Innate and Adaptive Immune Pathways to Tolerance

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Abstract

There is a vast scientific literature on the innate and adaptive immune responses that contribute to the development of tolerance with growing appreciation that innate and adaptive immunity do not function independently of each other. Innate immune pathways of current interest are those involving pattern recognition receptors, such as Toll-like receptors, particularly their expression by epithelial cells and dendritic cells at mucosal surfaces. The study of adaptive immune pathways has traditionally focused on specific IgA and the development of effector T-cell populations: the Th1/Th2 paradigm has evolved to encompass Th17 cells. Recent years have seen a dramatic resurgence in the investigation of regulatory T-cell populations that can modify a broad range of immunological activities. Dendritic cells play a key role in linking innate and adaptive immunity. The microenvironment of the dendritic cell at the time of antigen encounter modulates co-stimulatory molecule expression and cytokine production which orchestrate antigen-specific activity by T cells, especially the development of different effector T-cell populations (Th1/Th2/Th17) or regulatory T cells. Cascades of cytokines/chemokines play central roles in many types of immune responses. These include: (i) TGF-β; (ii) IL-10, and (iii) thymic stromal lymphopoietin (TSLP). Current paradigms of immunity and tolerance in relation to the development of allergy relate to the interplay between innate and immunoregulatory mechanisms.

Introduction

The development of allergic sensitization essentially reflects a failure of the host to establish effective immunological tolerance to a relatively non-pathogenic inhalant or dietary antigen. Therefore, understanding pathways leading to tolerance and how they might be perturbed to enable the establishment and maintenance of allergic sensitization clearly has implications for the design of disease treatment and prevention strategies. The mucosal
immune system must maintain a balance between immunity and tolerance in the face of exposure to potentially pathogenic microorganisms and non-pathogenic microorganisms such as commensal bacteria and other antigens such as food antigens. There is a vast scientific literature on how the innate and adaptive immune responses contribute to the development of tolerance. Moreover, there is growing appreciation of the interaction between these two major arms of our immune system in all types of immune responses and that innate and adaptive immunity do not function independently of each other.

To establish immunity or tolerance, the immune system must be able to distinguish self from non-self and determine which antigens constitute danger to the host. The innate immune response is rapid, nonspecific, nonanticipatory and nonclonal, and utilizes germ-line-encoded receptors. In contrast, the adaptive immune response is specific, anticipatory, clonal, and uses receptors that have been generated by somatic DNA rearrangement. Both types of immune responses are dependent on receptor-mediated responses: pattern recognition receptor-mediated innate immune responses and adaptive immune response dependent on rearranged antigen-specific receptors (TCR and BCR/antibody).

**The Innate Immune Response and the Development of Tolerance**

The gut mucosa is inhabited by a large commensal microflora that is required for the processing of nutrients and education of the gut immune system, conversely, innate and adaptive immune responses by the gut immune system shape the commensal flora. A central question is how tolerance of commensal microflora and innocuous dietary antigens is maintained while retaining the capacity to respond to pathogenic/harmful microorganisms/antigens.

**Pattern Recognition Receptors**

The pattern recognition strategy of these receptors is based on the detection of a limited set of conserved molecular patterns (pathogen-associated molecular patterns; PAMPs) that are virtually unique to the microbial world and signal to the host the presence of infection [1]. The Toll-like receptor (TLR) family is the best characterized class of mammalian pattern recognition receptors. TLRs are a family of proteins, consisting of 10 functional type-1 transmembrane receptor proteins in humans (fig. 1) that have emerged as key upstream mediators of inflammation at many tissue sites in humans and other organisms. Activation of TLRs results in an inflammatory immune response characterized by the production of cytokines and other antimicrobial factors. Through the regulation of co-stimulatory molecules, TLRs also facilitate the development of the adaptive immune response. Individual TLRs
respond to a limited number of ligands, yet collectively the family of TLRs can respond to a wide range of products associated with bacteria, viruses, fungi and protozoa. Interaction between PAMPs and TLRs initiates a signaling cascade upon recruitment of an adapter protein (e.g. MyD88) leading to activation of members of the MAP kinase family, NF-κB and interferon (IFN) regulatory factors amongst others [2]. Other pattern recognition receptors, such as nucleotide oligomerization domain (Nod) 1 and 2, also play a role in innate immune responses.

