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INTERVENTIONAL STUDIES ON IMMUNOLOGICAL BALANCE IN IBD AND ALLERGIC ASTHMA

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Stockholm 2011

Published by Karolinska Institutet.

ISBN 978-91-7457-191-2

Printed by
REPROPRINT AB
Stockholm 2010
www.reproprint.se
Gårdsvägen 4, 169 70 Solna

"… les étoiles sont éclairées afin que chacun puisse
un jour retrouver la sienne."
("…the stars are lit so everyone may one day find their own".)
A. de Saint-Exupéry

ABSTRACT

We have studied a role of immunological balance in connection to allergen challenge in asthma patients and in connection to apheresis treatment in inflammatory bowel disease (IBD) patients.

It has been suggested that T-helper type 2 cells become recruited into the bronchial mucosa and regulate allergic asthma reaction. Patients who mounted a late-phase reaction in connection to allergen challenge were designated dual responders opposite to single responders. Our finding that IL4+CD4+ cells decreased in the patient group and IFN-γ + CD4+ cells decreased in the single responders after allergen challenge suggests the active traffic of both Th1 and Th2 cells into bronchial mucosa. A diminished capacity to down-regulate the Th2 response by recruitment of sufficient number of IFN-γ positive CD4+ lymphocytes was suggested as explanation to the late phase symptoms in the dual responders.

A previously proposed mechanism of granulocyte- and monocyte adsorbing apheresis (GMA) is that in removing activated granulocytes and monocytes the production of pro-inflammatory cytokines, predominantly TNF-α will be reduced. Following GMA in patients with chronic, active IBD, IFN-γ-positive lymphocytes decreased in post-treatment biopsies in responders and appeared to predict the maintenance of long-term remission or response after 12 months.

The finding of down regulation of IFN-γ+CD4+ cells in post-treatment blood samples and IFN-γ+ cells in post-treatment biopsies indicates that the mechanisms of GMA are complex and may influence the Th-balance.

Given that FoxP3+ T regulatory cells and Toll Like Receptor (TLR) expression are key actors in mucosal immunoregulation we extended the previous study to identify the dynamics of these actors in the intestinal mucosa in relation to clinical improvement following GMA. The number of FoxP3+ cells and TLR-2 expression significantly decreased in post-treatment biopsies. The down regulation of FoxP3+ cells and TLR-2 expression mirrored clinical improvement in patients with active IBD after GMA. The results suggest a potential role of these cells in the pathogenesis of IBD and the induction of immunological tolerance in the mucosa.

The effect of apheresis system lines on soluble regulatory molecules (which are important for the immunological balance) has not been studied before, but was assessed on selected regulatory molecules during a safety study on a modified Cellsorba (device for leukacytapheresis (LCAP)). An important observation was that LL-37 increased at all apheresis sessions within the apheresis plastic lines. LL-37 is a major constituent of neutrophil granules. The peptide mediates a wide range of immunomodulatory actions (microbicidal and chemokine for granulocytes, monocytes, mastcells and T lymphocytes, it suppresses TLR-induced secretion of proinflammatory cytokines) and may therefore have a positive impact on the immunological tolerance and which may contribute to LCAP efficacy on UC.

In summary: We have suggested that differences in response to allergen can depend on different capacity to maintain and restore the immunological balance in bronchial mucosa, the Th-balance. Diminished capacity to recruit IFN-γ+ CD4+ lymphocytes is associated with development of an additional so called late-phase reaction. We have demonstrated that one plausible mechanism of GMA are immunomodulating involving down regulation of IFN-γ+ lymphocytes hereby influencing the Th-balance. The clinical improvement in IBD after GMA was associated with improved immunological tolerance mirrored by down regulation of FoxP3+ cells and TLR-2 expression. And finally, we have described generation of LL-37 in the plastic lines of apheresis system, which may also have a positive effect on the immunologic tolerance.

The results emphasize that the restoration of the immunological tolerance can be a key to future successful therapeutic strategy.

LIST OF PUBLICATIONS

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II. Muratov V, Lundahl J, Ulfgren AK, Elvin K, Fehrman I, Öst Å, Hittel N, Saniabadi A, Löfberg R. Downregulation of Interferon-γ Parallels Clinical Response to Selective Leukocyte Apheresis in Patients with Inflammatory Bowel Disease. A 12-Month Follow-up Study. Int J Colorectal Dis 2006; 21: 493-504.

III. Muratov V, Ulfgren A-K, Engström M, Elvin K, Winqvist O, Löfberg R, Lundahl J. Decreased numbers of FoxP3 positive and TLR-2 positive cells in intestinal mucosa are associated with improvement in patients with active inflammatory bowel disease following selective leukocyte apheresis. J Gastroenterology 2008; 43:277-282.

IV. Muratov V, Lundahl J, Mandic-Havelka A, Elvin K, Öst Å, Shizume Y, Furuya K, Löfberg R. Safety and tolerability of a modified filter-type device for leukocytapheresis using ACD-A as anticoagulant in patients with mild to moderately active ulcerative colitis. Results of a pilot study. J Clin Apheresis 2010; 25(5):287-293.

CONTENTS

LIST OF ABBREVIATIONS

ACD-A	acid citate dextrose solution A (citrate based anticoagulant)
APC	antigen presenting cell
CD	Crohn Disease
DAB	3,3´diaminobenzidinetetrahydrochloride
DC	dendritic cell
FcγR	receptor binding to Fc (fragment crystallizable) region of IgG
FEV1	Forced expiratory volume in 1 sec
GMA	granulocyte- and monocyte adsorbing apheresis (ADA®-column)
IBD	inflammatory bowel disease
LCAP	leukocyte adsorbing apheresis (CellsorbaTM)
MAb	monoclonal antibody
MNC	mononuclear cells
NM	nafamostat mesilate
PBMC	peripheral blood mononuclear cells
SRaw	Specific Airway resistance (reflects the overall dimensions of the airway)
T regs	T regulatory cells
TLR	Toll Like Receptors
TNF-R	TNF-receptor
UC	Ulcerative Colitis

1 INTRODUCTION

1.1 ALLERGIC ASTHMA AND IBD

Allergic asthma and inflammatory bowel diseases (IBD) are inflammatory conditions where a loss of the normal immunological tolerance is seen. In allergic asthma the tolerance to airborne normal environmental particles such as pollen (allergens) is lost and in IBD the tolerance to the normal gut flora is lost. The prevalence of these diseases has increased significantly since the 1970s, especially among the young and children. Allergic asthma is a chronic respiratory disease, arising from allergic inflammation, and characterized by recurring attacks of laboured breathing, reversible bronchoobstruction, airway remodelling and mucus hypersecretion. Inflammatory bowel disease (IBD) is a chronic inflammation of the intestine, which includes two major diagnoses, Crohn´s disease (CD) and ulcerative colitis (UC). IBD symptoms are like allergic asthma recurring in "flares". The dominating symptoms are diarrhoea and abdominal pain, while weight loss and rectal bleeding are common.

The inflammatory processes behind theses diseases are complex and involve a number of known and unknown mechanisms. By elucidating the underlying mechanisms involved, new therapeutic principles can be explored resulting in more effective and possibly individualized regimens.

1.2 IMMUNOLOGICAL TOLERANCE, T HELPER CELLS, TH1/TH2 CONCEPT

Under normal conditions, the regulatory processes maintain immunological tolerance. A loss of immunological tolerance *(or skewing of immunological balance)* seen in chronic inflammatory diseases can occur on different levels such as, impaired tolerance to normal gut flora [Okhusa et al, 2004; Duchman et al, 1996], breakdown of balance between effector cells and regulatory T cells [Maul et al, 2005], aberrant immune responses to the luminal antigens by activated helper T cells [Ibbotson et al, 1992] and defects in epithelial and leukocyte antimicrobial defence barrier [Wehkamp et al, 2007].

Inflammatory conditions are generally characterised by recruitment of inflammatory cells such as granulocytes, monocytes, lymphocytes and maintenance of the inflammatory processes. Chemokines and their receptors are key factors in attraction of leukocytes to sites of inflammation [Zhong et al, 2008]. Activated leukocytes express

specialized molecules for adhesion to vascular endothelium and transmigration into the inflammatory site. Mucosal macrophages produce cytokines (e.g. TNF-α), which stimulate endothelial cells to express adhesion molecules. Interaction of adhesion molecules on activated leukocytes and on endothelial cells mediates leukocyte recruitment from the blood into the inflamatory site. The infiltrating leukocytes produce pro-inflammatory cytokines and chemokines that facilitate the persistence of the disease, see image below.

