

# Progress in Understanding Postnatal Immune Dysregulation in Allergic Disease

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**Abstract:** It is increasingly unlikely that allergic disease is the result of isolated immune defects, but rather the result of altered gene activation patterns in intricate immune networks. This appears to be driven by complex environmental changes, including microbial exposure, diet, and pollutants, which are known to modify immune development in early life, beginning in pregnancy. The first models showing possible epigenetic mechanisms for these effects are beginning to emerge. This review focuses on recent advances in our knowledge of the consequent effects on postnatal immune development, highlighting recognized differences in children with and without allergies. Although we characterized essential differences in longitudinal T-cell development more than 10 years ago, new technologies using whole genome microarrays are now being used to examine for differential gene expression in T cells from individuals with allergies. We have also recently performed the first comprehensive study of the longitudinal development of innate toll-like receptor responses in children with and without allergies during the first 5 years of life, identifying significant differences in these pathways as well. Finally, although there are preliminary differences in regulatory T-cell function at birth, longitudinal studies are limited by difficulties isolating these cells in sufficient numbers from young children for functional studies. Thymic tissue isolated during cardiac surgery is a rich source of regulatory T-cell function in children and may provide further avenues for assessing differences in maturation of these cells in individuals with allergies. To further understand the pathogenesis of these altered patterns of immune response, future research needs to encompass the complexity of gene-environmental interactions, which confer individual susceptibility to environmental exposures.

**Key Words:** postnatal immune development, epigenetic mechanisms, children with and without allergies

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## INTRODUCTION

The modern era has been characterized by a dramatic rise in susceptibility to a wide range of immune-mediated diseases. Although the clinical manifestations of these autoimmune and allergic conditions vary widely, the common element to each is an underlying breakdown in immune regulation, with failed suppression of maladaptive immune responses directed toward either environmental or self-antigens. The individual variations in disease expression are likely to result from genetic differences in susceptibility to the effects of environmental change. This epidemic clearly also reflects the inherent plasticity of immune development in response to environmental pressures, and this same plasticity also implies opportunities for preventive intervention using environmental modification. There is no doubt that this problem is complex and multifactorial. It is also likely that the efficacy of prevention strategies will also vary according to individual differences in functional genetic polymorphisms. Thus, just as we are moving toward an era of individualized treatment, it is equally likely that prevention strategies will ultimately need to be individualized according to differences in genetic susceptibility.

Before any of this can be addressed, it is essential to have a better understanding of immune development and how aspects of these processes fail in the development of disease states. This will provide better insight into disease pathogenesis and potentially provide novel molecular targets for modifying these processes to prevent disease. It will also yield better early markers that will allow more accurate identification of individual susceptibility for more targeted and eventually more individualized interventions. Although these notions are currently almost in the realms of science fiction, this broad scope of vision is important to avoid narrowing our concepts. The purpose of this review is to consider the current understanding of immune development and the directions in which this needs to evolve.

## ANTENATAL EVENTS SET THE SCENE

Although the main focus here is on the postnatal development, it is important to recognize that antenatal events are critical in setting the stage for what happens thereafter. Well-recognized presymptomatic differences in immune function are present at birth in children who go on to develop allergic disease.<sup>1</sup> This includes differences in effector T-cell function, namely, interferon- $\gamma$  (IFN $\gamma$ ) production, and more

