
Crosstalk between autoreactive T cells and alveolar type II epithelial cells in inflammation and tolerance

Von der Fakultät für Lebenswissenschaften
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Gereke M, Westendorf AM, Buer J, and Bruder D. Induction of regulatory T cells by alveolar self antigen expression. (**Poster**). Keystone Symposia - Translational Medicine in Autoimmunity, Big Sky, Montana, USA, 2005.

Gereke M, Prettin S, Gröbe L, Kasper M, Buer J, and Bruder D. The role of type II alveolar epithelial cells for the induction of T cell tolerance and immune regulation. (**Vortrag**). Joint annual meeting of the German and Scandinavian Societies for Immunology, Kiel, Germany, 2005.

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Bruder D, Westendorf AM, Geffers R, Gereke M, Gruber A, and Buer J. Toleranz versus Autoimmunität: Autoreaktive CD4⁺ T-Zellen in der Pathogenese chronisch entzündlicher Erkrankungen der Lunge. (**Vortrag**). Tagung der Sektion Zellbiologie der Deutschen Gesellschaft für Pneumologie, Magdeburg, Germany, 2003.

Gereke M, Westendorf AM, Geffers R, Buer J, Bruder D. Alveolar self antigen expression leads to the induction of regulatory T cells. (**Vortrag**). Joint meeting of the Dutch and German Societies for Immunology, Maastricht, Netherlands, 2004.

Bruder D, Gereke M, Westendorf AM, Geffers R, Enelow RI, Buer J. Induction of regulatory T cells due to chronic self antigen stimulation in the lung. (**Poster**). 12th International Congress of Mucosal Immunology, Boston, USA, 2005.

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Erratum

Erratum zur Dissertation „Crosstalk between autoreactive T cells and alveolar type II epithelial cells in inflammation and tolerance“ vorgelegt von Marcus Gereke, 2006.

Während des Verfassens meiner Dissertation sind trotz mehrfacher und unabhängiger Korrekturen unabsichtliche Zitationsfehler aufgetreten, die hier innerhalb dieses Erratums korrigiert wurden. Originalpassagen aus der Dissertationsschrift sind dabei durch Anführungszeichen kenntlich gemacht und mit der dazugehörigen Quelle ergänzt worden.

Page 3

The following paragraph includes substantial material taken from the publication of Bienenstock and McDermott, 2005.

Bienenstock J, McDermott MR. Bronchus- and nasal-associated lymphoid tissues.
Immunol Rev. 2005 Aug; 206:22-31.

“The bronchus-associated lymphoid tissue (BALT) constitutes organized lymphoid aggregates of T and B cell that are capable to respond against to inhaled antigens. BALT, located mostly at bifurcations of the bronchus in animals and humans, is already present in the fetus and develops rapidly following birth, especially in the presence of antigens. Humoral immune responses elicited by BALT are based primarily in immunoglobulin A secretion, locally and by BALT-derived B cells that have trafficked to distant mucosal sites. Similarly, located T cell responses have been noted. On the basis of these findings, the BALT can be thought of as functionally analogous to mucosal lymphoid aggregates in the intestine and is deemed a member of the common mucosal immunologic system (Bienenstock and McDermott, 2005).”

Pages 7 and 8

The following paragraph includes substantial material taken from the publication of Lipscomb and Maasten, 2002. However, the source was omitted in the text unintentionally.

Lipscomb MF, Masten BJ. Dendritic cells: immune regulators in health and disease. *Physiol Rev.* 2002 Jan; 82(1):97-130.

“In the lung, DC reside within and beneath airway epithelium, in alveolar septae, in the connective tissue surrounding pulmonary veins and airway vessels, and with the lung capillaries of the lung parenchyma (Lipscomb et al., 1995). DC in the airway epithelium have an immature phenotype and exhibit a rapid turnover (Holt et al., 1994). DC that are resident within alveolar septae and in connective tissue surrounding vessels have a more mature phenotype than airway DC (Gong et al., 1992). In contrast to DC that are resident within the lung, in the vascular compartment circulating precursor DC are present (Suda et al., 1998). One role of lung DC is to provide protection against infectious agents by initiating immune response. An equally important role is to generate tolerance to inhaled allergens in normal noninflamed lungs. In this regard, immature DC continuously leave the peripheral blood and take over a surveillance position in lung tissue, avidly sampling the antigenic environment. In the steady state, lung DC likely remain relatively immature and constitutively migrate in low numbers into regional lymph nodes where they induce anergy, deletion of T cells, or a weak T_H2 -like response to air-borne antigens that is eventually downregulated (Stumbles et al., 1998). Active suppression of immature DC maturation by alveolar macrophages may explain why airway and intraepithelial DC remain immature during their steady-state migration to lung-associated lymph nodes (Holt, 1993; Lipscomb et al., 1993). Furthermore, autocrine production of IL-10 by immature DC can inhibit surface expression of MHC class-I and -II molecules and exert a generalized inhibitory effect on T cell proliferation (Stumbles et al., 1998). On exposure to inhaled allergens, the antigen may simply be insufficient in providing a danger signal to overcome suppression by alveolar macrophages and IL-10. However, if a danger signal is present at the tissue site, DC mature and migrate in greater number to draining lymph nodes to stimulate $CD4^+$ T cell clonal expansion and differentiation.”

Page 8

The following paragraph includes substantial material taken from the publication of Tschernig et al 1999. However, the source was omitted in the text unintentionally.

Tschernig T, Fliegert F, Westermann J, Pabst R. Increased expression of activation markers and adhesion molecules on lung T-cells compared with blood in the normal rat. *Eur Respir J.* 1999 Jan; 13(1):66-70.

“Lymphocytes play a significant role in lung disorders, for example sarcoidosis, asthma, and rejection after transplantation (Berman et al., 1990). T cells, B cells, and NK cells are present in various lung compartments (Agostini et al., 1993; Holt and Schon-Hegrad, 1987; Pabst, 1990; Stein-Streilein, 1988), for example the lung vascular bed, the lung interstitium, the epithelium and lamina propia of the bronchi and the bronchoalveolar space (Fliegert et al., 1996; Pabst et al., 1995). Since the lung has no afferent lymphatic vessels, the blood is the starting point for lymphocyte immigration. Lymphocytes migrate to the lung vascular endothelium (marginal pool) and enter the interstitial lung tissue (interstitial pool), where the lymphocyte composition is different from blood and bronchoalveolar lavages (BAL) (Fliegert et al., 1996). In contrast to peripheral blood lymphocytes, mainly activated T cells are found in BAL of humans and mice (Curtis et al., 1995; Saltini et al., 1990). This immunological status confers lymphocytes appropriate defense mechanisms to such a vulnerable organ. The expression of adhesion molecules depends on the compartment from which the cells are recruited, indicating that local activation and expression of adhesion molecules is induced by the microenvironment. Possible candidates for such activators are dendritic cells and macrophages or components of the extracellular matrix (Holt, 1993; van Haarst et al., 1994).”

Page 8

In the bibliography of this thesis two publications “Holt, 1993” are listed. The mentioned reference “Holt, 1993” within this paragraph above describes the following publication listed in the bibliography.

Holt PG. Regulation of antigen-presenting cell function(s) in lung and airway tissues. *Eur Respir J.* 1993 Jan; 6(1):120-9. Review.

The following paragraphs include substantial material taken from the publication of Knight and Holgate, 2003.

Knight DA, Holgate ST. The airway epithelium: structural and functional properties in health and disease.

Respirology. 2003 Dec;8(4):432-46.

“The epithelium constitutes the interface between the internal milieu and the external environment, and as such, it is the first point of contact for inhaled substances, in particular, respiratory viruses, airborne allergens, and environmental pollutants, as well as being a primary target for inhaled respiratory drugs (Folkerts et al., 1998; Gizycki et al., 1997).

At least eight morphologically distinct epithelial cell types are present in human respiratory epithelium, which can be classified in three different categories: basal, ciliated and secretory epithelial cells (Spina, 1998). Columnar ciliated epithelial cells are the predominant cell type within the airways, constituting more than 50% of all epithelial cells (Spina, 1998). The primary role of the ciliated apical surface is highlighted by the directional transport of mucus from the lung to the throat (Harkema et al., 1991). Mucus cells (goblet cells) are responsible for the control of the correct amount of mucus and the viscoelasticity of mucus for efficient mucociliary clearance by releasing acid mucins from their granules. These cells are thought to be capable of self-renewal and may also differentiate into ciliated epithelial cells (Evans et al., 1988; Harkema et al., 1991). Serous cells are also secreting cells and produce neutral mucin and a yet unidentified non-mucoid substance (Knight and Holgate, 2003). Basal cells are ubiquitous in the conducting epithelium, although the number of these cells decreases with airway size and the increasing thickness of the basal cell layer correlates with increasing size of the airway (Evans et al., 1988; Evans et al., 1990). Similar to the skin, the basal cell is thought to be the primary stem cell, giving rise to the mucus and ciliated epithelial cells. In smaller airways, where basal cells are sparse or absent, Clara cells perform the primary stem cell role.

In addition to their progenitor and structural roles, basal cells are also thought to secrete a number of bioactive molecules including neutral endopeptidase, 15-lipoxygenase products and cytokines (Knight and Holgate, 2003). In humans, Clara cells are located in large (bronchial) and small (bronchiolar) airways. The cells produce bronchiolar surfactant and are also characterized by agranular endoplasmic reticulum in the apical cytoplasm and granular endoplasmic reticulum basally. In addition to their role in secretion, Clara cells are believed to metabolize xenobiotic compounds by the action of p450 mono-oxygenases and may also produce specific antiproteases such as secretory leukocyte protease inhibitor (De Water et

al., 1986). More recent evidence suggests that these cells play important role for stem cells, serving as a progenitor for both ciliated and mucus secreting cells (Hong et al., 2001).

The major function of the respiratory epithelium was once thought to be primarily that of a physical barrier, but recent studies clearly indicate that it is metabolically very active with the capacity to modulate a variety of inflammatory processes through the agency of an array of receptor-mediated events. On activation, it has the capacity to produce a number of proinflammatory cytokines, proinflammatory or regulatory mediators including arachidonic acid products, nitric oxide, endothelin-1, transforming growth factor (TGF)- β , tumour necrosis factor (TNF)- α , and cytokines such as interleukin (IL)-1, IL-6 and IL-8 (Knight and Holgate, 2003).”

Page 11

The following paragraphs include substantial material taken from the publication Chen, 2004. However, the source was omitted in the text unintentionally.

Chen J, Chen Z, Narasaraju T, Jin N, Liu L. Isolation of highly pure alveolar epithelial type I and type II cells from rat lungs.

Lab Invest. 2004 Jun;84(6):727-35. Erratum in: Lab Invest. 2005 Sep;85(9):1181.

“Alveoli are the gas exchange units of the lung and the alveolar epithelium adapts to this functional role by developing two highly specialized alveolar epithelial cell types, which are morphologically and functionally different.”

“AECII consist of about 15% of the distal lung cells and occupy 5% of the alveolar surface (Crapo et al., 1978; Crapo et al., 1982; Haies et al., 1981).”

“AECII synthesize and secrete lung surfactant, a protein-lipid complex and surface-active material. Lung surfactant stabilizes alveoli by reducing the surface tension.”

“AECII also maintain the alveolar epithelium by cell proliferation and differentiation, minimize alveolar fluid by transport of sodium from the apical to the basolateral side, and alter the inflammatory process by secretion of growth factors and cytokines.

In contrast to AECII, AECl contribute 7% of total lung cells and cover more than 95% of the alveolar surface. This thin epithelium allows the easy diffusion of gases and forms a barrier against the indiscriminate leakage of fluid into alveolar spaces. It also regulates the

exchange of physiologically important solutes and water between circulating blood and the alveolar space.”

Page 12

The following sentence was taken from the publication of Fehrenbach et al, 2000. However, the source was omitted in the text unintentionally.

Fehrenbach H, Kasper M, Koslowski R, Tan P, Schuh D, Müller M, Mason RJ. Alveolar epithelial type II cell apoptosis in vivo during resolution of keratinocyte growth factor-induced hyperplasia in the rat.

Histochem Cell Biol 2000, 114:49–61.

“Notably, apoptotic AECII appeared to be removed not only by alveolar macrophages but also by AECII cell neighbors.”

Pages 12

The following paragraph includes substantial material taken from the publication of Fehrenbach, 2001. However, the source was omitted in the text unintentionally.

Fehrenbach H. Alveolar epithelial type II cell: defender of the alveolus revisited.

Respir Res. 2001; 2(1):33-46. Review.

“The alveolar epithelium can be classified as a continuously renewing tissue since it comprises a population of alveolar type II epithelial cells that are characterized by almost unlimited potential to proliferate. It is still a matter of debate whether all AECII or only a small population act as the alveolar epithelial stem cell population (Uhal, 1997). The concept of AECII as stem cells of the adult alveolar epithelium was proposed by Kapanci and colleagues, and is widely accepted today (Kapanci et al., 1969). During ontogenesis, the AECII may derive from precursor cell common to AECII and Clara cells (Wuenschell et al., 1996). Furthermore AECII proliferate and differentiate to AECI to repair the damaged alveolar epithelium after lung injury or during fetal lung development, thus contributing to epithelial repair, whereas AECI are terminally differentiated, lack mitotic activity, and are easily injured. The programmed cell death or apoptosis is an important mechanism of cell removal or renewing of tissue. AECII are known to express the membrane receptor Fas (CD95, APO-1), the ligation of which may initiate the apoptotic cascade (Fine et al., 1997).

This can be achieved by binding of Fas-ligand or the Fas-stimulating antibodies. There is some evidence that apoptosis of AECII is an integral mechanism of alveolar septal modelling in lung morphogenesis (Scavo et al., 1998; Schittny et al., 1998). Notably, apoptotic AECII appeared to be removed not only by alveolar macrophages but also by AECII cell neighbours (Fehrenbach et al., 2001).”

Pages 13, 14, 15, 16 and 17

The following paragraphs include substantial material taken from the publication of Fehrenbach, 2001. However, the source was omitted in the text unintentionally.

Fehrenbach H. Alveolar epithelial type II cell: defender of the alveolus revisited.

Respir Res. 2001; 2(1):33-46. Review.

“The best example for a cell-cell interaction between AECII and resident cells is the direct contact with AECl and during proliferation with AECII neighbours as well. These lateral cell-cell contacts within the alveolar epithelium are maintained by cell junction complex that includes gap junctions (Kasper et al, 1996). Additionally, AECII have direct contact to fibroblasts at the basal membrane or with capillary endothelial cells (Marin et al., 1982).

A strong evidence for a direct interaction of AECl and AECII was presented by Ashino and colleagues (Ashino et al., 2000). Mechanical stimulation of AECl is thought to result in Ca²⁺-oscillations, which were transmitted via intraepithelial gap junctions to AECII and modulate exocytosis rate of lamellar bodies. Direct inhibitory interactions between AECl and AECII have been postulated to suppress AECII proliferation. Loss of AECl during injury might then trigger the release of AECII from growth inhibition (Mason and McCormack, 1994). E-cadherin as a further candidate to mediate contact inhibition, has been localized to the basolateral membrane of AECII (Kasper et al., 1995; St. Croix et al., 1998).

But even an indirect cell-cell interaction for AECII to other AECII is possible by the negative feedback loop by which surfactant protein A (SP-A) upon release into the alveolar space inhibits surfactant exocytosis *in vitro* (Dobbs et al., 1987). Although AECII are equipped with membrane receptors for SP-A (Strayer et al., 1996), the *in vivo* relevance of this autocrine mechanism by which AECII may regulate their own action remains elusive, because mice that are deficient in SP-A did not show any defect in surfactant secretion nor any respiratory deficiency (Ikegami et al., 1998). Thus, some alternative mechanism must compensate the negative SP-A feedback loop.

Another potential feedback mechanism that has been postulated is the inhibition of AECII proliferation via AECII derived transforming growth factor (TGF)- β in bleomycin-induced experimental lung fibrosis (Khali et al., 1994). A number of growth factors are released by AECII, which might act in an autocrine way via the corresponding receptors expressed by AECII.

As mentioned before, fibroblasts are in contact to AECII. This reciprocal cell-cell interaction is relevant to the modelling of alveolus during lung morphogenesis as well as during remodelling associated with alveolar repair following lung injury (Kasper et al., 1996; O'Reilly et al., 1997; Shannon, et al., 1997). Both direct and indirect cell-cell interactions have been reported.”

“The interaction of alveolar epithelial and capillary endothelial cells is well examined. It was reported that from pulmonary endothelial cells conditioned medium stimulate fetal lung epithelial cell growth (Smith et al., 1986) and that endothelin-1 increases AECII surfactant secretion *in vitro* via a protein kinase C and Ca^{2+} -mediated pathway (Sen et al., 1994). As a source of endothelin-1, endothelial cells are therefore principally competent to act in a paracrine manner on AECII, which were reported to express the endothelin receptor A (Markewitz et al., 1995).

Recently, a very special mechanism of indirect intercellular communication between AECII and endothelial cells has been suggested. Stimulation of alveolar epithelial cells with tumour necrosis (TNF)- α was reported to increase epithelial Ca^{2+} influx and to activate epithelial cytoplasmic phospholipase A2, and results in basolateral release of arachidonic acid. Free arachidonic acid is thought to increase endothelial Ca^{2+} influx and expression of P-selectin (Kuebler et al., 2000), which is known to be crucial for initiation of leukocyte adherence. Thus, AECII could act as transducers of an inflammatory signal from the alveolus to the capillary bed to recruit granulocytes to the site of inflammation.

Alveolar macrophages are one of the mobile cell types that interact with AECII. Among the multitude of secretory products synthesized and released by alveolar macrophages (Kasper et al., 1996; Lohmann-Matthes et al., 1994) there are some factors that act as mitogens for AECII, such as hepatocyte growth factor (Mason et al., 1994) and heparin-binding epidermal growth factor (Leslie et al., 1997). Conversely, AECII were shown to express the chemokines RANTES and MCP-1, which chemotactically attract macrophages (O'Brien et al., 1998), as well as GM-CSF (Blau et al., 1994; Christensen et al., 1995), which in turn may stimulate macrophage growth (Worgall et al., 1999). Furthermore, SP-A released from AECII modulate

macrophage functions such as oxygen radical release (Weissbach et al. 1994) and nitric oxide production (Stamme et al., 2000).

Interactions of AECII with leukocytes have just recently come into focus. AECII synthesize some cytokines affecting leukocytes, such as interleukin (IL)-6 or IL-8. Via these cytokines, AECII might be involved in the induction of differentiation of basophil, eosinophil, and neutrophil granulocytes and maintenance of inflammatory reactions. Recent data support the idea that AECII have an accessory function in T lymphocyte activation (Zissel et al., 2000). This has been suggested on the basis of the findings that the cells bear MHC class-II molecules (Schneeberger et al., 1986)."

