# The Impact of a Germ Free Perinatal Period on the Variation in Animal Models of Human Inflammatory Diseases – A Review

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***Summary***

Bacteria prime the immune system in early life, which in the first place is relevant for the development of oral tolerance. For some disease models, such as those for inflammatory bowel disease, germ free status for an entire life span, leads to the absence of prominent disease symptoms, while for other models, such as the Type 1 diabetes-prone NOD mouse, germ free status in early life would increase the incidence to a maximum. Basically both reactions are dependent on how the immune system has been primed in early life, i.e. with which bacteria and at which age. After early life priming, the gut regulatory immunity seems to be stable and less prone to be influenced by the gut flora. However, disease development later in life will still be dependent on contact with microorganisms to induce the inflammatory response. The aim of this review is to analyze whether it is reasonable to assume that variation in animal models, and thereby reduced groups size in experiments, may be achieved if animals are reared germ free with subsequent inoculation of a standardized gut flora at a standard age.

***Introduction***

The establishment of the early life intestinal microbiota is essential for the development of the immune system and variation in animal models is likely to be caused by variation in this early life priming (*Tlaskalova-Hogenova et al., 2004*; *Hansen et al., 2009*). However, microbiological quality assurance of laboratory animals is solely focused on eradication of specific infectious agents (*Nicklas et al., 2002*); commonly illustrated by the use of the term *specific pathogen free (SPF)* animals. Such animals are produced by a combination of rederivation, barrier protection and health monitoring *(Hansen, 2002)*.

In present years there is an increasing demand for animal models for human autoimmune, inflammatory and allergic diseases, as the incidences of these diseases are rapidly increasing in developed as well as developing countries. The normal colonization of the mammalian intestine with commensal microbes is hypothesized to drive the development of humoral and cellular immune systems during neonatal life and to maintain the physiologically normal steady state of inflammation in the gut throughout life *(Cebra, 1999)*. It is therefore also reasonable to believe that the intestinal microbiota have an impact on the development and progress of these diseases in animal models (*Tian et al., 2001*; *Hansen et al., 2009*). However, the systems used to produce today's labo-

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Published in the Scandinavian Journal of Laboratory Animal Science - an international journal of laboratory animal science

ratory animals are not aimed at standardizing the 'normal microbiota' of animals as these are produced in open cages in huge facilities housing frequently more than 10,000 animals. Therefore, there is a need to develop systems aimed at more standardisation in the contact made with environmental microorganisms. However, it may be complicated to maintain a standardized microbial environment in animals for an entire life-time, e.g. by isolator maintenance, while it may be less complicated to have animals born under germ free (GF) conditions and after a limited period transfer them to a more traditional barrier protected environment. The aim of this review is therefore to consider the impact that GF conditions in early life may have on animal models of autoimmune and inflammatory diseases.

*The impact of commensal bacteria on immune system development*

The neonate gut is rapidly colonized by cutaneous, fecal and vaginal maternal bacteria initiated by facultative anaerobes, such as *Eschericia Coli*, streptococci and staphylococci inhabiting the gut, rapidly followed by anaerobic bacteria, such as *Bacteroides spp*, *Clostridium spp* and *Bifidobacterium spp*. (*Ouwehand et al., 2002*) (Figure 1). Bacterial surfaces express 'pathogen associated molecular pattern' (PAMP's), e.g. lipopolysaccharides, flaggelin and peptidoglycan, which are recognized and presented to other immune cells by antigen presenting cells (APC's), and in the first weeks after birth 'oral tolerance' to food antigens and commensal microorganisms develops (*Tlaskalova-Hogenova et al., 2004*), as gut bacteria inhibit systemic immune responses to these antigens (*Moreau and Corthier, 1988*; *Sudo et al., 1997*).

Two mechanisms are thought to be instrumental: The active suppression mediated by regulatory T-cells ($CD4^+CD25^+$ $T_{reg}$ cells) (*Fujiwara et al., 2008*) and their cytokines TFG-ß and IL-10, and deletion and anergy of T-cells following high doses of antigen (*Tlaskalova-Hogenova et al., 2004*). For example in GF mice it was only possible to induce oral $T_H2$ mediated tolerance to ovalbumin if these were also neonatally inoculated with *Bifidobacterium infantis* (*Sudo et al., 1997*).

Intestinal dendritic cells (DC's) in the lamina propria closely associated with the intestinal epithelial cells sample the antigen in the gut by one of three proposed mechanisms *(Mizoguchi and Mizoguch, 2008)*: 1) M cells overlaying the Peyer's pathces in the gut internalize the antigen and present it to DC's or T cells, 2) DC's in the lamina propria extend their dendrites for direct capture of the antigen, and break it into smaller peptides to be presented by major histocompatibility class II (MHC II) molecules on the cell surface, or 3) neonatal Fc receptors (FcRn) transport IgG from the lamina propria into the gut lumen to form immune complexes with the antigen, which are transported back into the lamina propria by the FcRn to bind with DC's. Such differences in DC stimulation decide whether the naive T cell is differentiated into various T-helper ($T_H$) cells or alternatively into $T_{reg}$. Specific pathogen free (SPF)

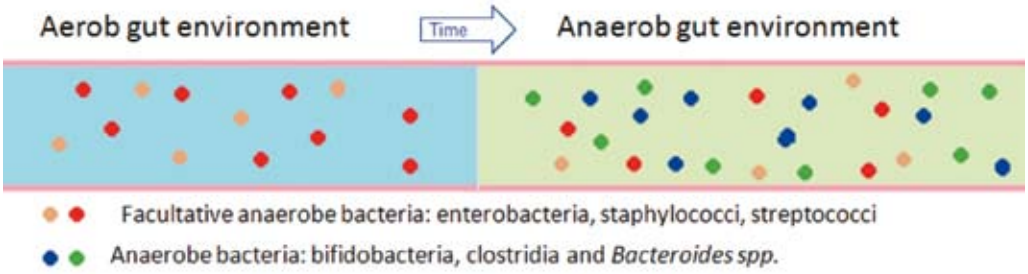


**Figure 1.** The colonization of the gut by bacteria leading to the development of an anaerobic environment suitable for the commensal flora.

and restricted flora (RF) status may shape the systemic DC population through a mechanism involving cytolytic $CD8^+$ T cells (*Fujiwara et al., 2008*), while DC's in both SPF and GF mice are of similar phenotype and have similar in vitro antigen presenting function (*Walton et al., 2006*).

