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IMMUNE REGULATION AND THE INFLUENCE OF MICROBES

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SUMMARY

Allergies, inflammatory bowel disease and celiac disease are common and increasing in Western societies. According to the hygiene hypothesis, they are caused by insufficient microbial exposure. This review will go through vital immunoregulatory mechanisms and discuss how these mechanisms may be influenced by microbes and microbial products.

INTRODUCTION

Allergy is the most common chronic disease amongst children in the Westernized parts of the world and affects as much as every third child. The increase in allergic diseases has become evident during the last twenty years and correlates with an increased living standard and excessive hygiene in the society. Accordingly, the hygiene hypothesis state that an exaggerated hygienic lifestyle give rise to an incorrect composition of the gut flora which leads to a defective maturation of the immune system which in turn give rise to immunoregulatory diseases such as allergy (*Wold*, 1998).

The reason to why some individuals suffer from diseases related to inadequate immune regulation, such as allergy, is not completely known. However, oral tolerance is a vital mechanism as it prevents untoward immune responses against food or airborne antigens, which get stuck in the upper airways and are subsequently swallowed, and thus prevents allergic reactions and inflammatory conditions in the gut. Oral tolerance is introduced by feeding of an antigen and result in development of regulatory T cells (Treg) and antigen-specific tolerance. Regulatory T cells suppress other immune cells and play a decisive role in maintaining the balance within the immune system. Regulatory T cells exist as natural Treg (nTreg) that are induced during thymic development and as induced Treg (iTreg), which arise from naïve cells and for instance during development of oral tolerance.

The gut flora is the major stimulus for the immune system and the absence of a gut flora result in an immature immune system, especially in the gut and also to a decreased ability of oral tolerance induction (*Moreau* and *Corthier*, 1988; *Moreau* and *Gaboriau-Routhiau*, 1996; *Sudo* et al., 1997). How microbial exposure can protect against allergy development remains elusive and no particular microbe(s) that better than others protect against development of allergy has yet been identified (*Adlerberth* et al., 2007). A complex microbiota, or rapid strain turnover, may be of importance, as a low com-

plexity of the comensal gut flora at one week of age predicted high risk of allergy development in a subsample of the ALLERGYFLORA cohort (*Wang* et al., 2008).

IMMUNO REGULATORY MECHANISMS

Mucosal tolerance

A large part of the immune system is localised to the gastrointestinal tract and it's also where the majority of all antigens encounter our body. We are readily exposed to foreign antigens from the food but also from airborne antigens that get stuck in airway mucus and transported to the throat and swallowed. It's crucial for the immune system to avoid aggressive immune responses such as allergic reactions towards these harmless substances.

Oral administration of soluble protein antigens result in the development of mucosal (oral) tolerance i.e. a state of immunological unresponsiveness. Even if extensive research has been performed during the last decades the mechanism or mechanisms behind mucosal tolerance remain unsolved. However, the work has revealed that almost all states of immunological immune responses can be suppressed by different regimens of oral Antigen administration. This includes *in vivo* responses such as DTH responses (*Miller* and *Hanson*, 1979; *Mowat* et al., 1982), formation of different Ig isotypes (*Vaz* et al., 1977; *Ngan* and *Kind*, 1978), changes in the clearance rate of Antigens from the circulation (*Hanson* et al., 1979) as well as *in vitro* assays such as lymphocyte proliferation (*Titus* and *Chiller*, 1981; *Hanson* and *Miller*, 1982), specific plaque forming cells and production of certain cytokines. Two main effectors mechanisms have been considered in oral tolerance and the outcome is generally thought to depend on the feeding strategies. The dogma state that feeding a low dose of Antigen results in the generation of regulatory T cells, whereas a high Antigen dose would result in clonal deletion and/or clonal anergy.

Tolerogenic antigen presenting cells

The antigen presenting cells residing both in the intestinal lamina propria and in the mesenteric lymph nodes have distinct tolerogenic properties and their antigen presenting ability favours development of regulatory T cells (*Chirdo* et al., 2005). The vitamin A metabolite retinoic acid (RA) has been discovered to be responsible for the up-regulation of the α4β7 integrin and CCR9, permitting newly formed T cells to accumulate preferentially in the GALT. The $CD103^+$ gut associated DCs express relatively high levels of retinal dehydrogenases, the enzymes required for the irreversible generation of RA from vitamin A (retinal). Inhibiting these enzymes reduced the expression of the α4β7 integrin on T cells and resulted in their depletion from the intestinal lamina propria (*Iwata* et al., 2004). These results explained why T cells stimulated by antigen on gut-associated DCs return to GALT.