"Additionally, AECII were reported to inhibit lymphocyte proliferation *in vitro* without altering their activation state (Paine et al., 1991). Moreover, AECII derived TGF- β (Zissel et al., 2000) could indirectly inhibit T cell proliferation via blockade of activating factors, such as IL-2. In contrast, granulocyte macrophage-colony stimulating factor (GM-CSF) released at the basolateral surface of AECII could increase the potential of dendritic cells to induce T-cell proliferation (Christensen et al., 1995)."

"The surface-active agent was characterized in numerous biochemical studies of BAL material and is now known to be composed of ~90% lipids (with ~80-90% phospholipids) and of ~10% proteins (Griese, 1999). Unlike most other lipid-rich components of cells and organs, the surfactant lipids are characterized by an unusually high level of saturated fatty acid chains, such as the predominant dipalmitoylphosphatidylcholines, which contribute substantially to the unique properties of pulmonary surfactant (van Golde et al., 1994). The protein fraction comprises a highly variable amount of serum proteins (Griese; 1999) and four apoproteins that are associated with surfactant and contribute to its specific function (Weaver and Whitsett, 1991)."

Page 17

The following passage includes substantial material taken from the publication of Wright, 2005.

Wright JR. Immunoregulatory functions of surfactant proteins.

Nat Rev Immunol. **2005** Jan; 5(1):58-68. Review.

“SP-B is essential for the ability of surfactant to reduce surface tension (Nogee, 2004), and SP-C has recently been shown to bind lipopolysaccharide (LPS) (Augusto et al., 2002; Augusto et al., 2003). In the absence of surfactant, surface tension is extremely high at end expiration and tends to collapse the lung. This makes breathing difficult to the extent that respiration is frequently impossible without ventilatory support and surfactant replacement. A deficiency of surfactant – which can result in “Respiratory-Distress Syndrome (RDS)” – occurs when infants are born prematurely, before their surfactant biosynthetic machinery has matured. Treatment of these premature infants with exogenous surfactant replacement reduces mortality and morbidity, because of this disease (Wright, 2005).”

Pages 17 and 18

The following paragraphs include substantial material taken from the publication of Fehrenbach, 2001.

Fehrenbach H. Alveolar epithelial type II cell: defender of the alveolus revisited. Respir Res. 2001; 2(1):33-46. Review.

“The other function of alveolar surfactant relies on the nature of SP-A and SP-D as collectins. Both proteins are able to bind to the surface of various pathogens, thus acting as opsonins to facilitate their elimination by alveolar macrophages. Therefore, alveolar surfactant is also responsible for host defence (Crouch, 2000; Pison et al., 1994; Wright, 1998).

Surfactant is synthesized by alveolar type II epithelial cells and released upon appropriate stimuli by exocytosis from special intracellular storage organelles termed lamellar bodies. Once released into the alveolar space, freshly secreted lamellar body material undergoes several steps of transformation that are necessary to establish the surface-active lining layer. Cyclic compression and expansion during ventilation result in a fraction of spent surfactant that will largely be recycled by AECII. Thus, single constituents of surfactant run through several cycles before being removed by alveolar macrophages and replaced by *de novo* synthesis (Fehrenbach, 2001).

Although the bronchiolar Clara cells and submucosal cells also synthesize and release the mature proteins SP-A, SP-B and SP-D (Kalina et al., 1992; Voorhout et al., 1992) the alveolar type II epithelial cell is the only type of pulmonary cell that produces all surfactant components including phospholipids as well as all four surfactant proteins. The mature 3.5 - 3.7kDa small SP-C is thought to be exclusively released by AECII cells (Beers et al., 1994; Phelps and Floros et al., 1991). About 85% of the secreted surfactant is taken up again, metabolised and re-secreted by AECII. Re-uptake and recycling have been demonstrated for all surfactant lipids and for all four surfactant proteins. The degradation of surfactant is accomplished by alveolar macrophages with only minimal contribution (Herbein et al., 2000; Nicholas, 1996; Young et al., 1993).”

Pages 18, 19, 20, 21 and 22

The following passages include substantial material taken from the publication of Wright, 2005. However, the source was omitted in the text unintentionally.

Wright JR. Immunoregulatory functions of surfactant proteins.
Nat Rev Immunol. **2005** Jan; 5(1):58-68. Review.

“2.3.1 Immunoregulatory functions of surfactant proteins

As mentioned above, the host defence functions of surfactant are primarily mediated by SP-A and SP-D, which are members of the collectin family of proteins. SP-A and SP-D have been also localized to non-pulmonary sites, including the trachea, brain, testes, salivary glands, lachrymal glands, heart, prostate, kidney, pancreas and the female urogenital tract (Leth-Larsen et al., 2004; Lin et al., 2000; Madsen et al., 2000; Rubio et al., 1995), although it is not yet clear whether all of these organs express sufficient amounts of protein for it to be physiologically effective.

Among their well-established role as opsonins, SP-A and SP-D also have functions in initiating parturition, facilitating clearance of apoptotic cells and directly killing bacteria.”

“ 2.3.2 Collectin structure

In addition to the two lung collectins SP-A and SP-D, serum collectins have been identified in humans (mannose-binding lectin, MBL) and in bovidae (conglutinin, CL-43 and CL-46) (Hansen and Holmskov, 2002).

SP-A and SP-D are synthesized as primary translation products of approximately 26-36kDa and 43kDa, respectively (figure 3). The collagen-like domain is N-terminal to a coiled-coil structure that precedes the C-terminal lectin domain. The lectin domains mediate the interaction of collectins with a wide variety of pathogens. The collagen domains vary greatly in length (Holmskov et al., 2003)."

"For example, both surfactant proteins, SP-A and SP-D, bind to mannose and glucose but bind only poorly to galactose (Haagsman et al., 1987; Lim et al.; 1994; Persson et al., 1990). The high affinity of the collectins for clustered oligosaccharides is thought to be important for their ability to distinguish non-self from self, as most carbohydrates in animals are terminated by sugars, such as galactose or sialic acid, that are poorly recognized by the collectins."

"2.3.3 Collectin regulation of immune cells

Surfactant proteins A and D bind to a variety of bacteria, viruses, allergens and apoptotic cells and thereby function as opsonins to enhance the uptake of these cells and particles. Binding of the collectins to pathogens occurs by various mechanisms. Some pathogens are aggregated by SP-A and/or SP-D and were phagocytized by immune cells like macrophages. SP-A and SP-D also have direct effects on immune cells and modulate the production of cytokines and inflammatory mediators.

Numerous studies have reported that SP-A mediates cellular functions through C1q receptors (Ferguson et al., 1999; Malholtra et al., 1994), including C1qR (also known as CD93) (Nepomuceno et al., 1997; Steinberger et al., 2002) and calreticulin (Malholtra et al., 1990; Malholtra et al., 1993). SP-A and SP-D are able to bind Calreticulin, which in turn binds to CD91. CD91 is a component of the binding complex (Gardai et al., 2003).

The binding of SP-A and/or SP-D to the signal-inhibitory regulatory protein- α (SIRP- α) modulates cellular functions in a similar way like the binding complex of surfactant proteins with the CD91-calreticulin complex. In the absence of a pathogen, SP-A binds through its lectin domain to SIRP- α , whereas in the presence of a foreign organism or cell debris, to which the lectin domain of SP-A binds, the free collagen-like region activates immune cells through CD91-calreticulin. Importantly, engagement of the different receptors elicits different responses. Upon binding of SP-A to SIRP- α , the inflammatory-mediator production is inhibited. By contrast, SP-A enhances inflammatory mediator like tumour-necrosis factor (TNF), CXCL12 and CCL2 production through its binding to the CD91-calreticulin complex.

Therefore, SP-A and SP-D both are able to enhance and inhibit inflammatory-mediator production to modulate the regulation of immune cells.

Another receptor that binds surfactant protein A was identified by Chroneos and colleagues and termed SP-R210 (Chroneos et al., 1996). Blocking of this receptor with specific antibodies leads to a loss of SP-A mediated functions, including inhibition of lymphocyte proliferation (Borron et al., 1998), enhanced uptake of bacteria by macrophages (Weikert et al., 1997) and mycobacterial killing by a nitric-oxide-dependent pathway (Weikert et al., 2000). Nevertheless, the molecular identity of SP-R210 is still unclear.

Glycoprotein 340 (gp340) is also discussed as a protein that binds SP-D through its CRD (Holmskov et al., 1997). Because of its localisation at the cell surface of alveolar macrophages, gp340 was suggested to be a SP-D receptor. It is identical to salivary agglutinin, a high-molecular-weight component of saliva that binds *Streptococcus mutans*, a bacterium that causes dental caries (Prakobphol et al., 2000). This putative receptor gp340 has no transmembrane domain so that it is suggested that it could interact with an adaptor molecule on the surface of the cell (Wright, 2005).

Additionally, a family of conserved cellular receptors that recognize pathogen-associated molecular patterns (PAMP) are discussed as binding-partners for SP-A and SP-D. This family of Toll-like receptors (TLRs) is activated by ligands like flagellin and CpG-containing DNA from bacteria, peptidoglycan from Gram-positive bacteria, LPS from Gram-negative bacteria, RNA from viruses and zymosan from yeast (Takeda et al., 2003). All these activation mechanisms end up in a series of conserved responses that culminate in inflammation and the production of inflammatory cytokines, such as TNF and interleukin-1 β .

The SP-A dependent binding to TLR4 results in an activation of the nuclear factor κ B (NF- κ B) signalling pathway and upregulation of cytokine synthesis (Guillot et al., 2002), whereas interaction of SP-A with TLR2 attenuates stimulation of TLR2 signalling and also stimulation of TNF secretion induced by zymosan or peptidoglycan (Sato et al., 2003).

In addition to phagocytosis, SP-A and SP-D have also the ability to regulate the production of inflammatory mediators by immune cells in a context-dependent manner. One example shows that inflammatory mediators, such as TNF, are both up- and downregulated by SP-A and SP-D (Crouch and Wright, 2001). The release of TNF that is induced by LPS or intact bacteria is inhibited by SP-A (Hickling et al., 1998; McIntosh et al., 1996; Rosseau et al., 1999). In contrast, SP-A enhances TNF production either when alone (Kremlev et al., 1994, Kremlev et al., 1997) or in presence of "rough" LPS (Sano et al., 1999).

A further effect of surfactant proteins SP-A and SP-D is the enhanced uptake of apoptotic cells by alveolar macrophages *in vitro* (Schagat, et al., 2001), which could be even shown for lungs of naïve mice in the case of SP-D (Vandivier et al., 2002).

Through the carbohydrate-recognition domains (CRD) and the collagen-like regions it is possible for SP-A and SP-D, as well as MBL, to bind DNA from a variety of origins, including mice and bacteria (Palaniyar et al., 2004). SP-D effectively binds and aggregates alveolar macrophages DNA and it enhances the uptake of DNA by human monocytic cells (Palaniyar et al., 2003). Binding of the collectins to cell-surface DNA might be one mechanism by which they mediate enhanced phagocytosis of apoptotic cells.

Uptake of apoptotic cells by macrophages results in release of anti-inflammatory mediators, such as transforming growth factors- β (TGF- β), IL-10 and prostaglandin E₂ (Fadok et al., 1998). This response is in contrast to the release of pro-inflammatory cytokines that occurs when phagocytes ingest microorganisms. In addition to enhancing the uptake of apoptotic cells, SP-A also enhances the release of TGF- β by macrophages (Reidy and Wright, 2003), indicating that SP-A can promote resolution of inflammation at several levels of the apoptotic-cell clearance process and that surfactant proteins can indirectly induce anti-inflammatory responses by phagocytes.

As discussed above, surfactant is linked to innate immunity. However, surfactant is also linked to adaptive immunity in the lung by modulating functions of both dendritic cells and T cells.

It has been shown that SP-A and SP-D have different effects on DC functions. The uptake and presentation of antigens is enhanced by SP-D (Brinker et al., 2001), but only SP-A can inhibit maturation of DC, as assessed by cell-surface marker expression, and functional activity, such as phagocytosis and chemotaxis (Brinker et al., 2003).

The proliferation of T cells stimulated with plant lectins, CD3-specific antibodies or phorbol esters is inhibited by SP-A and SP-D. It has been suggested that the inhibition of IL-2 production might mediate this process (Borron et al., 1996; Borron et al, 1998). In addition, both the collagen-like region and the CRD of SP-A have been implicated in the inhibition of lymphocyte function, probably due to inhibition of calcium signalling (Borron et al., 2002). These data indicate that SP-D and SP-A might provide an important link between innate and adaptive immunity, by modulation of both DC and T cell functions.”

Page 20

In the bibliography of this thesis two publications “Borron, 1998” are listed. The mentioned reference “Borron, 1998” within these paragraphs above describes the following publication listed in the bibliography.

Borron P, McCormack FX, Elhalwagi BM, Chroneos ZC, Lewis JF, Zhu S, Wright JR, Shepherd VL, Possmayer F, Inchley K, Fraher LJ. Surfactant protein A inhibits T cell proliferation via its collagen-like tail and a 210-kDa receptor.

Am J Physiol. **1998** Oct;275(4 Pt 1):L679-86.

“Blocking of this receptor with specific antibodies leads to a loss of SP-A mediated functions, including inhibition of lymphocyte proliferation (Borron et al., 1998), enhanced uptake of bacteria by macrophages (Weikert et al., 1997) and mycobacterial killing by a nitric-oxide-dependent pathway (Weikert et al., 2000).”

Page 22

In the bibliography of this thesis two publications “Borron, 1998” are listed. The mentioned reference “Borron, 1998” within these paragraphs above describes the following publication listed in the bibliography.

Borron PJ, Crouch EC, Lewis JF, Wright JR, Possmayer F, Fraher LJ. Recombinant rat surfactant-associated protein D inhibits human T lymphocyte proliferation and IL-2 production.

J Immunol. 1998 Nov 1;161(9):4599-603.

„The proliferation of T cells stimulated with plant lectins, CD3-specific antibodies or phorbol esters is inhibited by SP-A and SP-D. It has been suggested that the inhibition of IL-2 production might mediate this process (Borron et al., 1996; Borron et al, 1998).”

Page 24

The following paragraph includes substantial material taken from the publication of Bell & Bird, 2005. However, the source was omitted in the text unintentionally.

Elaine Bell & Lucy Bird. Autoimmunity

Nature 435, 583 (2 June 2005)

“3 Autoimmunity

The concept of autoimmunity was first predicted by Paul Ehrlich at the beginning of the twentieth century, and he described it as “horror autotoxicus”. His experiments led him to conclude that the immune system is normally focused on responding to foreign materials and has an inbuilt tendency to avoid attacking self tissues. But when this process is disturbed, the immune system can attack self tissues resulting in autoimmune diseases.”

Page 24

The following paragraph includes substantial material taken from the publication of Marrack et al, 2001. However, the source was omitted in the text unintentionally.

Marrack P, Kappler J, Kotzin BL. Autoimmune disease: why and where it occurs.

Nat Med. 2001 Aug; 7(8):899-905. Review.

“Autoimmune diseases occur in up to 3-5% of the population (Jacobson et al., 1997). Many of these diseases are classified according to what organs and tissues are targeted by the damaging immune responses. There is an autoimmune disease specific for nearly every organ in the body, usually involving responses to an antigen expressed only in that specific organ. In other autoimmune diseases, such as systemic lupus erythematosus (SLE), no particular cell type seems to be targeted; rather, the response seems to be directed against antigens that are widely expressed throughout the host. Nevertheless these diseases are antigen-specific; moreover, recognition of widely expressed antigens sometimes results unexpectedly in selective manifestations of the organ (Mathews et al., 1983; Yeaman et al., 1988). Autoimmune organ damage can be mediated by T cells, as in multiple sclerosis (MS) and type 1 diabetes (Steinman, 1996) and, furthermore, CD4⁺ and/or CD8⁺ T cells can have crucial roles (Haskins and McDuffie, 1990; Hutchings et al., 1992). In these diseases, autoantibodies are also produced and serve as markers of the antigen-specific T-cell responses, for example, antibodies to insulin or other pancreatic islet-cell antigens in type 1

diabetes (Yu et al., 1996). In other diseases, damage is actually mediated by autoantibodies and requires CD4⁺ T-helper cells. For example, nearly all SLE patients have elevated levels of autoantibodies to nuclear antigens.”

Page 27

The following paragraph includes substantial material taken from the publication of O’Gara and Vieira, 2004. However, the source was omitted in the text unintentionally.

O’Gara A, Vieira P. Regulatory T cells and mechanisms of immune system control.
Nat Med. 2004 Aug; 10(8):801-5. Review.

“Regulatory T cells may be defined as CD4⁺ T cells that inhibit immunopathology or autoimmune disease *in vivo*. Specifically, T_{reg} cells include those able to suppress naïve T cell proliferation *in vitro* and to control CD4⁺ or CD8⁺ T cell numbers *in vivo*, in lymphopenic hosts. Two major T_{reg} populations have been described so far,“

Page 27

The following paragraph includes substantial material taken from the publication of Bluestone and Abbas, 2003. However, the source was omitted in the text unintentionally.

Bluestone JA, Abbas AK. Natural versus adaptive regulatory T cells.
Nat Rev Immunol. 2003 Mar; 3(3):253-7.

“The two subsets of regulatory T cells might function in different immunological settings, depending on the context of antigen exposure, the nature of the inflammatory response and the T cell receptor (TCR) repertoires of the individual cells. The natural T_{reg} cells are probably most effective at suppressing autoreactive T cell responses locally, in non-inflammatory settings – circumstances in which antigen specific, self limiting reactions are required to achieve a fine homeostatic balance. In contrast, during self-damaging inflammatory reactions to microbes or transplanted tissue, or settings (for example inflammatory bowel disease), adaptive T_{reg} cells might be induced to suppress the pathological immune responses.”

Page 28

The following paragraph includes substantial material taken from the publication of O’Gara and Vieira, 2004. However, the source was omitted in the text unintentionally.

O’Gara A, Vieira P. Regulatory T cells and mechanisms of immune system control. Nat Med. 2004 Aug; 10(8):801-5. Review.

“4.1 Naturally occurring CD4⁺CD25⁺ regulatory T cells

The CD4⁺CD25⁺ regulatory T cells are currently the focus of intensive research and were first described in the early 1970s by Gershon and colleagues (Gershon et al., 1974). These cells represent 5-10% of the CD4⁺ T lymphocytes in healthy adult mice and humans and are thought to perform a specialized role in controlling both the innate and the adaptive immune system. Although easily identified and isolated from unmanipulated mice and humans on the basis of CD25 expression, this chain of the IL-2(R) receptor is also expressed on activated T cells (Maloy et al., 2003; Sakaguchi et al., 2001; Shevach, 2002). So far, no characteristic stable surface marker has been assigned to T_{reg} cells. Additional markers expressed by these cells include cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) (Read et al., 2000; Takahashi et al., 2000) and glucocorticoid induced tumor necrosis factor receptor (TNFSR18) (McHugh et al., 2002; Shimizu et al., 2002), which were initially implicated in the mechanism of T_{reg} action. However, both of these molecules are also expressed by nonregulatory T cells after activation. Various groups identified the forkhead/winged helix transcription factor Foxp3 as a marker for both CD25⁺ T_{reg} cells and CD25⁻ cell that have regulatory activity (Fontenot et al., 2003; Hori et al., 2003).”