Different toll-like receptors (TLR) present on the DC surface recognize different microbial antigens, and mediate the antigenic stimulation (*Ozinsky et al., 2000*). TLR's binding activates nuclear factor κB (NF-κB), a transcription factor required for proinflammatory signalling, and mitogen-activated protein kinases (MAPK), which again stimulate the expression of a number of pro- and anti-inflammatory genes *(Sartor, 2006)*. Immature DC's internalize the antigen, break it into smaller peptides and

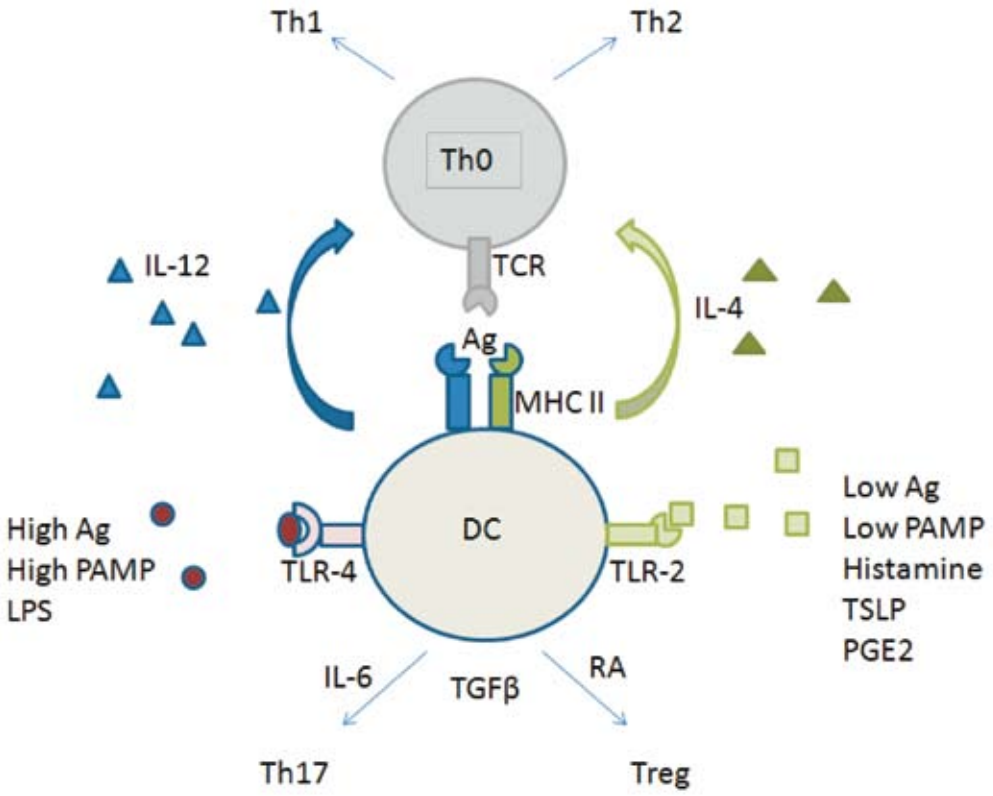


**Figure 2.** Different stimulation of the dendritic cells (DCs) promotes different T cell subtypes and T regulatory cells, in the presence of different co-stimulatory molecules like interleukines (IL) or surface molecules like ICAM-1 or CD40. The DCs recognise antigen epitopes on different receptors – LPS is recognized by toll-like receptor 4 (TLR 4) and prime a $T_H1$ cell development, whereas TLR 2 recognize Gram positive, fungal and mycobacterial components and prime a $T_H2$ response. In the presence of TGFß and IL-6, the development will skew towards effector $T_H17$ and IL-23 is required to maintain the $T_H17$. If on the other hand RA or no IL-6 are present $T_{reg}$ will preferentially develop. Abbreviations: DC dendritic cell, TCR T cell receptor, LPS lipopolysaccharide, PAMP pathogen associated molecular pattern, TSLP thymic stromal lymphopoietin (produced by intestinal epithelial cells), RA retinoic acid, Ag antigen, TGFß transformin growth factor ß *(Mizoguchi and Mizoguchi 2008, Eisenbarth et al. 2003, Ozinsky et al. 2000).*

**Table 1.** Factors influencing T cell development and favouring either $T_H1$ or $T_H2$ development. ICAM-1. Abbreviations: Inter cellular adhesion molecule, OX40L: a ligand present on the dendritic cell, interacts with OX40 on the T cell, CD 40 and B7: membrane proteins - interacts with CD40L and CD28 on the T cell respectively, STAT: signal transducer activator of transcription, IL: interleukine.