New studies (*Benson* et al., 2007; *Coombes* et al., 2007; *Sun* et al., 2007) support this idea by showing that $CD103^+$ DCs in GALT are specially equipped for converting antigen-specific T cells into $Foxp3^+$ Treg cells in an RA- and TGF-β–dependent manner. The RA-enhanced conversion process also leads to the up-regulation of α4β7 integrin and CCR9 permitting the newly formed Treg cells to accumulate preferentially in GALT. Finally, RA

can reduce the negative impact of co-stimulation on the TGF-β–dependent conversion of T cells into Foxp3$^+$ Treg cells.

Tolerogenic processing

Studies performed by the group of Stephan Strobel, revealed that a tolerogenic moiety is present in the circulation of mice that were fed OVA and that induced tolerance upon transfer into naïve recipients (*Strobel* et al., 1983). The tolerogenic processing was also shown to be different from physiochemical alteration of the protein, since neither the native or chemically altered protein could induce tolerance in naïve recipients (*Bruce* and *Ferguson*, 1986). At that point the features of the tolerogenic moiety i.e. serum factor, were left unsolved. However E. Telemo and his colleagues could for the first time identify the serum factor and it was shown to consist of a supra-molecular structure that sedimented at 70.000 x g. The structures sized 40-50 nm, showed similarity to exosomes that are released from B cells and DCs and due to their tolerogenic function were named tolerosomes (*Karlsson* et al., 2001).

Tolerogenic processing involve sampling of luminal antigens by the small intestinal epithelial cells, co-localisation of antigenic peptides with MHC II molecules in vesicular structures of the intestinal epithelial cells, production of multivesicular structures by invagination of the vesicular membrane and finally the release of MHC class II expressing small vesicular structures on the basolatheral side of the intestinal epithelial cells (*Zimmer* et al., 2000). This tolerogene trafficking is of vital importance for the oral tolerance mechanisms and studies has shown that germfree animals (*Moreau* and *Corthier*, 1988; *Moreau* and *Gaboriau-Routhiau*, 1996; *Sudo* et al., 1997) and severe combined immunodeficiency (SCID) mice (*Ostman* et al., 2005), that both have an immature gut and lack of MHC class II expression in the intestinal epithelium, have a defective tolerogen trafficking and impaired oral tolerance.

Regulatory T cells

Naturally occurring CD4$^+$CD25$^+$ regulatory T cells

Nishizuka and Sakakura were the first to show that T cells are involved in the control of autoreactions, by demonstrating that mice, thymectomized at day tree of life developed spontaneous organ specific autoimmunity (*Nishizuka* and *Sakakura*, 1969). Then, 30 years later, Sakaguchi and colleagues demonstrated that the adoptive transfer of T cells, depleted of the IL-2 receptor a-chain (CD25) expressing cells, induced multiorgan autoimmunity in immunodeficient recipient animals (*Sakaguchi* et al., 1995), thus positioning regulatory T cells as part of the natural and essential tolerance system acting to control autoreactions.

The fundamental need for functional nTregs become evident in humans suffering from IPEX, which is a fatal disorder characterized by immune dysregulation, polyendocrinopathy, enteropathy and X-linked inheritance and caused by mutations of the human gene FOXP3, the ortholog of the gene mutated in scurfy mice forkhead box p3 (Foxp3). Recent findings have demonstrated an important role of the forkhead transcription factor FoxP3, for development and regulatory function of nTreg (*Fontenot* et al., 2003; *Hori* et al., 2003; *Setoguchi* et al., 2005).