Page 28

In the bibliography of this thesis two publications “Sakaguchi et al., 2001” are listed. The mentioned reference “Sakaguchi et al., 2001” within this paragraph above describes the following publication listed in the bibliography.

Sakaguchi S, Sakaguchi N, Shimizu J, Yamazaki S, Sakihama T, Itoh M, Kuniyasu Y, Nomura T, Toda M, Takahashi T. Immunologic tolerance maintained by CD25⁺ CD4⁺ regulatory T cells: their common role in controlling autoimmunity, tumor immunity, and transplantation tolerance. Immunol Rev. 2001 Aug;182:18-32. Review.

Pages 28, 29 and 30

The following paragraphs include substantial material taken from the publication of Bluestone and Abbas, 2003. However, the source was omitted in the text unintentionally.

Bluestone JA, Abbas AK. Natural versus adaptive regulatory T cells.

Nat Rev Immunol. 2003 Mar; 3(3):253-7.

“The resident regulatory cells that develop in the thymus are generated in a burst of activity during the early stages of fetal and neonatal T cell development (Sakaguchi et al., 2001). They are polyclonal on the basis of diverse TCR usage (Shevach, 2002), and they are potentially capable of recognizing diverse self-antigens.

The promiscuous gene expression of many self tissue-specific proteins in the medullar epithelial cells of the thymus is described as a potential mechanism to ensure central tolerance to peripheral self-antigens, because this self-antigen expression in the thymus might lead, among other things, to the deletion of immature autoreactive T cells (Derbinski et al., 2001). However, it is possible that these self proteins are expressed at low levels and, additionally, by only some of the epithelial cells, making clonal deletion a rather ineffective means of inducing tolerance to peripheral antigens. An alternative mechanism of inducing self tolerance in the thymus might be the localized antigen presentation, resulting in a more robust regulation of autoreactivity. Once generated, the thymic T_{reg} cells are exported in the peripheral tissues, where they may function normally to prevent the activation of other, self reactive T cells that have the potential of developing into effector cells (Salomon et al., 2000).

These regulatory T cells were described as a “normal” population of suppressor cells, because they are always present in normal individuals and carry out their regulatory function during normal surveillance of self-antigens. Furthermore, because of their development in the thymus, the natural regulatory T cells are expected to be specific for self-antigens.

Recent studies indicate that CD28 controls both thymic development and peripheral homeostasis of natural T_{reg} cells. Ligation of CD28 is expected to act at two stages during T_{reg} cell development (Boden et al., 2003). In addition, once the natural T_{reg} cells emerge from the thymus, costimulation through CD28 is required to maintain a stable pool of these cells in the periphery by promoting their self renewal through homeostatic proliferation and by supporting their survival (Boden et al., 2003; Salomon et al., 2000). The development and maintenance functions of CD28 are not mediated through IL-2. It is possible that signalling through CD28 stimulates the production of a response to an yet unknown cytokine that functions as a growth and survival factor of these cells. The absence of CD80/CD86 or CD28 results in a

reduction of the number of regulatory cells in peripheral lymphoid tissues and an unexpected exacerbation of natural T_{reg} cells, which plays an important role controlling autoimmunity (Lenschow et al., 1996; Salomon et al., 2000).”

“4.2 Adaptive regulatory T cells

These cells are generated from mature T cell populations under certain conditions of antigenic stimulation, and they can be induced *ex vivo* by culturing mature CD4⁺ T cells with antigen or polyclonal activators in the presence of immunosuppressive cytokines, namely IL-10 (Barrat et al., 2002; Levings et al., 2001). Similar to natural T_{reg} cells, adaptive T_{reg} cells originate from thymus, but they might be derived from classical T cell subsets or natural T_{reg} cells. The level of expression of CD25 by adaptive T_{reg} cell is variable, depending on the disease setting and the site of regulatory activity. Of note, adaptive T_{reg} cells function *in vivo* in a cytokine dependent manner (Barrat et al., 2002; Chatenoud et al., 1997; Maloy and Powrie, 2001), so that these regulatory T cells are distinguished from natural T_{reg} cells not by their origin (the thymus), but by their requirement for further differentiation as a consequence of exposure to antigen in a distinct immunological context.”

“Another possibility to induce regulatory T cells is antigen exposure by certain routes, including intranasal or oral administration. This strategy seems to induce selectively the appearance of T cells with this regulatory phenotype (Chen et al., 1994)”

Page 30

The following paragraph includes substantial material taken from the publication of Shevach, 2002. However, the source was omitted in the text unintentionally.

Shevach EM. CD4⁺ CD25⁺ suppressor T cells: more questions than answers.

Nat Rev Immunol. 2002 Jun; 2(6):389-400. Review.

“Several different *in vitro* protocols have been described over the past few years that result in the generation of suppressor T cells. The activation of mouse or human CD4⁺ T cells *in vitro* in the presence of IL-10 has been shown to result in the generation of T cell clones with a cytokine profile different from that of T helper 1 (T_H1) or T helper 2 (T_H2) cells. Functionally, these T cell clones have inhibitory effects on antigen specific activation of naïve T cells that are mediated partially by IL-10 and TGF-β, and were termed T regulatory 1 (T_R1) cells (Groux et al., 1997). A related approach for the generation of suppressor T cells *in vitro*

involves the stimulation of naïve T cells with immature (im)DC. Surprisingly, although these cells produce IL-10, their suppressor phenotype resembles that of CD25⁺ T cells, as it is contact dependent, antigen non-specific and APC-independent. Immature DC are the ideal population to prime regulatory T cells as they are deficient in costimulatory molecules, and priming with antigen-imDC complexes might even be able to downregulate pre-existing antigen specific immune responses (Dhodapkar et al., 2001). Exposure to TGF- β has also been reported to facilitate the differentiation/expansion of suppressor T cell populations *in vitro* (Yamagiwa et al.; 2001).”

Page 31 and 32

The following paragraphs include substantial material taken from the publication of Bluestone and Abbas, 2003. However, the source was omitted in the text unintentionally. The subsequent figure and figure legend are adapted from Bluestone and Abbas, 2003.

Bluestone JA, Abbas AK. Natural versus adaptive regulatory T cells.

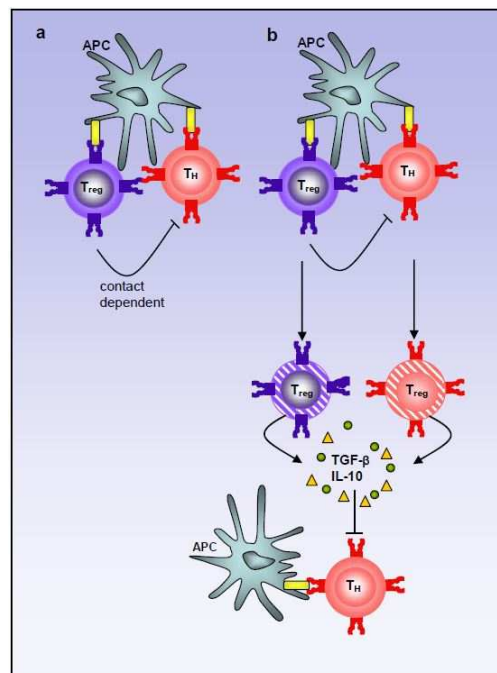
Nat Rev Immunol. 2003 Mar; 3(3):253-7.

“Moreover, in contrast to natural T_{reg} cell, which are fully functional at the time of thymic export as a consequence of strong TCR engagement, the development of adaptive T cells in the periphery might be triggered by low-affinity antigen or altered TCR signal transduction. These antigen-stimulated adaptive T cells are not functional without activation by further exposure to antigens – such as during infection, organ transplantation under cover of certain immunomodulatory therapies, or ectopic expression of non-self-antigens (Apostolou et al., 2002; Belkaid et al., 2002; Fuss et al., 2002; Kingsley et al., 2002; Powrie, 2003). Concerning the antigen specificity of adaptive T cells, it is interesting to speculate that these cells have a diverse repertoire, which might be expanded as a consequence of fortuitous cross-reactivities with foreign proteins. It is possible that the TCR repertoire of adaptive T cells is self-antigen specific, but that these cells are triggered in an inflammatory environment to promote bystander suppression through the production of suppressive cytokines.

It is important to note that unlike natural T_{reg} cells, adaptive T_{reg} cells might not require costimulation through CD28 for their development or function (Taylor et al., 2002). Interestingly, IL-2 might promote the development and function of both types of T_{reg} cells, on the basis of studies showing the total absence of T_{reg} cells in IL-2 receptor-deficient mice (Malek et al., 2002; Furtado et al., 2002).

4.3 Mechanism of suppression

In addition to potential differences in terms of TCR repertoire and differentiation state, it is proposed that natural and adaptive subsets of T_{reg} cells differ in their mechanism of action. Adaptive T_{reg} cells mediate their inhibitory activities by producing immunosuppressive cytokines, such as TGF- β and IL-10 (Kingsley et al., 2002; Nakamura et al., 2001). In contrast, natural T_{reg} cells, at least *in vitro*, function by a cytokine-independent mechanism, which presumably involves direct interactions with responding T cells or antigen-presenting cells (Shevach, 2002). This contact-dependent mechanism of suppression has been shown most convincingly by $CD4^+CD25^+$ natural T_{reg} cells employed in *in vitro* models of suppression, whereas cytokine-mediated suppression has been best established for peripheral adaptive T_{reg} cells *in vivo*. However, the adaptive T_{reg} cell subset, although it suppresses in a cytokine-dependent manner, might still require direct cell-cell contact to initiate the suppressive cascade.”



“**Figure 6: Two classes of regulatory T cells can be envisioned.** **a** In this hypothetical model, the natural regulatory T (T_{reg}) cells (blue) suppress immune response in a contact-dependent manner and function in general homeostasis to block the actions of autoimmune T cells (red) in noninflammatory settings. **b** The adaptive T_{reg} cell subset enhances the robust nature of suppression in an inflammatory milieu. Importantly, adaptive T_{reg} cells can develop either $CD4^+CD25^+$ natural T_{reg} cells (blue striped) or by altering the activity of T helper (T_H) cells (red striped). APC, antigen presenting cell; interleukin (IL)-10, transforming growth factor (TGF)- β , regulatory T cell (T_{reg}) (adapted from Bluestone and Abbas, 2003).”

Page 55

The following paragraph includes substantial material taken from the publication of Fehrenbach, 2001. However, the source was omitted in the text unintentionally.

Fehrenbach H. Alveolar epithelial type II cell: defender of the alveolus revisited. *Respir Res.* 2001; 2(1):33-46. Review.

“Already 1977 Mason and Williams developed the concept of the alveolar type II epithelial cell (AECII) as a defender of the alveolus (Mason and Williams, 1977). AECII may act as immunoregulatory cells and can interact with resident and mobile cells, either directly by membrane contact or indirectly via cytokines/growth factors and their receptors. Thus alveolar type II epithelial cells represent an integrative unit of immune responses within the alveolus.”

Page 57

The following paragraphs include substantial material taken from the publication of Roper et al, 2003. However, the source was omitted in the text unintentionally.

Roper JM, Staversky RJ, Finkelstein JN, Keng PC, O'Reilly MA. Identification and isolation of mouse type II cells on the basis of intrinsic expression of enhanced green fluorescent protein.

Am J Physiol Lung Cell Mol Physiol. 2003 Sep;285(3):L691-700. Epub 2003 May 9.

“Alveolar type II epithelial cells (AECII) are critical for normal lung development, homeostasis, and repair after injury. AECII produce pulmonary surfactant lipids and proteins required for reducing alveolar surface tension (Finkelstein et al., 1983; Shannon et al., 2001). As essential progenitors for type I epithelial cells, they are also critical for normal alveolar development and tissue remodelling after injury (Adamson and Bowdenet, 1974; Adamson and Bowden, 1975). The ability to investigate organogenesis and disease progression by overexpressing and deleting genes in mice, particularly genes expressed by alveolar type II epithelial cells, has recently favoured the use of mouse models in pulmonary research. Although mice are advantageous for manipulating genes, they have not been useful for isolating alveolar type II epithelial cells for *ex vivo* study so far. In contrast, rat and rabbit AECII have successfully been isolated using velocity centrifugation through a gradient of

albumin (Dobbs and Mason, 1979 and Finkelstein et al., 1983). Isolation of mouse AECII by this method has been less successful.”

Page 57

In the bibliography of this thesis two publications “Adamson and Bowden, 1974” are listed. The mentioned reference “Adamson and Bowden, 1974” within this paragraph above describes the following publication listed in the bibliography.

Adamson IY, Bowden DH. The type 2 cell as progenitor of alveolar epithelial regeneration. A cytodynamic study in mice after exposure to oxygen. Lab Invest. **1974** Jan;30(1):35-42.

Pages 79 and 80

The following paragraphs include substantial material taken from the publication of Wright, 2005. However, the source was omitted in the text unintentionally.

Wright JR. Immunoregulatory functions of surfactant proteins. Nat Rev Immunol. 2005 Jan; 5(1):58-68. Review.

„Pulmonary surfactant was initially identified as a lipoprotein complex that reduces surface tension at the air-liquid interface of the lung (Clements, 1957; Pattle, 1955). This definition has been reassessed in light of recent studies that show that surfactant also functions in pulmonary host defence and that surfactant proteins are expressed also in non-pulmonary sites. The host defence functions of surfactant are primarily mediated by SP-A and SP-D, which are members of the collectin family of proteins. An emphasis is placed on recent studies showing that, in addition to their well-established role as opsonins, SP-A and SP-D also have novel functions in initiating parturition, facilitating clearance of apoptotic cells and directly killing bacteria. Furthermore, immunoregulatory functions of the surfactant proteins A and D on T cells are discussed (Wright, 2005).”

Page 86

The following paragraph includes substantial material taken from the publication of Boyton and Openshaw, 2002.

Boyton RJ, Openshaw PJ. Pulmonary defences to acute respiratory infection.

Br Med Bull. 2002;61:1-12.

“The respiratory tract is a fragile tissue with architecture that is finely designed for gas exchange. Due to this main function the lung is exposed to numerous pathogens and other harmful air pollutions and developed many mechanisms to prevent infectious and inflammations. In the first line of defence are structural mechanisms coming from barriers such as epithelial cell layers, mucus and cilia, which avoid the invasion of pathogens or antigens. A battery of mediators that constitute the innate response including lactoferin, lysozyme, collectins and defensins is followed. Activation of these molecules can lead directly to lysis of pathogens, or to destruction through opsonisation or the recruitment of inflammatory cells (Boyton et al., 2002).”

Page 90

The following sentence includes substantial material taken from the publication of Herrath and Harrison, 2003. However, the source was omitted unintentionally.

von Herrath MG¹, Harrison LC. Antigen-induced regulatory T cells in autoimmunity.

Nat Rev Immunol. 2003 Mar;3(3):223-32.

“Therefore, physiological regulatory functions cannot be distinguished easily from effects that are caused by homeostatic proliferation and clonal expansion of transferred cells (Bach, 2003; Barthlott et al., 2003).”

Page 90

In the bibliography of this thesis two publications “Bach, 2003” are listed. The mentioned reference “Bach, 2003” within this paragraph above describes the following publication listed in the bibliography.

Bach JF. Regulatory T cells under scrutiny.

Nat Rev Immunol. 2003 Mar;3(3):189-98. Review. Erratum in: *Nat Rev Immunol.* 2003 Jun; 3 (6): 509.

The following paragraphs include substantial material taken from the publication of Moyron-Quiroz et al, 2004.

Moyron-Quiroz JE, Rangel-Moreno J, Kusser K, Hartson L, Sprague F, Goodrich S, Woodland DL, Lund FE, Randall TD. Role of inducible bronchus associated lymphoid tissue (iBALT) in respiratory immunity.

Nat Med. 2004 Sep;10(9):927-34. Epub 2004 Aug 15.

“The role of BALT in mouse and humans are controversially discussed and it is reported that infection or inflammation triggers the organization of lymphoid structures in the lung of both species (Chvatchko et al., 1996; Delventhal et al., 1992; Tschernig and Pabst, 2000). These structures do not fit the classical definition of BALT, as they are not formed independently of antigen (Bienenstock and Johnston, 1976; Plesch et al., 1983). Because the inducible BALT (iBALT) appears in the lung only after infection or inflammation, it is generally assumed that iBALT is simply an accumulation of effector cells that were initially primed in conventional lymphoid organs. The neo-formation of iBALT is caused by inflammatory responses, which directly promote the recruitment, priming and expansion of antigen-specific lymphocytes (Moyron-Quiroz et al., 2004).”

Als zweiten Teil meines Erratums möchte ich folgende Abbildungen innerhalb meiner Dissertation ersetzen bzw. zur Untermauerung der in der Dissertation getroffenen Aussage ergänzen. Diese Ersetzungen und Ergänzungen haben keinen Einfluss auf die Gesamtaussage meiner Dissertation.

Figure 11 (continued) page 41

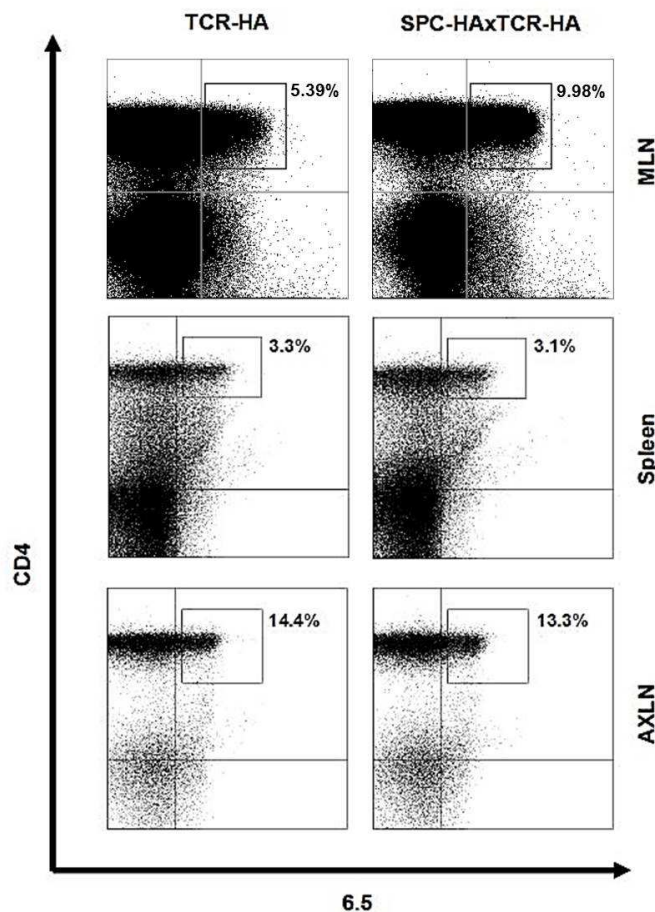


Figure 11 (continued): HA-specific CD4⁺ T cells are present in the periphery of SPC-HA x TCR-HA mice. SPC-HA x TCR-HA and TCR-HA control mice were sacrificed. Lung, BLN, MLN, Spleen, AXLN, INLN and CVLN were isolated and stained for CD4 and 6.5 expression to measure the percentage of transgenic T cells in the different compartments. These results are representative of two experiments with similar outcome.