| | $T_H1$ | $T_H2$ | References |
|---|---|---|---|
| Co-stimulatory molecules on dendritic cell | ICAM-1, OX40L, CD40, B7 | OX40L, B7 | Eisenbarth et al. 2003 |
| Stimulatory IL | IL-12, IL-18, IL-27 | IL-4, IL-5, IL-13 | Eisenbarth et al. 2003 |
| Transcription factors activated | STAT-4, T-bet | STAT-6, Gata 3 | Shimada et al. 2006 |
| Secrete | IFNγ, TNF, IL-12 | IL-4, IL-5, IL-10 | Flohé et al. 2003<br>Ménard et al. 2008 |

present them on MHC-II. Naive T-cells that specifically recognize the antigen presented will then differentiate into the appropriate effector T-cell depending on the co-stimulatory molecules co-represented on the surface of the DC (Figure 2 and Table 1). In the steady state DC's will internalize commensals and preferentially induce $T_H2$ and $T_{reg}$ response after migration to the mesenteric lymph node priming a small number of $T_H1$ and $T_H17$ cells as well. Furthermore, the intestinal epithelial cells produce thymic stromal lymphopoietin (TSLP) that stimulate $T_H2$ differentiation *(Coombes and Powrie, 2008)*. Some pathogenic bacteria present in the gut make DC's induce an inflammatory immune response dominated by $T_H1$ and $T_H17$ *(Coombes and Powrie, 2008).* Cytokines produced by the different T cell populations have stimulatory as well as inhibitory functions (*Nakae et al., 2003*; *Eisenbarth et al., 2003*; *Shimada et al., 2006*; *Denning et al., 2007*; *Mizoguchi and Mizoguch, 2008*). Different subsets of DC's, such as myeloid-, plasmacytoid- and $CD8^+$DC's, also diversify the immune response to microbial antigens. Plasmacytoid DC's (pDC) have been shown to induce $CD4^+CD25^+$ $T_{reg}$ cells or $T_H1$/ $T_H2$ cells depending on the antigen present (*Colonna et al., 2004*), and have also been proposed to play an important role in inducing tolerance via the activation of $CD4^+CD25^+$ $T_{reg}$ cells (*Fujiwara et al., 2008*). Absence of $T_{reg}$ suppression may cause harmful induction of $T_H$ by first time PAMP exposure later in life (*Strachan, 1989*; *Romagnani, 2004*; *Hansen et al., 2009*). There are at least three different types of $T_{reg}$ cells: $CD4^+CD25^+$ $T_{reg}$ cells or Foxp3 $T_{reg}$ acting in a cell-cell mediated mechanism; Tr1 producing IL-10; and $T_H3$ producing TGFß (*Romagnani, 2004*; *Shevach, 2006*). There is some disagreement about whether the DC's (*Coombes and Powrie, 2008*) or the macrophages (*Denning et al., 2007*) are responsible for the induction of $T_{reg}$. $T_H2$ cell differentiation is the "default" pathway as this in the cell type most heavily represented in GF animals *(Mazmanian and Kasper, 2006)*.

Specific bacteria have been found to preferentially prime the different T-cells. Kopitar et al. found that the commensal oral bacteria *Bacterioides fragilis*, *Streptococcus mitis* and *Propionibacterium acnes* prime human DC to induce $T_H1$, $T_H2$ and $T_{reg}$ respectively, and therefore these bacteria would also be likely to influence the development of inflammatory diseases (*Kopitar et al., 2006*). Microbial glycolipids are taken up by DCs and presented to natural killer T-cells (NKT) cells. These are divided into invariant NKT (iNKT or type 1 NKT) cells, defined by their reaαction to α-galactosyl ceramide

(α-GalCer), and into non-invariant NKT (non-iNKT or type 2 NKT) cells reacting with other glycolipids including the mammalian ß forms. Glycosphingolipid molecules cannot be presented by the MHC complex, which only binds peptides. Instead, presentation is achieved through the MHC-like CD1 molecules (*Porcelli and Modlin, 1999*). The CD1 molecule contains a groove with two large hydrophobic pockets able to anchor the lipid tails of a glycosphingolipid (*Zeng et al., 1997*). The human DC's can express five kinds of CD1 molecules a, b, c, d and e, of which only c is expressed in mice. The most investigated CD1 ligand is α-GalCer, which is isolated from marine sponges. There are clear indications that the development of oral tolerance is time dependent, i.e. there is an "immunological window" that plays a role in the development of autoimmune and inflammatory diseases (*Sudo et al., 1997*; *Liu et al., 2005*). It is generally assumed that this window is open sometime between birth and weaning during which period regulatory immune priming is dominant and after which adaptive reactions take over *(Butler and Sinkora, 2007)*. However, the precise duration in different animal species and humans is not fully known. $T_{reg}$ development seems to start already in the neonatal state (*Samy et al., 2008*), while later in life intestinal based regulatory immunology seems to be less influenced by microbial stimulation (*Min et al., 2007*).

Three checkpoints to control autoimmunity have been proposed (*Darabi et al., 2004*): (1) Some naive self-reactive T cells avoid negative selection, and (2) after a TLR-activating co-stimulation from a microorganism develop into pathogenic $T_H1$ cells, which (3) due to a high serum level of cytokines from inflammation in a remote organ, e.g. the intestine, make harmful autoimmune sterile tissue injury in e.g. the beta cells.

*Immunological characteristics of germ free rodents*

GF mice have underdeveloped intestinal lymphatic constituents, such as decreased lymphocyte number in organized and diffuse lymphatic gut tissues (*Tlaskalova-Hogenova et al., 1983*; *Pleasants et al., 1986*; *Cebra, 1999*), decreased phagocyte activity and macrophage chemotaxis. $CD4^+$ in Peyer's patches, and $CD8^+$ or natural killer cells in intraepithelial leukocyte spaces of GF animals are quiescent. Furthermore, it has been observed that GF animals have a low concentration of IgA plasmablasts in lamina propria *(Kramer and Cebra, 1995)*, as well as less diversified immunoglobulins and that lymphocytes react with low intensity to mitogens and polyclonal stimuli (*Tlaskalova-Hogenova et al., 1983*; *Bos et al., 2001*). Cells spontaneously secreting natural antibodies against LPS and tissue antigens appear delayed compared to conventional animals (*Tlaskalova-Hogenova et al., 1983*; *Cebra, 1999*). GF sentinels infected with *Clostridium piliforme* in young age do not produce a significant level of antibodies compared to barrier bred rats (*Hansen et al., 1994*). Maternal antigen experience may be thought to be instrumental in activating fetal T cells present in the gut mucosa, as in the GF maternal environment foetal $CD3^+ß_7^+$ T cells of both GF and bacteriologically reconstituted mice expressed the naive T cell selectin CD26L (*Williams et al., 2006*). These naive T cells may be involved in the induction of oral tolerance and thus are an important factor in the development of immune bias in early life and subsequent development of immune-mediated mucosal allergic and inflammatory disease (*Williams et al., 2006*).

