[104]. Thus, the independent contributions of prenatal and postnatal factors may be difficult to tease apart.

Autoimmune diseases may be affected by prenatal glucocorticoid exposure. Children exposed prenatally to stress or corticosteroid therapy are at increased risk of developing type 1 diabetes (Table 2). Type 1 diabetes results from the destruction of the beta cells in the pancreatic islets by infiltrating autoreactive lymphocytes[246]. Transcripts encoding insulin, the main autoantigen in diabetes, are expressed in the thymus, driven by Aire [247]. Thus, a glucocorticoid-induced depletion of mTEC[209], or a direct effect of glucocorticoids on the regulation of Aire[208], could impair negative selection of lymphocytes recognizing insulin[248]. In addition, as we have mentioned before, by antagonizing TCR signaling, glucocorticoids modulate the threshold between positive and negative selection of thymocytes[185,80]. Excessive glucocorticoid signaling (due to prenatal corticosteroid treatment or as a result of HPA hyper-responsiveness) could improve the survival of T cells prone to autoreactivity that otherwise would have been eliminated in the thymus. Beyond effects on the immune system, GR signaling determines beta cell differentiation during a critical developmental window through the regulation of the pancreatic master transcriptional regulator, Pdx-1[141]. A better maintenance of beta cells could slow down development of the disease.

In summary, mechanisms for glucocorticoid mediated programming of immune diseases may include short and long term changes in the immune system that interact with HPA axis hyper-activity and potentially with prenatally programmed altered function of the affected organs. Figure 1 depicts a hypothetical scenario, where the known or potential interactions between these players are illustrated, as well as their relation to postnatal immunity. The complexity of this scenario explains the difficulties of dissecting out the roles and understanding the hierarchical action of the individual players.

Final remarks

Epidemiological data unambiguously reveal prenatal life as a relevant period for programming of immune diseases. We have restricted this review to intrauterine immune development and the maternal, placental and fetal factors that may be modulated by glucocorticoid over-exposure in the settings of prenatal stress or synthetic glucocorticoid administration. However, maternal stress effects on offsprings' immunity exceed the pregnancy period, and maternal prenatal stress is associated to postnatal stress/anxiety, which can also programme i.e. the risk for asthma[249]. Not to be forgotten is the influence of postnatal care[250] and lifestyle of the child, which may exert further effects on the immune system.

Despite all the evidence, very little is known about the mechanisms that lead to immune disorders, and how they are programmed during fetal life. Moreover, crucial milestones of fetal immune development are achieved comparatively earlier in humans compared to mice and, consequently, the windows of sensitivity to glucocorticoids during fetal life are also likely to be different. For these reasons, there is an acute need for analysis of large cohorts, in which women are recruited early during pregnancy with follow-up of their offspring beyond puberty. In addition to strict documentation of the time window at which stressful events or treatment occurred, such studies should include a broad assessment of the child's immune compartment and function at birth and later in life. This strategy should provide the much sought-after biomarkers to assess disease risk, and identify targets for mechanistic research in glucocorticoid-mediated programming of the immune system.

Given the steady increase in atopy and autoimmune disease over the last few decades[251], investigating the windows of sensitivity to maternal stress/antenatal glucocorticoids and the multiplicity of factors involved in the programming of the immune system will permit the design of prevention strategies and constitute a true investment in our health.

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Table 1: Changes in the innate and adaptive immune systems observed at birth and in early postnatal life