recently described differences in regulatory T-cell (Treg) function<sup>2</sup> and innate immune function.<sup>3</sup> Although this may have been previously attributed to genetically inherited predisposition, there is growing recognition that this may also reflect the first signs of environmental impact. There is now good evidence that a number of maternal environmental exposures in pregnancy have the capacity to modify fetal immune development and perinatal immune function.<sup>1</sup> The emerging field of epigenetics also provides a new perspective on the mechanisms by which environmental changes can be inducing changes in gene expression and disease susceptibility in subsequent life.<sup>4</sup> Epigenetic regulation refers to the control of gene expression by changes in DNA and histone methylation, histone acetylation, and chromatin structure, as reviewed in detail elsewhere.<sup>5</sup> These induce conformational changes, which activate or silence genes by determining the degree of DNA compaction and accessibility for gene transcription. There are now many elegant models showing how environmental changes at critical times during development can profoundly alter the phenotype of genetically identical animals, through epigenetic modification (reviewed in<sup>5,6</sup>). Although a number of maternal exposures in pregnancy (such as infection,<sup>7</sup> maternal diet,<sup>8</sup> and smoking<sup>9</sup>) are known to modify neonatal immune function, the first models demonstrating epigenetic mechanisms in allergic disease are now beginning to emerge. Notably, an animal model using maternal supplementation with dietary methyl donors (folate) in pregnancy was shown to induce patterns of gene methylation (silencing), which promoted an allergic phenotype in the offspring, and that this effect was heritable to the next generation.<sup>10</sup> The significance of this is not clear in humans; however, one recent study has reported that folate supplements in pregnancy are associated with increased childhood wheezing<sup>11</sup> and another epidemiological study reported increasing incidence of asthma in 3.5-year-old children of mothers taking folic acid supplements during pregnancy.<sup>12</sup> Although this clearly warrants further investigation, these observations highlight the importance of considering antenatal events in subsequent patterns of immune development and disease susceptibility.

## UNDERSTANDING EARLY POSTNATAL EVENTS

In humans our opportunities to examine immune development in detail are generally limited to peripheral blood, which provides the context for most of our existing knowledge. However, it now seems likely that the most critical events in postnatal immune development occur in the gastrointestinal tract, which houses the largest reservoir of immune cells in the body. Immediately after birth infants are exposed to a vast array of new antigens, mostly from food and commensal flora, and must quickly learn to distinguish harmless from pathogenic. As most of these antigens are harmless, the gastrointestinal-associated lymphoid tissue has evolved to provide strong anti-inflammatory responses to luminal contents. Thus, “immune tolerance” is the default response to oral exposures (reviewed recently by<sup>13</sup>). Microbial colonization appears to be essential for the establishment and homeostasis of this tolerogenic microenvironment.<sup>14</sup>

The immunosuppressive milieu of the gut prevents unwanted local inflammation, and also provides optimal conditions for the development of highly regulated systemic immune responses.<sup>13</sup> Dendritic cells (DC) play a central role in these processes. Depending on the specific patterns of surface marker expression and cytokine production, intestinal DC can induce a range of antigen-specific regulatory T-cell populations including interleukin-10 (IL-10) producing Treg (Tr1), transforming growth factor- $\beta$  producing Treg (Th3), and CD4<sup>+</sup>CD25<sup>+</sup>FOXP3<sup>+</sup> Treg. These inhibit differentiation of potential inflammatory allergen-specific effector T cells and are essential for systemic immunosurveillance and tolerance.

However, these tolerogenic pathways are also matched with the capacity for DC to active inflammatory pathways, when the inflammatory signals of an infectious threat outweigh tolerogenic signals. In that setting, subgroups of DC produce IL-12 and promote IFN $\gamma$ -producing T<sub>H</sub>1 effector T cells to assist with pathogen clearance. Other DC subpopulations can also induce T<sub>H</sub>2 differentiation under some conditions. Ideally, acute effector responses are efficiently terminated after an antigenic encounter, but lead to the generation of long-lived memory T cells in preparation for future defense. Disease states seem to arise when these responses are directed inappropriately (toward nonpathogenic antigens) and are ineffectively regulated. This appears to underlie both allergic and autoimmune conditions. At this stage the mechanisms are still poorly understood. Further investigation into the development and regulation of tolerance pathways in the gastrointestinal tract remains an important research priority.