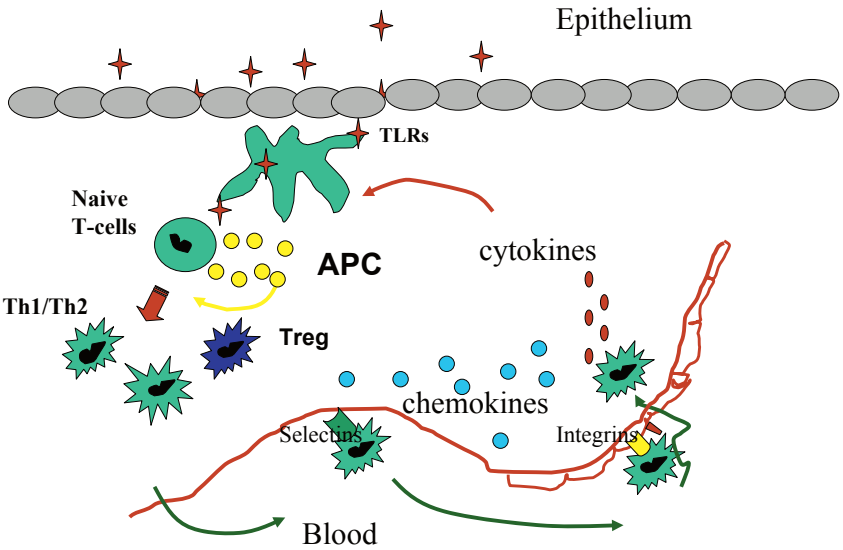


Image 1. Break-down of immunological tolerance in the mucosa and maintenance of the chronic inflammatory process. This image illustrates chemokine release, leukocyte recruitment, skewed Th1/Th2 and T regulatory cells balance. Multiple adhesion molecules include different selectins (e.g. L-selectin) and integrins (e.g. VLA-4), which support rolling and adhesion respectively. APC- antigen presenting cell. TLR - Toll Like Receptor. Antigens (macromolecules or microorganisms) - marked as red stars.

T helper cells are necessary for activation of both B cells and T killer cells and produce different cytokines for these purposes. Mosman and Coffman have shown that there are

different groups of T helper cells involved in the activation of T killer cells - T helper cells type 1(Th1) and B cells - T helper cells type 2(Th2). These groups suppress each other. For example, Th2 cells are suppressed by IFN-γ produced by Th1 cells, while Th1 cells are down regulated by IL-10 produced by Th2 cells. Immune deviation towards Th1 or Th2 is characterized mainly by production of the cytokines IFN-γ and IL4 respectively- "the signature" cytokines [Glimcher and Murphy, 2000].
Mosmann and Coffman postulated a concept of Th1/Th2 in 1986, where two different subsets of T helper cells are distinguished by different subsets of cytokines and possessing different effector functions [Mosmann et al, 1986; Mosmann and Coffman, 1989].
Th1/Th2 imbalance is considered pivotal in the pathophysiology in both allergic asthma and inflammatory bowel diseases (IBD (Image, see below).

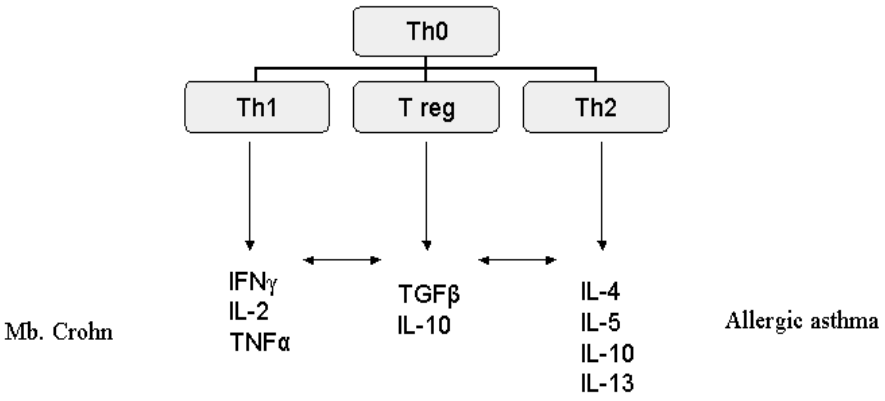


Image 2. Th1/Th2 concept, balance and dysregulation.

Regarding allergic asthma there is a generally accepted concept of deviation towards a Th2-response [Truyen et al, 2006].
A recruitment of eosinophils and lymphocytes into the bronchial mucosa is

characteristic for allergic asthma. The Th2 hypothesis indicates a relative IFN-γ deficiency, but at the same time the numbers of IFN-γ positive cells and IFN-γ levels in serum of asthmatics are increased and correlate to asthma activity [Sahid El-Radgi et al, 2000].

Imbalance towards a Th1-response pertains to Crohn Disease (CD), whereas the circumstances are more complex for ulcerative colitis (UC). A general overproduction of inflammatory cytokines is seen in both CD and UC [Fiocchi,1998, MacDonald et al, 2000]. This disease group is also characterized by the recruitment of white blood cells into intestinal mucosa.
The Th1 / Th2 concept became up-dated after the recent discovery of Th17 and T regs, where T regs can suppress / regulate the effector cells.
There are three types of effector T helper cells, Th1 which are protective against intracellular bacteria, Th2 which play a role in protection against nematodes, but are also responsible for allergic reactions and the more recently discovered Th17 cells which are probably protective against extracellular bacteria, but are also involved in autoimmune disorders [Harrington et al, 2005].
Kugathasan et al have showed that production of IL-4 increases in late phases of CD, which indicates that the regulation at the T helper cell level may be changing depending on the disease phases. At onset of CD mucosal T cells appear to mount a typical Th1 response that resembles an acute infectious process, but this response is lost with progression of CD [Kugathasan et al, 2007].
Nowadays it is considered that both Th1 and Th17 are important mediators of inflammation in Crohn Disease (CD) [Brand et al, 2009].
Indeed, the Th-concept became more complicated with the discovery of Th17 and T regulatory cells, but the concept gave the notion that the subsets of T cells could negatively regulate the functions of each other [Steinman, 2007].

1.3 T REGULATORY CELLS

Regulatory T cells (T regs) are a subset of T cells with a capacity to balance immune responses and maintain tolerance. [Sakaguchi et al, 1995; Suri-Payer et al, 2006].

T regs are divided into naturally occurring thymically derived and peripherally induced. The induced regulatory T cells originate from CD25 negative T lymphocytes. The natural T regs are defined as CD4+CD25+^{high}. Both types express X-linked forkhead /winged-helix transcription factor box P3 (FoxP3) [Rondacor et al, 2005], which is important for their development and function. FoxP3 is now recognized as the most reliable marker of regulatory T-cell lineage irrespective of CD25 expression [Fontenot et al, 2005], and even transient expression of FoxP3 is associated with regulatory properties [Pillai et al, 2007]. Mutations in FoxP3 gene are associated with a severe immunodeficiency syndrome IPEX (immune dysregulation, polyendocrinopathy, enteropathy, X-linked) [McMurchy et al, 2010].

T regs use various mechanisms to affect effector cells. Some mechanisms are unknown. To mention a few known mechanisms T regs can by secretion of IL-10 and TGFβ inhibit T cell function. By a granzyme mediated way T regs can induce apoptosis of effector cells [Grossman et al, 2004 and Grossman et al, 2004], they can even suppress the function of antigen presenting cells (when their CTLA-4 interacts with co-stimulatory molecules on APCs) and hereby prevent activation of naïve T cells [Misra et al, 2004; Onishi et al, 2008], T regs may also inhibit induction of inflammatory cytokines through TNF-R shedding [van Mierlo et al, 2008].

1.4 TOLL LIKE RECEPTORS (TLRS), IMPACT ON TH1/TH2

Toll Like Receptors (TLRs) were first described in the end of the 90-ies. These molecules play a front line role in recognition of microbial structures and for tolerance maintenance.

TLR are expressed mainly on antigen presenting cells (such as dendritic cells and macrophages) and epithelial cells. Bacterial lipoproteins and Gram-positive bacterial peptidoglycan are activators of TLR2, while TLR4 is the main receptor for Gram-negative bacterial lipopolysaccharide (LPS)[Furrie et al, 2005; Erridge et al, 2004; Opitz et al, 2001]. Stimulation of different TLRs leads to different cytokine and chemokine gene transcription [Re et al, 2001] and different cytokine secretion with impact on Th1/Th2 balance [Schaub et al, 2004]. For example, Sieling et al demonstrated that IFN-γ (but not IL-4) production was mediated via TLR2 and not TLR4 [Sieling et al, 2003]. Inhalation of LPS, a TLR4 agonist, leads to increased TNF-α, IL-1β, IL-6 and IL-8 expression [Mookherjee et al, 2006].

The composition of microbial flora affects mucosal immunological balance [Ivanov et al, 2008].

A breakdown in the appropriate regulation of the TLR pathway can cause chronic inflammatory disorders [Mookherjee et al, 2006; Pierik et al, 2006].

1.5 IMMUNO REGULATORY MOLECULES AND IMMUNOLOGICAL BALANCE.

Some mediators are stored in cell granules and can be released within seconds, i.e. histamine in mast cells. Another group of molecules will be generated during minutes, i.e. free oxygen radicals and NO. Prostaglandins, leukotriens, tromboxanes on the other hand are mediators generated from lipids of the cell membrane. Cytokines need a time to be synthesized and secreted by the cells. Membrane associated structures may become soluble forms after shedding as for example CD30 ligand (see below).