In the original figure 11 two identical dot plots for mesenteric lymphnodes (MLN) were used unintentionally. In the revised figure 11 above the two identical dot plots were replaced by new dot plots from a similar experiment with a similar outcome suggesting no physiological relevant difference in the relative numbers of HA-specific CD4⁺ T cells in peripheral tissues between chronic diseased mice or healthy control mice. These data were generated following the protocols described in the chapter Materials and Methods.

Figure 13 page 47

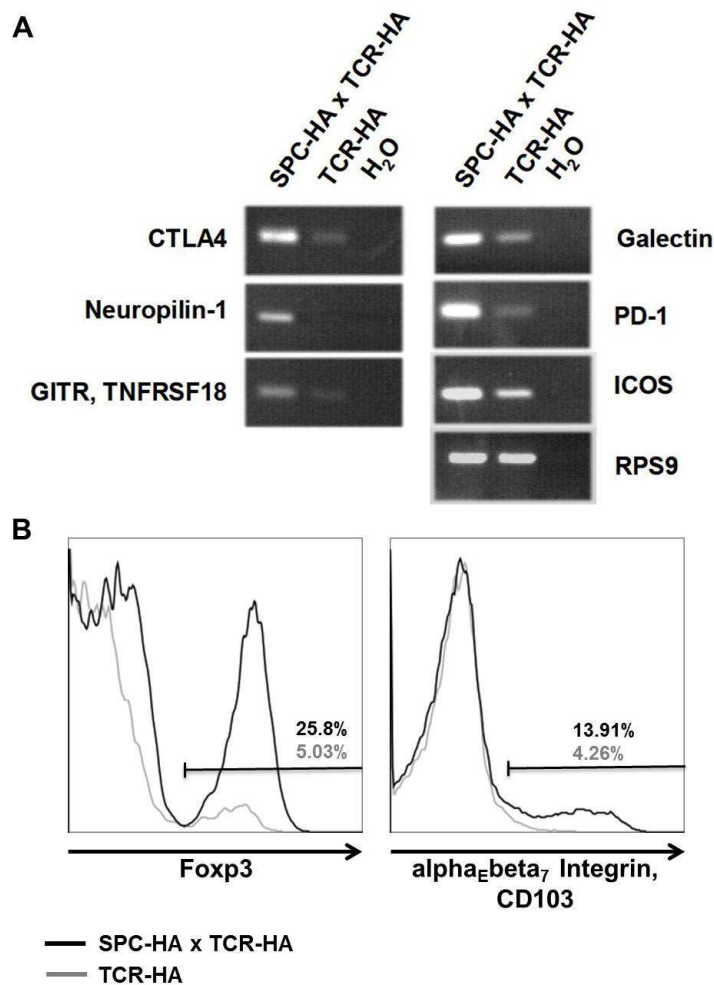


Figure 13: Semi-quantitative RT-PCR analysis of *ex vivo* isolated 6.5⁺CD4⁺ lung lymphocytes from SPC-HA x TCR-HA mice and TCR-HA control mice. (A) The expression of different molecular marker genes for regulatory T cells as CTLA4, Neuropilin-1, GITR (TNFRSF18), Galectin, PD-1 and ICOS was analyzed. RPS9 was used as a housekeeping gene. (B) In addition the expression of Foxp3 and alpha_Ebeta₇, CD103 were analyzed by flow cytometry on isolated 6.5⁺CD4⁺ lung lymphocytes from SPC-HA x TCR-HA mice (black line) and TCR-HA control mice (grey line).

In the original figure 13 the pictures of the semi-quantitative RT-PCR analysis of Foxp3 and CD103 in *ex vivo* isolated 6.5⁺CD4⁺ lung lymphocytes from SPC-HA x TCR-HA mice and TCR-HA control mice were interchanged unintentionally. These pictures were replaced in the revised figure 13 by histograms representing flow cytometric analysis of Foxp3 and CD103 expression in 6.5⁺CD4⁺ lung lymphocytes derived from SPC-HA x TCR-HA mice and TCR-HA control mice (Figure 13 B). Isolation of lung lymphocytes and surface staining for flow cytometry follows the instructions described in the chapter Materials and Methods and the manual of instructions by eBioscience for surface target staining for flow cytometry¹. The used antibody clone for CD103 (alpha_Ebeta₇) is 2E7. The intracellular Foxp3 staining follows

the instructions described by eBioscience for intracellular (nuclear) proteins² with the Foxp3/Transcription Factor Staining Buffer Set and the Foxp3 antibody clone FJK-16s.

References:

- 1 Staining Cell Surface Antigens for Flow Cytometry Protocol – Protocol A: Cell Suspensions <http://www.ebioscience.com/resources/best-protocols/flow-cytometry-protocols.htm>)
- 2 Staining Intracellular Antigens for Flow Cytometry Protocol - Protocol B: One step protocol for (nuclear) intracellular proteins <http://www.ebioscience.com/resources/best-protocols/flow-cytometry-protocols.htm>)

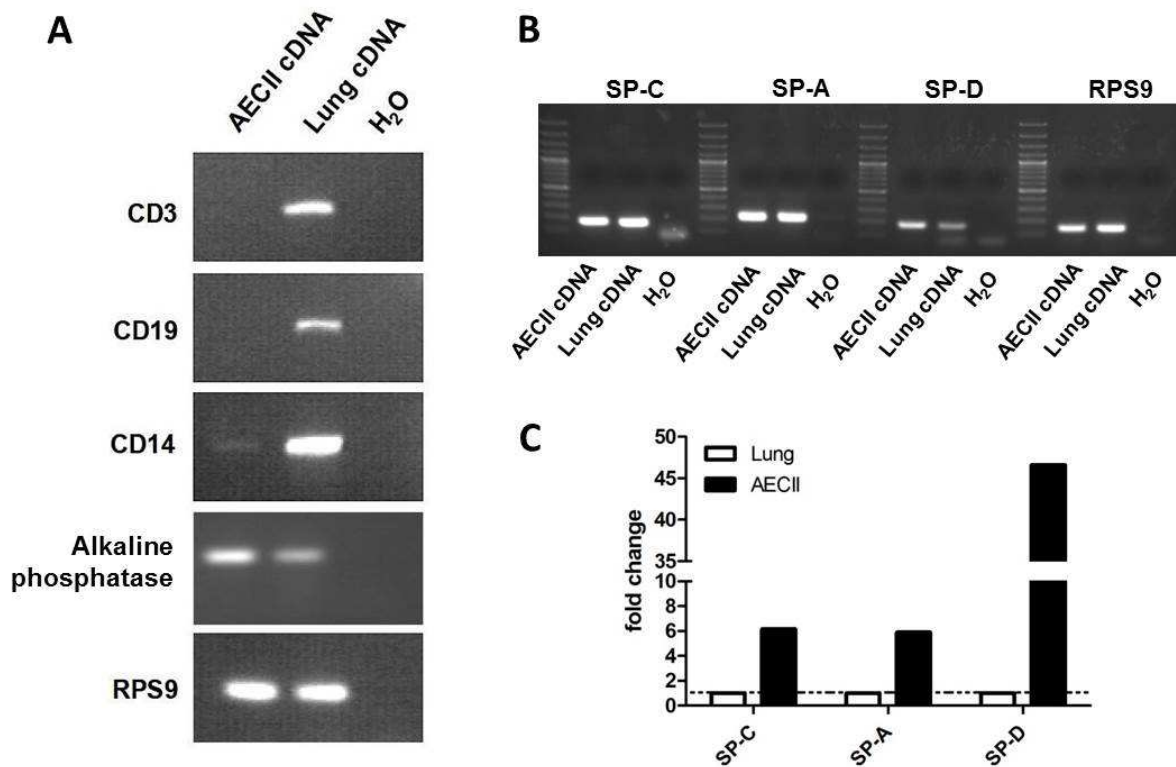


Figure 21: Analysis of hematopoietic cell contamination of post-sorted AECII population by PCR. (A) RNA from freshly isolated alveolar type II epithelial cells was compared with RNA obtained from complete lung tissue. Primer pairs for CD3, CD19 and CD14 were chosen to test for hematopoietic cells in the type II cell preparation. (B) Primer pairs for SP-A, SP-C, SP-D and alkaline phosphatase (Figure 21 A) were chosen to test the RNA for AECII-specific genes. (C) Quantitative RT-PCR analysis on cDNA of freshly isolated alveolar type II epithelial cells were performed to demonstrate the enriched expression of AECII marker genes SP-C, SP-A and SP-D in comparison to cDNA of whole lung tissue. RPS9 represents the housekeeping gene expression and was used in all cases to proof cDNA quality.

In the original figure 21 the picture of the semi-quantitative RT-PCR analysis of SP-C with cDNA derived from alveolar type II epithelial cells were interchanged with the RPS9 control from figure 24 unintentionally. This picture was replaced by new pictures of a semi-quantitative RT-PCR analysis of AECII specific genes (SP-C, SP-A and SP-D) with cDNA derived from freshly isolated AECII shown in figure 21 B. Moreover, the quantitative RT-PCR analyses of AECII-specific genes (SP-C, SP-A and SP-D) with cDNA from freshly isolated AECII in comparison with cDNA of whole lung homogenates (figure 21 C) were added. Isolation of RNA and cDNA synthesis follows the protocols described in chapter Materials and Methods. Figure 21 C clearly shows the elevated level of AECII-specific gene transcripts in comparison to whole lung homogenates suggesting a successful AECII isolation.

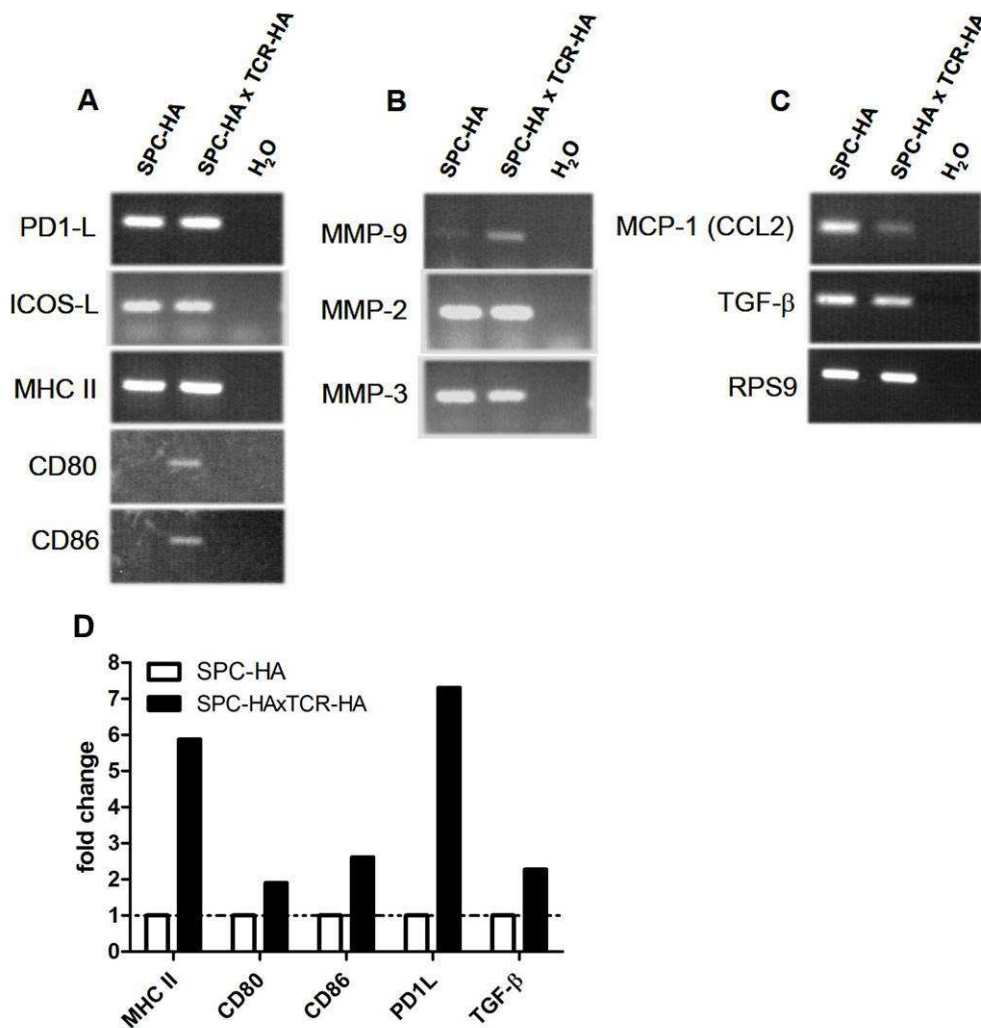


Figure 24: Gene expression in AECII derived from SPC-HA x TCR-HA double transgenic mice and SPC-HA transgenic mice. Different genes were selected to analyze their expression level in AECII by RT-PCR. (A) Genes encoding costimulatory molecules (PD1-L, ICOS-L, MHC II, CD80 and CD86) were used to determine the stimulatory capacity of AECII on the molecular level. (B) represents selected genes for Matrix metalloproteinase (MMP-9, MMP-2 and MMP-3) and (C) MCP-1 and TGF-β. The housekeeping gene RPS9 was used to estimate the quality and quantity of used cDNA. (D) Quantitative RT-PCR analysis of AECII derived from diseased SPC-HA x TCR-HA and healthy SPC-HA control mice reveal elevated expression levels of MHC II, CD80, CD86, PD1L and TGF-β in AECII isolated from SPC-HA x TCR-HA mice.

To underline the relative expression data from figure 24 A-C selected genes (MHCII, CD80, CD86, PD1L and TGF-β) were analyzed again by quantitative RT-PCR. Isolation of RNA and cDNA synthesis follows the descriptions in chapter Material & Methods. All tested genes show an increased expression in AECII derived from SPC-HAxTCR-HA mice in comparison to SPC-HA mice suggesting an inflammation mediated gene expression profile in AECII from

chronic diseased SPC-HA x TCR-HA mice. Isolation of RNA and cDNA synthesis follows the protocols described in chapter Materials and Methods.

Figure 34 page 82

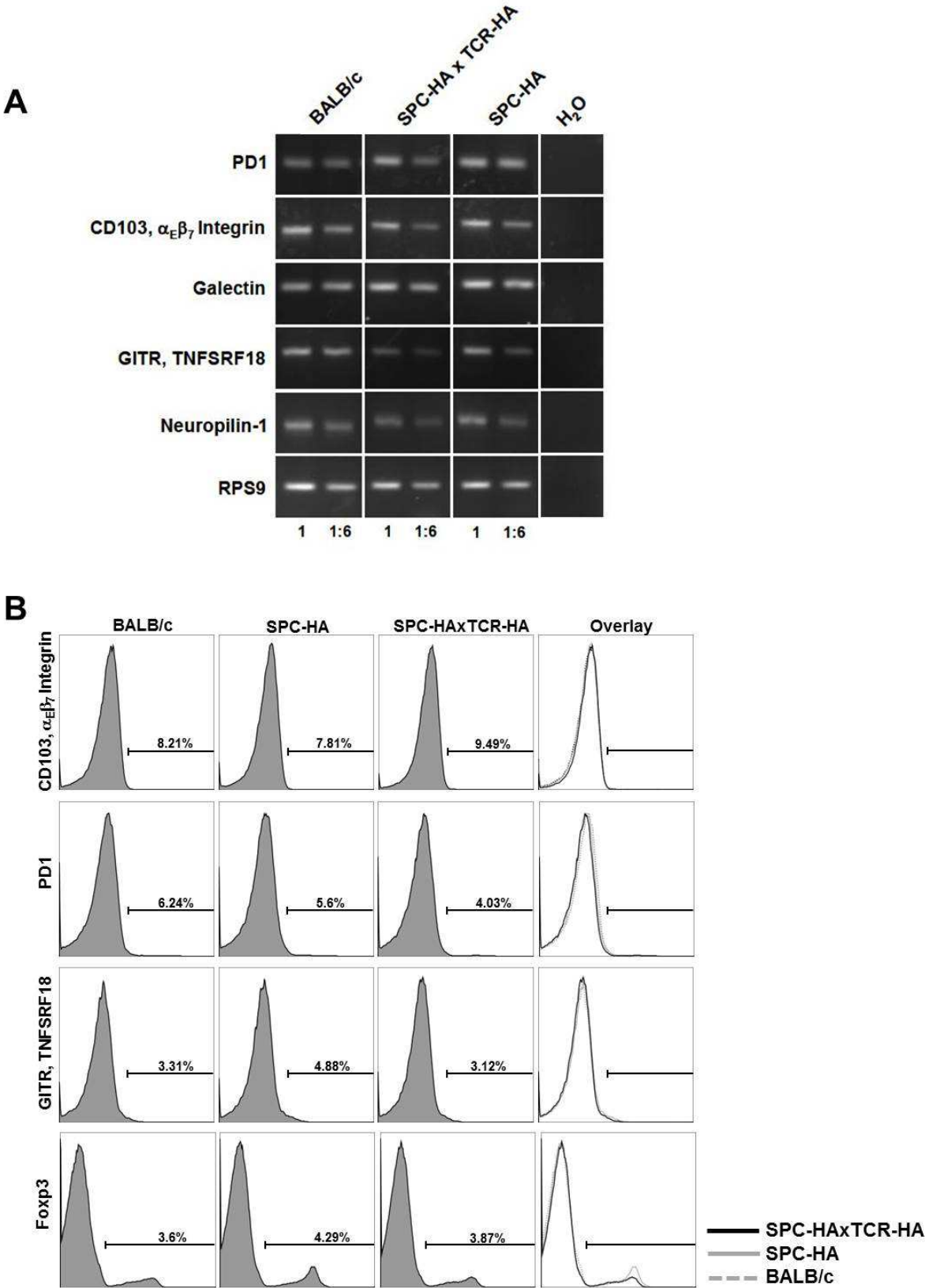


Figure 34: Semi-quantitative RT-PCR analysis of re-isolated CD4⁺T cells after prior coculture with AECII from SPC-HA x TCR-HA, SPC-HA and BALB/c mice. (A) To assess the effect of AECII derived from SPC-HA x TCR-HA double transgenic mice, SPC-HA transgenic mice and BALB/c control mice on the phenotype of antigen-specific CD4⁺ T cells the expression of different molecular marker genes for regulatory T cells including CD103 (alpha_Ebeta₇), Neuropilin-1, GITR (TNFRSF18), Galectin and PD-1 were analyzed. RPS9 was used as a housekeeping gene. Different amounts of cDNA (undiluted (1) and diluted (1:6) were used for the semi-quantitative RT-PCR analyses. (B) In parallel re-isolated HA-specific CD4⁺ T cells after prior coculture with AECII were analyzed by flow cytometry for the regulatory marker proteins CD103 (alpha_Ebeta₇), PD-1, GITR (TNFRSF18) and Foxp3.