**TLRs and Epithelial Cells**

Commensal bacteria are recognized by TLRs under normal steady-state conditions and this interaction plays a role in the maintenance of intestinal epithelial homeostasis [3]. Spatial and functional localization of pattern recognition receptors is pivotal to ensuring a tolerant response to commensal flora: expression and activity of TLRs in the gut epithelium is regulated in multiple ways as summarized in table 1. For example, in the healthy gut TLR5 expression is restricted to the basal surface of the epithelium where flagellin-bearing bacteria will not be encountered. Once the epithelial barrier is breached, activation via TLR5 stimulates chemokine release and recruitment of immature

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**Fig. 1.** TLRs expressed by human cells are located at the extracellular membrane and on intracellular vesicles. The various adapter molecules utilized to initiate intracellular signaling are shown as differently shaded squares and circles. The outcome of type-I interferon (IFN) production or inflammatory cytokine production is shown in relation to each TLR.
dendritic cells (DCs) that can send dendrites through disrupted epithelial tight junctions and sample luminal antigens prior to migrating to local lymph nodes for presentation to T cells.

**TLRs and DCs**

Contiguous networks of DCs are present in the epithelium of the gut, skin and airways where they play pivotal roles in surveillance for environmental antigens. DCs orchestrate antigen-specific activity by T cells via the provision of major histocompatibility complex (MHC)/antigenic peptide, co-stimulatory molecules and cytokines (fig. 2). DC expression of co-stimulatory molecules, such as the B7 family, and cytokine production are tightly regulated. Expression is particularly controlled by environmental stimuli that interact with receptors on DCs [4]. These environmental stimuli arise endogenously (e.g. stress hormones, neuropeptides) or exogenously (e.g. diet or microbiota) and have a dramatic effect on whether immunity or tolerance occurs after initial antigen exposure.

There is particular interest in the patterns of cytokines produced by DCs upon encounter with microbial stimuli as these cytokines drive the development of effector T-cell populations. Different TLR ligands are reported to preferentially favor different effector T-cell populations, e.g. TLR2 ligands generally favor Th2 responses whereas TLR9 ligands inhibit Th2 effector activities [5]. Activation of TLR4 with lipopolysaccharide (LPS; endotoxin) can promote either Th1 (high doses) or Th2 (low doses) responses [5]. Little is known about the Th1/2-biasing properties of other TLR ligands.

**Mast Cells**

Mast cells can be activated via several non-FceRI-mediated stimuli, including TLR ligands and are commonly found at mucosal surfaces where they will encounter numerous antigenic stimuli [6]. For many years the focus of mast cell immunology has been their role in allergy/anaphylaxis although they have been long recognized to play a role in the protective host immune response to

Table 1. Mechanisms that control TLR activity in epithelial cells of the gastrointestinal tract

<table>
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<th>Mechanism</th>
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<td>Reduced expression in areas of higher bacterial load – e.g. colon vs. small intestine</td>
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<td>Preferential expression on basal surface of epithelium (e.g. TLR5)</td>
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<td>Limited TLR repertoire</td>
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<td>Endotoxin (and other PAMPs) tolerance</td>
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<td>Expression of inhibitory molecules</td>
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parasites. The ability of mast cells to interact directly and indirectly with DCs and to regulate T cells is of increasing interest. As mast cells have the capacity to differentially secrete preformed and inducible mediators (e.g. TGF-β, IL-10, discussed below) they might have the capacity to fine tune the immune response including the potential to modulate the microenvironment in which differentiation of naïve T cells into effector cells occurs [6, 7].

**NK Cells**

Natural killer (NK) cells are recognized for their role in the surveillance for tumors and virally infected cells. More recently their potential to regulate and be regulated by DCs, macrophages, T cells and endothelial cells has emerged [8]. NK cells express an array of activating and inhibitory receptors at the cell surface that regulate NK cell activity: activating NK cell receptors detect infectious non-self ligands, e.g. TLR ligands and signals from stressed cells, whereas inhibitory receptors determine the absence of constitutively expressed self molecules such as MHC class I. As for different effector T-cell populations, the cytokine microenvironment (e.g. IL-12 is a potent activator of NK cells) and interactions with other cells (e.g. DCs) determine the nature of the response by NK cells.