Induced CD4$^+$ T regulatory cells

Feeding of protein antigens induce a state of immunological tolerance i.e. oral tolerance and Weiner and colleagues were the first to describe that oral feeding of myelin basic protein induced a distinct subset of Ag specific

T cells (Th3) that secreted IL-4 and tumour growth factor-beta (TGF-β) and prevented development of EAE (*Chen* et al., 1994). In addition, Roncarolo and colleges reported that repetitive stimulation of naïve T cells, from OVA TCR-transgenic mice, with OVA and IL-10 resulted in T cell clones with a unique cytokine profile that were distinct from Th0, Th1 or Th2 cells (*Groux* et al., 1997). These Tr1 cells produce IL-10 and some IL-5 and interferon-γ (IFN-γ), with or without TGF-β, but with little or no IL-2 and IL-4 production and show immunosuppressive properties *in vivo*. In contrast to nTregs, induced Tregs have been shown to be specific for antigens not present in the thymus such as food antigens, bacterial flora antigens (*Cong* et al., 2002; *McGuirk* et al., 2002; *Satoguina* et al., 2002) and some self-antigens such as insulin (*Chen* et al., 2003) or altered self-peptides (*Wildbaum* et al., 2002). These cells arise from naïve precursor cells and achieve regulatory function only after proper induction in the presence of endogenous local factors, such as cytokines.

Suppressive mechanisms of regulatory T cells

nTregs are the most well studied Tregs and despite intense research the mechanisms behind the suppressive properties of nTreg remain unclear. nTreg can suppress cells from both the innate and adaptive immune system (*Maloy* et al., 2003; *Fehervari* and *Sakaguchi*, 2004) require TCR and IL-2 stimulation to achieve suppressive function (*Nelson*, 2004). In contrast to nTreg, iTregs mediates their suppressive function primarily by cytokine dependent mechanisms. Tr1 mediated suppression depend on IL-10 and debatably on TGF-β and suppress the proliferation and cytokine production of naive $CD4^+CD25^-$ T, Th1 and Th2 cells. Th3 cells are dependent on TGF-β and mediate suppression of Th1 and Th2 cells and can also stimulate plasma cells to secrete IgA.

Microbial interactions with the immune system

Newborn children are brought from the sterile uterus to an environment that is crowded with microbes. Colonization of the newborn intestinal mucosa and epithelium of the skin starts immediately after birth. Several studies have provided information of the host-microbe interaction and have revealed a mutual beneficial relationship. The bacteria benefit from the stable habitat that is rich in energy sources from the food we eat. Bacteria in the normal gut flora have been shown to provide the mammalian host with essential nutrients, defence against pathogens and contribution to the intestinal architecture and development of the immune system.

Intestinal microorganisms

The mammalian microflora is largely composed of bacteria but other organisms such as, protozoa and fungi are also present. The colon harbours the majority of bacteria (10^{10}-10^{12} organisms per gram or ml of luminal content), which can comprise 400 different species. Anaerobes predominate in the lower intestine, particularly bacteroides, bifidobacteria, fusobacteria and peptostreptococci, while aerobes and facultative aerobes including enterobacteria and lactobacilli are more rare. The distal portion of the small intestine, the ileum, has less dense colonization (10^8 per gram) and limited colonization is observed in the stomach and the proximal small intestine (10^3-10^5 per gram), consisting mainly of acid-tolerant lactobacilli and streptococci.

The intestine of a newborn is rich in oxygen and therefore, facultative aerobes, such as *Escherichia coli* and

other enterobacteria, enterococci and staphylococci are the first colonizers of the human intestine. The oxygen is gradually consumed and the aerobe bacteria receive competition from the thus favoured anaerobic bacteria, including bifidobacteria, bacteriodes, clostridia and lactobacilli that start to colonize. The microbiota of infants is rapidly developed during the first week and remains unstable for the first year of life, becoming more stable later on. The colonization is largely influenced by the maternal microbiota, environmental factors and by infant feeding patterns. Alterations in the established microflora, such as turnover of individual strains occur constantly and are influenced by the presence of microbes in the environment, intestinal infections and exposure to antibiotic. Accordingly, the turnover of individual bacterial strains is much higher in developing countries, as compared to developed countries (*Nowrouzian* et al., 2003). Moreover, colonization with bacterial strains, such as *E. coli*, enterococci and lactobacilli is less frequent and occurs later in life of children in westernized countries as compared with children in developing countries or former socialist countries in East Europe (*Bennet* et al., 1991; *Sepp* et al., 1997; *Adlerberth* et al., 1998).