Condition	Sample analyzed	n	Innate immunity	Adaptive immunity			References
				Cells	Function	Ab resp.	
Betamethasone (RDS Tx)	CB *In vitro*	18	↓ Neutr. migration ↓ Neutr. chemotaxis				Fuenfer, 1987[122]
Betamethasone (RDS Tx)	CB	84	↑ Neutr. counts ↑ Immature neutr.				Barak, 1992[123]
Betamethasone (RDS Tx)	CB serum	125	↓ IL-6 ↓ ROS				Caldas, 2012[117]
Betamethasone (RDS Tx)	CB serum	200	⇔ IL-1β, IL-6, TGF-β ⇔ IL-10, IL-8, IL-4				Kumar, 2011[118]
Betamethasone (RDS Tx)	CB cells *In vitro*	51	⇔ IL-6	⇔ T cells ⇔ NK cells	↓ T cell prolif. ↑ NK activation		Kavelaars, 1999[119]
Antenatal steroids (not specified)	Cord blood	42		↓ Lymphoc. ↓ $CD4^+$ ↓ $CD25^+$			Chabra, 1998[121]
Betamethasone (RDS Tx)	CB cells	100		⇔ Lymphoc.	⇔ Apoptosis		Agakidis, 2009[252]
Dexamethasone (RDS Tx)	Chest X-ray at <36h life	50		No thymic shadow			Michie, 1998[115]
Betamethasone (RDS Tx)	Serum after vaccination	54				↑ Hib	Tsuda, 2012[112]
Betamethasone (RDS Tx)	Serum after vaccination	130				↓ Tetanus	Slack, 2004[113]
Maternal anxiety (20 &32 WOG)	CB serum Blood at 2m	1209			↓ T cells ↓ IFN-γ ↑ IL-4	↓ HBV	O'Connor, 2013[114]
Maternal stress (end of pregnancy)	CB cells *In vitro*	557	↑ IL-8, ↑ IFN- γ after TLR stimulation		↑ IL-13 after mite dust stim. ↑ IFN-γ after PHA stim.		Wright, 2010[125]
Maternal stress (2nd trimester)	CB serum	43	↑ IL-1β, IL-6, ⇔IL-12, TNF-α ↑ IL-8 ↑ IL-4, IL-5				Andersson, 2016[120]
Maternal negative life events (29 WOG)	CB serum	403				↑ IgE	Peters, 2012[126]
Maternal selfreported psychosocial stress	CB serum	334				↑ IgE	Lin, 2004[127]

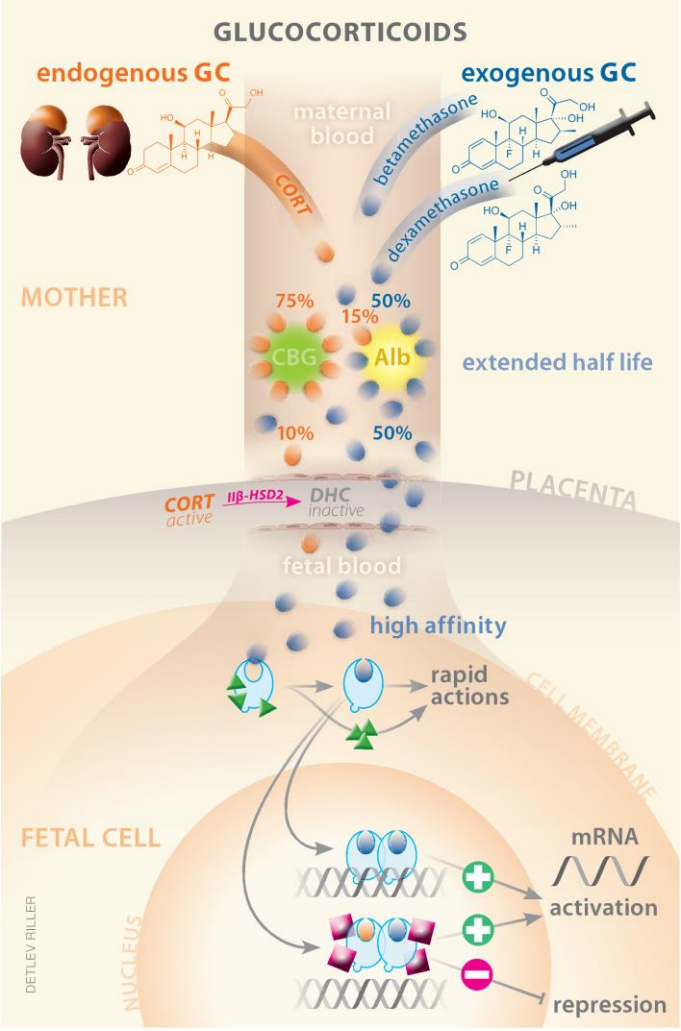
CB: Cord blood; ROS: Reactive oxygen species; n. sp. Not specified which; WOG: weeks of gestation; Neutr.: Neutrophil.; PHA: phytohaemagglutinin; IgE, immunoglobulin E; HBV, hepatitis B virus; Hib, *Haemophilus influenzae type b;* m: months; Ab resp., antibody response

Table 2: Epidemiological studies showing association between prenatal stress or steroids and immune diseases