It appears that the balance between proinflammatory cytokines and their endogenous inhibitors, such as IL-1 and IL-1-receptor-antagonist (IL-1Ra) is disturbed in inflammatory bowel disease [Casini-Raggi et al, 1995; Noguchi et al, 1998]. IL-1Ra is a member of the interleukin 1 cytokine family. IL-1Ra blocks the biologic activity of IL-1 and since two years is availabe as a medication (in recombinant form, Anakinra®) in rheumatoid arthritis.

CD30 ligand (CD30L, CD153) and its receptor CD30 are members of the TNF-α / TNF-receptor superfamily. CD30 is expressed on subpopulations of T and B cells, while CD30L is expressed on activated T cells and dendritic cells. CD30 has a function to regulate lymphocyte survival and differentiation. Most members of the TNF-superfamily expressed on membranes can be cleaved by specific proteases and exist in a soluble form, exerting its activity on the corresponding receptor. Both CD30 and CD30L(CD153) exist in soluble forms and may prevent interaction between CD30+ cell and CD30L+ cell [Kennedy et al, 2006]. It has been demonstrated in murine models that blocking of CD30/CD30L signaling reduces inflammation in allergic asthma [Polte et al, 2006] and in IBD [Sun et al, 2008]. Sun and co-workers have suggested recently that CD30L is involved in pathogenesis of intestinal inflammation and may prevent development of UC [Sun et al, 2008].

The sole cathelicidin peptide in humans, hCAP-18/LL-37 is found at high concentrations in its unprocessed form in azurophilic granules of neutrophils and is

processed to the antimicrobial peptide LL-37 upon degranulation. hCAP-18/LL-37 is also produced by epithelial cells and keratocytes. LL-37 gets into active configuration first in contact with its target membrane [Johansson et al, 1998; Oren et al, 1999]. LL-37 mediates a wide range of immunomodulatory actions [Davidson et al, 2004; Akashi-Takimura and Miyake, 2008]. It is microbicidal [Ouhara et al, 2005] and a chemokine for granulocytes, monocytes, mast cells and T lymphocytes [Nijnik and Hancock, 2009] and suppresses TLR-induced (TLR-2, 4 and 9) secretion of proinflammatory cytokines [Mookherje et al, 2006]. Low expression of LL-37 is believed to be associated with infections and low concentration of LL-37 in neutrophil granules was recently described in patients with acute myeloid leukemia [An et al, 2005]. Neutrophilic granulocytes (60 % of all circulating white blood cells) have four types of granules and can release one or all type granules depending on the received signal.

The idea to use some compounds of the blood cells as therapeutic agents was described in the 90`s after the finding that transfusions of non-filtered blood had some immunomodulatory effects. These effects were connected to apoptosis-inducing molecules, such as FasL and soluble HLA [Ghio et al, 1999]. This finding is interesting in the context of IBD, since this condition can be associated with prolonged survival of activated intestinal T -cells [Souza et al, 2005]. FasL and soluble HLA were described as released by the white blood cells during blood storage [Puppo et al, 2001; Ottonello et al, 2007].

A way to stimulate generation of regulatory agents is the exposure of white blood cells to foreign materials. One example of earlier experiments is how MNC *in vitro* were stimulated for production of IL-1Ra in presence of human IgG adhered to plastic [Andersen et al 1995]. Later, it was reported that IL-1Ra [Saniabadi et al 2005, Tozawa et al, 2008] and soluble TNF- RI/RII were generated during GMA in the apheresis device [Hanai H et al, 2004] by the cells adhered on the cellulose acetate beads. The apheresis system is closed, and once generated these molecules return back to the blood circulation of the patient. It has been suggested that IL-1Ra and soluble TNF-RI/II have anti-inflammatory effects when returned into the patient circulation.

2 INTERVENTIONS

2.1 BACKGROUND FOR INTERVENTION BY ALLERGEN CHALLENGE IN ASTHMA

Allergic asthma is the most common type of asthma, affecting over 50% of asthma patients. Patients with allergic asthma develop airway inflammation and broncho obstruction depending on specific airborne allergens. Birch, mugworth and timothy are the most common allergens in Sweden [Rönmark et al, 2003].

When exposed to allergens to which they are sensitive one half of allergic asthmatics develop only one allergic reaction (early reaction) with broncho constriction and another half develops an additional late-phase reaction [Matsumoto et al, 2002]. The early reaction occurs when mast cells and basophils release histamin from the granules and produce leukotrienes and prostoglandines after IgE molecules on the surface of these cells have bound to the corresponding allergens. Both histamine and leukotrienes cause the bronchoconstriction [Roquet et al, 1997]. Moreover, the degranulation initiates inflammatory cells recruitment. The early reaction begins within 30 minutes and usually ends by two hours. The late-phase reaction can begin three to seven hours after exposure to the allergen [Matsumoto et al, 2002].

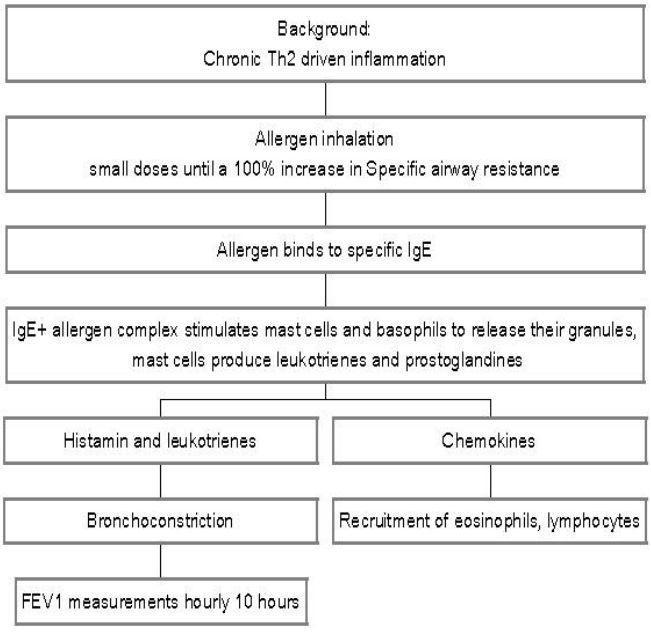


Image 3. Summary image for allergen challenge.

High numbers of IL-4+ and IL-5 + cells in bronchial submucosa in allergic asthma patients reflect conditions with a Th-balance deregulated towards Th2 compared to healthy controls [Brightling et al, 2002]. Relative IFN-γ deficiency was suggested in the Th2 hypothesis of allergic asthma, but the role of IFN-γ is believed to be more complex [Smart et al, 2002; Wong et al, 2001; Cho et al, 2002]. Earlier described differences in circulating T cell populations were mostly based on comparison between healthy individuals and asthmatic patients. An active trafficking between circulating blood and the lungs was suggested as an explanation for different results in stable asthma and its exacerbation. [Hamid et al, 2000].

To study the role of balance between IFN-γ+ and IL4+ T lymphocytes we have suggested a model when the subsets of the lymphocytes are measured before and after allergen challenge.
Allergen challenge is a well-recognized instrument in allergic asthma research [Scott, 1989]. An allergen challenge is performed until a certain degree of an asthmatic reaction is reached, measured by increasing airway resistance and diminished forced expiratory volume.
We hypothesized that an allergen challenge induces an active recruitment of lymphocytes into the bronchial mucosa, which is mirrored by a decrease in corresponding circulating population.

2.2 BACKGROUND FOR INTERVENTION BY APHERESIS TREATMENT IN IBD

2.2.1 Disease activity scores in clinical studies on IBD

Separate scoring systems were developed for UC and CD to use during clinical trials - clinical disease activity indices (see Methods, paper II, IV). The clinical outcomes are classified as “worsening”, “no response” or “response” depending on how many points the score is changed. If the score is lower than the value for remission, the outcome will be called “remission”.

2.2.2 Leukocyte adsorption apheresis techniques GMA and LCAP, adsorbed cells

The word “apheresis” originates from Greek “to remove”. There are many different apheresis treatment techniques used to remove or replace different blood components. To remove any blood components the blood of a patient or donor is collected continuously under procedure time, passed through an apparatus that separates the desired constituent and returnes the remainder to the circulation, see image below.