The hygiene hypothesis

Certain diseases are on the increase in Western countries. This has been clearly demonstrated for allergies (*Williams* et al., 1994; *von Mutius* et al., 1994), inflammatory bowel diseases (*Langholz* et al., 1991; *Munkholm* et al., 1992; *Farrokhyar* et al., 2001) and autoimmune disorders such as insulin-dependent type I diabetes and multiple sclerosis (*Rosati* et al., 1988; *Patterson* et al., 1996; *Parslow* et al., 1997; *Pundziute-Lycka* et al., 2002). The reasons for the increase in these diseases are not known, but they may all be related to the increased hygienic lifestyle in the modern Western society. Good housing standard, small families (*von Mutius* et al,, 1994) have all been linked to the high risk of developing allergies, while exposure to early day-care (*Kramer* et al., 1999; *McKinney* et al., 2000), pets (*Hesselmar* et al., 1999) or a live-stock farming environment (*Braun-Fahrlander* et al., 1999) all protect against allergies. In 1989, David Strachan formulated the hygiene hypothesis (*Strachan*, 1989) that was based on previous studies. He proposed that microbial stimulation was required in order to educate the immune system properly and that a decreased microbial exposure would lead to failure of such stimulation and development of allergic disease.

There is also an inverse correlation with previous infections with hepatitis A, Toxoplasma gondii or Helicobacter pylori and a decrease in the risk of developing allergy (*Matricardi*, 1997; *Matricardi* et al., 2000). One suggested explanation for the observed protection against allergy is that interaction with certain microorgansims causes immune deviation, thereby skewing immune responses away from the neonatal Th2, bias towards Th1 cell responses (*Busse* and *Lemanske*, 2001) However, this explanation cannot account the observation that infections with helminths, that are known to induce Th2 cytokines, are connected with protection from asthma (*van den Biggelaar* et al., 2004) or that humans with an immunodeficiency that affect Th1 cell cytokine pathways do not have an increased incidence of allergies (*Lammas* et al., 2000). The observed increase in allergic diseases, seen during the last decade has been accompanied by similar increase in autoimmune diseases that are considered to be Th1 biased (*Kero*

et al., 2001; *Stene* and *Nafstad*, 2001) again arguing against a general Th1 skewing by a westernised life style.

Several studies aim to link microbial infections with various autoimmune disorders, however, no epidemiological, statistically relevant associations have been observed so far. In opposition, several experimental studies support the hygiene hypothesis. For example, injection with coxsackievirus cannot only enhance (*Horwitz* et al.m 2000) but also prevent disease in none obese diabetic (NOD) mice (Tracy, Drescher et al. 2002). Furthermore, IFN-γ and TNF-α have protective effects in EAE or diabetes models (*Jacob* et al., 1990; *Christen* et al., 2001). Inflammation caused by viruses, bacteria and especially by parasitic worms can shift the T cell balance towards a more immunosuppressive state that would favour induction of regulatory T cells. Indeed, studies have provided evidence for regulatory T cells with specificity for the pathogens to occur in Leishmania major (*Mendez* et al., 2004), HSV (*Toka* et al., 2004) and Friend retrovirus (murine leukaemia virus) infections (*Dittmer* et al., 2004). Infection might also cause hyperactivation of autoaggressive lymphocytes, which may lead to activation induced cell death and diminish the systemic load of autoreactive T cells (*Christen* et al., 2001; *Qin* et al., 2004). Infection at a site away from the autoimmune reaction might keep autoaggressive cells from reaching the site of autoimmune destruction and prevent disease (*Christen* et al., 2001).

Infections result in an immune response that is partly specific (T cell clones specific for pathogenic peptides and high affinity neutralizing antibodies specific for surface epitopes) and partly non-specific (class switch recombination of natural antibody specificities, resulting from bystander help by specific T cell clones) and could cause an alteration in the T cell repertoire. In addition, cells from both the innate and adaptive immune system can recognize microbial structures, as shown by the finding of several TLRs expressed on T cells (*Caramalho* et al., 2003), thus indicating that the immune system has evolved to be highly susceptible for microbial stimulation. A cleaner environment results in a reduced and more stable microflora and fewer infections, which seem to disturb the immune homeostasis. One possible explanation for this could be that encounters with microbes helps to maintain the Treg population via an IL-2 dependent mechanism (*Maloy* and *Powrie*, 2005). Certain comensal bacteria has been proposed to aid in the hosts health and has resulted in recent years development of probiotics i.e. bacterial preparations that impart clinically verified beneficial effects on the health of the host when consumed orally(*Salminen* et al., 1998). Most probiotics are currently either lactic acid bacteria or bifidobacteria. The probiotic action includes competitive exclusion of pathogens, effects on the composition of the microbiota as well as modulation of the intestinal and systemic immune responses.