It still has to be confirmed whether changes in the neonatal environment not corresponding to maternal antigen experience may result in defective regulation of the neonatal mucosal T cell repertoire and immune biasing (*Williams et al., 2006*). GF status has been shown to favour the development of a $T_H2$ bias (*Mazmanian and Kasper, 2006)*. Bacterial polysaccharide A (PSA) contains both positive and negative charges, thereby being of a different composition to other bacterial polysaccharides which are normally either neutral or negatively charged. *Bacillus fragilis* PSA will stimulate the normal development of $T_H1$ vs. $T_H2$ cells and thereby correct the imbalance in $T_H1/T_H2$ that arises in GF animals. It will also correct the systemic T cell deficiency and direct lymphoid organogenesis (*Mazmanian et al.,*

*2005*), as mice inoculated with *B. fragilis* with the PSA gene proceeded to develop the immune system normally, whereas mice inoculated with *B. fragilis* without the PSA gene did not support the same differentiation of the immune system, and did not differ from the GF mice (*Mazmanian et al., 2005*).

*Germ free disease models*

The importance of maternal antigen experience and development of fetal T cells has also been proposed as a factor in the development of harmful inflammatory responses later in life (*Williams et al., 2006*). As GF status favours the development of a $T_H2$ bias it is most likely to assume a lower incidence of $T_H1$ dependent inflammatory diseases such as Type 1 diabetes (T1D) or $T_H1$ mediated colitis (a model for Crohn's disease) and a higher incidence of $T_H2$ dependent inflammatory diseases such as allergies in GF animal models. This is, however, more complicated (Table 2).

*Type 1 diabetes (T1D) models*

Functional mediators of ß-cell destruction in T1D are thought to be pro-inflammatory $T_H1$ cells (*Tian et al., 2001*). Incidence in female NOD mice is about 60-80% in barrier bred colonies but frequently less than half in conventional colonies (*Pozzilli et al., 1993*). The incidence is influenced by the environment and the occurrence of infection with Coxsackievirus B early in life will exert a protective effect (*Drescher et al., 2004*). Animals reared under GF conditions have higher cumulative incidence, up to 100% in females (*Tlaskalova-Hogenova et al., 2004*). Treatment with the anti-Gram positive antibiotic fusidic acid (*Buschard et al., 1992*), and a shift to a less Gram positive microflora induced by a gluten free diet have an incidence lowering effect (*Hansen et al., 2006*), which is accompanied by an increase in intestinal $T_{reg}$ (*Ejsing-Duun et al., 2008*). On the other hand, treatment with *Lactobacilli* later in life also reduces T1D incidence (*Tabuchi et al., 2003*). Brugman et al. showed that the intestinal

**Table 2.** Summary of the effect of germ free status on the disease incidence in animal models. Abbreviations: T1D Type 1 diabetes, IBD Inflammatory bowel disease, RA rheumatoid arthritis, MS multiple sclerosis, KO knock-put, TG trans-gene, EAE experimental allergic encephalomyelitis. ? = no studies have been performed, but the fact that when injecting the CNS antigen, co-injection of adjuvant is what starts the inflammation, bacterial status might be less important than in other models. Adapted from *Hansen et al. 2009*.

| **Disease** | **Organ specificity** | **T- cell** | **Animal model** | **Impact of germ free status on incidence** | **References** |
|---|---|---|---|---|---|
| T1D | ß-cells | $T_H1$ | NOD mouse, | Higher | Suzuki et al. 1987 |
| | | | BB rat | Same | Like et al. 1982 |
| IBD | Bowel | $T_H1$ or $T_H17$ | IL-2, IL-10 KO mice<br>HLA-B7 TG rats | Lower | Taurog et al. 1994<br>Song et al. 1999 |
| | | ($T_H2$) | TCRα | | Contractor et al. 1998 |
| RA | Joints | $T_H1$ | Collagen-/adjuvant Induced arthritis | Lower | Taurog et al. 1994<br>Lu et al 1999<br>Bigazzi 2005 |
| MS | CNS | $T_H1$ | EAE | ? | Darabi et al 2004 |

microbiota were an influencing factor in the development of diabetes (*Brugman et al., 2006*). They found that the faecal microbiota differed before the onset of diabetes and that those animals developing diabetes had a higher binding to a *Bacteroides*-specific probe than the rats that did not develop diabetes. Furthermore there was a protective effect in the groups treated with antibiotics, an effect improved by feeding a hydrolysed casein diet, reducing the observed incidence of diabetes in this study from approx. 90% to 0%. Both SJL and NOD mice have defects in NKT cell development and/or function (*Yoshimoto et al., 1995*; *Gombert et al., 1996*), and in humans with autoimmune diseases NKT cell numbers are reduced (*van der Vliet et al., 2001*). α-GalCer-specific activation of NKT cells protects against diabetes in NOD mice (*Sharif et al., 2001*; *Hong et al., 2001*), providing strong evidence that CD1d-reactive NKT cells suppress autoreactive T cells. Overexpression of NKT cells protects transgenic NOD mice against diabetes (*Lehuen et al., 1998*), whereas a shortage of NKT cells in CD1d knock-out mice leads to exacerbation of Type 1 diabetes (*Shi et al., 2001*). Finally, upregulation of CD1d expression within the beta cells restores the immunoregulatory function of NKT cells and prevents diabetes in NOD mice (*Falcone et al., 2004*).