## EFFECTOR T-CELL DEVELOPMENT

Patterns of effector T-cell response have been of central interest because their role was recognized in promoting IgE production. There has been clear evidence that most T<sub>H</sub>2 cytokines (IL-4, IL-5, IL-9, and IL-13) are implicated in the expression and development of airways inflammation and airway hyperactivity.<sup>15–17</sup> Reciprocal inhibition between IFN $\gamma$ -producing T<sub>H</sub>1 cells and IgE-promoting “pro-allergic” T<sub>H</sub>2 lead to the earlier proposal that allergic responses were the result of a “skewing” of T-cell responses.<sup>18,19</sup> Although this remains true to some extent, this model has evolved to recognize a more central role for regulatory T cells and antigen-presenting cells in dictating the pattern of effector T-cell differentiation. Although there are clear differences in the allergen-specific effector of individuals with and without allergies, so far allergic disease cannot be attributed to specific defects in any of these pathways. It appears more likely that allergic disease is the result of complex gene-environmental interactions that, under modern environmental conditions, are enhancing the T<sub>H</sub>2 differentiation at a number of developmental stages.

It is certain that T<sub>H</sub>2 genes must have been conserved for an evolutionary advantage. Although these pathways are important in defense from parasitic infection, they are also crucial much earlier in development for fetal survival. During pregnancy there is a prevailing “T<sub>H</sub>2 skew” to protect the

fetus from maternal T<sub>H</sub>1 tissue rejection. This T<sub>H</sub>2 milieu has a significant influence on fetal immune responses and is reflected in the T<sub>H</sub>2 dominant responses detected in cord blood to a range of stimuli.<sup>20</sup> Although T<sub>H</sub>1 responses can still be induced at this age, they are relatively immature and there is hypermethylation (epigenetic silencing) of the IFN $\gamma$  gene promoter.<sup>21</sup> Although this pattern of immune response is adaptive in pregnancy, it is critical that this T<sub>H</sub>2 propensity is suppressed in favor of T<sub>H</sub>1 maturation to adapt to the postnatal environment.<sup>22</sup> Notably, newborns who subsequently develop allergic disease seem to have a greater relative immaturity of effector T-cell function, most notably in T<sub>H</sub>1 function.<sup>23,24</sup> This has been proposed to contribute to the persistence of T<sub>H</sub>2 propensity in children who develop allergic disease.<sup>22</sup> More recently, noted perinatal differences in Treg function may also contribute to this.<sup>25,26</sup> Factors that promote T<sub>H</sub>1 maturation (and Treg maturation) such as microbial exposure are believed to be central to this process,<sup>27</sup> and these concepts have provided additional support for the hygiene hypothesis of allergic disease.

This model had provided a useful framework for understanding the role of T cells in the early development of allergic disease; however, we still have not uncovered the core pathways in pathogenesis. It is possible that this may vary between individuals, particularly as these conditions are so heterogeneous.

Newer approaches are using whole genome microarray technology to examine for differential gene expression in T cells from individuals with allergies (compared with individuals without allergies). This has identified novel genes that may be involved in the allergic response.<sup>28</sup> We are now using the same technology in children with food allergy to identify patterns of early T-cell gene expression, not only during symptomatic disease but also in the same children during the perinatal period before disease has developed. We anticipate that this will identify novel genes that could shed further light on disease pathogenesis and may also provide early predictors of allergic propensity.

## DEVELOPMENT OF INNATE IMMUNE FUNCTION

Antigen-presenting cells and other cells of the innate immune system provide the first line of defense against pathogenic environmental exposures. Among these, DC play the most critical role in programming naive T-cell responses. Antigen encounter in peripheral tissues induces migration and maturation in regional nodes.<sup>29</sup> The development of these responses is naturally difficult to study in humans. Animal studies suggest that resting DC stimulate T<sub>H</sub>2 immune development unless they receive obligatory T<sub>H</sub>1-trophic signals during antigen processing.<sup>30</sup> These signals may occur under conditions of infection or other local stress,<sup>31,32</sup> which evoke protective T<sub>H</sub>1 effector T-cell responses. Foreseeably, variations in local inflammation (such as with infection) and the subsequent pattern of local T-cell responses could have a key role in determining DC maturation. Animal studies confirm that local airway DC networks are less developed in infant animals and display markedly attenuated responses to inflam-

matory triggers.<sup>33,34</sup> Similarly, human infants do not typically show DC in the airways in the absence of inflammation.<sup>35</sup> However, mature DC do appear in association with severe respiratory infection even at this age.<sup>35</sup> This suggests that local tissue events in infancy can influence the maturation of DC and modify downstream T-cell programming in early life.