**Fig. 2.** The development of a particular effector (Th1, Th2, Th17) or regulatory T-cell population after interaction between major histocompatibility complex (MHC)/antigen on dendritic cells and the TCR depends on co-stimulatory molecule (CSM) expression and cytokine profile produced. Various stimuli, such as bacteria interacting with pattern recognition receptors (PRR) on DCs determine CSM and cytokine expression and therefore T-cell differentiation.
Breast Milk

Some consideration of the potential role of breast milk in the development of immunity and tolerance in early infancy is warranted. Human milk contains an array of nutrients, hormones, growth factors and immunoactive molecules that influence the growth, development and immune status of the newborn. Immunoactive mediators include immunoglobulins (e.g. IgA), cytokines (e.g. TGF-β), lysozyme, lactoferrin, complement, and nutrients. These mediators orchestrate the development of the mucosal immune system, especially at the gastrointestinal tract, and probably serve to compensate for developmentally programmed downregulation of many immune functions during infancy [9]. Breast- versus bottle-fed babies have a lower incidence of gastrointestinal infections and inflammatory conditions but the protective effect of breastfeeding on the development of allergic diseases remains divisive: studies that find a positive effect, no effect or even a negative effect of breastfeeding on atopic sensitization, eczema, allergic rhinoconjunctivitis and/or asthma continue to be published.

Adaptive Immune Response and Development of Tolerance

Dendritic Cells

DCs are central to the initiation of specific immunological reactivity to allergens and other antigens, i.e. adaptive immune responses. DCs modulate the development of different effector T-cell populations, namely Th1, Th2, Th17 or regulatory T (Treg) cells. This differentiation is orchestrated by signals via the interaction between MHC/antigen on the DC with T-cell receptor (signal 1), co-stimulatory molecules (signal 2) and cytokine/receptor interactions (signal 3; fig. 2). Mucosal DCs are generally considered immunosuppressive and the production of retinoic acid by these cells, including effects of retinoic acid on TGF-β-dependent immune responses, might underlie some of these effects [10–12]. The local microenvironment, often as created by prevailing commensal/infectious microflora, therefore plays a critical role in determining the T-cell phenotype developed upon antigen encounter via TLR-dependent and TLR-independent pathways operating in DCs.

Regulatory T Cells

Initially identified by their ability to suppress autoimmune disease, Treg cells have since been shown to modify a broad range of immunological activities associated with inflammatory and infectious diseases, cancer and transplantation. Moreover, abnormalities in the activity of these cells have been
implicated in susceptibility to many conditions with underlying immune etiology, including allergy. Several types of Treg cells have been described and these can be broadly subdivided into natural and inducible Treg (iTreg) cells. Natural CD4+ Treg cells can be identified by the expression of CD25 and low expression of CD127 (IL-7 receptor α chain) [13]. These cells also express FoxP3, deemed the master regulator of development/function of this population [14]. Their functional properties have been attributed to CTLA-4, TGF-β and IL-10 but this remains an area of ongoing investigation and controversy.

Additional Treg populations, including CD8+CD25+ Treg cells, iTreg cells and NKT cells, are also of interest but are comparably under-investigated. The relative ease of identification of natural Treg cells (CD4+VD25+/CD127lo) in peripheral and umbilical cord blood has accelerated investigation of the role of these cells in health and disease. There is a burgeoning literature on the contribution of these cells to allergic disease. Whilst there are increasing numbers of publications identifying perturbations in the activity of CD4+CD25+ T cells in allergic adults, a direct causal relationship between these cells and allergic inflammation in humans has not been demonstrated. Of great interest is the possibility that perturbations in Treg cell activity could be identified and targeted prior to disease onset, i.e. at birth. Differences in Treg activity that precede the development of egg allergy have been identified: CD4+CD25+CD127lo Treg cells can suppress IFN-γ production by conventional CD4+ T cells (both populations prepared from umbilical cord blood) and this ability is less often detectable and, when detectable, is reduced in neonates who went onto develop allergy [15]. There was no significant difference in the number of Treg cells or their FoxP3 expression at birth in the 2 groups. Maternal atopy has also been implicated to have a negative effect on natural Treg cells [16].

iTreg cells are generated preferentially by CD103+ DCs in the gut-associated lymphoid tissues (GALT). This DC population expresses high levels of retinal dehydrogenase that converts vitamin A to retinoic acid: inhibiting this enzyme or neutralizing TGF-β attenuates the development of iTreg and adding retinoic acid enhances TGF-β-induced iTreg differentiation [10, 11]. This pathway might be particularly important for the induction of tolerance to innocuous mucosal antigens including those from the commensal flora and dietary origin. GALT DCs also imprint locally developed effector T cells for preferential migration back to the gut via induction of specific homing receptors, also a retinoic acid-mediated affect [17].