In the original figure 34 the corresponding water controls of each semi-quantitative RT-PCR has been pasted in together with pictures of the corresponding SPC-HA (AECII/T cell) coculture experiment. In the new figure 34 (see above) the corresponding water controls were depicted from the corresponding SPC-HA (AECII/T cell) coculture experiment separately to indicate the different gels.

Moreover, the results for Foxp3 and IL10 in the original figure were removed since the results of the SPC-HA (AECII/T cell) coculture were interchanged unintentionally. As a substitute flow cytometric analysis of cocultured CD4⁺6.5⁺ T cells from a similar experiment were shown in figure 34 B. Here, the expression pattern of the regulatory marker proteins CD103 (alpha_Ebeta₇), PD-1, GITR (TNFRSF18) and Foxp3 were analyzed for CD4⁺6.5⁺ T cells after coculture with the indicated AECII. The results show no differences within the groups and underline the findings from the original figure 34 A. Surface staining for flow cytometry follows the instructions described by the chapter Materials and Methods and the manual of instructions by eBioscience for surface target staining for flow cytometry¹. The used antibody clones are for CD103 (alpha_Ebeta₇) / 2E7, PD-1 / J43 and GITR (TNFRSF18) / 108,619. The intracellular Foxp3 staining follows the instructions described by eBioscience for intracellular (nuclear) proteins² with the Foxp3/Transcription Factor Staining Buffer Set and the Foxp3 antibody clone FJK-16s.

References:

- 1 Staining Cell Surface Antigens for Flow Cytometry Protocol – Protocol A: Cell Suspensions <http://www.ebioscience.com/resources/best-protocols/flow-cytometry-protocols.htm>
- 2 Staining Intracellular Antigens for Flow Cytometry Protocol - Protocol B: One step protocol for (nuclear) intracellular proteins <http://www.ebioscience.com/resources/best-protocols/flow-cytometry-protocols.htm>

CHAPTER I

Introduction

1 The mucosal immune system

The mucosal surfaces of gastrointestinal and respiratory tracts, like the skin, are colonized by lymphocytes and accessory cells in order to respond optimally to ingested and inhaled antigens. Although the presence of lymphocytes in the mucosa and submucosa of the gastrointestinal and respiratory tracts has been recognized for many decades, the idea of a specialized mucosal immune system is relatively new. Like the skin, these mucosal epithelia are barriers between the internal and external environments and are, therefore, an important first line of defence against infections.

Much of our knowledge of mucosal immunity is based on studies of the gastrointestinal tract. In comparison, little is known about immune responses in the respiratory mucosa, even though the airways are a major portal of antigen entry. It is likely, however, that the features of immune responses are similar in all mucosal lymphoid tissues. The two major functions of the lung are the transport of air and subsequent gas exchange. The air-conducting parts are covered by a barrier-forming mucosa, while the gas-exchange parts, such as the alveoli, are devoid of such a barrier, as a mucosa would severely impede the rapid and effective gas exchange. Thus, the protective mechanisms are essentially different in these two compartments of the lung. The mucosa has a further function, which is to take up antigen in order to initiate an effective immune response against possible infections by tolerance, humoral or cellular immune reactions (Brandtzaeg, 1992; Brandtzaeg et al., 1997; Ogra et al., 1994).

In the intestine exists specialized structures for antigen uptake. These comprise Peyer's patches (PP) and other compartments for the effector side of the immune system, such as plasma cells in the lamina propria and effector T lymphocytes in the epithelium. The immune system of the lung can be compared with that of the intestine; even when major differences exist (see also table 1).

Table 1: Lymphocyte compartments: a comparison between lung and gut.

Lung	Gut
Central airways (mucosal lining)	All parts of the gut
Intraepithelial lymphocytes (IEL)	Intraepithelial lymphocytes (IEL)
Lamina propria	Lamina propria
Bronchus-associated lymphoid tissue (BALT)	Peyer's patches (gut-associated lymphoid tissue-GALT)
Peripheral airways (without mucosa)	
Intestinal lymphocytes	No real equivalent
Lymphoid aggregations	No real equivalent
Lymphocytes in the bronchoalveolar space	No real equivalent
Large intravascular lymphocyte pool	Undefined intravascular lymphocyte pool

(adapted from Pabst, 2000)

The bronchus-associated lymphoid tissue (BALT) constitutes organized lymphoid aggregates of T and B cell that are capable to respond against to inhaled antigens. BALT, located mostly at bifurcations of the bronchus in animals and humans, is already present in the fetus and develops rapidly following birth, especially in the presence of antigens. Humoral immune responses elicited by BALT are based primarily in immunoglobulin A secretion, locally and by BALT-derived B cells that have trafficked to distant mucosal sites. Similarly, located T cell responses have been noted. On the basis of these findings, the BALT can be thought of as functionally analogous to mucosal lymphoid aggregates in the intestine and is deemed a member of the common mucosal immunologic system (Bienenstock and McDermott, 2005).

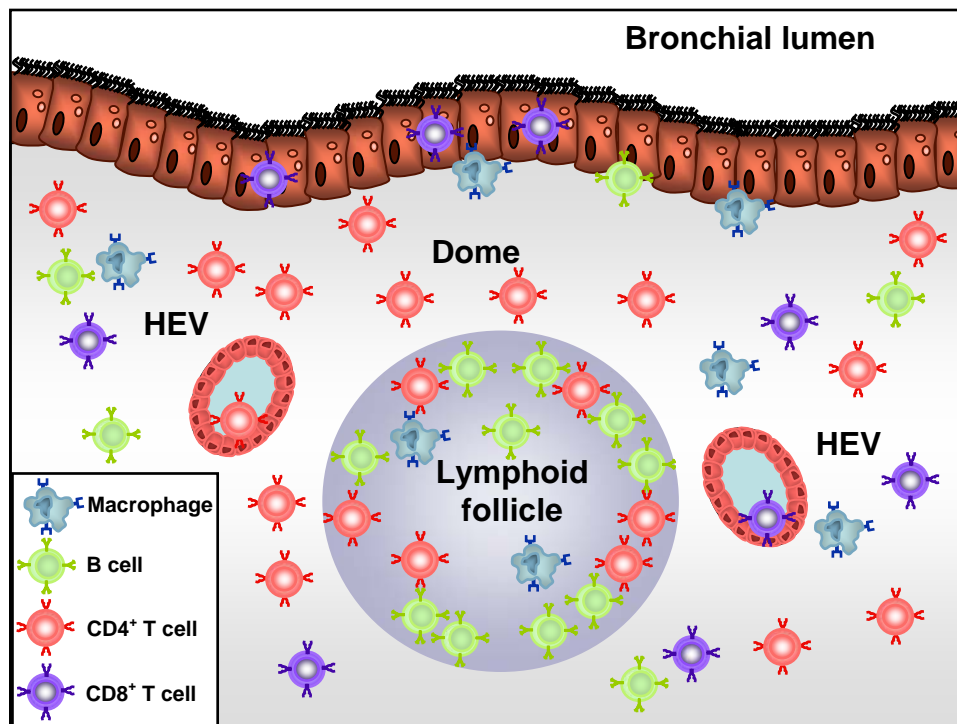


Figure 1: The basic structure of BALT and their distribution of lymphocyte subsets and macrophages. In BALT the most prominent structure is a follicular-like accumulation of lymphocytes, partly forming a classical germinal centre. This is surrounded by more mature, small lymphocytes. High endothelial venuels (HEV) are located even more peripherally. In the walls of the HEV, lymphocytes can be seen leaving the blood and thus entering BALT. As there are no afferent lymphatic vessels, these HEV are the only site of entry for lymphocytes to migrate into the BALT. In the direction of the bronchial epithelium, a dome-like protrusion (similar to Peyer's Patches) into the bronchial lumen is sometimes obvious. This basic structure seems to be valid for all species, but there are many differences in details (adapted from Tschernig and Pabst, 2000).

2 The respiratory tract in context of immunity

Due to its function, i.e. to allow gas-exchange in organisms, the lung has a very exposed position to the external environment. The human lung is exposed daily to over 10,000 litres of inspired, ambient air and the continuous aspiration of small amounts of nasopharyngeal secretions during sleep. Depending on the quality of air in the environment or the resident flora in the nasopharynx, the respiratory tree faces the enormous task of oxygenating blood across a moist, thin alveolar-capillary wall (approximately 1 μ m) and yet resisting infection. Mechanical mechanisms and other innate host defences and adaptive immune responses are important in preventing infection.

The lung, similar to other epithelial surfaces that face with the environment, has developed several strategies to avoid infection. One important feature of the host immune system is the ability to downregulate both non-specific and immune-mediated inflammation. Hence, the immune apparatus of the lung, as the lung itself, represents a sensitive system that coordinates immune responses to avoid immunological collapses by creating a tolerant and suppressive milieu.

2.1 Immune cells and structures of the lung

Considering the development of pulmonary immunity in a spatial context, it is useful to divide the progression of an immune response in the lung into three distinct but overlapping phases: (1) in the afferent phase, the antigen reaches the lung, is taken up by antigen-presenting cells (APC), and is presented to naïve T cells expressing the relevant T cell receptors (TCR); (2) in the central processing phase, specific lymphocyte clones expand and differentiate; and (3) in the effector phase, effector T cells and B lymphoblasts find their way to pulmonary sites requiring expression of a specific immune response. At each phase, events must be tightly regulated to allow an effective immune response yet to avoid excess or a potentially destructive inflammation (Lipscomb et al., 1995). The place in the respiratory tract, where each of these phases occurs, is somewhat controversial. The majority of evidences indicate that in the normal host after reaching the lungs, antigen is carried on APC, in phagocytes, or free in lymphatic fluid to draining lung-associated lymph nodes

(Lauweryns and Baert, 1976-1977; Lehnert, 1992) where central processing occurs. Effector cells are released into the efferent lymph and reach the blood stream recruiting from the vasculature into the lung (Berman et al., 1990).

The cells that are the major initiators and regulators of immunity in the lung include macrophages, dendritic cells (DC), and lymphocytes. However, other cells in the milieu, for example epithelial cells, fibroblasts, mast cells and various recruited blood leukocytes also play important regulatory roles.

2.1.1 Macrophages

Lung macrophages reside within the airways at all levels of the respiratory tract, in the lamina propria, the interstitium, the alveolar regions and pleura, and within pleural spaces (Brain, 1992; Lehnert, 1992). All lung macrophages originate from the bone marrow. Most of the clearance of small inhaled particles and microorganisms reaching the periphery of the lung relies on the phagocytic system (Jonsson et al., 1985). Macrophages are the primary phagocytes in the healthy lung. The macrophages interact with other cells and molecules through the release of numerous secretory products and the expression of several surface receptors (Delacourt et al., 1997). Pulmonary macrophages are both phenotypically and functionally diverse, so that at least three different groups of pulmonary macrophages can be observed: alveolar macrophages (AM), intraluminal macrophages and interstitial macrophages. Many *in vitro* studies examined the role of AM in pulmonary immunity and in particular their capacity to support T and B cell activation. Nevertheless, AM are inefficient in providing accessory cell activity *in vitro* compared to macrophages derived from other sources or monocytes (Holt, 1993). In fact, AM have been shown to be efficient suppressors of T cell activation and antibody production by B cells (Thepen et al., 1994). Furthermore, intraepithelial DC from lung have shown a downmodulation of antigen-presenting capacities of DC in the bronchial and alveolar environment by the respective macrophage population (Bilyk and Holt, 1993). Therefore, not only T cells, but also DC are maintained in a downmodulated state in the airways, tightly controlled by high numbers of nearby macrophages.

The factors involved in T cell and DC downmodulation by AM are partly known: In mice, nitric oxide (NO) produced by AM is the major source of this immunomodulation (Strickland et al., 1996). Several other immunomodulating factors produced by AM have been isolated, the most important being prostaglandins and cytokines. Prostaglandin E₂ (PGE₂) enhances interleukin (IL)-10 transcription and protein production by peripheral blood lymphocyte (Huang et al., 1996) which offers possibilities for potent immunomodulation. Finally, PGE₂ has been ascribed as substance deactivating AM and T cell, and it is produced by AM (Kawano et al., 1999).

2.1.2 Dendritic cells

The other important group of APC include the pulmonary dendritic cells (DC). DC play a critical role in sampling and presenting antigen in the lung, which leads to activation and expansion of CD4⁺ T cells and preferential production of T helper subset 2 (T_H2)-based adaptive immune responses (Kearney et al., 1994; Lambrecht et al., 2000). In the lung, DC reside within and beneath airway epithelium, in alveolar septae, in the connective tissue surrounding pulmonary veins and airway vessels, and with the lung capillaries of the lung parenchyma (Lipscomb et al., 1995). DC in the airway epithelium have an immature phenotype and exhibit a rapid turnover (Holt et al., 1994). DC that are resident within alveolar septae and in connective tissue surrounding vessels have a more mature phenotype than airway DC (Gong et al., 1992). In contrast to DC that are resident within the lung, in the vascular compartment circulating precursor DC are present (Suda et al., 1998). One role of lung DC is to provide protection against infectious agents by initiating immune response. An equally important role is to generate tolerance to inhaled allergens in normal noninflamed lungs. In this regard, immature DC continuously leave the peripheral blood and take over a surveillance position in lung tissue, avidly sampling the antigenic environment. In the steady state, lung DC likely remain relatively immature and constitutively migrate in low numbers into regional lymph nodes where they induce anergy, deletion of T cells, or a weak T_H2-like response to air-borne antigens that is eventually downregulated (Stumbles et al., 1998). Active suppression of immature DC maturation by alveolar macrophages may explain why airway and

intraepithelial DC remain immature during their steady-state migration to lung-associated lymph nodes (Holt, 1993; Lipscomb et al., 1993). Furthermore, autocrine production of IL-10 by immature DC can inhibit surface expression of MHC class-I and -II molecules and exert a generalized inhibitory effect on T cell proliferation (Stumbles et al., 1998). On exposure to inhaled allergens, the antigen may simply be insufficient in providing a danger signal to overcome suppression by alveolar macrophages and IL-10. However, if a danger signal is present at the tissue site, DC mature and migrate in greater number to draining lymph nodes to stimulate CD4⁺ T cell clonal expansion and differentiation.

2.1.3 Lymphocytes

Lymphocytes play a significant role in lung disorders, for example sarcoidosis, asthma, and rejection after transplantation (Berman et al., 1990). T cells, B cells, and NK cells are present in various lung compartments (Agostini et al., 1993; Holt and Schon-Hegrad, 1987; Pabst, 1990; Stein-Streilein, 1988), for example the lung vascular bed, the lung interstitium, the epithelium and lamina propria of the bronchi and the bronchoalveolar space (Fliegert et al., 1996; Pabst et al., 1995). Since the lung has no afferent lymphatic vessels, the blood is the starting point for lymphocyte immigration. Lymphocytes migrate to the lung vascular endothelium (marginal pool) and enter the interstitial lung tissue (interstitial pool), where the lymphocyte composition is different from blood and bronchoalveolar lavages (BAL) (Fliegert et al., 1996). In contrast to peripheral blood lymphocytes, mainly activated T cells are found in BAL of humans and mice (Curtis et al., 1995; Saltini et al., 1990). This immunological status confers lymphocytes appropriate defense mechanisms to such a vulnerable organ. The expression of adhesion molecules depends on the compartment from which the cells are recruited, indicating that local activation and expression of adhesion molecules is induced by the microenvironment. Possible candidates for such activators are dendritic cells and macrophages or components of the extracellular matrix (Holt, 1993; van Haarst et al., 1994).

2.1.4 Airway epithelium

In addition to the aforementioned migrating cells of the respiratory tract, the resident structural cells building the airway epithelium have important functions in modulation of immune responses in the lung (figure 2). The epithelium constitutes the interface between the internal milieu and the external environment, and as such, it is the first point of contact for inhaled substances, in particular, respiratory viruses, airborne allergens, and environmental pollutants, as well as being a primary target for inhaled respiratory drugs (Folkerts et al., 1998; Gizycki et al., 1997).

At least eight morphologically distinct epithelial cell types are present in human respiratory epithelium, which can be classified in three different categories: basal, ciliated and secretory epithelial cells (Spina, 1998). Columnar ciliated epithelial cells are the predominant cell type within the airways, constituting more than 50% of all epithelial cells (Spina, 1998). The primary role of the ciliated apical surface is highlighted by the directional transport of mucus from the lung to the throat (Harkema et al., 1991). Mucus cells (goblet cells) are responsible for the control of the correct amount of mucus and the viscoelasticity of mucus for efficient mucociliary clearance by releasing acid mucins from their granules. These cells are thought to be capable of self-renewal and may also differentiate into ciliated epithelial cells (Evans et al., 1988; Harkema et al., 1991). Serous cells are also secreting cells and produce neutral mucin and a yet unidentified non-mucoid substance (Knight and Holgate, 2003). Basal cells are ubiquitous in the conducting epithelium, although the number of these cells decreases with airway size and the increasing thickness of the basal cell layer correlates with increasing size of the airway (Evans et al., 1988; Evans et al., 1990). Similar to the skin, the basal cell is thought to be the primary stem cell, giving rise to the mucus and ciliated epithelial cells. In smaller airways, where basal cells are sparse or absent, Clara cells perform the primary stem cell role.

In addition to their progenitor and structural roles, basal cells are also thought to secrete a number of bioactive molecules including neutral endopeptidase, 15-lipoxygenase products and cytokines (Knight and Holgate, 2003). In humans, Clara cells are located in large (bronchial) and small (bronchiolar) airways. The cells produce bronchiolar surfactant and are also characterized by agranular endoplasmic reticulum in the apical cytoplasm and granular endoplasmic reticulum basally. In addition to their role in secretion, Clara cells are believed to metabolize xenobiotic

compounds by the action of p450 mono-oxygenases and may also produce specific antiproteases such as secretory leukocyte protease inhibitor (De Water et al., 1986). More recent evidence suggests that these cells play important role for stem cells, serving as a progenitor for both ciliated and mucus secreting cells (Hong et al., 2001).

The major function of the respiratory epithelium was once thought to be primarily that of a physical barrier, but recent studies clearly indicate that it is metabolically very active with the capacity to modulate a variety of inflammatory processes through the agency of an array of receptor-mediated events. On activation, it has the capacity to produce a number of proinflammatory cytokines, proinflammatory or regulatory mediators including arachidonic acid products, nitric oxide, endothelin-1, transforming growth factor (TGF)- β , tumour necrosis factor (TNF)- α , and cytokines such as interleukin (IL)-1, IL-6 and IL-8 (Knight and Holgate, 2003).