Disease	Condition	Study cohort	Association to disease	Reference
Atopy	Maternal high stress	N= 1264 Until 2 yr	↑ risk atopic disease	Wen, 2011[253]
	Prenatal stress: negative life events	N= 653 Until 2 yr	↑ wheezing in children born to mothers nonsensitized (low IgE)	Mathilda Chiu, 2012[254]
	Prenatal community violence	N= 708 Until 2 yr	↑ association with wheezing	Chiu, 2014[255]
	Prenatal maternal distress (a), depression and anxiety (b)	N= 1531 (a) + 973 (b) Until 4yr	↑ risk for atopic dermatitis (a,b) *in prenatal stress + atopic dermatitis:* ↑ serum IgE levels at 1 year of age	Chang, 2016[25]
	Prenatal demoralization (i.e. psychol. distress)	N= 279 Until 5 yr	↑ transient and persistent wheeze ⇔ IgE CB/blood	Reyes, 2011[256]
	Maternal psychological distress (20 WOG)	N= 4848 1-6 yrs	↑ odds of wheezing in 1-4 yrs ↑ asthma and eczema at age 6 years	Guxens, 2014[257]
	Prenatal (and postnatal) stress	N= 765 6 yrs	↑ asthma in boys prenatally or postnatally stressed ↑ asthma in prenatally + postnatally stressed girls	Lee, 2016[249]
	Maternal psychosocial job strain	N=32.104 Until 7 yr	↑ atopic dermatitis in high strain job; ↑ asthma in active jobs	Larsen, 2014[258]
	Maternal anxiety (18 and 32 WOG)	N= 5810 Until $7^1/_2$ yr	↑ likelihood for asthma if high anxiety at WOG 32	Cookson, 2009[6]
	Antenatal steroids	N=80448 Until 8 yr	↑ risk of asthma between 3-5 years of age (HR: 1.19)	Pole , 2010[131]
	Maternal bereavement	N=3.2 million 1->9 yrs	↑ risk of asthma hospitalization	Khashan, 2012[259]
	Maternal bereavement	N = 426.334 (1-4 yrs) N = 493.813 (7-12 yrs)	↑ risk of asthma at 1–4 yrs in boys exposed (2^{nd} trimester maternal bereavement) ↑ risk of asthma attack 7–12 yrs in boys	Fang, 2011[135]
	Prenatal adverse life events	N= 1587 Until 14 yr	↑ asthma and eczema at age 14 yrs ⇔ asthma in children aged 7 yrs	Hartwig, 2014[260]
	Maternal bereavement	N = 750.058 Until 15 yrs	↑ risk of asthma events in children aged 0-3 years ⇔ asthma in children aged 4-15 years	Liu, 2015[134]
Infection	Antenatal Betamethasone	N=453 Infants (days after birth	↑ Early-onset neonatal sepsis (OR 1.25) and ↑ death (OR 1.70), if multiple courses	Vermillion, 2000[124]
	Antenatal steroids	N=2994 Infants 48h after birth	↓ systemic infections (RR 0.56)	Roberts, 2000[32]
	Relationship dissatisfaction Stressful life events (30 WOG)	N= 58530 Until 1 yr	↑ frequency/variety infectious diseases	Henriksen, 2015[245]
	Maternal anxiety (37 WOG)	N= 147 Until 1 yr	↑ infant antibiotic use	Beijers, 2010[7]
	Antenatal steroids	N=102 10-12 yr old	↑ hospital admissions because of infectious diseases	Smolders-de Haas, 1990[136]

	Prenatal stress: negative life events	N= 1.7 million Until 14 yr	↑71% increased risk of severe infectious disease hospitalization.	Nielsen, 2011[137]
Type I diabetes	Antenatal steroids	N=505386 Until 10 yr	↑ risk of type 1 (HR: 1.20) ↑ risk of type 2 diabetes (HR: 1.51)	Greene, 2013[139]
	Maternal bereavement	N = 1.548.746 2-27 yrs	↑ type-1 diabetes, mainly in girls	Virk, 2010[138]
Cancer	Maternal bereavement (spouse or child)	N=6143772 Until 14 yr	↑ 30% risk of any cancer, especially non-Hodgkin disease and hepatic cancer	Li, 2014[142]
	Maternal bereavement (parents)	N= 39002 vs. > 11 million (database) 0-43 yr	↑ leukemia, Hodgkin's disease (lymphoma) independent bereavement timing ↑ testicular cancer, ↑ in 3rd trimester bereavement	Bermejo, 2007[143]