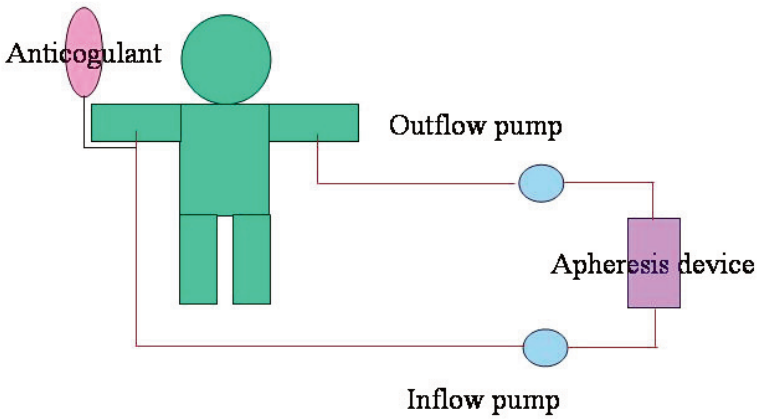


Image 4. This image illustrates how the apheresis system is connected to the patient’s blood circulation. The GMA apheresis device (Adacolumn) consists of a plastic column with cellulose acetate beads. The LCAP apheresis device (CellSorba) consists of a plastic column with polyester filter.

More than 10 years ago, two leukocyte adsorption apheresis devices became available for treatment of inflammatory conditions in Japan. Granulocyte and monocyte apheresis (GMA) device, ADA®-column, contents cellulose acetate beads, while leukocytapheresis (LCAP) device Cellsorba™ contents nonwoven polyester fabric.

Previous case studies showed beneficial effects of removal granolocytes and monocytes using non-pharmacological treatment by GMA [Kashiwagi N et al, 1998,

Shimoyama T et al, 2001; Hanai et al, 2004] and LCAP [Sawada et al, 1995; Sawada et al, 2003; Sawada et al, 2005; Emmrich et al, 2007; Matsumoto et al, 2008].

Both GMA and LCAP devices are used to remove white blood cells, which are considered as a major source of inflammatory cytokines and appropriate target for therapies [Hiraishi K et al, 2003; Ohara M et al, 1997].

GMA adsorbs on its cellulose acetate beads cells expressing both Fcγ receptor and complement receptors [Saniabadi et al, 2005]. These features belongs to granulocytes and monocytes [Hanai, et al, 2004], see image below. Neutrophils and monocytes constitute a majority of circulating leukocytes, 60 respectively 10 % (eosinophils –1-3%, basophils -<1%). Totally one column adsorbs 65% of circulating granulocytes and 55% of circulating monocytes. Thus, a mechanism of GMA suggested earlier was that by removal of activated granulocytes and monocytes, the production of pro-inflammatory cytokines, predominantly TNF-α was reduced[Hiraishi et al, 2003; Mitsuyama et al, 2005].

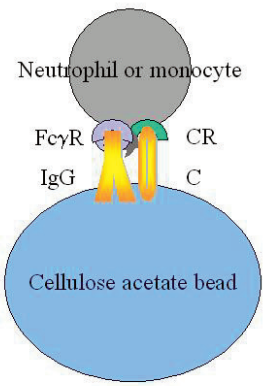


Image 5. This image reflects a hypothesis of adsorption of granulocytes and monocytes on cellulose acetate beads. CR-complement receptor (e.g. CR3), C-complement fragment (e.g. C3b). FcγR -Fcγ receptor.

LCAP adsorbs on its polyester filter higher cell numbers compared to GMA, nearly 100% of circulating granulocytes and monocytes and 64% of circulating lymphocytes, even platelets are influenced significantly [Abreu et al, 2007]. It may lead to some differences regarding immunological effects between GMA and LCAP.

There are no publications at the moment about which adhesion molecules mediate the adsorption in the LCAP filter. Common mechanisms of leukocyte removal by filters were suggested earlier to be associated with complement and adhesion molecules [Fehr, 1977; Dzik, 1993; Kohgo et al, 2003].
We suggest that similarities in adsorption mechanisms of GMA and LCAP exist. This suggestion is based on studies, which show that both treatments reduce numbers of CD14+CD16+DR+ monocytes [Hanai et al, 2008; Kanai et al, 2007]. These CD14+CD16+DR+ cells are FcγRIII (=CD16) positive activated monocytes and a major source of TNF-α [Belge et al, 2002]. The cell removal changes neutrophil populations to immature profile both after GMA (CD10 negative [Kashiwagi et al, 2002]; down regulated L-selectin [Saniabadi et al, 2005]) and LCAP (down regulated VLA-4 [Okawa-Takatsuji et al, 2007]).

GMA and LCAP appear to be safe, and the depletion of cells does not lead to fall in total white blood cells counts below the normal range [Saniabadi et al, 2003; Pineda, 2006].

2.2.3 Specific background for paper 2:
A safety study on GMA. Th1/Th2.

There was a need for a pilot safety study in European setting before GMA could be introduced. Primarily our study was aimed to provide information on safety, tolerability and impact on clinical symptoms when using GMA in patients with chronic active IBD. We hypothesized that in addition to the removal granolocytes and monocytes (mentioned in the previous part) some immunoregulatory mechanisms involving other cell groups such as the lymphocyte populations exist and may contribute to restore of the immunological tolerance.

2.2.4 Specific background for paper 3:
An extension of the previous study. T regs, TLRs.

Human inflammatory bowel disease (IBD) is driven by expansion of effector T cells (Teff) that overwhelm regulatory T cells (Tregs) and propagate innate immune responses [Maul et al, 2005; Hart et al, 2005; Kelsen et al, 2006; Holmen et al, 2006]. Toll Like Receptors are widely distributed on and inside the cells of immune system in order to recognize and respond to diverse molecules, and were shown be responsible for bacterial recognition and tolerance to normal gut flora [Hart et al, 2005]. Szebeni et

al, 2008 described elevated expression of TLR-2 and TLR-4 in inflamed colonic mucosa of children with IBD. To further elucidate immunological response on GMA we extended the second study by analysing FoxP3+ T regulatory cells and expression of Toll Like Receptors in the colonic biopsies.

2.2.5 Specific background for paper 4:

A safety study on modified LCAP. Regulatory molecules.

Nafamostat mesilate (NM) is used as anticoagulant in previous LCAP devices (in Japan). NM suppresses bradykinin generation during apheresis [Kojima et al, 1991] but accumulates in the kidney and induces hyperkalemia [Muto et al, 1994; Li et al, 2004]. In the present study ACD-A is introduced as anticoagulant in LCAP for the first time, as well the filter material was treated to diminish the risks for bradykinin generation [Hild et al, 1998, Schaefer et al, 1993, Iwama, 2001]. Therefore a safety study on this new device was performed.

There are no earlier studies on how the apheresis system lines could impact the immunoregulatory molecules, which are potentially important for restoration of the immunological balance.

During the apheresis treatments co-interactions of blood components and the foreign materials of the apheresis system occur, these co-interactions are of interest for future investigations.

We hypothesized that contact of blood with the apheresis system lines or filter might be modulating the immune system by influencing (selected) immunoregulatory molecules.

3 AIMS OF THE STUDY

The general objective of this thesis was to elucidate the role of immunological balance in connection to interventions by allergen challenge in asthma patients and apheresis treatment in inflammatory bowel disease patients.

Paper I. The aim was to investigate the impact of allergen challenge on the cytokine profile of circulating lymphocyte populations (in allergic asthma) and thereby study role of Th1/Th2 balance in relation to the clinic.

Paper II. The objective of this study was to provide information on safety, tolerability, feasibility, and impact on clinical symptoms when using a selective granulocyte- and monocyte apheresis device in patients with chronic active IBD in a European setting. Aims in my project were to study correlation between clinical response to granulocyte- and monocyte- apheresis and cytokine profile changes in peripheral blood and in colonic mucosal tissue in patients with IBD.

Paper III. Given that regulatory T cells and TLR expression constitute additional key actors in mucosal immune regulation this study was aimed to identify the dynamics of these actors in the intestinal mucosa in relation to clinical improvement following GMA treatment.

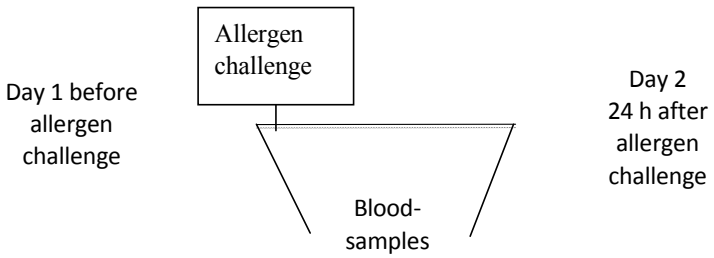
Paper IV. The primary aim of this pilot trial was to evaluate safety and tolerability of the Cellsorba™ EX filter in combination with ACD-A in the treatment of UC patients by monitoring clinical adverse effects, whole blood cell counts, bradykinin as well as IL-6. Potential therapeutic efficacy was evaluated using the composed clinical and endoscopical disease activity index (Mayo score). The aim of my project was to evaluate the effect of the LCAP filter and apheresis system lines on selected regulatory molecules.

4 METHODS

Methods paper 1

Subject characterization and sampling procedure

Ten subjects underwent a bronchial allergen challenge and peripheral blood samples were collected before and 24 hours after allergen provocation (Schedule 1, below).