Table 2: Immunomodulatory cell-cell interactions in the lower respiratory tract.

Effector cell	Target cell	Target cell function	Effector cell mechanism	Stimulation (+) Suppression (-)
Macrophage	T cell	proliferation	NO secretion	-
	T cell	IL-10 secretion	PGE ₂ secretion	-
	T cell	cytokine production	TGF- β secretion	-
	B cell	antibody production	NO secretion	-
	DC	antigen presentation	NO secretion	-
	DC	maturation	NO secretion	-
	macrophage	apoptosis	IL-10 & TGF- β	-
Epithelial cell	T cell	proliferation	NO secretion	-
	eosinophil	apoptosis	NO secretion	-
	macrophage	apoptosis	TGF- β secretion	-
	macrophage	TNF- α secretion	NO secretion	-
T helper 2 cell	macrophage	metalloproteinase	IL-4 secretion	-
	macrophage	PGE ₂ secretion	IL-4 secretion	-
	macrophage	apoptosis	IL-10 secretion	-
	T helper 2 cell	IL-12 secretion	IL-4 secretion	-
T helper 1 cell	macrophage	NO secretion	IFN- γ secretion*	+
	T helper 2 cell	IL-4 secretion	IFN- γ secretion	-
AM & epithelial	T helper 1 cell	IFN- γ secretion	NO secretion	-

Summary of immunomodulatory cell-cell interactions, which can occur in lower respiratory tract. *IFN- γ secretion by T helper 1 cells is not a directly immunomodulatory interaction between T helper 1 cells and macrophages. This mechanism has been included for its pivotal role activating NOS2 transcription via STAT1 in macrophages and epithelial cells (adapted from Bingisser and Holt, 2001).

2.1.5 Alveolar epithelium – Type II and Type I alveolar epithelial cells

Alveoli are the gas exchange units of the lung and the alveolar epithelium adapts to this functional role by developing two highly specialized alveolar epithelial cell types, which are morphologically and functionally different. Alveolar epithelial type II cells (AECII, granular pneumocyte, type II pneumocyte, giant corner cell) are cuboidal, with a diameter of $\sim 10\mu\text{m}$ and a volume of $\sim 450\text{-}900\mu\text{m}^3$. Alveolar epithelial type I cells (AECI) are squamous in shape, with a diameter of $\sim 50\text{-}100\mu\text{m}$ and a volume of $\sim 2000\text{-}3000\mu\text{m}^3$ (Crandall et al., 2001). AECII consist of about 15% of the distal lung cells and occupy 5% of the alveolar surface (Crapo et al., 1978; Crapo et al., 1982; Haies et al., 1981). The alveolar type II cells perform a variety of important functions within the lung, including regulation of surfactant metabolism, ion transport, and alveolar repair in response to injury. AECII synthesize and secrete lung surfactant, a protein-lipid complex and surface-active material. Lung surfactant stabilizes alveoli by reducing the surface tension. Ultrastructural criteria to identify alveolar type II epithelial cells are the presence of lamellar bodies, apical microvilli and cell-cell junctions. AECII also maintain the alveolar epithelium by cell proliferation and differentiation, minimize alveolar fluid by transport of sodium from the apical to the basolateral side, and alter the inflammatory process by secretion of growth factors and cytokines.

In contrast to AECII, AECI contribute 7% of total lung cells and cover more than 95% of the alveolar surface. This thin epithelium allows the easy diffusion of gases and forms a barrier against the indiscriminate leakage of fluid into alveolar spaces. It also regulates the exchange of physiologically important solutes and water between circulating blood and the alveolar space. Recent studies indicate the presence of transport proteins such as $\text{Na}^+\text{-K}^+\text{-ATPases}$, epithelial sodium channels and aquaporin (Aqp) water channels in AECI, therefore implying an active role in regulation of lung fluid homeostasis (Borok et al., 2002; Johnson et al., 2002; Matthay et al., 2002). These cells also might be involved in the transport of macromolecules due to the abundance of microvesicles (caveolae) (Gumbleton et al., 2003).

The alveolar epithelium can be classified as a continuously renewing tissue since it comprises a population of alveolar type II epithelial cells that are characterized by almost unlimited potential to proliferate. It is still a matter of debate whether all AECII or only a small population act as the alveolar epithelial stem cell population (Uhal, 1997). The concept of AECII as stem cells of the adult alveolar epithelium was proposed by Kapanci and colleagues, and is widely accepted today (Kapanci et al., 1969). During ontogenesis, the AECII may derive from precursor cell common to AECII and Clara cells (Wuenschell et al., 1996). Furthermore AECII proliferate and differentiate to AECl to repair the damaged alveolar epithelium after lung injury or during fetal lung development, thus contributing to epithelial repair, whereas AECl are terminally differentiated, lack mitotic activity, and are easily injured. The programmed cell death or apoptosis is an important mechanism of cell removal or renewing of tissue. AECII are known to express the membrane receptor Fas (CD95, APO-1), the ligation of which may initiate the apoptotic cascade (Fine et al., 1997). This can be achieved by binding of Fas-ligand or the Fas-stimulating antibodies. There is some evidence that apoptosis of AECII is an integral mechanism of alveolar septal modelling in lung morphogenesis (Scavo et al., 1998; Schittny et al., 1998). Notably, apoptotic AECII appeared to be removed not only by alveolar macrophages but also by AECII cell neighbours (Fehrenbach et al., 2001).

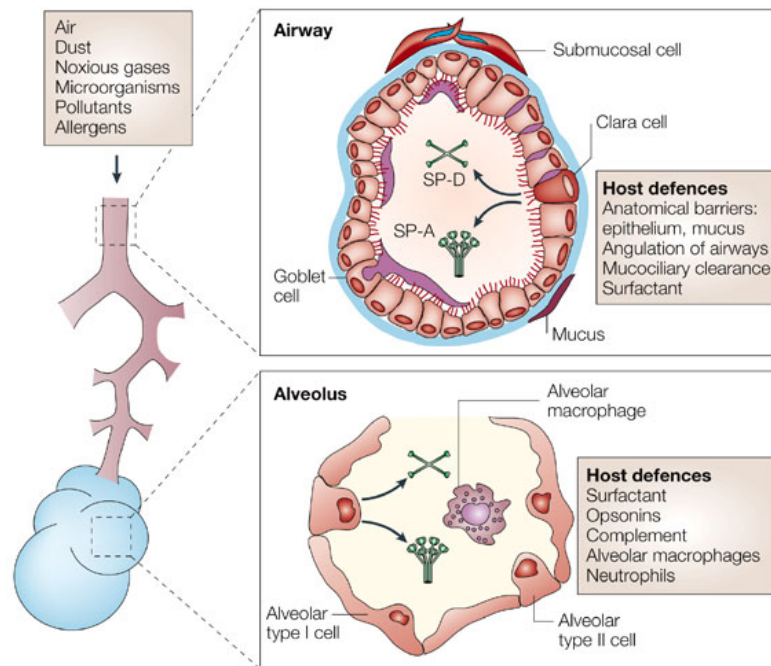


Figure 2: Schematic structure of airway and alveolus and their host-defence mechanisms. The lung is constantly challenged by inhaled pathogens, pollutants and particles. Several different defence mechanisms contribute to lung defence. These include filtration in the naso-oropharynx and conducting airways, sneezing, coughing and mucociliary clearance. Small particles might reach the alveolar gas-exchange regions of the lung. Host-defence functions in the peripheral air-spaces include surfactant, other opsonins (such as immunoglobulins) and innate immune cells (including alveolar macrophages and neutrophils). Surfactant protein A (SP-A); surfactant protein D (SP-D) (adapted from Wright, 2005).

2.2 The alveolar type II epithelial cell as an integrative unit of the alveolus

It is essential for AECII to interact with its resident as well as its mobile neighbour cells. AECII express a number of molecules necessary for the perception as well as the generation of signals involved in cell-cell as well as in cell-matrix interactions. Cell-cell interactions may be direct via contact of the cell membranes, or indirect via secreted and diffusible signals. Consequently, alveolar type II epithelial cells were termed as integrative units of the alveolus (Fehrenbach, 2001).

The best example for a cell-cell interaction between AECII and resident cells is the direct contact with AECl and during proliferation with AECII neighbours as well. These lateral cell-cell contacts within the alveolar epithelium are maintained by cell junction complex that includes gap junctions (Kasper et al, 1996). Additionally, AECII

have direct contact to fibroblasts at the basal membrane or with capillary endothelial cells (Marin et al., 1982).

A strong evidence for a direct interaction of AECI and AECII was presented by Ashino and colleagues (Ashino et al., 2000). Mechanical stimulation of AECI is thought to result in Ca^{2+} -oscillations, which were transmitted via intraepithelial gap junctions to AECII and modulate exocytosis rate of lamellar bodies. Direct inhibitory interactions between AECI and AECII have been postulated to suppress AECII proliferation. Loss of AECI during injury might then trigger the release of AECII from growth inhibition (Mason and McCormack, 1994). E-cadherin as a further candidate to mediate contact inhibition, has been localized to the basolateral membrane of AECII (Kasper et al., 1995; St. Croix et al., 1998).

But even an indirect cell-cell interaction for AECII to other AECII is possible by the negative feedback loop by which surfactant protein A (SP-A) upon release into the alveolar space inhibits surfactant exocytosis *in vitro* (Dobbs et al., 1987). Although AECII are equipped with membrane receptors for SP-A (Strayer et al., 1996), the *in vivo* relevance of this autocrine mechanism by which AECII may regulate their own action remains elusive, because mice that are deficient in SP-A did not show any defect in surfactant secretion nor any respiratory deficiency (Ikegami et al., 1998). Thus, some alternative mechanism must compensate the negative SP-A feedback loop.

Another potential feedback mechanism that has been postulated is the inhibition of AECII proliferation via AECII derived transforming growth factor (TGF)- β in bleomycin-induced experimental lung fibrosis (Khali et al., 1994). A number of growth factors are released by AECII, which might act in an autocrine way via the corresponding receptors expressed by AECII.

As mentioned before, fibroblasts are in contact to AECII. This reciprocal cell-cell interaction is relevant to the modelling of alveolus during lung morphogenesis as well as during remodelling associated with alveolar repair following lung injury (Kasper et al., 1996; O'Reilly et al., 1997; Shannon, et al., 1997). Both direct and indirect cell-cell interactions have been reported. Secreted factors from fibroblasts or AECII or the direct cell-cell contact from fibroblasts to AECII had influence to proliferation behaviour of AECII or fibroblasts (Adamson et al., 1991).

The interaction of alveolar epithelial and capillary endothelial cells is well examined. It was reported that from pulmonary endothelial cells conditioned medium stimulate fetal lung epithelial cell growth (Smith et al., 1986) and that endothelin-1 increases AECII surfactant secretion *in vitro* via a protein kinase C and Ca^{2+} -mediated pathway (Sen et al., 1994). As a source of endothelin-1, endothelial cells are therefore principally competent to act in a paracrine manner on AECII, which were reported to express the endothelin receptor A (Markewitz et al., 1995). Furthermore, alveolar type II epithelial cells themselves may synthesize endothelin-1 and stimulate endogenous prostaglandin E_2 synthesis in an autocrine fashion (Markewitz et al., 1995).

Recently, a very special mechanism of indirect intercellular communication between AECII and endothelial cells has been suggested. Stimulation of alveolar epithelial cells with tumour necrosis (TNF)- α was reported to increase epithelial Ca^{2+} influx and to activate epithelial cytoplasmic phospholipase A₂, and results in basolateral release of arachidonic acid. Free arachidonic acid is thought to increase endothelial Ca^{2+} influx and expression of P-selectin (Kuebler et al., 2000), which is known to be crucial for initiation of leukocyte adherence. Thus, AECII could act as transducers of an inflammatory signal from the alveolus to the capillary bed to recruit granulocytes to the site of inflammation.

Alveolar macrophages are one of the mobile cell types that interact with AECII. Among the multitude of secretory products synthesized and released by alveolar macrophages (Kasper et al., 1996; Lohmann-Matthes et al., 1994) there are some factors that act as mitogens for AECII, such as hepatocyte growth factor (Mason et al., 1994) and heparin-binding epidermal growth factor (Leslie et al., 1997). Conversely, AECII were shown to express the chemokines RANTES and MCP-1, which chemotactically attract macrophages (O'Brien et al., 1998), as well as GM-CSF (Blau et al., 1994; Christensen et al., 1995), which in turn may stimulate macrophage growth (Worgall et al., 1999). Furthermore, SP-A released from AECII modulate macrophage functions such as oxygen radical release (Weissbach et al. 1994) and nitric oxide production (Stamme et al., 2000).

Interactions of AECII with leukocytes have just recently come into focus. AECII synthesize some cytokines affecting leukocytes, such as interleukin (IL)-6 or IL-8. Via

these cytokines, AECII might be involved in the induction of differentiation of basophil, eosinophil, and neutrophil granulocytes and maintenance of inflammatory reactions. Recent data support the idea that AECII have an accessory function in T lymphocyte activation (Zissel et al., 2000). This has been suggested on the basis of the findings that the cells bear MHC class-II molecules (Schneeberger et al., 1986). Furthermore, the expression of costimulatory molecules by human type II alveolar cells and their ability to deliver costimulatory signals for T cells could be demonstrated, providing evidence that AECII are able to act as antigen-presenting and immunoregulatory cells of the lung. Additionally, the accessory function of AECII is under the control of endogenously released TGF- β (Zissel et al., 2000).

For murine type II alveolar epithelial cells it was demonstrated that after infection with pulmonary pathogens AECII became activated and had increased cell surface expression of MHC class-II, CD54, and CD95. AECII used the MHC class-II pathway to process and present mycobacterial antigens to primed CD4⁺ T cells and indicating participation in the effector phase of the immune response (Debbabi et al., 2005).

Additionally, AECII were reported to inhibit lymphocyte proliferation *in vitro* without altering their activation state (Paine et al., 1991). Moreover, AECII derived TGF- β (Zissel et al., 2000) could indirectly inhibit T cell proliferation via blockade of activating factors, such as IL-2. In contrast, granulocyte macrophage-colony stimulating factor (GM-CSF) released at the basolateral surface of AECII could increase the potential of dendritic cells to induce T-cell proliferation (Christensen et al., 1995).

2.3 The alveolar type II epithelial cell as the source of alveolar surfactant

Pulmonary surfactant was initially identified as a lipoprotein complex that reduces surface tension at the air-liquid interface of the lung (Clements, 1957; Pattle, 1955). The surface-active agent was characterized in numerous biochemical studies of BAL material and is now known to be composed of ~90% lipids (with ~80-90% phospholipids) and of ~10% proteins (Griese, 1999). Unlike most other lipid-rich components of cells and organs, the surfactant lipids are characterized by an

unusually high level of saturated fatty acid chains, such as the predominant dipalmitoylphosphatidylcholines, which contribute substantially to the unique properties of pulmonary surfactant (van Golde et al., 1994). The protein fraction comprises a highly variable amount of serum proteins (Griese; 1999) and four apoproteins that are associated with surfactant and contribute to its specific function (Weaver and Whitsett, 1991). These surfactant associated proteins were termed surfactant proteins (SP) -A, -B, -C and -D whereas SP-B and SP-C are extremely hydrophobic. SP-B is essential for the ability of surfactant to reduce surface tension (Nogee, 2004), and SP-C has recently been shown to bind lipopolysaccharide (LPS) (Augusto et al., 2002; Augusto et al., 2003). In the absence of surfactant, surface tension is extremely high at end expiration and tends to collapse the lung. This makes breathing difficult to the extent that respiration is frequently impossible without ventilatory support and surfactant replacement. A deficiency of surfactant – which can result in “Respiratory-Distress Syndrome (RDS)” – occurs when infants are born prematurely, before their surfactant biosynthetic machinery has matured. Treatment of these premature infants with exogenous surfactant replacement reduces mortality and morbidity, because of this disease (Wright, 2005).

The other function of alveolar surfactant relies on the nature of SP-A and SP-D as collectins. Both proteins are able to bind to the surface of various pathogens, thus acting as opsonins to facilitate their elimination by alveolar macrophages. Therefore, alveolar surfactant is also responsible for host defence (Crouch, 2000; Pison et al., 1994; Wright, 1998).

Surfactant is synthesized by alveolar type II epithelial cells and released upon appropriate stimuli by exocytosis from special intracellular storage organelles termed lamellar bodies. Once released into the alveolar space, freshly secreted lamellar body material undergoes several steps of transformation that are necessary to establish the surface-active lining layer. Cyclic compression and expansion during ventilation result in a fraction of spent surfactant that will largely be recycled by AECII. Thus, single constituents of surfactant run through several cycles before being removed by alveolar macrophages and replaced by *de novo* synthesis (Fehrenbach, 2001).

Although the bronchiolar Clara cells and submucosal cells also synthesize and release the mature proteins SP-A, SP-B and SP-D (Kalina et al., 1992; Voorhout et al., 1992) the alveolar type II epithelial cell is the only type of pulmonary cell that

produces all surfactant components including phospholipids as well as all four surfactant proteins. The mature 3.5 -3.7kDa small SP-C is thought to be exclusively released by AECII cells (Beers et al., 1994; Phelps and Floros et al., 1991).

About 85% of the secreted surfactant is taken up again, metabolised and re-secreted by AECII. Re-uptake and recycling have been demonstrated for all surfactant lipids and for all four surfactant proteins. The degradation of surfactant is accomplished by alveolar macrophages with only minimal contribution (Herbein et al., 2000; Nicholas, 1996; Young et al., 1993).

2.3.1 Immunoregulatory functions of surfactant proteins

As mentioned above, the host defence functions of surfactant are primarily mediated by SP-A and SP-D, which are members of the collectin family of proteins. SP-A and SP-D have been also localized to non-pulmonary sites, including the trachea, brain, testes, salivary glands, lachrymal glands, heart, prostate, kidney, pancreas and the female urogenital tract (Leth-Larsen et al., 2004; Lin et al., 2000; Madsen et al., 2000; Rubio et al., 1995), although it is not yet clear whether all of these organs express sufficient amounts of protein for it to be physiologically effective.

Among their well-established role as opsonins, SP-A and SP-D also have functions in initiating parturition, facilitating clearance of apoptotic cells and directly killing bacteria.

2.3.2 Collectin structure

In addition to the two lung collectins SP-A and SP-D, serum collectins have been identified in humans (mannose-binding lectin, MBL) and in bovidae (conglutinin, CL-43 and CL-46) (Hansen and Holmskov, 2002).

SP-A and SP-D are synthesized as primary translation products of approximately 26-36kDa and 43kDa, respectively (figure 3). The collagen-like domain is N-terminal to a coiled-coil structure that precedes the C-terminal lectin domain. The lectin domains mediate the interaction of collectins with a wide variety of pathogens. The collagen domains vary greatly in length (Holmskov et al., 2003).