OR: odds ratio; RR: relative risk; HR: hazard ratio; RDS Tx: Respiratory Distress Syndrome Treatment; WOG: weeks of gestation;
Open cells depict prenatal stress exposure and shadowed cells antenatal steroid treatment. Only studies with more than 100 participants were included



BOX 1 Steroid hormones play a fundamental role during pregnancy. Endogenous glucocorticoids (CORT: cortisol in humans, corticosterone in mouse and rats) rise during the second half of pregnancy (top left), and might be further increased by maternal stress perception. Additionally, exogenous synthetic steroids, such as betamethasone or dexamethasone (top right) and prednisolone may be administrated during gestation to promote fetal lung maturation or to treat autoinflammatory diseases of the mother. Endogenous and exogenous glucocorticoids exhibit differential binding to plasma globulins: endogenous glucocorticoids appear mostly bound to corticosteroid binding globulin (CBG) and only in a minor fraction bound to albumin (Alb) or free, whereas exogenous glucocorticoids appear equitably bound to Alb or free. In contrast to dexamethasone and betamethasone, endogenous glucocorticoids and prednisolone are good substrates for inactivation by placental 11β-HSD2 enzyme, which limits their transplacental passage. As steroid compounds, glucocorticoids readily cross the fetal cell membrane to bind the intracellular glucocorticoid receptor (GR, light blue). Noteworthy, dexamethasone and betamethasone display a higher affinity to GR than the other glucocorticoids. In the cytoplasm, inactive GR appear associated to a multiplicity of chaperon proteins (green triangles), which are released upon glucocorticoid binding. Engagement of the receptor elicits rapid non-genomic GR and chaperone protein signalling and allows GR translocation to the nucleus. GR can modulate gene expression by binding as homodimeric transcription factors to the palindromic glucocorticoid response elements (GRE) in the DNA. Additionally, GR monomers or dimers can interact with other transcription factors (pink squares) to activate or repress gene expression. Altogether, these regulation of gene expression is called (trans)activation or (trans)repression (reviewed in[261-263]). Whilst most glucocorticoid induced pathways are mediated through binding to the widely expressed GR, glucocorticoids can bind even with higher affinity the mineralocorticoid receptor (MR), whose distribution is restricted to fewer fetal cell types than GR. Moreover, the effect of glucocorticoids signalling through MR and GR is often dampened by the local co-expression (i.e. in the fetal cells) of the glucocorticoid inactivating enzyme 11β-HSD2.