Overall, the outcome of antigen encounter reflects the interplay between innate and immunoregulatory mechanisms. The role of TLRs and other relevant receptors on DCs has already been discussed, however Treg cell populations also express TLRs and are therefore responsive to microbial stimuli. CD25-expressing Treg cells express TLR2, TLR4, TLR5 and TLR8, and activation via these receptors has variable effects on survival, proliferation and the suppressive activity of these cells [18]. There are no data concerning the
expression and activity of TLRs on neonatal and pediatric Treg cell populations but this would clearly be of great interest and might potentially have therapeutic implications. The next few years should see some exciting developments in our understanding of Treg cell activity in early childhood and its impact on IgE sensitization and allergic disease outcomes.

**Other T-Cell Subsets – Th1/Th2/Th17**

The microenvironment in which DCs encounter antigen has dramatic effects on co-stimulatory molecule expression and cytokine production which determine the effector T-cell populations (Th1, Th2 or Th17) that develop. The signature features of each effector T-cell population are the cytokine profile produced and the transcription factors that orchestrate development (fig. 3). Th2 cells have been long recognized to play a central role in the development of allergic sensitization and the ensuing clinical symptoms. Until recently, IFN-γ-producing Th1 cells had been considered one of the major effector T cells in the development and/or progression of autoimmune diseases but Th17...
cells have emerged as critical effector cells in autoimmunity. This paradigm shift was mediated by the discovery of IL-23 [19]. The Th17 subset of CD4+ effector T cells is defined by its ability to preferentially produce IL-17/IL-17A as well as IL-17F, IL-21 and IL-22 [20]. The specific effector functions of Th17 cells include host defense against extracellular bacteria and fungi, organ-specific autoimmunity, and the regulation of granulopoiesis under physiological stress [20]. The physiological role of Th17 cells is not yet clear although they are present constitutively in the intestinal lamina propria of the mouse.

**B Cells**

The contribution of B cells to the development of tolerance has been attributed largely to their capacity to produce IgA as discussed below. More recently, B cells with regulatory activity, including the capacity to modulate the development and/or activity of Treg populations have been identified in animal models but so far there are little data about these populations in humans. Key to elucidating regulatory B-cell activity in humans is consideration of the maturation stage of the B cells being studied [21].

**IgA**

A key question in mucosal immunology is how coexistence with a dense and diverse commensal microflora is maintained in the absence of inflammatory intestinal pathology in the majority of individuals. IgA plays a role in generating noninflammatory immune protection via a number of mechanisms as summarized in table 2. Retention of small numbers of live commensal bacteria by intestinal DCs has been implicated in the selective induction of IgA that limits mucosal penetration by commensals [22]. IgA might also play a role in driving diversification of bacterial surface structures and contribute to strain diversity in the intestine [23]. The pathway by which IgA is produced is very primitive and IgA can be produced both dependently and independently of T-cell help. IgA dimers secreted by intestinal B cells are actively transported across the epithelium cells generating secretory IgA that is released into the gut lumen. TGF-β, IL-10 and TSLP and retinoic acid, all discussed elsewhere, play roles in the development of antigen-specific IgA-secreting plasma cells.

The transfer of IgA from mother to infant via breast milk provides transient immune protection against gastrointestinal pathogens. IgA in breast milk requires local IgA-secreting plasma cells which must migrate into the lactating mammary gland, presumably from the gastrointestinal tract. Mucosal epithelial chemokine (CCL28) has been implicated as a key regulator of the trafficking of IgA-secreting cells into the mammary gland and IgA secretion into the milk in animals [24]. This type of study is more challenging in humans.
but we have found very high levels of mucosal epithelial chemokine in human breast milk collected in the first 2 weeks postpartum [Thornton et al., unpublished observation] and are continuing functional studies.