*Inflammatory bowel disease (IBD) models*

Both $T_H1$/ $T_H2$ and more recently $T_H17$ have been demonstrated to be involved in the pathogenesis of IBD. The role of a given molecule in the development of IBD in the many different animal models of IBD varies greatly depending on several factors, including the mechanism of disease induction, the target cell type, phase of disease and the environment *(Mizoguchi and Mizoguch, 2008)*. The presence of commensals in the gut are an important factor in the pathogenesis of IBD (*Blumberg et al., 1999*). Tolerance mechanisms, such as active suppression by $T_{reg}$ (*Fujiwara et al., 2008*), prevent inappropriate responses to non-pathogenic, commensal bacteria in the gut lumen, and loss of these may lead to the development of IBD (*Brimnes et al., 2001*). IBD-associated $CD4^+$T cells from diseased *SCID* mice did not react to faecal extracts from GF mice, nor to extracts from food antigens, indicating that bacterial antigens are responsible for activating the $CD4^+$T cells (*Brimnes et al., 2001*). If reared under GF conditions knockout (KO) mice for IL-10, IL-2 and TCRα, HLA-B7 transgenic rats and SAMP1/Yit mice (a spontaneous model for IBD) will not develop mucosal inflammation (*Taurog et al., 1994*; *Contractor et al., 1998*; *Song et al., 1999*). On the other hand, GF IL-2-deficient mice in the absence of clinical symptoms histologically showed mild focal intestinal inflammation at 13 weeks of age, suggesting that the mechanisms behind IBD were present in GF mice, but exacerbated in SPF or conventional mice (*Schultz et al., 1999*). In HLA-B7 transgenic rats, which develop not only intestinal inflammation, but skin, joint and genital inflammatory lesions, a GF state prevented IBD and peripheral joint lesions, but not skin and genital inflammation (*Taurog et al., 1994*; *Contractor et al., 1998*). When the animals were treated with a bacterial flora, IBD occurred, but no joint inflammation developed in the relatively short observation period of this study (*Taurog et al., 1994*). The Gram positive flora including *Lactobacilli* seems to be protective against IBD (*Schultz et al., 2002*).

*Pristane (tetramethylpentadecane) induced murine lupus and rheumatoid arthritis (RA) model*

The pristane model is primarily a model of human systemic lupus erythematosus in SLE mice, while in BALB/c, CBA and DBA mice, inflammatory joint disease (pristane-induced arthritis, PIA), which makes this an interesting model for rheumatoid arthritis (RA) *(Lu and Holmdahl, 1999).* The development of PIA is dependent on contact with microorganisms, as animals reared in GF environment do not develop PIA *(Bigazzi, 2005)*.

*Experimental allergic encephalomyelitis (EAE) multiple sclerosis model*

Injection of neuroantigen in a TLR-activating adjuvant is enough to induce a vigorous neuroantigen

specific $T_H1$ response in EAE susceptive mice. However, in other murine models there is also the need for pertussis toxin (PTX) to be co-injected in order to develop EAE (*Darabi et al., 2004*). PTX promotes the $T_H1$ differentiation, and in addition, it is also thought to break down the blood-brain-barrier and thereby grant the autoantigen-specific $T_H1$ access to the brain (*Darabi et al., 2004*). In these models the impact of the commensal intestinal flora would seem redundant as the method for inducing the disease is entirely dependent on the injection of antigen, adjuvant and other co-stimulatory molecules like PTX. However, according to checkpoint 3 as described by (*Darabi et al., 2004*) and mentioned previously, the presence of inflammation in the intestine could influence the development of EAE by massive serum IL-12, which would also make PTX unnecessary. Treatment of mice with sulfated galactosyl ceramide (sulfatide) prevents EAE, which seem to involve an increase in the number of $T_{reg}$ (*Jahng et al., 2004*).

***Discussion***

It seems to be clear that bacteria prime the immune system in early life during which microbial contact with $T_{reg}$, DC and NKT cells is important. In the first place this is relevant for the development of oral tolerance. For some disease models, such as those for IBD, GF status leads to the absence of prominent disease symptoms, which in most cases would invalidate their use as animal models (*Taurog et al., 1994*; *Contractor et al., 1998*; *Schultz et al., 1999*; *Song et al., 1999*). For other models, such as the T1D-prone NOD mouse, GF status in early life would increase the incidence to a maximum (*Tlaskalova-Hogenova et al., 2004*).

However, both model reactions may be explained from the 'hygiene hypothesis' *(Strachan, 1989)*: contact with microorganisms in early life primes the immune system in a regulatory direction, while contact with microorganisms later in life primes the immune system in a more inflammatory direction. IBD-prone transgenic mice and NOD mice are both examples of genetically based models, in which the genetic drive towards disease is very strong. However, early life priming in IBD mice is not strong enough to prevent IBD from developing when direct exposure to bacteria is encountered in the intestine. Furthermore, it is also evident that in some mice subclinical symptoms develop even in the GF state (*Schultz et al., 1999*), while $T_{reg}$ probably are the only defence against T1D in NOD mice and responsible for the absence of the disease in those 20 % of the mice not developing T1D. Therefore the lack of early life priming removes the last defence against the genetic inevitability also in these mice.

After early life priming the gut regulatory immunity seems to be stable and less prone to be influenced by the gut microbiota (*Min et al., 2007*). However, disease development later in life will still be dependent on contact with microorganisms to induce the inflammatory response (*Darabi et al., 2004*). Hence it is reasonable to hypothesize that disease development in animal models will be enhanced by early life GF status reliant on the reduced regulatory immune response, followed by a strong microbial contact later in life. Variation in this contact is very likely to induce variation in the development of disease and thereby increase the group sizes needed to achieve statistical significance (*Hansen et al., 2009*).