Microbial products, arguably the most powerful immunostimulants in the early environment, are likely to play a particularly key role in the maturation of innate pathways, Treg and T<sub>H</sub>1 responses, which may all act together to prevent inappropriate allergic T<sub>H</sub>2 responses. This has logically lead to interest in possible differences in microbial recognition pathways in infants with and without allergies, namely, toll-like receptor (TLR) function. This family of receptors recognizes a broad range of microbial agents<sup>36</sup> with different TLR signaling the presence of different microbial components. It has been proposed that TLR-mediated activation of both APC and regulatory T cells may play an important role in reducing the risk of T<sub>H</sub>2-mediated allergic responses (<sup>27,37</sup> and others). Several groups have now examined neonatal TLR responses, comparing in children with allergic predisposition (family history) with those deemed at low risk. Schaub et al have observed lower neonatal IFN $\gamma$  and IL-10 responses to TLR activation.<sup>26</sup> Although this study examined only TLR2 responses, it is consistent with a much smaller study that previously showed that newborns of women with allergies ( $n = 9$ ) had significantly lower in vitro responses after TLR2 ligation (with peptidoglycan) compared with those of women without allergies ( $P = 0.03$ ).<sup>38</sup> This is in contrast to our own observations in a larger population of infants with known allergic outcomes. Using a much wider range of ligands (TLR2–TLR9) to assess neonatal innate responses, we demonstrated significantly higher TLR responses at birth in relation to both maternal atopy and subsequent infant allergic outcomes.<sup>3</sup> The significance of these contrasting findings still needs to be clarified. It is well recognized that the effect of host-environmental interactions can depend on genetic polymorphisms and that the effects of functional TLR polymorphisms<sup>39</sup> (and other microbial recognition pathways<sup>40</sup>) vary with the level of microbial exposure.<sup>39</sup> These complex interactions may explain some of the inconsistencies between studies performed in different environmental contexts.<sup>41</sup>

In a separate cohort we have also performed the first longitudinal study of these responses (to TLR2–TLR9) during the first 5 years of life. We again observed that the group with allergies had higher IL-10, TNF $\alpha$ , and IL-6 TLR responses at birth compared with children without allergies. We also observed that children who go on to develop allergic disease have significantly higher proportion of TLR2<sup>+</sup> pDCs at birth, compared with children who do not develop allergies. Although children without allergies showed an age-related increase in these innate responses, this was not seen in the group with allergies and by 5 years of age children with allergies had significantly reduced innate function compared with control children (Tulic and Prescott, submitted for publication). This is consistent with a recent cross-sectional comparison of TLR2 responses in 5-year-old children, which

found that monocytes from children with allergies had impaired regulation of TLR2 upon peptidoglycan stimulation, suggesting a relatively hyporesponsive state.<sup>42</sup>

Despite the increased inflammatory responses to microbial responses in the early postnatal period, these children showed persistently weaker T<sub>H</sub>1 responses to allergens with a consistent T<sub>H</sub>2 dominance which consolidated with age. These observations suggest that individuals with allergies have increased inflammatory responses to microbial products during the early perinatal period of immune programming, but this fails to result in sufficient T<sub>H</sub>1 maturation to suppress allergen-specific T<sub>H</sub>2 responses. This focuses attention on the role of inflammatory cytokines, such as IL-6, in unfavourably altering the early balance between tolerance and inflammation. Further studies are required to determine the significance of this.