**Cytokines (TGF-β, IL-10 and TSLP)**

Cascades of cytokines and chemokines play central roles in many types of immune responses, although elucidating the role of any individual cytokine in an immune response is challenging. Still, a number of cytokines have emerged as critically important to the development of immunity and tolerance.

**TGF-β**

TGF-β plays a role in peripheral T-cell homeostasis, tolerance to self or innocuous environmental antigens and T-cell differentiation during immune responses [25]. Active immune suppression by TGF-β is a pivotal mechanism of tolerance and occurs via direct effects of TGF-β or via its effects on effector T cells and Treg populations. TGF-β converts naïve T cells into regulatory cells via induction of FoxP3 expression, but in the presence of IL-6 favors, at least in animal models, the differentiation of naïve T cells into IL-17-producing Th17 effector cells [26, 27]. The involvement of TGF-β in the development of Treg and Th17 cells is emerging as mutually exclusive: conditions favoring the differentiation of one population inhibit that of the other although the molecular mechanisms underlying this reciprocal relationship remain poorly understood. Three types of TGF-β are found in mammals with TGF-β1, the most critical in immunoregulation. TGF-β1 is produced by many cell types and must be released from the constraints of binding proteins that confer latency if it is to exert any biological effect. This complexity provides a unique mechanism to integrate signals from multiple cell types to regulate T-cell development, survival, proliferation and differentiation in a TGF-β-dependent fashion.
**IL-10**

A major immunological role for IL-10 is the control of exuberant responses to microbial antigens. IL-10 also has emerged as an important immunoregulatory cytokine via its ability to modulate the regulatory activity of various T- and B-cell populations. B cells and monocytes are the major source of IL-10 in humans. IL-10 inhibits production of both Th1 and Th2 cytokines and acts as an anti-inflammatory cytokine able to downregulate the production of a range of proinflammatory mediators (e.g. TNF-α, IL-6) [28]. There is also a growing number of IL-10-related genes, the products of which form the IL-10 super-family (IL-10, IL-19, IL-20, IL-22, IL-24, IL-26, IL-28 and IL-29). There is not a great deal known about many of these family members but they play a role in skin development (IL-19 and IL-20), Th2 immune deviation (IL-19), mucosal and cutaneous immunity (IL-26) and antiviral immunity (IL-28 and IL-29) [28].

**Thymic Stromal Lymphopoietin**

Thymic stromal lymphopoietin (TSLP) is an IL-7-like cytokine produced predominantly by epithelial cells, including those of the skin and airways. Production of TSLP by epithelial cells is driven by exposure to microbial products (e.g. double-stranded RNA), and by the combined action of proinflammatory (e.g. TNF-α) and Th2 cytokines [29]. TSLP has been identified as pivotal to the production and expansion of CD4+CD25+ Treg cells within the human and murine thymus, and has emerged as a potential key mediator of both the initiation and maintenance of Th2 responsiveness [30]. Consequently, there is much interest in the biological roles of TSLP and those identified to date are summarized in figure 4. Given the role that TSLP plays in promoting Th2-biased responses, understanding the potential contribution of TSLP to the development of atopic sensitization in very early infancy is clearly of great importance. We have shown that human breast milk contains immunoreactive TSLP for at least the first 2 weeks postpartum [Macfarlane et al., manuscript submitted] and are pursuing further studies on the role of TSLP in the induction of tolerance and Th2 sensitization in the perinatal period.

**Conclusions**

Current paradigms to explain the impact of microbial exposures, especially during infancy, on allergic outcomes relates these to the interplay between innate and immunoregulatory mechanisms. Understanding how the innate and adaptive immune responses interact in the perinatal period, how this activity leads to immunological tolerance, and how innate and adaptive immunity might be perturbed in the development of allergic and other immune-mediated diseases is of critical importance if allergic disease is to be prevented or novel therapeutic strategies to treat the disease developed.
**Fig. 4.** Thymic stromal lymphopoietin (TSLP) is emerging as a cytokine of interest. The biological effects ascribed to TSLP so far are summarized here.

### References

Immunity and Tolerance


Discussion

Dr. Björkstén: When you talk about IgA, is that total IgA or is it secretory IgA, and where did you measure it?