Several authors have introduced pro- and pre-biotics in potential preventive therapies against allergies and autoimmune diseases, and the Gram positive flora, including *Lactobacilli*, indeed seems to be protective/preventive against IBD (*Schultz et al., 2002*), reduce T1D incidence (*Tabuchi et al., 2003*) and ameliorate symptoms of RA (*Nenonen et al., 1998*). The explanation may be that the presence of e.g. microaerophilics, suppress those bacteria, which express the PAMP's that induce the inflammatory response, and it underlines that variation in bacterial contact may lead to variation in disease expression. It is reasonable to assume that the impact that different groups of bacteria will have on the immune system is very much age-dependent, so microaerophilics may have another impact if given at another age. Therefore, the lowest variation in the model may be achieved if GF status is com-

bined with inoculation with a standardized flora at a standardized age. However, which flora to use for a high disease incidence is a complicated matter.

In conclusion, it is likely to assume that early life GF status combined with subsequent inoculation of a standardized gut flora would be an applicable tool to reduce variation in animal models, but the questions still need further elucidation to be applied in practice.

***References***

*Bigazzi PE*: Murine lupus induced by tetramethylpentadecane: an animal model of systemic human autoimmunity. Clin.Immunol. 2005, *114*, 97-9.

*Blumberg RS, LJ Saubermann & W Strober*: Animal models of mucosal inflammation and their relation to human inflammatory bowel disease. Curr.Opin.Immunol. 1999, *11*, 648-56.

*Bos NA, HQ Jiang & JJ Cebra*: T cell control of the gut IgA response against commensal bacteria. Gut 2001, *48*, 762-4.

*Brimnes J, J Reimann, M Nissen & M Claesson*: Enteric bacterial antigens activate CD4(+) T cells from scid mice with inflammatory bowel disease. Eur.J.Immunol. 2001, *31*, 23-31.

*Brugman S, FA Klatter, JTJ Visser, ACM Wildeboer-Veloo, HJM Harmsen, J Rozing & NA Bos*: Antibiotic treatment partially protects against type 1 diabetes in the Bio-Breeding diabetes-prone rat. Is the gut flora involved in the development of type 1 diabetes? Diabetologia 2006, *49*, 2105-8.

*Buschard K, C Pedersen, SV Hansen, I Hageman, K Aaen & K Bendtzen*: Anti-Diabetogenic Effect of Fusidic Acid in Diabetes Prone Bb Rats. Autoimmunity 1992, *14*, 101-4.

*Butler JE & M Sinkora*: The isolator piglet: a model for studying the development of adaptive immunity. Immunologic Research 2007, *39*, 33-51.

*Cebra JJ*: Influences of microbiota on intestinal immune system development. Am J Clin Nutr 1999, *69*, 1046S-51S.

*Colonna M, G Trinchieri & YJ Liu*: Plasmacytoid dendritic cells in immunity. Nat.Immunol. 2004, *5*, 1219-26.

*Contractor NV, H Bassiri, T Reya, AY Park, DC Baumgart, MA Wasik, SG Emerson & SR Carding*: Lymphoid hyperplasia, autoimmunity, and compromised intestinal intraepithelial lymphocyte development in colitis-free gnotobiotic IL-2-deficient mice. J.Immunol. 1998, *160*, 385-94.

*Coombes JL & F Powrie*: Dendritic cells in intestinal immune regulation. Nat.Rev.Immunol. 2008, *8*, 435-46.

*Darabi K, AY Karulin, BO Boehm, HH Hofstetter, Z Fabry, JC LaManna, JC Chavez, M Tary-Lehmann & PV Lehmann*: The third signal in T cell-mediated autoimmune disease? J.Immunol. 2004, *173*, 92-9.

*Denning TL, YC Wang, SR Patel, IR Williams & B Pulendran*: Lamina propria macrophages and dendritic cells differentially induce regulatory and interleukin 17-producing T cell responses. Nat.Immunol. 2007, *8*, 1086-94.

*Drescher KM, K Kono, S Bopegamage, SD Carson & S Tracy*: Coxsackievirus B3 infection and type 1 diabetes development in NOD mice: insulitis determines susceptibility of pancreatic islets to virus infection. Virology 2004, *329*, 381-94.

*Eisenbarth SC, DA Piggott & K Bottomly*: The master regulators of allergic inflammation: dendritic cells in Th2 sensitization. Curr.Opin.Immunol. 2003, *15*, 620-6.

*Ejsing-Duun M, J Josephsen, B Aasted, K Buschard & AK Hansen*: Dietary Gluten Reduces the Number of Intestinal Regulatory T Cells in Mice. Scandinavian Journal of Immunology 2008, *67*, 553-9.

*Falcone M, F Facciotti, N Ghidoli, P Monti, S Olivieri, L Zaccagnino, E Bonifacio, G Casorati, F Sanvito & N Sarvetnick*: Up-regulation of CD1d expression restores the immunoregulatory function of NKT cells and prevents autoimmune diabetes in nonobese diabetic mice. J.Immunol. 2004, *172*, 5908-16.

*Fujiwara D, B Wei, LL Presley, S Brewer, M McPherson, MA Lewinski, J Borneman & J Braun*: Systemic control of plasmacytoid dendritic cells by CD8+ T cells and commensal microbiota. J.Immunol. 2008, *180*, 5843-52.

*Gombert JM, A Herbelin, E TancredeBohin, M Dy, C Carnaud & JF Bach*: Early quantitative and functional deficiency of NK1(+)-like thymocytes in the NOD mouse. European Journal of Immunology 1996, *26*, 2989-98.

Hansen AK: Health Status and Health monitoring. In: J Hau, GL van Hoosier (eds.): Handbook of Laboratory Animal Science CRC Press, Boca Raton 2002.

*Hansen AK, HV Andersen & O Svendsen*: Studies on the diagnosis of Tyzzer's disease in laboratory rat colonies with antibodies against Bacillus piliformis (Clostridium piliforme). Lab Anim Sci 1994, *44*, 424-9.