Figure 1

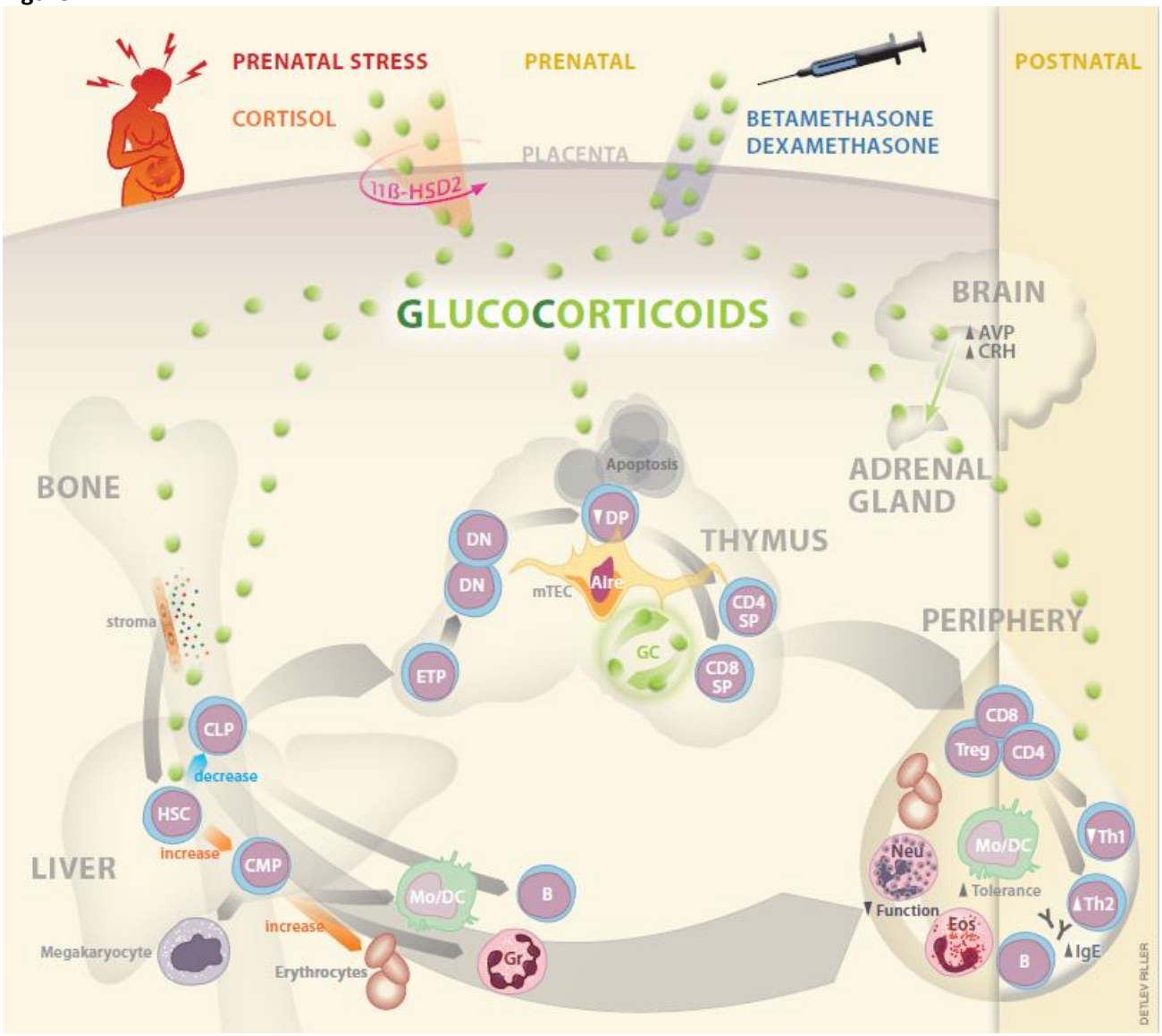


Figure 1. Hypothetical scenario depicting mechanisms by which excessive fetal glucocorticoid exposure may affect the fetal immune ontogeny resulting in altered postnatal immune responses. Excessive fetal glucocorticoid exposure can result from overwhelming maternal glucocorticoid levels, antenatal corticosteroid treatment, or from fetal HPA hyperactivity. Glucocorticoids may favour liver (murine E12-E16) or bone marrow (E16.5-birth) erythropoiesis and myeloid hematopoiesis, by promoting (orange arrows) haematopoietic stem cell (HSC) differentiation to common myeloid progenitors (CMP) in detriment (blue arrows) of common lymphoid progenitors (CLP). Moreover, glucocorticoids may directly affect the bone marrow stromal cells, i.e. osteoblast, which through the secretion of soluble factors can modulate HSC migration, proliferation and differentiation activities. Altered hematopoiesis might be associated to impaired perinatal neutrophil (Neu) function and humoral (B cell derived) responses. In the thymus, where endogenous glucocorticoids are also locally produced towards the end of pregnancy, an excess of glucocorticoids results in increased apoptosis of immature doble positive (DP) thymocytes and forces an accelerated maturation of doble negative (DN) precursors to fill the vacant niche. By antagonizing TCR signalling or by blunting AIRE-mediated autoantigen transcripts, glucocorticoids may affect the process of negative selection, allowing the export of autoreactive CD4 and CD8 single positive (SP) T cells. In addition, prenatal glucocorticoids program CD4 T helper (Th) cells towards a Th2 profile. Finally, programming of postnatal HPA axis hyperactivity, exhibiting increased levels of corticotropin-releasing hormone (CRH) and arginine-vasopressin (AVP), potentiate altered innate and adaptive immune responses, i.e. monocyte (Mo), macrophages or/and dendritic cell (DC) tolerance towards pathogens or excessive mast cell degranulation, which would in turn contribute to the prenatal programming of immune function to enhance the risk for infection, asthma and other immune diseases. 11β-HSD2, 11β-hydroxysteroid dehydrogenase 2; ETP, Early Thymocyte Precursors; IgE, Immunoglobulin E; mTEC, medullary Thymic Epithelial Cells; Treg, regulatory T cell.