Dr. Thornton: We haven’t done any IgA measurement studies; I was summarizing what is in the literature. So they would variably be secretory IgA and total IgA that people are talking about in these studies. I think most of those studies looking at biological mechanisms of IgA relate to secretory IgA.

Dr. Björkstén: As mentioned in my talk, in Estonia most of the IgA in saliva is secretory IgA, while in Sweden the development of secretory IgA is delayed although salivary IgA is not.

Dr. Thornton: All the studies looking at the roles of IgA in the gut are focused on secretory IgA, so they are studies with emerging interest in the driving diversity of bacterial strains like secretory IgA which actually function in the lumen of the gut.
**Dr. Gibson:** What sort of levels of thymic stromal lymphopoietin (TSLP) are found in breast milk? Are they in the range that might be biologically significant?

**Dr. Thornton:** It is really hard to gage the physiologically active levels of this; it is about 100–200 mg/ml in 3- or 5-day postpartum breast milk. We've got to start thinking about the volumes of TSLP that the babies drink at that time, despite the fact there are lower levels per milliliter of breast milk. How much milk does a neonate drink in one feed, 7–10 ml? It depends on the age when you start fracturing it up, and it also depends on how it is presented in the breast milk. We don't know if it's native TSLP or associated with macromolecules or anything like that, but I would suspect it is physiological.

**Dr. Renz:** You have nicely dissected all the various events in this highly complicated paradigm. You have shown us that some of these Toll-like receptors induce interferon type-1 mediators, while others, depending on the signaling cascade, induce poor inflammatory cytokines. All this depends somewhat on the dosing and the timing of this. When it comes to real life there is usually not stimulation of only one selected individual Toll-like receptor. This morning we talked about the microbial situation in the gut where we have billions of microbes and maybe thousands of different species which probably trigger all these Toll-like receptors at the same time. By looking at the literature in this field, there is very little information on what happens if a combination of different microbes, bacteria or fungi stimulate Toll-like receptors simultaneously in terms of the outcome of the signaling response.

**Dr. Thornton:** I think one of the shortcomings in the literature is that people tend to just use a single stimulus and look at the single Toll-like receptor and the output associated with that. There are very few studies where different microbes were mixed. I can't think of a study where for example Candida was put in with a Toll-like receptor 2 or Toll-like receptor 4 ligand, and also there are different cells responding. When I was going over these this morning I was thinking about the Langerhan's cells in the skin because the Langerhan's cells sit amongst sites which express different Toll-like receptors. They are more likely to initiate a much a more limited innate immune response to protect the skin. In the gut it is incredibly complex, and I don't know how to start teasing all those apart.

**Dr. Makrides:** Listening to you it made me wonder whether the environment or what happens to the mother during pregnancy has any effect on predicting how things work out. For example, over the years pregnant women have increased in BMI with the excess weight gain during pregnancy, or the high rates of gestational diabetes that are now seen. We know that fat is not just a storage organ with metabolic activity. Is there any information about these extra stresses in pregnancy, and do they have any influence on predicting how things work out?

**Dr. Thornton:** They are the kind of studies we are just starting to do, and I am really looking forward to Dr. Isolauri's talk later today because of the obesity aspect. Dr. Björkstén mentioned earlier the study looking at maternal antibiotic use during pregnancy and it's association with an increased prevalence of allergic disease in the children. That is one of the few studies that has actually looked in any detail at what goes on in the mother during pregnancy. Obviously we could go to the hygiene hypothesis and the sibling effect. The sibling effect might not relate to having all the siblings per se in the family, it might relate to the fact that the mothers had any number of pregnancies prior to the pregnancy by which you were born, and immune function in pregnancy might change with the success of pregnancies. Nobody has really looked at the potential impact of obesity and gestational type 2 diabetes during this time, and I think that definitely we should start looking at this.

**Dr. Sartor:** Going back to TSLP, could you tell us a little bit about how TSLP is regulated? Is it a compensatory response to inflammation, say IFN-γ upregulating
and then stimulating counter-regulatory cells? You mentioned that it was impressively upregulated with labor, for example. Is it responsive to cytokines or hormones; how is this regulated?