Schedule 1. This schedule illustrates the study design and shows the time points for sampling.

Heparinized blood from 10 healthy blood donors served as control. The patients used no *per os* or inhaled steroids during the study period and no patient had an on-going infection. All patients inhaled β_2- agonists as needed.

Bronchial allergen challenge

The allergen challenge was done with a dosimeter jet nebulizer. Standardized and freeze-dried
birch or timothy allergen extracts were diluted and used at a maximum of four concentrations: 1000, 4000, 16000 and 64000 SQ (Standardized Quality) allergen units x mL^{-1}. The nebulizer was set to nebulize for 0.5 s giving an output of 7.1 µL/breath. At each concentration, first 2 and then 4 breaths could be taken, and if needed followed by 8 and 16 breaths at the highest concentration providing doses from 14 to 7040 SQ allergen units. Specific airway resistance (SRaw) and thoracic gas volume were measured in a constant-volume whole-body plethysmograph 15 min after each dose of allergen. The challenge proceeded until a 100 % increase in SRaw was reached.

Median provocative dose of allergen required to cause 100% increase of SRaw ($PD_{SRaw100\%}$) was calculated by linear interpolation on a logarithmic scale. Forced expiratory volume in one second (FEV_1) and SRaw were recorded immediately before and 15 min after each single dose of the allergen.

Spirometry

The measurements of FEV_1 were made hourly using a portable computerized spirometer.

The maximal fall in FEV_1 from allergen challenge to 3-10 hours after allergen challenge, as well as the average fall in FEV_1 during this period, was used to measure the asthmatic reaction during the late phase.

Definition of single- and dual-responders

Single responders had only an early reaction 15 min after the allergen challenge with a 100% increase in SRaw. Late phase reaction was defined as at least 15% decline in FEV_1 3-10 hours after the last dose of allergen challenge. Dual responders had a late phase reaction in addition to the initial 100% increase in SRaw.

Stimulation of peripheral blood lymphocytes

Whole blood samples were incubated for 4 hours with mitogen Phorbol 12-Myristate-13-Acetate (PMA) and Ionomycin in presence of Brefeldin (BFA) and Hepes-RPMI. Unstimulated cells, which served as controls, remained in BFA and medium only for 4 hours. BFA was used to interrupt the intracellular Golgi mediated transport and to allow the cytokines to be accumulated inside the cells.

Surface immunostaining

In vitro activation of CD3+ cells was assessed by CD69 up-regulation, and 90% CD69+CD3+ was set as the lowest acceptable level. To identify the CD4 and CD8 positive lymphocytes by flow cytometry the cells were stained with specific fluorescent-conjugated antibodies.

Cell membrane fixation, permeabilization and immunofluorescence staining of intracellular IFN-γ and IL-4

After cell membrane fixation and permeabilization (using a commercial kit) fluorescent-conjugated antibodies to IFN-γ respectively IL-4 were added. Samples were finally analysed in an EPICS XL flow cytometer.

Flow cytometric analysis

Discrimination gates were set around the respective cell population and a minimum of 3000 lymphocytes were accumulated during analysis. The cells were identified both as CD3, CD69, CD4, CD8-positive and positive for the respective cytokine.

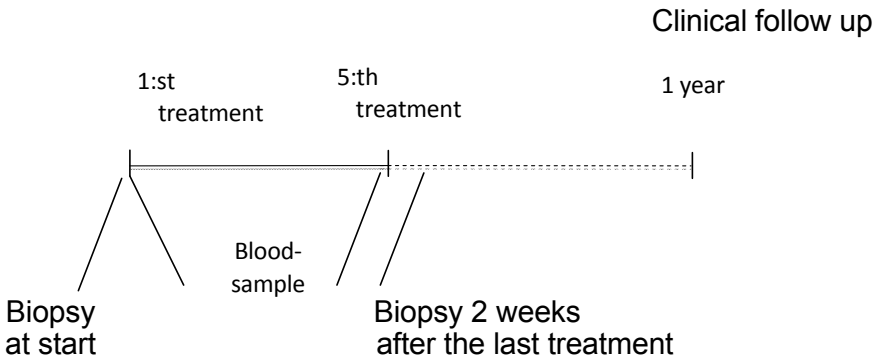
Statistical methods

Results are given as median and range values or Δ -values which represent the percentage change between two observations within the same individual. The Wilcoxon test was used to analyse the differences between paired measurements before and after the allergen provocation. And the Mann-Whitney U Test was used to analyse the differences of Δ -values between single and dual responders.

Methods paper 2

Study design

An initial assessment included colonoscopy with biopsies and blood samples. The active treatment period was five weeks with one treatment per week and a new overall evaluation, including endoscopy with biopsies, was made two weeks after the last apheresis had been carried. Following the active part of the study, the patients were then followed as out-patients on a bi-monthly schedule with clinical assessment, recording of possible adverse events and side effects until week 52, when a final clinical assessment of tolerability and response was made. (Schedule 2, below)



Schedule 2. This schedule illustrates the study design and shows the time points for sampling.

Patients

All 10 patients (7 with Crohn Disease; 3 with Ulcerative Colitis, median age: 31 yrs, 3 male and 7 female) had a diagnosis of mild to moderately active IBD. Apheresis was then performed with a perfusion rate of 30 mL/minute, the duration of each session was 60 minutes and anticoagulation was performed using a bolus dose of heparin of 4000 units. Altogether, each patient received once weekly apheresis treatments for five consecutive weeks. The dosages of all anti-inflammatory drugs were kept constant during the study period, with the exception of oral prednisolone. The dose of prednisolone was decreased after the second apheresis if the patient had improved. Tapering was then gradually performed with 2.5 mg per week.

Clinical assessment

The patients' symptoms were assessed using the modified Clinical Activity Index (CAI) [Rachmilewitz et al, 1989], and for the CD-patients the CD activity index-score (CDAI) [Best et al, 1979] was also calculated. A reduction of the CAI by three points or more compared with baseline (week 0) indicated clinical response, and a score of four or less was considered as clinical remission. A reduction of the CDAI by 70 points or more was indicating response and a reduction to less than 150 was considered as clinical remission.

Intracellular cytokines

Monoclonal antibodies suitable for intracellular staining and flow cytometry were used for detection of intracellular human IL-4, TNF-α, IL-10 and IFN-γ as described in the first paper.

ELISpot assay for detection of cells producing IFN-γ, IL-10, TNFα, IL-12, IL-4, IL-6

Ninety six-well membrane plates were coated with mAb to IFN-γ; IL-10; TNF-α; IL-12; IL-4 and IL-6. PBMC were separated from blood samples by gradient centrifuging. A total of 30,000 and 100,000 PBMC were added to each well in culture medium containing mitogen PHA. ELISpot plates were incubated for 21-23 h. Plates were then washed and biotinylated detection mAb to IFN-γ; IL-10; TNF-α ; total IL-12; IL-4; IL-6 was respectively added. Plates were washed and streptavidin-ALP was added before incubation for 1 h at RT. The plates were washed and substrate was added. After development of spots for 15 min at RT, the plates were washed with tap water and dried. The number of spots per well was counted by an automated ELISpot reader.

ELISA determination of IFN-γ, IL-6 and TNF-α plasma levels

Analyses were performed with commercial ELISA kits according to the manufacturer's instructions. The lowest detectable concentration of cytokine was 8, 0.7 and 0.1 pg/mL for the IFN-γ, IL-6 and TNF-α assays, respectively.

Immunohistochemistry staining of biopsies

Sections for CD3 and CD8 staining were fixed with acetone and sections for cytokine staining (TNF-α, IL-1β, IFN-γ, IL-4, IL-15) were fixed with formaldehyde.

A mixture of H_2O_2 and NaN_3 in PBS for one hour in the dark in room temperature was used for blocking endogenous peroxidase activity. Primary monoclonal antibodies (mAbs) where in a mixture of PBS, BSA, NaN3 and saponin for the cytokine staining. Saponin was used as a detergent to make intracellular compartments available for antibodies. The primary antibodies were incubated over night in a humid chamber. Incubation with normal horse serum (NHS) was used as blocking procedure. The secondary antibody was

diluted in a mixture of PBS and NHS for incubation. An avidin-biotin-horseradish peroxidase was diluted in PBS or PBS-saponin. Sections were incubated in the solution. A staining reaction was developed with a Peroxidase Substrate Kit containing 3,3´diaminobenzidinetetrahydrochloride (DAB).

The sections were assessed on two different occasions by two independent observers in all cases with concordant results.

Examinations were performed in a blind manner with regard to patient response, biopsy site and time point. The assessment of CD3, CD8 as well as for all cytokines was performed by semiquantative evaluation. For CD3 and CD8 assessment a grading scale ranging from four (massive infiltration: > 1000 positive cells per view), three (between 100 and 1000 cells), two (between 10 and 100), one (between 1 and 10) to zero (no visible staining) was used. An almost similar, but four graded scale was used for all cytokine assessments; three (massive infiltration, > 100 positive cells per view), two (between 10 and 100), one (between 1 and 10), zero (no visible staining). Negative controls were processed on each slide.