*Hansen AK, M Ejsing-Duun, B AASTED, J Josephsen, GB Christensen, FK Vogensen, M Hufeldt & K Buschard*: The impact of the post natal gut microbiota on animal models. Proceedings of the Tenth FELASA Symposium and the XIV ICLAS General Assembly & Conference, Cernobbio, Italy, 11-14 June 2007 2009, 95-9.

*Hansen AK, F Ling, A Kaas, DP Funda, H Farlov & K Buschard*: Diabetes preventive gluten-free diet decreases the number of caecal bacteria in non-obese diabetic mice. Diabetes-Metabolism Research & Reviews 2006, *22*, 220-5.

*Hong S, MT Wilson, I Serizawa, L Wu, N Singh, OV Naidenko, T Miura, T Haba, DC Scherer, J Wei, M Kronenberg, Y Koezuka & KL Van*: The natural killer T-cell ligand alpha-galactosylceramide prevents autoimmune diabetes in non-obese diabetic mice. Nat.Med. 2001, *7*, 1052-6.

*Jahng A, I Maricic, C Aguilera, S Cardell, RC Halder & V Kumar*: Prevention of autoimmunity by targeting a distinct, noninvariant CD1d-reactive T cell population reactive to sulfatide. J.Exp.Med. 2004, *199*, 947-57.

*Kopitar AN, HN Ihan & A Ihan*: Commensal oral bacteria antigens prime human dendritic cells to induce Th1, Th2 or Treg differentiation. Oral Microbiol.Immunol. 2006, *21*, 1-5.

*Kramer DR & JJ Cebra*: Early appearance of "natural" mucosal IgA responses and germinal centers in suckling mice developing in the absence of maternal antibodies. J.Immunol. 1995, *154*, 2051-62.

*Lehuen A, O Lantz, L Beaudoin, V Laloux, C Carnaud, A Bendelac, JF Bach & RC Monteiro*: Overexpression of natural killer T cells protects Valpha14- Jalpha281 transgenic nonobese diabetic mice against diabetes. J.Exp.Med. 1998, *188*, 1831-9.

*Liu Z, N Li & J Neu*: Tight junctions, leaky intestines, and pediatric diseases. Acta Paediatr. 2005, *94*, 386-93.

*Lu S & R Holmdahl*: Different therapeutic and bystander effects by intranasal administration of homologous type II and type IX collagens on the collagen-induced arthritis and pristane-induced arthritis in rats. Clin.Immunol. 1999, *90*, 119-27.

*Mazmanian SK & DL Kasper*: The love-hate relationship between bacterial polysaccharides and the host immune system. Nature Reviews Immunology 2006, *6*, 849-58.

*Mazmanian SK, CH Liu, AO Tzianabos & DL Kasper*: An immunomodulatory molecule of symbiotic bacteria directs maturation of the host immune system. Cell 2005, *122*, 107-18.

*Min B, A Thornton, SM Caucheteux, SA Younes, K Oh, J Hu-Li & WE Paul*: Gut flora antigens are not important in the maintenance of regulatory T cell heterogeneity and homeostasis. European Journal of Immunology 2007, *37*, 1916-23.

*Mizoguchi A & E Mizoguch*: Inflammatory bowel disease, past, present and future: lessons from animal models. Journal of Gastroenterology 2008, *43*, 1-17.

*Moreau MC & G Corthier*: Effect of the gastrointestinal microflora on induction and maintenance of oral tolerance to ovalbumin in C3H/HeJ mice. Infect.Immun. 1988, *56*, 2766-8.

*Nakae S, S Saijo, R Horai, K Sudo, S Mori & Y*

*Iwakura*: IL-17 production from activated T cells is required for the spontaneous development of destructive arthritis in mice deficient in IL-1 receptor antagonist. Proc.Natl.Acad. Sci.U.S.A 2003, *100*, 5986-90.

*Nenonen MT, TA Helve, AL Rauma & OO Hanninen*: Uncooked, lactobacilli-rich, vegan food and rheumatoid arthritis. Br.J.Rheumatol. 1998, *37*, 274-81.

*Nicklas W, P Baneux, R Boot, T Decelle, A Deeny, M Fumanelli & B Illgen-Wilcke*: Recommendations for the health monitoring of rodent and rabbit colonies in breeding and experimental units. Laboratory Animals 2002, *36*, 20-42.

*Ouwehand A, E Isolauri & S Salminen*: The role of the intestinal microflora for the development of the immune system in early childhood. Eur.J.Nutr. 2002, *41 Suppl 1*, I32-I37.

*Ozinsky A, DM Underhill, JD Fontenot, AM Hajjar, KD Smith, CB Wilson, L Schroeder & A Aderem*: The repertoire for pattern recognition of pathogens by the innate immune system is defined by cooperation between toll-like receptors. Proc. Natl.Acad.Sci.U.S.A 2000, *97*, 13766-71.

*Pleasants JR, MH Johnson & BS Wostmann*: Adequacy of chemically defined, water-soluble diet for germfree BALB/c mice through successive generations and litters. J.Nutr. 1986, *116*, 1949-64.

*Porcelli SA & RL Modlin*: The CD1 system: antigen-presenting molecules for T cell recognition of lipids and glycolipids. Annu.Rev.Immunol. 1999, *17*, 297-329.

*Pozzilli P, A Signore, AJK Williams & PE Beales*: Nod Mouse Colonies Around the World - Recent Facts and Figures. Immunol Today 1993, *14*, 193-6.

*Romagnani S*: The increased prevalence of allergy and the hygiene hypothesis: missing immune deviation, reduced immune suppression, or both? Immunology 2004, *112*, 352-63.

*Samy ET, KM Wheeler, RJ Roper, C Teuscher & KS Tung*: Cutting edge: autoimmune disease in day 3 thymectomized mice is actively controlled by endogenous disease-specific regulatory T cells. J.Immunol. 2008, *180*, 4366-70.

*Sartor RB*: Mechanisms of disease: pathogenesis of Crohn's disease and ulcerative colitis. Nat.Clin. Pract.Gastroenterol.Hepatol. 2006, *3*, 390-407.