The collectins are assembled as trimeric subunits, which multimerize to varying degrees. SP-A is mainly an octadecamer and forms a bouquet-like structure that is similar to MBL, whereas SP-D forms a dodecamer (Wright, 2005).

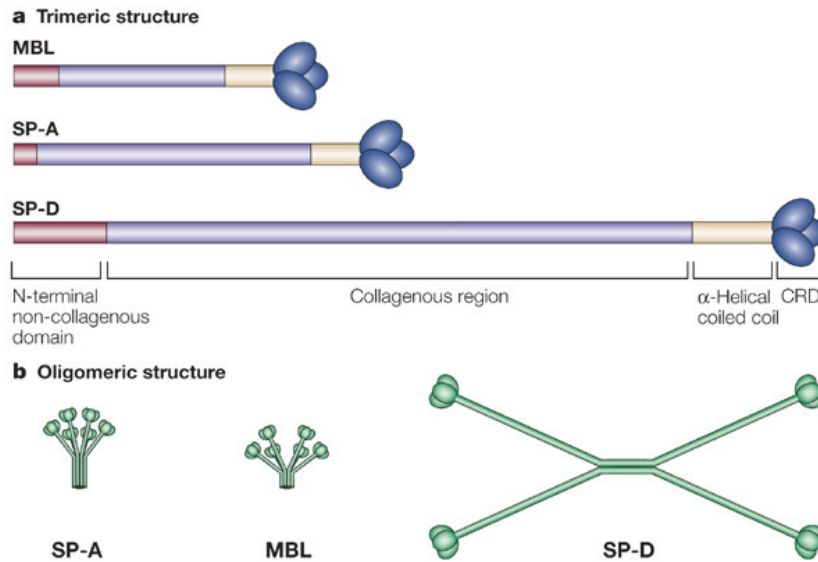


Figure 3: Collectin and C1q structure. Surfactant protein A (SP-A) and SP-D are members of a family of proteins known as collectins. **a** Collectins have collagen-like amino (N)-terminal regions and C-type (calcium dependent) carbohydrate-recognition domains (CRDs). Collectins consist of structural subunits that are composed of trimeric polypeptide chains, which are identical except for human SP-A. The trimers are assembled into oligomers. **b** SP-A and mannose-binding lectin (MBL) are octadecamers (18-mers), consisting of six trimeric subunits. SP-D is a dodecamer (12-mer), consisting of four trimeric subunits. Although C1q is structurally homologous to SP-A and MBL, it is not a collectin as it does not have a lectin domain (CRD) (adapted from Wright, 2005).

The lung collectins have both distinct and common carbohydrate-binding activities (Wright, 2005). For example, both surfactant proteins, SP-A and SP-D, bind to mannose and glucose but bind only poorly to galactose (Haagsman et al., 1987; Lim et al.; 1994; Persson et al., 1990). The high affinity of the collectins for clustered oligosaccharides is thought to be important for their ability to distinguish non-self from self, as most carbohydrates in animals are terminated by sugars, such as galactose or sialic acid, that are poorly recognized by the collectins.

2.3.3 Collectin regulation of immune cells

Surfactant proteins A and D bind to a variety of bacteria, viruses, allergens and apoptotic cells and thereby function as opsonins to enhance the uptake of these cells and particles. Binding of the collectins to pathogens occurs by various mechanisms. Some pathogens are aggregated by SP-A and/or SP-D and were phagocytized by immune cells like macrophages. SP-A and SP-D also have direct effects on immune cells and modulate the production of cytokines and inflammatory mediators.

Numerous studies have reported that SP-A mediates cellular functions through C1q receptors (Ferguson et al., 1999; Malholtra et al., 1994), including C1qR (also known as CD93) (Nepomuceno et al., 1997; Steinberger et al., 2002) and calreticulin (Malhotra et al., 1990; Malholtra et al., 1993). SP-A and SP-D are able to bind Calreticulin, which in turn binds to CD91. CD91 is a component of the binding complex (Gardai et al., 2003).

The binding of SP-A and/or SP-D to the signal-inhibitory regulatory protein- α (SIRP- α) modulates cellular functions in a similar way like the binding complex of surfactant proteins with the CD91-calreticulin complex. In the absence of a pathogen, SP-A binds through its lectin domain to SIRP- α , whereas in the presence of a foreign organism or cell debris, to which the lectin domain of SP-A binds, the free collagen-like region activates immune cells through CD91-calreticulin. Importantly, engagement of the different receptors elicits different responses. Upon binding of SP-A to SIRP- α , the inflammatory-mediator production is inhibited. By contrast, SP-A enhances inflammatory mediator like tumour-necrosis factor (TNF), CXCL12 and CCL2 production through its binding to the CD91-calreticulin complex. Therefore, SP-A and SP-D both are able to enhance and inhibit inflammatory-mediator production to modulate the regulation of immune cells.

Another receptor that binds surfactant protein A was identified by Chroneos and colleagues and termed SP-R210 (Chroneos et al., 1996). Blocking of this receptor with specific antibodies leads to a loss of SP-A mediated functions, including inhibition of lymphocyte proliferation (Borron et al., 1998), enhanced uptake of bacteria by macrophages (Weikert et al., 1997) and mycobacterial killing by a nitric-oxide-dependent pathway (Weikert et al., 2000). Nevertheless, the molecular identity of SP-R210 is still unclear.

Glycoprotein 340 (gp340) is also discussed as a protein that binds SP-D through its CRD (Holmskov et al., 1997). Because of its localisation at the cell surface of alveolar macrophages, gp340 was suggested to be a SP-D receptor. It is identical to salivary agglutinin, a high-molecular-weight component of saliva that binds *Streptococcus mutans*, a bacterium that causes dental caries (Prakobphol et al., 2000). This putative receptor gp340 has no transmembrane domain so that it is suggested that it could interact with an adaptor molecule on the surface of the cell (Wright, 2005).

Additionally, a family of conserved cellular receptors that recognize pathogen-associated molecular patterns (PAMP) are discussed as binding-partners for SP-A and SP-D. This family of Toll-like receptors (TLRs) is activated by ligands like flagellin and CpG-containing DNA from bacteria, peptidoglycan from Gram-positive bacteria, LPS from Gram-negative bacteria, RNA from viruses and zymosan from yeast (Takeda et al., 2003). All these activation mechanisms end up in a series of conserved responses that culminate in inflammation and the production of inflammatory cytokines, such as TNF and interleukin-1 β . The SP-A dependent binding to TLR4 results in an activation of the nuclear factor κ B (NF- κ B) signalling pathway and upregulation of cytokine synthesis (Guillot et al., 2002), whereas interaction of SP-A with TLR2 attenuates stimulation of TLR2 signalling and also stimulation of TNF secretion induced by zymosan or peptidoglycan (Sato et al., 2003).

In addition to phagocytosis, SP-A and SP-D have also the ability to regulate the production of inflammatory mediators by immune cells in a context-dependent manner. One example shows that inflammatory mediators, such as TNF, are both up- and downregulated by SP-A and SP-D (Crouch and Wright, 2001). The release of TNF that is induced by LPS or intact bacteria is inhibited by SP-A (Hickling et al., 1998; McIntosh et al., 1996; Rosseau et al., 1999). In contrast, SP-A enhances TNF production either when alone (Kremlev et al., 1994, Kremlev et al., 1997) or in presence of "rough" LPS (Sano et al., 1999).

A further effect of surfactant proteins SP-A and SP-D is the enhanced uptake of apoptotic cells by alveolar macrophages *in vitro* (Schagat, et al., 2001), which could be even shown for lungs of naïve mice in the case of SP-D (Vandivier et al., 2002).

Through the carbohydrate-recognition domains (CRD) and the collagen-like regions it is possible for SP-A and SP-D, as well as MBL, to bind DNA from a variety of origins, including mice and bacteria (Palaniyar et al., 2004). SP-D effectively binds and aggregates alveolar macrophages DNA and it enhances the uptake of DNA by human monocytic cells (Palaniyar et al., 2003). Binding of the collectins to cell-surface DNA might be one mechanism by which they mediate enhanced phagocytosis of apoptotic cells.

Uptake of apoptotic cells by macrophages results in release of anti-inflammatory mediators, such as transforming growth factors- β (TGF- β), IL-10 and prostaglandin E₂ (Fadok et al., 1998). This response is in contrast to the release of pro-inflammatory cytokines that occurs when phagocytes ingest microorganisms. In addition to enhancing the uptake of apoptotic cells, SP-A also enhances the release of TGF- β by macrophages (Reidy and Wright, 2003), indicating that SP-A can promote resolution of inflammation at several levels of the apoptotic-cell clearance process and that surfactant proteins can indirectly induce anti-inflammatory responses by phagocytes.

As discussed above, surfactant is linked to innate immunity. However, surfactant is also linked to adaptive immunity in the lung by modulating functions of both dendritic cells and T cells.

It has been shown that SP-A and SP-D have different effects on DC functions. The uptake and presentation of antigens is enhanced by SP-D (Brinker et al., 2001), but only SP-A can inhibit maturation of DC, as assessed by cell-surface marker expression, and functional activity, such as phagocytosis and chemotaxis (Brinker et al., 2003).

The proliferation of T cells stimulated with plant lectins, CD3-specific antibodies or phorbol esters is inhibited by SP-A and SP-D. It has been suggested that the inhibition of IL-2 production might mediate this process (Borron et al., 1996; Borron et al., 1998). In addition, both the collagen-like region and the CRD of SP-A have been implicated in the inhibition of lymphocyte function, probably due to inhibition of calcium signalling (Borron et al., 2002). These data indicate that SP-D and SP-A might provide an important link between innate and adaptive immunity, by modulation of both DC and T cell functions.

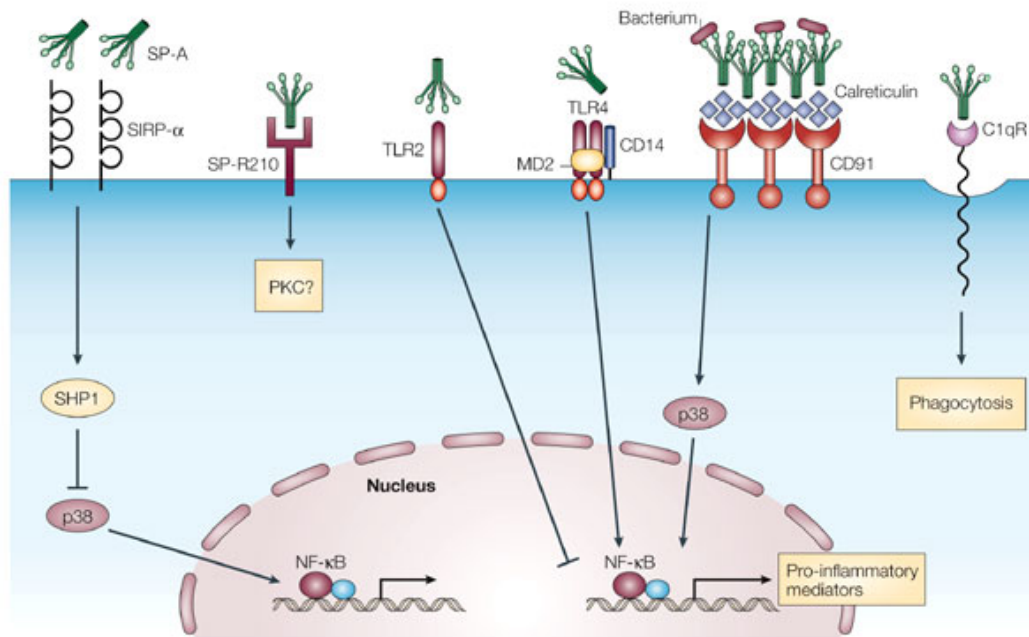


Figure 4: Collectin receptors. Surfactant protein A (SP-A) and SP-D potentially bind several receptors, including SP-R210, Toll-like receptor 2 (TLR2), TLR4, signal-inhibitory regulatory protein- α (SIRR- α) and CD91-calreticulin. SP-A binds SIRR- α and inhibits production of inflammatory mediators. In contrast, when SP-A is bound to a pathogen or cellular debris, its collagen-like region is bound to CD91-calreticulin and induces inflammatory-mediator production. Nuclear factor κ B, (NF- κ B); protein kinase C (PKC); SRC homology 2 (SH2)-domain-containing protein tyrosine phosphatase 1 (SHP1) (adapted from Wright, 2005).

3 Autoimmunity

The concept of autoimmunity was first predicted by Paul Ehrlich at the beginning of the twentieth century, and he described it as “horror autotoxicus”. His experiments led him to conclude that the immune system is normally focused on responding to foreign materials and has an inbuilt tendency to avoid attacking self tissues. But when this process is disturbed, the immune system can attack self tissues resulting in autoimmune diseases.

Autoimmune diseases occur in up to 3-5% of the population (Jacobson et al., 1997). Many of these diseases are classified according to what organs and tissues are targeted by the damaging immune responses. There is an autoimmune disease specific for nearly every organ in the body, usually involving responses to an antigen expressed only in that specific organ. In other autoimmune diseases, such as systemic lupus erythematosus (SLE), no particular cell type seems to be targeted; rather, the response seems to be directed against antigens that are widely expressed throughout the host. Nevertheless these diseases are antigen-specific; moreover, recognition of widely expressed antigens sometimes results unexpectedly in selective manifestations of the organ (Mathews et al., 1983; Yeaman et al., 1988). Autoimmune organ damage can be mediated by T cells, as in multiple sclerosis (MS) and type 1 diabetes (Steinman, 1996) and, furthermore, CD4⁺ and/or CD8⁺ T cells can have crucial roles (Haskins and McDuffie, 1990; Hutchings et al., 1992). In these diseases, autoantibodies are also produced and serve as markers of the antigen-specific T-cell responses, for example, antibodies to insulin or other pancreatic islet-cell antigens in type 1 diabetes (Yu et al., 1996). In other diseases, damage is actually mediated by autoantibodies and requires CD4⁺ T-helper cells. For example, nearly all SLE patients have elevated levels of autoantibodies to nuclear antigens. Examples for organ-specific and systemic autoimmune diseases with known auto antigen targets are shown in table 3.

Table 3: Examples for organ-specific and systemic autoimmune diseases with known autoantigen targets.

Diseases	Organ	Examples of known autoantigens	Mechanism of damage	Prevalence (%)
<u>Organ-specific autoimmune diseases</u>				
Thyroiditis (autoimmune)	thyroid	thyroglobulin, thyroid peroxidase	T cells/antibody	1.0 - 2.0
Gastritis	stomach	H ⁺ /K ⁺ ATPase, intrinsic factor	T cells/antibody	1 - 2% in > 60-y old
Celiac disease	small bowel	transglutaminase	T cells/antibody	0.2 - 1.1
Type 1 diabetes	pancreas β cells	insulin, glutamic acid decarboxylase	T cells	0.2 - 0.4
Multiple sclerosis	brain/spinal cord	myelin basic protein, proteolipid protein	T cells	0.01 - 0.15
Hepatitis (autoimmune)	liver	hepatocyte antigens (cytochrome P450)	T cells/antibody	< 0.01
<u>Systemic autoimmune diseases</u>				
Rheumatoid arthritis	joint, lung, heart etc.	IgG, filaggrin, fibrin etc.	T cells/antibody	0.8
Systemic lupus (SLE)	skin, joint, kidney	nuclear antigens (DNA, histones, ribonucleoproteins), others	antibody	0.1
Polymyositis/dermatomyositis	skeletal muscle (predominant), lung, heart, joint, others	muscle antigens, aminoacyl-tRNA synthetases, other nuclear antigens	T cells/antibody	< 0.01

Diseases are listed by category (organ-specific versus systemic) and then by prevalence (adapted from Marrack et al., 2001).

The potential for autoimmunity exists in all individuals because during their development lymphocytes may express receptors specific for self-antigens and many self-antigens are readily accessible to the immune system. Tolerance to self-antigens is normally maintained by selection processes in the thymus that prevent the maturation of some self-antigen-specific lymphocytes (Sakaguchi et al., 2000) and, in addition, by mechanisms in the periphery those inactivate or delete self-reactive lymphocytes that do mature. Loss of self-tolerance may result from abnormal selection or regulation of self-reactive lymphocytes and by abnormalities in that way that self-antigens are presented to the immune system. Self tolerance can be divided into central tolerance and peripheral tolerance. In central tolerance, immature lymphocytes that happen to recognize self-antigens in generative lymphoid organs die by apoptosis. In contrast, in peripheral tolerance, mature self-reactive

lymphocytes encounter self-antigens in peripheral tissues and are killed or shut off. The principle mechanisms of peripheral tolerance are anergy (functional unresponsiveness) (Schwartz, 1996), deletion (apoptotic cell death) (Miller and Basten, 1996), and suppression by regulatory T cells (Piccirillo and Shevach, 2004).

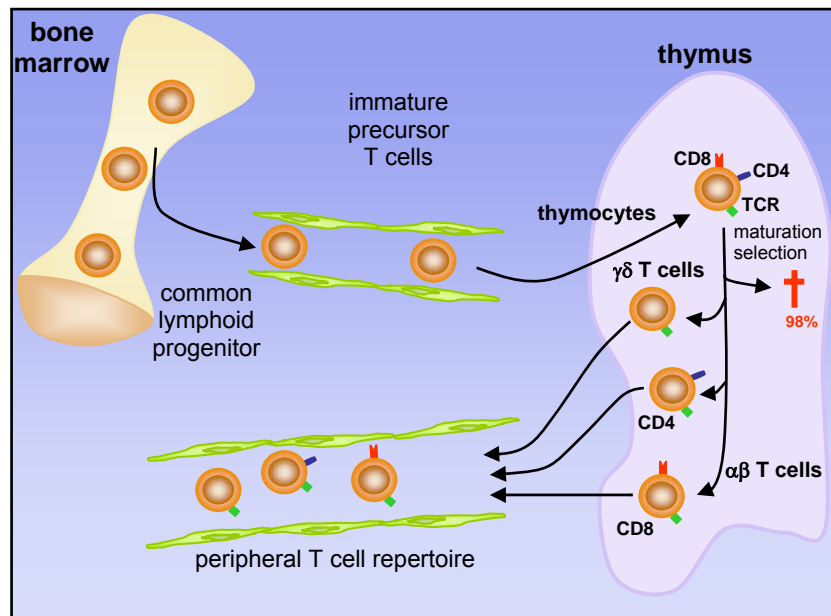


Figure 5: Schematic representation of T cell development. T cells originate from the common lymphoid progenitor cells in the bone marrow. They migrate as immature precursor T cells via bloodstream into thymus, which they populate as thymocytes. The thymocytes go through a series of maturation steps including distinct changes in the expression of cell surface receptors, such as the CD3 signalling complex (not shown) and the coreceptors CD4 and CD8, as well as the arrangement of their antigen receptor (T cell receptor, TCR) genes. More than 98% of the thymocytes die during maturation by apoptosis (†), as they undergo positive selection for their TCR's compatibility with self-major histocompatibility molecules, and negative selection against those T cells that express TCRs reactive to autoantigenic peptides. In humans, the vast majority of peripheral blood T cells express TCRs consisting of α and β chains ($\alpha\beta$ T cells). A small group of peripheral T cells bear an alternative TCR composed of γ and δ chains ($\gamma\delta$ T cells). $\alpha\beta$ and $\gamma\delta$ T cells diverge early in T cell development. Whereas $\alpha\beta$ T cells are responsible for the classical helper or cytotoxic T cell response, the function of the $\gamma\delta$ T cells within the immune system is largely unknown. $\alpha\beta$ T cells that survive thymic selection lose expression of either CD4 or CD8, increase the level of expression of the TCR, and leave the thymus to form the peripheral T cell repertoire (adapted from Skapenko et al., 2005).