### DEVELOPMENT OF REGULATORY T-CELL FUNCTION

Although regulatory T cells have become a leading focus in the development of allergic disease, they are among the hardest cells to study. First, these cells are difficult to identify and isolate because of the lack of specific conventional surface markers, and second, their presence in such small numbers makes it logistically difficult to perform functional studies on the small volumes of blood that are available from children. Thus, to our knowledge, there are no longitudinal studies of Treg function in children with allergies versus children without allergies.

Currently, the main developmental studies of Treg have been with cord blood, and several have shown differences in neonatal Treg function in relation to allergic disease.<sup>26,43</sup> Schaub and colleagues have shown impaired neonatal Treg function in infants at high risk of allergy.<sup>26</sup> An earlier preliminary study found that high-risk neonates had reduced capacity to generate CD4<sup>+</sup>CD25<sup>+</sup> cells after stimulation<sup>43</sup>; however, regulatory function was not examined directly. In our forerunner studies,<sup>2</sup> we examined neonatal T-regulatory function in relation to subsequent allergic outcomes (IgE-mediated food allergy). We used the more definitive strategy for identifying CD4<sup>+</sup>CD25<sup>+</sup> CD127<sup>lo/-</sup> regulatory cells to examine presymptomatic differences in the activity and frequency of Tregs in cord blood. We observed that neonates who developed subsequent allergy had reduced capacity to suppress selected effector responses compared with the group that did not develop allergies.<sup>2</sup> Although this was not seen for all cytokines, this provides further preliminary evidence that allergic disease may be associated with developmental difference in these pathways.

The thymus offers a novel opportunity to document the maturation of these pathways, which are otherwise difficult to study in the small volumes of peripheral blood available from young infants. Our preliminary studies show age-related changes in the cytokine milieu in the thymus parallel changes in peripheral immune function (Tulic, and Prescott, submitted for publication). Specifically, the thymic microenvironment is similarly “T<sub>H</sub>2-skewed” in the early postnatal period (with higher IL-5 and IL-13 cytokine production), and this under-

goes age-related suppression as T<sub>H</sub>1 (IFN $\gamma$ ) production increases. Thymic IL-10 responses also increase with age. CD4<sup>+</sup>CD25<sup>+</sup>CD127<sup>lo/-</sup>FOXP3<sup>+</sup> T regulatory (Treg) cells are readily identified and comprise ~5% of the total proportion of thymocytes in neonates. This proportion increases with age in parallel to an age-related increase in thymic FOXP3 mRNA expression. We have examined the percentage of Ki67<sup>+</sup> cells as a measure of the proliferative expansion of CD4<sup>+</sup>CD25<sup>+</sup>CD127<sup>lo/-</sup>Treg, presumably mediated by a combination of self- and environmental antigens. We observed greater Treg expansion in the older children compared with the younger children, suggesting that there may be increased thymic Treg expansion with age. These data suggest that the developmental changes in the thymus parallel recognized changes in peripheral blood responses, and support our hypothesis that there are also age-related changes in regulatory function. Similar technologies will now be used to compare children with allergic and nonallergic phenotypes.

### CONCLUSIONS

Although there has been steady progress in our understanding of immune development, there are still considerable gaps in our knowledge. It is increasingly unlikely that allergic disease is the result of isolated immune defects, but rather the result of altered activation patterns in intricate immune networks. This appears to be driven by complex environmental changes, and the diverse increase in immune dysregulation (autoimmunity and allergy) suggests effects on common regulatory pathways. Future research needs to encompass the complexity of gene-environmental interactions, which confer individual susceptibility to environmental exposures. This complexity implies that there may be different causal pathways and different prevention strategies according to a multitude of functional genetic polymorphisms. At this stage, this complexity appears overwhelming, but the development of new research methodologies, mathematical modeling and network theory, may provide the capacity to study these complex interactions. The current research characterizing development pathways in more detail will be an essential platform for these future approaches.

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