*Schultz M, SL Tonkonogy, RK Sellon, C Veltkamp, VL Godfrey, J Kwon, WB Grenther, E Balish, I Horak & RB Sartor*: IL-2-deficient mice raised under germfree conditions develop delayed mild focal intestinal inflammation. Am.J.Physiol 1999, *276*, G1461-G1472.

*Schultz M, C Veltkamp, LA Dieleman, WB Grenther, PB Wyrick, SL Tonkonogy & RB Sartor*: Lactobacillus plantarum 299V in the treatment and prevention of spontaneous colitis in interleukin-10-deficient mice. Inflam Bowel Dis 2002, *8*, 71-80.

*Sharif S, GA Arreaza, P Zucker, QS Mi, J Sondhi, OV Naidenko, M Kronenberg, Y Koezuka, TL Delovitch, JM Gombert, M Leite-De-Moraes, C Gouarin, R Zhu, A Hameg, T Nakayama, M Taniguchi, F Lepault, A Lehuen, JF Bach & A Herbelin*: Activation of natural killer T cells by alpha-galactosylceramide treatment prevents the onset and recurrence of autoimmune Type 1 diabetes. Nat.Med. 2001, *7*, 1057-62.

*Shevach EM*: From vanilla to 28 flavors: multiple varieties of T regulatory cells. Immunity. 2006, *25*, 195-201.

*Shi FD, M Flodstrom, B Balasa, SH Kim, GK Van, JL Strominger, SB Wilson & N Sarvetnick*: Germ line deletion of the CD1 locus exacerbates diabetes in the NOD mouse. Proc.Natl. Acad.Sci.U.S.A 2001, *98*, 6777-82.

*Shimada K, JK Park & H Daida*: T helper 1/T helper 2 balance and HMG-CoA reductase inhibitors in acute coronary syndrome: statins as immunomodulatory agents? Eur.Heart J. 2006, *27*, 2916-8.

*Song F, K Ito, TL Denning, D Kuninger, J Papaconstantinou, W Gourley, G Klimpel, E Balish, J Hokanson & PB Ernst*: Expression of the neutrophil chemokine KC in the colon of mice with enterocolitis and by intestinal epithelial cell

lines: effects of flora and proinflammatory cytokines. J.Immunol. 1999, *162*, 2275-80.

*Strachan DP*: Hay-Fever, Hygiene, and Household Size. Br Med J 1989, *299*, 1259-60.

*Sudo N, S Sawamura, K Tanaka, Y Aiba, C Kubo & Y Koga*: The requirement of intestinal bacterial flora for the development of an IgE production system fully susceptible to oral tolerance induction. J.Immunol. 1997, *159*, 1739-45.

*Tabuchi M, M Ozaki, A Tamura, N Yamada, T Ishida, M Hosoda & A Hosono*: Antidiabetic effect of Lactobacillus GG in streptozotocin-induced diabetic rats. Bioscience Biotechnology and Biochemistry 2003, *67*, 1421-+.

*Taurog JD, JA Richardson, JT Croft, WA Simmons, M Zhou, JL Fernandezsueiro, E Balish & RE Hammer*: The Germ-Free State Prevents Development of Gut and Joint Inflammatory Disease in Hla-B27 Transgenic Rats. Journal Of Experimental Medicine 1994, *180*, 2359-64.

*Tian J, D Zekzer, L Hanssen, YX Lu, A Olcott & DL Kaufman*: Lipopolysaccharide-activated B cells down-regulate Th1 immunity and prevent autoimmune diabetes in nonobese diabetic mice. Journal Of Immunology 2001, *167*, 1081-9.

*Tlaskalova-Hogenova H, R Stepankova, T Hudcovic, L Tuckova, B Cukrowska, R Lodinova-Zadnikova, H Kozakova, P Rossmann, J Bartova, D Sokol, DP Funda, D Borovska, Z Rehakova, J Sinkora, J Hofman, P Drastich & A Kokesova*: Commensal bacteria (normal microflora), mucosal immunity and chronic inflammatory and autoimmune diseases. Immunology Letters 2004, *93*, 97-108.

*Tlaskalova-Hogenova H, J Sterzl, R Stepankova, V Dlabac, V Veticka, P Rossmann, L Mandel & J Rejnek*: Development of immunological capacity under germfree and conventional conditions. Ann.N.Y.Acad.Sci. 1983, *409*, 96-113.

*van der Vliet H, BM von Blomberg, N Nishi, M Reijm, AE Voskuyl, AA van Bodegraven, CH Polman, T Rustemeyer, P Lips, AJ van den Eertwegh, G Giaccone, RJ Scheper & HM Pinedo*: Circulating V(alpha24+) Vbeta11+ NKT cell numbers are decreased in a wide variety of diseases that are characterized by autoreactive tissue damage. Clin.Immunol. 2001, *100*, 144-8.

*Walton KL, J He, BL Kelsall, RB Sartor & NC Fisher*: Dendritic cells in germ-free and specific pathogen-free mice have similar phenotypes and in vitro antigen presenting function. Immunol.Lett. 2006, *102*, 16-24.

*Williams AM, CS Probert, R Stepankova, H Tlaskalova-Hogenova, A Phillips & PW Bland*: Effects of microflora on the neonatal development of gut mucosal T cells and myeloid cells in the mouse. Immunology 2006, *119*, 470-8.

*Yoshimoto T, A Bendelac, J Hu-Li & WE Paul*: Defective IgE production by SJL mice is linked to the absence of CD4+, NK1.1+ T cells that promptly produce interleukin 4. Proc.Natl. Acad.Sci.U.S.A 1995, *92*, 11931-4.

*Zeng Z, AR Castano, BW Segelke, EA Stura, PA Peterson & IA Wilson*: Crystal structure of mouse CD1: An MHC-like fold with a large hydrophobic binding groove. Science 1997, *277*, 339-45.