Immune cell activation by intestinal microbes

The intestinal microbes are separated from the underlying tissue by a single epithelial cell layer. Mucus produced by specialized cells in the epithelium i.e. goblet cells and epithelial cell derived defensins, supports the mucosal barrier and diminish the entry of microbes. Even so, certain bacterial species can penetrate this barrier to reach into the tissue and other bacteria make their entry via the M-cells in the PP. Recent studies have also shown that lamina propria DCs can make a way

through tight junctions and pick up luminal bacteria(*Rescigno* et al., 2001). The majority of comensal bacteria that pass over the mucosal barrier are rapidly killed by macrophages but they can survive for several days inside DCs (*Macpherson* and *Harris*, 2004). DCs that have been loaded with comensal bacteria in the Peyer´s patches or lamina propria migrate only as far as to the mesenteric lymph nodes (*Macpherson* and *Harris*, 2004) and priming by live comensals is presumably restricted to the mucosal immune system. This *in vivo* priming induces IgA production that in turn, aggravates penetration of additional comensals by means of exclusion. However, bacterial degradation products contaminate the systemic circulation and act as stimulators of the peripheral immune system.

Natural adjuvants

Several animal studies have suggested a role for microbial stimuli or so called natural adjuvants, like LPS or the B subunit of CT, for the effective induction of mucosal tolerance (*Michalek* et al., 1982; *Wannemuehler* et al., 1982; *Khoury* et al., 1990; *Rask* et al., 2000; *Bregenholt* et al., 2003). Epidermological studies have shown that there is an association with high levels of endotoxin in the sleeping mattress and protection against allergy development (*Braun-Fahrlander* et al., 2002). It was also found in a subsample of the ALLERGYFLORA cohort that infants colonized in the first week(s) of life with *Staphylococcus aureus* (*S. aureus*) had lower risk of developing food allergy than other children (*Lundell* et al., 2007). There was also a correlation between early colonization with enterotoxin producing *S. aureus* and the expansion of putative Tregs ($CD4^+CD25^+CTLA\text{-}4^+$) in the blood of 4 months old children in this cohort (*Karlsson* et al*.,* unpublished).

S. aureus is foremost a skin bacterium, but we it has become a quite common inhabitant of the neonatal gut in Swedish infants (*Lindberg* et al., 2000; *Lindberg* et al., 2004), probably as a result of decreased competition from "classical" faecal bacteria whose circulation has decreased strongly in today's highly hygienic hospitals and homes. Of *S. aureus* strains colonizing the neonatal gut, approximately 45% have the capacity to produce a toxin with superantigenic function. *S. aureus* enterotoxins have previously been incriminated in the pathogenesis of eczema, as eczematous skin lesions are often colonized by *S. aureus*, and since *S. aureus* colonization aggravates the lesion. However, data from animal experiments indicate that exposure to *S. aureus* superantigen may in fact favour development and functional activity of Treg. Thus, repeated i.v. injections of SE to mice result in development of T cells with regulatory function (*Sundstedt* et al., 1997; *Feunou* et al., 2003) and increased *in vitro* suppressive ability of isolated regulatory T cells (*Grundström* et al., 2003) as well as increased serum levels of the cytokine IL-10 (*Sundstedt* et al., 1997) which down regulates T cell activation and IFN-γ production . $CD4^+$ T cells from mice injected with SEA are about 3-fold more potent suppressors of SEA-induced T cell proliferation and IL-2 production compared to natural $CD4^+CD25^+$ regulatory T cells from untreated mice (*Grundström* et al., 2003).

CONCLUSION

Allergies are the most common chronic diseases amongst children in the Western society and affect as much as every third child. Many people believe the underlying reason for the increase in allergic diseases is due to an incorrect stimulation of the immune system during early infancy. Strategies to improve the development and maturity of the infant immune system by exposure to bacteria or bacterial products such as superantigens during early infancy is a possible way for allergy prevention and if this would succeed, the positive health effect would be enormous.

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