**Dr. Thornton:** Most of the regulation is done in the skin, the airways and the synovial fibroblast, and it tends to be upregulated de novo by Toll-like receptor ligands in particular, but the inflammatory cytokines like TNF-α and IL-1 would exacerbate that response. For example in the mast cell you want to get this TSLP response in the presence of these other inflammatory cytokines. In terms of the pregnancy-related work, as far as we know we are the first to do this and so what I have shown you is about as far as we have got. We would like to go back now and look at different cytokines inducing this and what might regulate this, and whether hormones do have an effect particularly in the placenta and breast milk.

**Dr. Sartor:** Then it's effect is downstream. You mentioned that it will stimulate both Th2 and regulatory cells. What determines which pathway? Is it the presence of other cytokines such as IL-4, TGF-β that might turn it one way or the other?

**Dr. Thornton:** I think it's going to be something like that. At the moment the prevailing literature suggests that it's really the site, so the induction of regulatory T cells by TSLP is restricted to the thymus. So it's the medullary epithelial cells making TSLP act on the local dendritic cells, and therefore you are talking about a much more immature thymocyte rather than naive T cell; whereas the induction of Th2 cells is being predominantly done using myeloid dendritic cells, using Langerhan's cells and using naive T cells, and generating the cytokine response. It is the maturity of the T-cell population in the immune response, and I suppose it is the type of dendritic cell, and it must be the local environment of the thymus versus where the myeloid dendritic cells are working.

**Dr. Brandtzaeg:** I have a couple of comments about IgA. As you say secretory IgA can affect the expression of antigens expressed by the commensal flora; but I think it’s important to stress that secretory IgA antibodies do not eliminate commensal bacteria. They just contain them in the lumen or in a biofilm and most of them are coated by IgA but stay there and proliferate. We need them. You asked how IgA plasma cells go from the gut to the mammary glands. First of all, plasma cells do not migrate, so you meant to say the precursors for IgA plasma cells. The receptor for MEC or CCL28 is CCR10, and that receptor is expressed also on the B-cell trigger in tonsils or Waldeyer's ring all together, as well as on GALT-derived B cells. The beauty of mammary glands is that they take in memory/effector B cells both from Waldeyer's ring and from GALT structures in the gut, so the lactating glands can produce antibodies both against respiratory antigens and gut antigens. Such shared homing of B cells is very important for the content of antibodies in breast milk.

**Dr. Prescott:** A comment in response to Dr. Makrides’ question about antenatal programming and the differences according to in utero exposures. In the work we are doing comparing babies born in Papua New Guinea versus those born in Perth, there really are quite striking differences in the neonatal TLR expression and functional responses, indicating that there are in utero differences. Now is this due to genetic factors? It probably has more to do with the very different environments. Obviously microbial burden is one major difference but there are really many differences. The point to be made here is that those differences are present before colonization has occurred. So clearly there are factors in utero that are having a major effect.

**Dr. Stanley:** The maternal changes over the decades are amazing, and one of the big things that has changed is of course maternal age. Could you comment on the immunological effects in the babies?

**Dr. Thornton:** I am much more familiar with the literature related to the impact of maternal age on diabetes rather than allergy. I know in diabetes it has quite an
important effect, but in allergy I can’t think of a really decent study where they have looked at that.

**Dr. Björkstén:** There are older studies indicating that the risk of atopic disease is increased in young mothers.

**Dr. Gibson:** How is it that we seem to have so many tools to tackle the gut-mediated reactions and there are so few in the airways? It seems as though it is a harder problem to tackle. Do you have any sort of explanation or do I have it wrong?

**Dr. Thornton:** It is the issue of antigen load in the gut. We’ve already talked about the volume of food that passes through the gut every day and the burden of the commensal flora. So it is much greater in the gut than it is in the airways, and there is also different developmental timing and things like that.

**Dr. Brandtzaeg:** Just a question about the role of TSLP. Is it as simple as you actually said? It is not just the classical Th2 cells. Studies on the so-called inflammatory Th2 cell population with TNF-α production show that the TSLP-simulated OX40 ligand engagement is extremely important to change an ordinary Th2 to an inflammatory Th2 response as we see in asthma. Do you agree with that?

**Dr. Thornton:** Yes, that is certainly the case in those studies but there are studies showing that a naïve T-cell population will also produce TNF-α. They upregulate IL-4 but they also upregulate TNF-α, so it is not a classic Th2 response. It is this inflammatory Th2 population that is also producing TNF-α.