Statistics

Statistical analyses were done by using the Wilcoxon non-parametric tests. A P-value <0.05 was considered significant.

Methods paper 3

Patients

Ten patients (7 with Crohn Disease; 3 with Ulcerative Colitis, median age: 31 yrs, 3 male and 7 female) with mild to moderately chronic active IBD were studied. Eight out of 10 patients were improved clinically with good long-term effect after GMA , which was done once weekly over five consecutive weeks.

Immunohistochemistry

Immunohistochemical staining of biopsy materials taken before start of treatment and two weeks after last treatment was performed on acetone fixed sections (CD25 and CD163 (macrophage marker)) and on formaline fixed sections (FoxP3, BDCA-2, -3, -4 (dendritic cells marker), TLR-2, TLR-4) as described in the second paper.
All examinations were done in a blinded manner with regard to patient response, biopsy site and time point. The assessments were done by semi-quantitative evaluation with a grading scale ranging from 3 (> 50 cells), 2 (10 - 50), 1 (1 -10) to 0 (no visible staining).

Statistics

Statistical analyses were done by using the Wilcoxon non-parametric tests.
P-values <0.05 were considered significant.

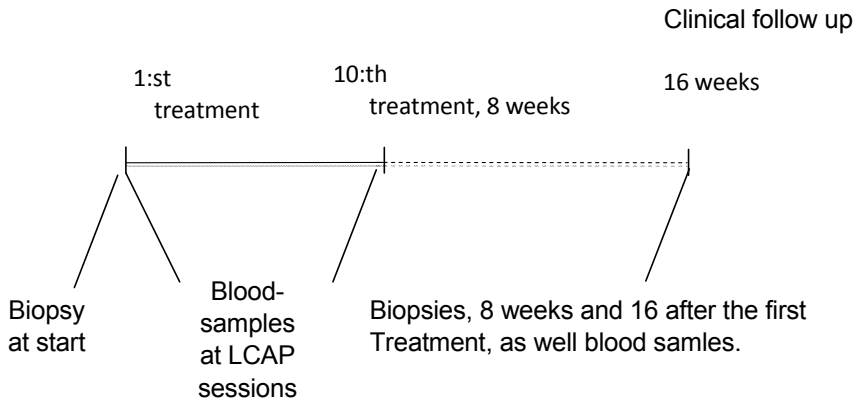
Methods paper 4

Patients

Patients with mild to moderate active UC (Mayo Score: 6 – 10) despite treatment with 5-ASA equivalent to minimum of 4 g sulfasalazine / day for at least six weeks were eligible to enter this study (Table I). Aminosalicylate treatment was continued without any change during the current study. Analgesic or spasmolytic drugs were allowed as concomitant medication, while, long-term antibiotic treatment or bowel motility modifying drugs (e.g. loperamide) were not permitted during the study.

Treatment course

Two sessions of LCAP each week were carried out for the first three weeks, followed by one session a week for the subsequent four weeks (10 sessions totally). A follow- up visit was held in week 16 (Schedule 3, below).



Schedule 3. This schedule illustrates the study design and shows the time points for sampling.

Safety and tolerability.

Vital signs including pulse rate and blood pressure were recorded before, during and after each LCAP session. Measurements of differential blood flow pressure between the inlet and outlet lines of the Cellsorba^{TM} EX device were also done. Any adverse event and/or device or procedure related incidents were registered.

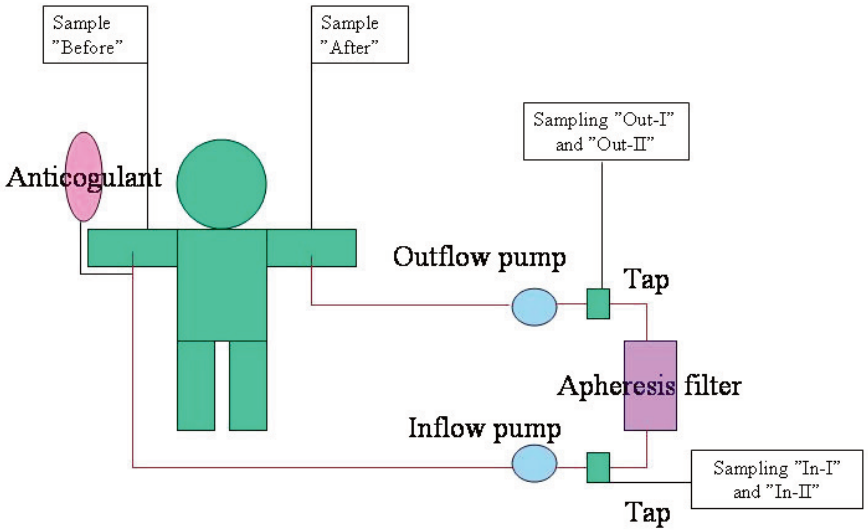
Clinical follow up and efficacy

Clinical activity was evaluated by means of disease activity index – the Mayo score [Lewis et al, 2008] before start of LCAP, at week 8 (after 1 week of the last LCAP) and at week 16. Videocolonoscopy with pictures and biopsies were performed at entry and at weeks 8 and 16. A reduction of Mayo score to ≤ 2 was taken as a clinical remission, while a drop in Mayo score ≥ 3 points was considered as a clinical response. Histological assessment of biopsies was performed in a blinded fashion using the grading by Geboes et al [Geboes et al, 2000]. The worst score was used for the evaluation.

Laboratory analyses

Peripheral venous samples for whole blood counts were collected before and directly after each LCAP, designated "Before" and "After" respectively. The samples taken before LCAP # 1, 3 and 10 were in addition subjected to analysis of CRP and ESR. Samples were also collected from the inlet and outlet lines of the Cellsorba^{TM} EX

device at two occasions during a treatment session, namely at time of first blood return (I), and at before rinse back (II). The inlet samples were taken from taps on plastic lines located 190 cm after vein puncture and 180 cm before the column, while the outlet samples were taken from taps located 49 cm after the column. These samples were designated “In-I”, “Out-I”, “In-II” and “Out-II”, respectively and subjected to analysis of WBC and platelets as well as soluble markers. IL-6 and bradykinin were analysed at the first LCAP. (Schedule 4, below).



Schedule 4. The image illustrates where and when the samples were taken. Samples were also collected from the inlet (In) and outlet (Out) lines device at two occasions during a treatment session, namely at time of first blood return (I), and at before rinse back (II).

Commercial ELISA kits were used and the analysis procedures were done according to manufacturers´ instructions for assessment of bradykinin, IL-6, IL-1ra, LL-37, CD30L (CD153).

Statistics

The Wilcoxon non-parametric tests were used for the statistical analyses,

P- values <0.05 were considered as significant.

5 RESULTS AND DISCUSSION

First paper

Allergen challenge was used as a way to study allergic inflammatory responses. The patients got symptoms of allergic asthma and lymphocyte profiles became changed. IL-4 positive CD4+ cells decreased in peripheral blood after the allergen challenge. IFN-γ positive CD4+ cells decreased in peripheral blood in single responders, but had a tendency to increase in dual responders. There was a significant difference between single and dual responders regarding changes in numbers of IFN-γ positive CD4+ cells.

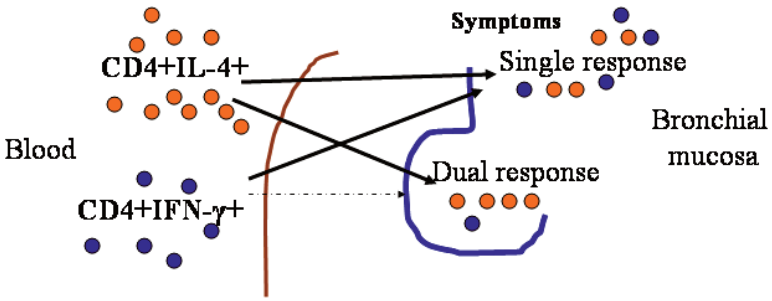


Image 6. Recruitment of Th2 and Th1 lymphocytes into bronchial mucosa in response to allergen challenge. Reduced recruitment of CD4+IFN-γ+ (Th1) cells in dual responders is illustrated by a dotted line.

Existence of an active traffic of T lymphocytes into bronchial mucosa in order to participate in the immune response was suggested earlier [Durham et al. 2000]. It has been hypothesized that an active recruitment of lymphocytes into the bronchial mucosa is reflected by a decrease of the corresponding circulating population.

The allergic inflammatory response is associated with IgE-mediated mast cell

activation and followed by recruitment of leucocytes into the airway tissue. It has been suggested that T-helper type 2 cells become recruited into the lungs and regulate allergic asthma reaction. [Durham et al, 2000]. Our finding that IL4+CD4+ cells decreased in peripheral blood in the patient group and IFN-γ + CD4+ cells decreased in the single responders after allergen challenge suggests the active traffic of both Th1 and Th2 cells.