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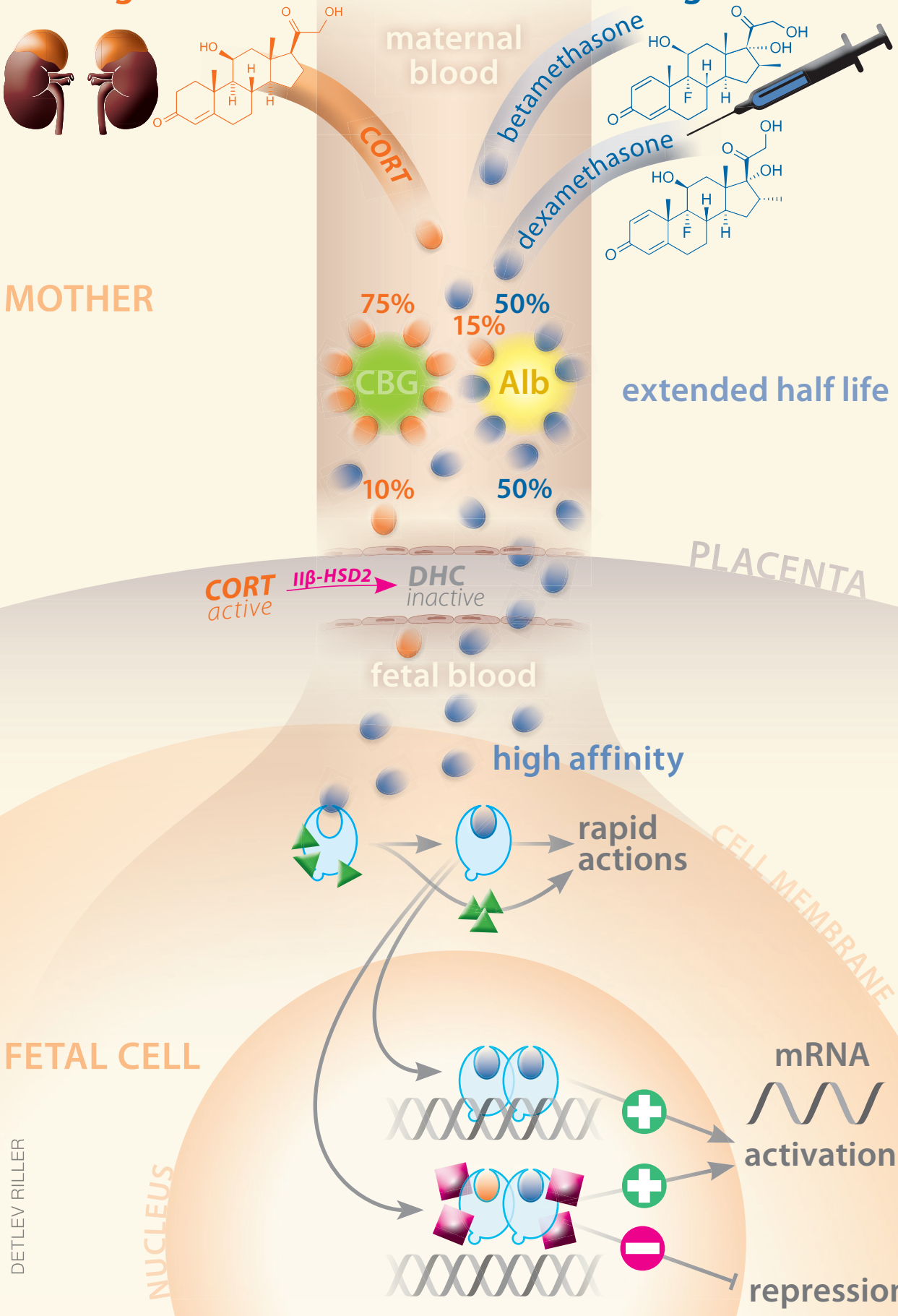
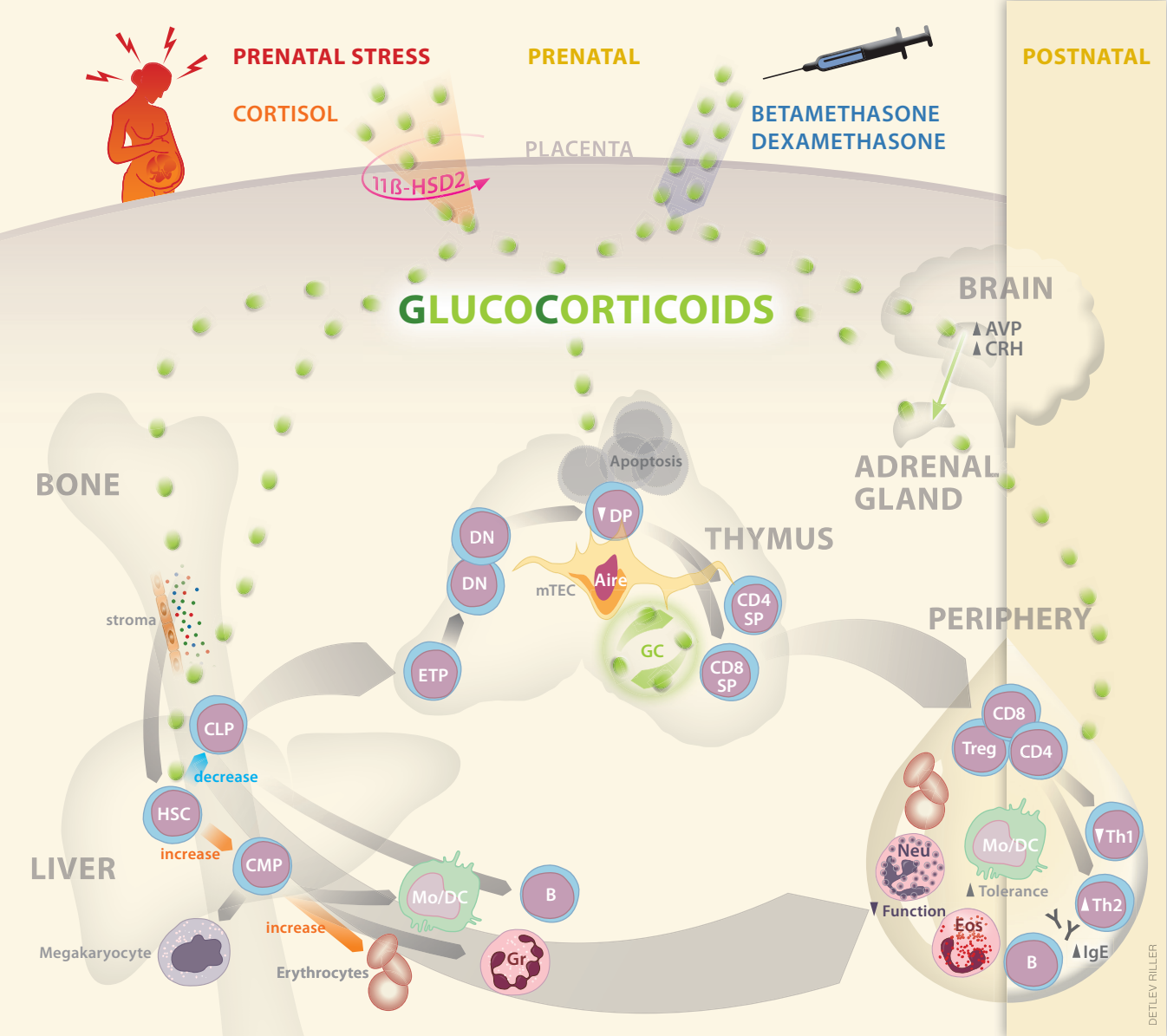
GLUCOCORTICOIDS

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CD4 SP
CD8 SP
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CD8
Treg
CD4
Neu
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Response to reviewers

We have addressed the comments of **reviewer#1** by adding at different points of the manuscript if the comment stated or the work reviewed refers to animal or human research. In addition, we have added a comment referring to differences in the timing of development of the immune system in mice and human, and the consequences on the windows of susceptibility (highlighted).

As suggested by **reviewer#2**, we have shortened the manuscript by cutting some less relevant paragraphs and sentences, which are highlighted in yellow and crossed in the revised version. Among other, we have eliminated the section '11beta-HSD1 expression in the uterus' because, even of importance, it is more related to embryo implantation, and not so much to the development of the fetal immune system. In total, we have now 715 words less in the new version.