**Dr. Brandtzaeg:** But the problem with the TSLP in the gut, for example, is that it has only been shown as a message so far; it is very difficult to detect it as a protein except when you have squamous epithelium as in the skin and upper respiratory tract.

**Dr. Thornton:** Yes, most of the work has been done in the respiratory tract and the skin; there are only one or two papers looking at the gut.

**Dr. Smith:** We had a discussion earlier about IL-10 being a surrogate marker that we use for T-regulatory cells, but it is a lot more complicated. Would you like to discuss this further?

**Dr. Thornton:** I was really just picking up on the point that Dr. Brandtzaeg made earlier this morning. There is a vast array of cell types that make IL-10, with monocytes macrophages probably being their predominant source. People have a tendency to measure IL-10, and thereby detect an immunoregulatory mechanism. But without showing which cell types are producing IL-10, no comment can be made on whether it is a regulatory T-cell population. Again we saw a nice example of a non-regulatory cell population upregulating IL-10. One of the key roles of IL-10 is to break immune responses of all types and so different cell types will upregulate IL-10 production to feed back in as an anti-inflammatory. So we need to know which cell types are making IL-10 before we go down the track of assuming it is a regulatory T cell.

**Dr. Sinn:** Breast milk is protective of necrotizing enterocolitis, yet it is not sterile and may have pathogenic organisms in it. How does an immunologically suppressed premature infant respond to these organisms in terms of the gut IgA response?

**Dr. Thornton:** Breast milk contains bacteria, particularly of the mother’s skin.

**Dr. Björkstén:** Dr. Hernell and I and some collaborators went through the literature and published an article in the *British Medical Journal* in 1980 [1] showing that unpasteurized fresh breast milk could contain almost any bacteria including salmonella without propagating disease to the babies. Every single case of propagation through breast milk was through pasteurized milk, so it seems that it could contain live bacteria as Dr. Brandtzaeg has pointed out, but they did not propagate disease. So this was the basis of my question to Dr. Brandtzaeg, whether exposure to antigens under
the protection of breast milk is actually a nice and normal way of inducing immune responses in the babies? The only reason we had to pasteurize milk was that HIV arose and people were put into a psychological situation where unpasteurized milk could not even be considered.

*Dr. Simmer:* I agree with Dr. Björkstén’s comment, and now in many countries we are beginning to use unpasteurized donor milk for premature babies for all the reasons he and Dr. Hernell demonstrated in 1980. With regard to Dr. Sinn’s comment, you are assuming that the mother gave the group B streptococcal sepsis (GBS) to the baby, but it could be that the baby gave the GBS to the mother, because we usually culture the mother’s breast milk after we diagnosed GBS in the baby.

*Dr. Sinn:* How does the baby give the mother GBS?

*Dr. Simmer:* It’s the same way within *Candida*, we know that the mother and the baby will always have *Candida* but it’s not necessarily the mother who gave it to the baby.

*Dr. B. Koletzko:* Can I just ask how strong the evidence is on which we base these conclusions? We have seen a lot of studies showing that under conditions of poor hygiene breastfed babies have less risk of infection, particularly GI infection. I was a bit disturbed by that simple conclusion after seeing the results from a multicentric study in Europe where, in a large population of more than 1,000 babies with a low risk of infection, there was on average 1 day of fever in the first year. In fact the breastfed population had a significantly higher risk of fever episodes and infection than the formula-fed population, which is counterintuitive if we look at studies from developing countries. This year a study from Sweden was published on 450 women who donated milk for a breast milk bank and were screened for about 2 years [2]. In healthy women without any signs of mastitis, extremely high levels of bacterial contamination (10^6–10^7 bacteria) and all imaginable pathogens were found. I am not saying that this is causing infection, I am just asking if we really have the evidence to base our conclusions that this is without any biological effect.

*Dr. Björkstén:* In our study in 1980 summarizing the literature [1] we arbitrarily defined the threshold of acceptable numbers of bacteria in milk to be <10^6/ml. This was totally arbitrary, and as I said the literature is full of reports of various pathogens in milk samples but we couldn’t find a single epidemic propagated by fresh milk.

*Dr. Hernell:* This was the situation at that time and I think the reason, as you mentioned, was HIV and CMV. Those are the reasons why we need to pasteurize milk.

**References**