We suggest that a difference in recruitment of IFN-γ positive CD4+ lymphocytes can explain differences between clinical response in patients with late-phase reaction and patients without late-phase reaction. A diminished capacity to down-regulate the Th2 response by recruitment of sufficient number of IFN-γ positive CD4+ lymphocytes was suggested as explanation to the late phase symptoms in the dual responders. Matsumoto and co-workers [Matsumoto et al, 2002] have reported that IL-10+CD4+ lymphocytes increase in single responders and decrease in dual responders after an allergen challenge. This finding goes in line with our IFN-γ-data, because an existing antagonistic balance between IFN-γ and IL-10 has been suggested [Romagnani, 1991].

In the present study we also compared T lymphocyte subpopulations in patients with allergic asthma and in healthy blood donors. Our findings that the patients have lower IFN-γ + and higher IL-4+ lymphocyte counts compared to the healthy controls were consistent with results reported by other research groups. This confirms the accuracy of the applied laboratory method in this study and encourages us to apply this method again in other patient group.

Second paper

Patients with inflammatory bowel disease (IBD), both Crohn´s disease and ulcerative colitis underwent a series of five consequent one per week treatments by selective granulocyte- and monocyte- apheresis (GMA). Eighth of ten patients reached good clinical response and even maintained a long-term response.

A significant decrease of IFN-γ-producing CD4+ cells after five apheresis sessions (P=0.046) was found by flow cytometric analyses. This result was further supported by the ELISpot analyses from eight cases where IFN-γ-producing cells also decreased significantly between week 0 and 7 (P=0.037).

The median IFN-γ-positive cell staining score decreased from 2.0 to 0.0 for all patients. However, when biopsies from the patients attaining a significant clinical response at week 7 (7/9) were analyzed separately, the median IFN-γ-positive cell staining score

significantly decreased from 3.0 to 0.0 (P=0.027). At week 52, eight patients had attained clinical response and when the reduction in IFN-γ-positive cells measured at week 7 was then reassessed, the result fell just short of significance (P=0.052). The result was influenced by one patient (#10) who, at week seven, had only achieved limited clinical benefit and interestingly had a slight increase of IFN-γ-positive staining in the biopsy from week 7 compared to baseline. However, this patient still had clinical improvement at week 52 (CDAI score drop by almost 70 points).
The immunohistochemistry staining for other cytokines (i.e., TNF-α, IL-1 β, Il-4, and IL-15) did not show an statistically significant change, although some patients had a marked reduction of TNF-α staining. There was a tendency towards a decrease of IL-15-positive cells at week 7 (P=ns). The analyses of CD3+ and CD8+ did not show significant changes at week 7 (NK cells were few in pre- and post-treatment biopsies and there were no decrease in their numbers, unpublished data). Apart from IFN-γ, no significant changes were measured by ELISpot (IL-10, TNF-α, IL-12, IL-4, and IL-6) or by flow cytometry (IL-4, IL-10, and TNF-α).
A significant increase of soluble (measured by ELISA) TNF-α in the serum after the apheresis treatment were noted for the whole group of patients (P=0.007), but there was no appreciable difference between median values before and after 4.0 (range: 1.3–51.1) vs 5.3 (1.4–76.9). In eight out of ten patients, TNF-α levels were almost unchanged. No significant changes were observed for IL-6 and IFN-γ when measured by ELISA.
The clinical response was parallel to down regulation of IFN-γ+ CD4+ cells in peripheral blood and IFN-γ+ cells in colonic mucosa biopsies.

Common feature for the first and second trials was that lymphocyte subpopulations were influenced. Tendency to normal balance would lead to symptom relief. In the asthma trial, IFN-γ+ (Th1) are involved in limiting of IL-4+ (Th2) response. And the scarcity in this capacity deteriorates the inflammatory response. In this case late-phase reaction will be observed.
In the second study the finding of down regulation of IFN-γ+ T helper cells in the blood and IFN-γ+ cells in the biopsies confirmed our suggestions that mechanisms of column apheresis were complex and immunomodulatory involving more cells types in addition to the absorbed cell type.

In the IBD patients who maintained a long-term response (for 12 months) IFN-γ positive lymphocytes were decreased already in the post-treatment biopsies at week 7.

week 7. Therefore we suggested that down regulation of these cells might be a predictive marker for long-term response.
IFN-γ can be both pro-inflammatory and regulatory, but if the immunological balance has turned to the tolerance state it might be predictive for a better clinical outcome. Our opinion is shared in recent review by Bosani et al, 2009, who have written that with anti-IFN-γ agents "we are moving closer to the epicentre of immune reaction. Only agents restoring T cell tolerance long term or repairing the basic dysfunction in the innate immune system may provide the perspective of cure in chronically remitting and flaring diseases…"

Third paper
When we expanded the second study by analysing T regulatory cells and expression of Toll Like Receptors in the colonic biopsies the findings became following:
The number of FoxP3-positive cells decreased significantly in the mucosa. Furthermore, the number of TLR-2 positive cells decreased significantly in the lamina propria, but not in the epithelium. TLR-4 positive cells were unchanged. No significant differences were noticed for BDCA-2, -3, -4 neither for CD3 and CD25. In the patients who experienced a long-term effect of the treatment (n=8), the number of CD163 positive cells decreased significantly (P=0.046), but not significantly when all patients were included (n=9). UC and CD groups were also analysed separately showing equal response in measured parameters.

We did not find a strict relationship between FoxP3 and CD25 staining, and FoxP3+ cells were slightly more numerous than CD25+ cells at the start of treatment. FoxP3 expression is not exclusively linked to CD25 expression, which is supported by recent studies [Pillai et al, 2007; Fontenot et al, 2005].

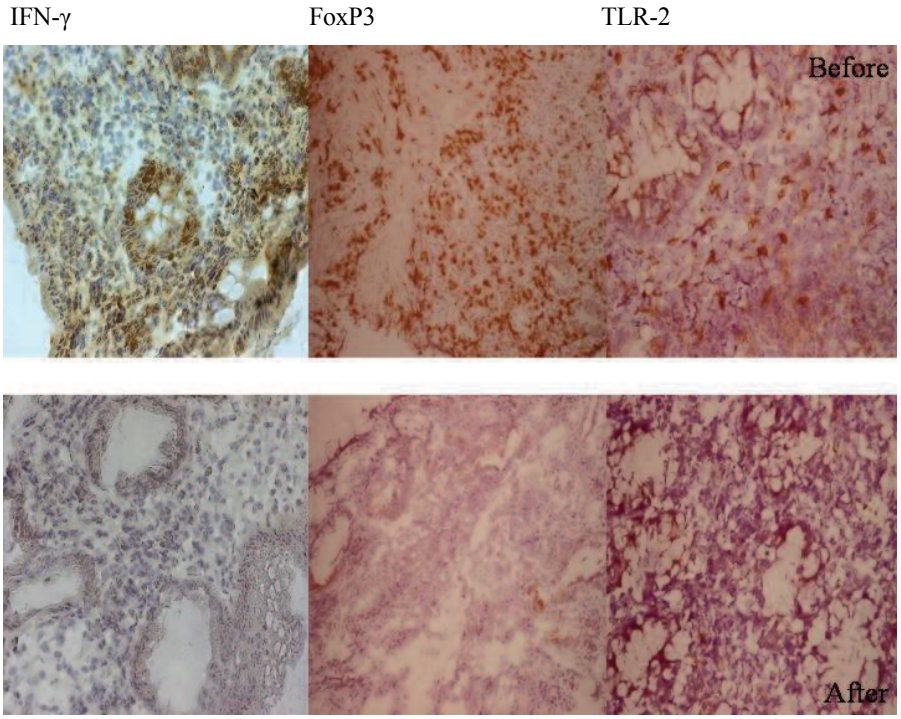


Image 7. Summary immunohistochemistry image from second and third paper

It was a novel message that the number of FoxP3+ T regs can diminish parallel to clinical improvement, because of belief that the higher number of T regs the better protection against inflammation [Maul et al, 2005; Denning et al, 2003; Uhlig et al, 2006]. Our finding suggests existence of an ongoing balance process with dynamic tendencies. According to our suggestion, diminished numbers of T regs reflects a state of improved tolerance (T regs *vs* effector cells).

The selective decrease in TLR-2 expression may reflect differences in regulation of TLR-2 and TLR-4. TLR-2 expression is induced in bacterial infections, while TLR-4 is constantly expressed [Matsuguchi et al, 2000]. According to previous reports induction of tolerance to bacterial lipoprotein might be associated with down-regulation of TLR-2 expression [Wang et al, 2002]. Thus, down regulated expression of TLR-2 in our study may reflect a state of improved tolerance to intestinal flora.

It has been speculated that in spite of increased numbers T regs suppressive capacity is down regulated in inflamed mucosa [Yu et al, 2007]. Expression of TLRs on T regs was described recently [Caramalho et al, 2003]. Sutmuller et al have demonstrated in experiments on mice T regs that TLR-2 signalling directly stimulates T regs expansion

and T regs stimulated through TLR-2 have diminished capacity to suppress proliferation of T helper cells *in vitro* [Sutmuller et al, 2006]. Their results are in line with simultaneous decrease of TLR-2 expression and T regs number in our study. Further research is required to elucidate the role of TLR triggering on T regs [van Maren et al, 2008].

Fourth paper

The modified LCAP using ACD-A as anticoagulant was found to be a safe and well-tolerated procedure, regarding both the vital signs and IL-6/ bradykinin generation.
Four patients were responders, of whom two patients went into remission. Median histological scores decreased from 3.5 to 2.0 in these four patients.
The analyses of CD30L, IL-1Ra and LL-37 have shown that the LCAP filter does not hinder the passage of these important soluble molecules. Some absorbance of IL-1Ra in the plastic lines could not be excluded.
There was no increase of IL-1Ra during LCAP, which is indicative of a difference between LCAP and GMA in this aspect.
An important observation regarding the analyzed molecules was that LL-37 increased at all sessions within the apheresis plastic lines.

Disturbed tolerance to the intestinal bacteria is considered important in the pathogenesis of IBD [Ohkusa et al, 2004]. Since LL-37 affects the cellular response to bacteria and is known for its antibacterial properties it plays an important role for maintaining the tolerance to the bacteria [Mookherje et al, 2006].
Wehlin et al reported earlier that Mac-1 (CD11b/CD18) up-regulation occurs when blood samples are kept in plastic tubing [Wehlin et al, 1998]. Mac-1 up-regulation is associated with neutrophil degranulation and is complement dependent [Borregaard et al, 1994]. Thus, we assume that LL-37 (contained in the granules of neutrophil granulocytes [Sörensen et al, 1997]) is released from neutrophils within the plastic apheresis lines and may perform its regulatory functions when returned back into the patient.
Nafamostat mesilate used in earlier types of CellSorba (LCAP) and heparin used in GMA inhibit complement activation [Keck et al, 2001; Weiler et al, 1992]. While, significant complement activation was described in connection to apheresis with ACD-A (the most widely used in the apheresis systems citrate based anticoagulant) [Ptak et al, 2005]. Lever et al have reported that neutrophil degranulation is inhibited by heparin

[Lever et al, 2007]. Thus, the anticoagulants have different properties regarding their capacity to inhibit the complement activation and the neutrophil degranulation. Our own unpublished data indicates that heparin (used as anticoagulant) inhibits degranulation of neutrophils and generation of LL-37. The anticoagulant (ACD-A) used in the present study did not prevent the neutrophil degranulation.
LL-37 was recently shown to have potential therapeutic properties against cancers, HIV [Chuang et al, 2009; Bergman et al, 2007], for wound healing, against bacterial biofilm and methicillin-resistant bacteria [Nijnik and Hancock, 2009; Komatsuzawa et al, 2006]. There is a need of a method to stimulate the endogenous production of LL-37 which may find therapeutic applications [Nijnik and Hancock, 2009]. Mookherje et al, 2006, showed that even small increase in concentration of LL-37 enhances the cellular tolerance to bacterial components and suppresses TLR-induced secretion of proinflammatory cytokines, which supports our suggestion that generation of LL-37 in the lines might have a positive impact on the immunologic tolerance and contribute to the efficacy of the apheresis treatment.

Conclusions

We have suggested that differences in response to allergen can depend on different capacity to maintain immunologic balance (Th-balance) – diminished capacity to recruit IFN-γ+ CD4+ lymphocytes seems to be associated with development of an additional so called late-phase reaction. We have shown that mechanisms of leukocyte adsorbing apheresis were immunomodulating involving down regulation of IFN-γ+ lymphocytes hereby influencing the Th-balance. The clinical improvement in IBD was associated with improved immunologic tolerance mirrored by down regulation of FoxP3+ cells and TLR-2 expression in the gut mucosa. And finally, we have described generation of LL-37 in plastic lines of apheresis system, which may have a positive impact on the immunologic tolerance.
The restoration of the immunologic tolerance can be the key to successful therapeutic strategies.

6 FUTURE PERSPECTIVES

GMA and LCAP were reported to have promising clinical results in many prospective studies. The absence of serious side effects is an advantage of the apheresis treatments. However, their role in the treatment of IBD is still debated. Only two sham-controlled studies have been published. One smaller study on LCAP showed a better outcome in active moderate UC [Sawada et al, 2005]. More recently, a larger study on GMA was published [Sands et al, 2008]. This study did not show any significant difference in the effect on UC between the sham-control and GMA. In the later study however, blood of sham-control patients was circulating through plastic tubing and then returned back into the patient circulation. This approach can be challenged since different cascade systems can be activated when in contact with foreign surfaces as the complement and the coagulation systems. We have also shown a generation of LL-37 in the plastic lines. Therefore, there is a need to further investigate the role of complement system activation as well as other immunomodulators for the treatment outcomes.

We do not know why some patients are treatment responders and others are not. We speculate that different phases of the disease might be of importance for induction of response to the treatment [Kugathasan et al, 2007]. We also suggest that treatment frequency and interval between the sessions must be adjusted to each patient.

Today there are various treatments for inflammatory conditions, but one cannot know in advance whether the patient will benefit from a certain treatment or develop serious side effects. One example is the use of biologics that are expensive and can cause serious side effects. Moreover, not all patients respond to these treatments. It is important to be able to choose the right treatment and spare the patients from the treatments with serious adverse reactions. Identification of immunological predictive markers for treatment response would help to give the right individual treatment and even save a lot of money. At best, predictors could help to choose tailored [Scaldaferri and Fiocchi, 2007] treatment in the early phase of inflammatory diseases. The future challenge is thus to find such predictors.

7 ACKNOWLEDGEMENTS

I would like to thank everyone who has supported me through this study and especially:

My supervisor, professor Joachim Lundahl, for sharing your profound knowledge, your enthusiasm, for teaching me a lot, for generous help whenever needed.
My co-suprvosor, professor Robert Löfberg, for sharing your profound knowledge, your positive spirit and ideas, for valuable and stimulating discussions.
My co-supervisor, Dr Kerstin Elvin, for sharing your profound knowledge, for valuable and stimulating discussions, availability and support.
My mentor, Ann-Charlotte Wikström for your advices.
The whole staff at the Department of Clinical immunology and Transfusion medicine for all kinds of help.
All members of the "Inflammatory unit" for your warm support.
Titti Nieminen for teaching me flow cytometry and many more. Josefin Paulsson for being a wonderful roommate. Michaela Cassmer for your help.
My co-authors, especially Marianne van Hage, Ann-Kristin Ulfgren, Ola Winqvist, Aleksandra Mandic-Havelka for fruitful collaboration.
Abraham Mamo, Eva Lindroos, Marianne Malmström, Petra Jones for helping me in labs.
My family for love and support.

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9 SAMMANFATTNING PÅ SVENSKA

Allt fler drabbas av inflammatoriska sjukdomar som allergisk astma och inflammatoriska tarmsjukdomar. Denna ökning är märkbar speciellt bland de yngre. Vi har studerat hur den immunologiska balansen förändras vid exponering för allergen hos patienter med allergisk astma och i samband med aferesbehandling som avlägsnar en del av vita blodkroppar ur blodet hos patienter med inflammatorisk tarmsjukdom. Mellan undergrupper av T hjälparceller, Th1 och Th2, existerar en balans. Denna balans är förskjuten vid inflammatoriska sjukdomar som allergisk astma och inflammatorisk tarmsjukdom. Man vet att vid allergisk reaktion alternativt vid allergisk provokation hos allergiska astmatiker Th2 celler går in i bronkvävnad och styr inflammatoriska processer. Vi har påvisat att även Th1 celler rekryteras in i bronkvävnad och om denna rekrytering inte är tillräcklig utvecklar patienterna en ytterliggare allergisk reaktion med bronkoobstruktion.
Antal Th1 celler (IFN-γ+) minskar i tarmslemhinna och i blod hos patienter med inflammatorisk tarmsjukdom efter aferesbehandling som avlägsnar vita blodkroppar, vilket tyder på att behandlingsmekanismerna påverkar immunologisk balans. Även minskning av uttryck av molekyler som känner igen bakteriella strukturer och minskat antal T celler som har förmåga att nedreglera inflammatoriska celler har samband med förbättrad immunologisk tolerans hos patienter med inflammatorisk tarmsjukdom som genomgått denna behandling.
Vi har upptäckt generering av LL-37 i aferesslangarna, vilket kan ha positiv inverkan på immunologisk tolerans och verka därmed mot inflammatorisk tarmsjukdom.

Resultaten tyder på att återställandet och upprätthållandet av immunologisk tolerans/balans kan vara en nyckel till framtida framgångsrika behandlingsstrategier.