4 Regulatory T lymphocytes

As mentioned above, many possibilities exist to regulate immune responses mediated by T cells, by cytokine release and cell-cell interaction to effector cells with immune modulating ability like macrophages and DC. The following sections deal with the suppression of autoimmune responses by regulatory T (T_{reg}) lymphocytes and will give an overview about the subpopulations of T_{reg} cells and their mechanism to suppress autoreactive immune responses.

The immune system employs several strategies to maintain its own homeostatic balance. The thymus plays an important role in this regulation, because of its particular role in clonal deletion of self-reactive T cells, but other mechanisms preventing immunological self attacks are also involved in the maintenance of self tolerance. These latter mechanisms occur in the periphery and include - among other things - T cell anergy and the effects of regulatory cells. Regulatory T cells may be defined as $CD4^+$ T cells that inhibit immunopathology or autoimmune disease *in vivo*. Specifically, T_{reg} cells include those able to suppress naïve T cell proliferation *in vitro* and to control $CD4^+$ or $CD8^+$ T cell numbers *in vivo*, in lymphopenic hosts. Two major T_{reg} populations have been described so far, one part is termed naturally occurring T_{reg} and includes anergic T cells that require cell-cell contact for their function, and the other part is designated adaptive T_{reg} cells including T_{R1} and T_{H3} cells, functioning by the secretion of inhibitory cytokines (Roncarolo and Levings, 2000).

The two subsets of regulatory T cells might function in different immunological settings, depending on the context of antigen exposure, the nature of the inflammatory response and the T cell receptor (TCR) repertoires of the individual cells. The natural T_{reg} cells are probably most effective at suppressing autoreactive T cell responses locally, in non-inflammatory settings – circumstances in which antigen specific, self limiting reactions are required to achieve a fine homeostatic balance. In contrast, during self-damaging inflammatory reactions to microbes or transplanted tissue, or settings (for example inflammatory bowel disease), adaptive T_{reg} cells might be induced to suppress the pathological immune responses.

4.1 Naturally occurring CD4⁺CD25⁺ regulatory T cells

The CD4⁺CD25⁺ regulatory T cells are currently the focus of intensive research and were first described in the early 1970s by Gershon and colleagues (Gershon et al., 1974). These cells represent 5-10% of the CD4⁺ T lymphocytes in healthy adult mice and humans and are thought to perform a specialized role in controlling both the innate and the adaptive immune system. Although easily identified and isolated from unmanipulated mice and humans on the basis of CD25 expression, this chain of the IL-2(R) receptor is also expressed on activated T cells (Maloy et al., 2003; Sakaguchi et al., 2001; Shevach, 2002). So far, no characteristic stable surface marker has been assigned to T_{reg} cells. Additional markers expressed by these cells include cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) (Read et al., 2000; Takahashi et al., 2000) and glucocorticoid induced tumor necrosis factor receptor (TNFSR18) (McHugh et al., 2002; Shimizu et al., 2002), which were initially implicated in the mechanism of T_{reg} action. However, both of these molecules are also expressed by nonregulatory T cells after activation. Various groups identified the forkhead/winged helix transcription factor Foxp3 as a marker for both CD25⁺ T_{reg} cells and CD25⁻ cell that have regulatory activity (Fontenot et al., 2003; Hori et al., 2003). In the mouse, Foxp3 is preferentially expressed by CD25⁺ cells, and mutations of the Foxp3 gene are associated with a broad range of hypersensitivity conditions in both mouse and man (Ramsdell, 2003). Additional markers are required, and it has been shown, that Neuropilin-1 could be used to distinguish CD25⁺ T_{reg} cells from recently activated CD25⁺ non-regulatory cells (Bruder et al., 2004).

The resident regulatory cells that develop in the thymus are generated in a burst of activity during the early stages of fetal and neonatal T cell development (Sakaguchi et al., 2001). They are polyclonal on the basis of diverse TCR usage (Shevach, 2002), and they are potentially capable of recognizing diverse self-antigens.

The promiscuous gene expression of many self tissue-specific proteins in the medullar epithelial cells of the thymus is described as a potential mechanism to ensure central tolerance to peripheral self-antigens, because this self-antigen expression in the thymus might lead, among other things, to the deletion of immature autoreactive T cells (Derbinski et al., 2001). However, it is possible that these self proteins are expressed at low levels and, additionally, by only some of the epithelial

cells, making clonal deletion a rather ineffective means of inducing tolerance to peripheral antigens. An alternative mechanism of inducing self tolerance in the thymus might be the localized antigen presentation, resulting in a more robust regulation of autoreactivity. Once generated, the thymic T_{reg} cells are exported in the peripheral tissues, where they may function normally to prevent the activation of other, self reactive T cells that have the potential of developing into effector cells (Salomon et al., 2000).

These regulatory T cells were described as a “normal” population of suppressor cells, because they are always present in normal individuals and carry out their regulatory function during normal surveillance of self-antigens. Furthermore, because of their development in the thymus, the natural regulatory T cells are expected to be specific for self-antigens.

Recent studies indicate that CD28 controls both thymic development and peripheral homeostasis of natural T_{reg} cells. Ligation of CD28 is expected to act at two stages during T_{reg} cell development (Boden et al., 2003). In addition, once the natural T_{reg} cells emerge from the thymus, costimulation through CD28 is required to maintain a stable pool of these cells in the periphery by promoting their self renewal through homeostatic proliferation and by supporting their survival (Boden et al., 2003; Salomon et al., 2000). The development and maintenance functions of CD28 are not mediated through IL-2. It is possible that signalling through CD28 stimulates the production of a response to an yet unknown cytokine that functions as a growth and survival factor of these cells. The absence of CD80/CD86 or CD28 results in a reduction of the number of regulatory cells in peripheral lymphoid tissues and an unexpected exacerbation of natural T_{reg} cells, which plays an important role controlling autoimmunity (Lenschow et al., 1996; Salomon et al., 2000).

4.2 Adaptive regulatory T cells

These cells are generated from mature T cell populations under certain conditions of antigenic stimulation, and they can be induced *ex vivo* by culturing mature CD4⁺ T cells with antigen or polyclonal activators in the presence of immunosuppressive cytokines, namely IL-10 (Barrat et al., 2002; Levings et al., 2001). Similar to natural T_{reg} cells, adaptive T_{reg} cells originate from thymus, but they might be derived from classical T cell subsets or natural T_{reg} cells. The level of expression of CD25 by adaptive T_{reg} cell is variable, depending on the disease setting and the site of regulatory activity. Of note, adaptive T_{reg} cells function *in vivo* in a cytokine dependent manner (Barrat et al., 2002; Chatenoud et al., 1997; Maloy and Powrie, 2001), so that these regulatory T cells are distinguished from natural T_{reg} cells not by their origin (the thymus), but by their requirement for further differentiation as a consequence of exposure to antigen in a distinct immunological context. Several different *in vitro* protocols have been described over the past few years that result in the generation of suppressor T cells. The activation of mouse or human CD4⁺ T cells *in vitro* in the presence of IL-10 has been shown to result in the generation of T cell clones with a cytokine profile different from that of T helper 1 (T_H1) or T helper 2 (T_H2) cells. Functionally, these T cell clones have inhibitory effects on antigen specific activation of naïve T cells that are mediated partially by IL-10 and TGF- β , and were termed T regulatory 1 (T_R1) cells (Groux et al., 1997). A related approach for the generation of suppressor T cells *in vitro* involves the stimulation of naïve T cells with immature (im)DC. Surprisingly, although these cells produce IL-10, their suppressor phenotype resembles that of CD25⁺ T cells, as it is contact dependent, antigen non-specific and APC-independent. Immature DC are the ideal population to prime regulatory T cells as they are deficient in costimulatory molecules, and priming with antigen-imDC complexes might even be able to downregulate pre-existing antigen specific immune responses (Dhodapkar et al., 2001). Exposure to TGF- β has also been reported to facilitate the differentiation/expansion of suppressor T cell populations *in vitro* (Yamagiwa et al.; 2001). Another possibility to induce regulatory T cells is antigen exposure by certain routes, including intranasal or oral administration. This strategy seems to induce selectively the appearance of T cells with this regulatory phenotype (Chen et al., 1994). These induced T_H3 cells produce high concentration of TGF- β and can inhibit the development of immune pathology in

several animal models (Chen et al., 1994; Neurath et al., 1996). Moreover, in contrast to natural T_{reg} cells, which are fully functional at the time of thymic export as a consequence of strong TCR engagement, the development of adaptive T_{reg} cells in the periphery might be triggered by low-affinity antigen or altered TCR signal transduction. These antigen-stimulated adaptive T_{reg} cells are not functional without activation by further exposure to antigens – such as during infection, organ transplantation under cover of certain immunomodulatory therapies, or ectopic expression of non-self-antigens (Apostolou et al., 2002; Belkaid et al., 2002; Fuss et al., 2002; Kingsley et al., 2002; Powrie, 2003). Concerning the antigen specificity of adaptive T_{reg} cells, it is interesting to speculate that these cells have a diverse repertoire, which might be expanded as a consequence of fortuitous cross-reactivities with foreign proteins. It is possible that the TCR repertoire of adaptive T_{reg} cells is self-antigen specific, but that these cells are triggered in an inflammatory environment to promote bystander suppression through the production of suppressive cytokines.

It is important to note that unlike natural T_{reg} cells, adaptive T_{reg} cells might not require costimulation through CD28 for their development or function (Taylor et al., 2002). Interestingly, IL-2 might promote the development and function of both types of T_{reg} cells, on the basis of studies showing the total absence of T_{reg} cells in IL-2 receptor-deficient mice (Malek et al., 2002; Furtado et al., 2002).

4.3 Mechanism of suppression

In addition to potential differences in terms of TCR repertoire and differentiation state, it is proposed that natural and adaptive subsets of T_{reg} cells differ in their mechanism of action. Adaptive T_{reg} cells mediate their inhibitory activities by producing immunosuppressive cytokines, such as TGF- β and IL-10 (Kingsley et al., 2002; Nakamura et al., 2001). In contrast, natural T_{reg} cells, at least *in vitro*, function by a cytokine-independent mechanism, which presumably involves direct interactions with responding T cells or antigen-presenting cells (Shevach, 2002). This contact-dependent mechanism of suppression has been shown most convincingly by CD4⁺CD25⁺ natural T_{reg} cells employed in *in vitro* models of suppression, whereas cytokine-mediated suppression has been best established for peripheral adaptive T_{reg}

cells *in vivo*. However, the adaptive T_{reg} cell subset, although it suppresses in a cytokine-dependent manner, might still require direct cell-cell contact to initiate the suppressive cascade.

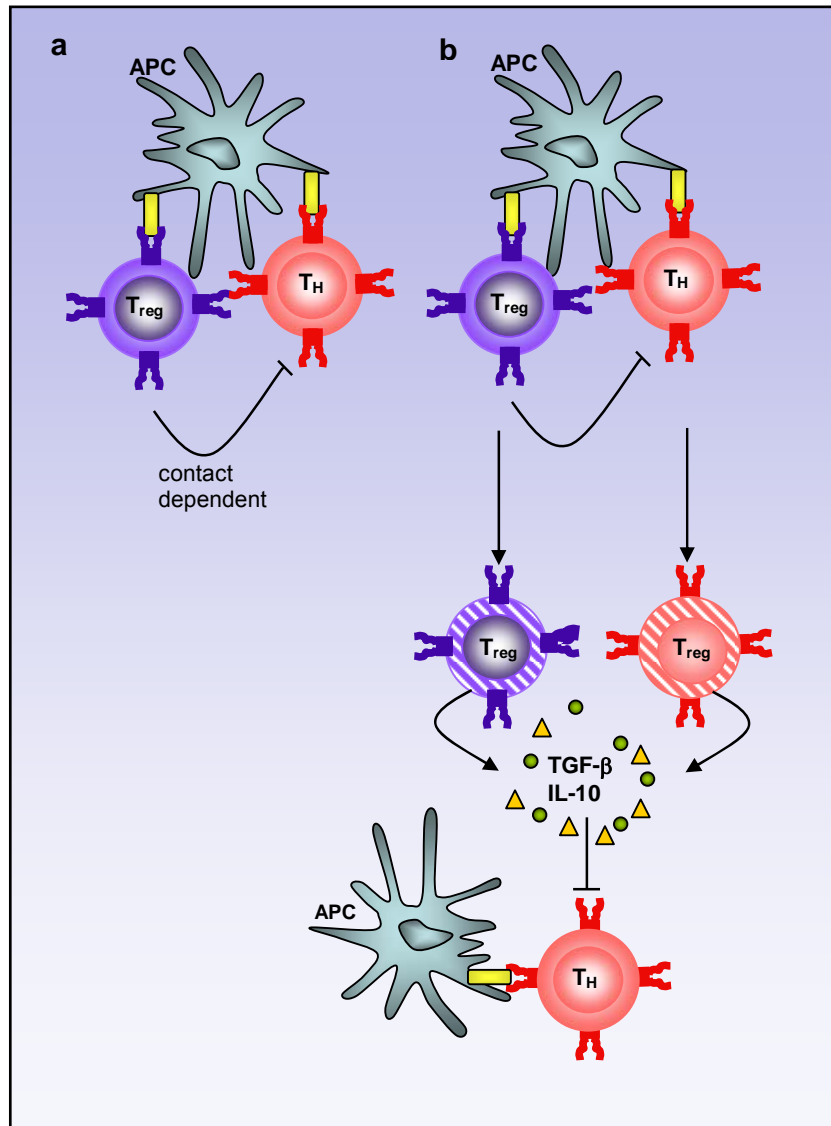


Figure 6: Two classes of regulatory T cells can be envisioned. a In this hypothetical model, the natural regulatory T (T_{reg}) cells (blue) suppress immune response in a contact-dependent manner and function in general homeostasis to block the actions of autoimmune T cells (red) in noninflammatory settings. **b** The adaptive T_{reg} cell subset enhances the robust nature of suppression in an inflammatory milieu. Importantly, adaptive T_{reg} cells can develop either $CD4^+CD25^+$ natural T_{reg} cells (blue striped) or by altering the activity of T helper (T_H) cells (red striped). APC, antigen presenting cell; interleukin (IL)-10, transforming growth factor (TGF)- β , regulatory T cell (T_{reg}) (adapted from Bluestone and Abbas, 2003).

5 SPC-HA transgenic mouse model

To get further insights into the mechanisms underlying the induction and regulation of T cell mediated immune reactions in the lung, a new SPC-HA transgenic mouse line has been established prior to this work (Bruder et al., 2004).

The SPC-HA transgenic mouse expresses hemagglutinin (HA) of the influenza strain A/PR8/34 under the transcriptional control of the surfactant protein C (SP-C) promoter specifically in type II alveolar epithelial cells. High levels of HA expression could be detected in the lung and relatively low levels in the thymus of SPC-HA mice, but not in other organs. Immunohistochemical stainings of lung sections from transgenic mice and non-transgenic littermates revealed HA expression exclusively in surfactant protein C producing type II alveolar epithelial cells in SPC-HA transgenic mice. This expression of the neo-self-antigen HA specifically in the lung tissue represents the basic condition to establish a new mouse model for autoimmune-mediated lung disease.

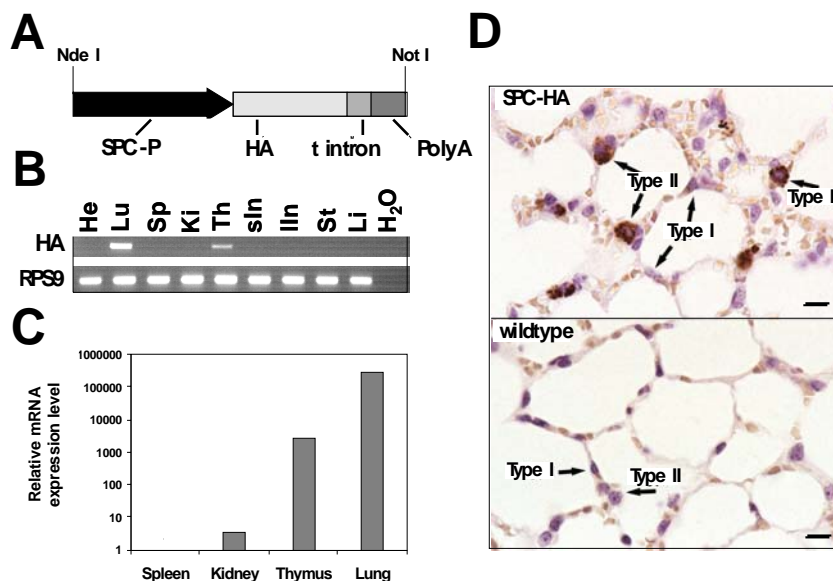


Figure 7: Targeted expression of hemagglutinin in the lung. (A) Construct used for the generation of surfactant protein C (SPC)-hemagglutinin (HA) transgenic mice. PolyA = polyadenylic acid (B) Real-time RT-polymerase chain reaction (PCR) to detect HA mRNA expression in different tissues of SPC-HA transgenic mice (He: heart; Lu: lung; Sp: spleen; Ki: Kidney; Th: thymus; sIn: small intestine; lIn: large intestine; St: stomach; Li: Liver). RPS9 housekeeping gene specific primer served as internal control. (C) Quantification of HA mRNA expression levels in selected tissues by RT-PCR. (D) Immunohistochemical detection of influenza HA antigen on lung sections taken from SPC-HA transgenic (upper panel) and wild type (lower panel) animals using DAB stain (brown) as chromogen and hematoxylin (blue) as nuclear counterstain. Bar = 30µm (adapted from Bruder et al., 2004).

Crossing SPC-HA transgenic mice into TCR-HA mice, which bear a major histocompatibility complex class-II-restricted T cell receptor specific for the peptide HA110-120 (Kirberg et al., 1994) results in the development of a progressive interstitial pneumonitis characterized by a massive lymphocytic and plasmacytic infiltration of interalveolar septa of SPC-HA x TCR-HA double transgenic mice.

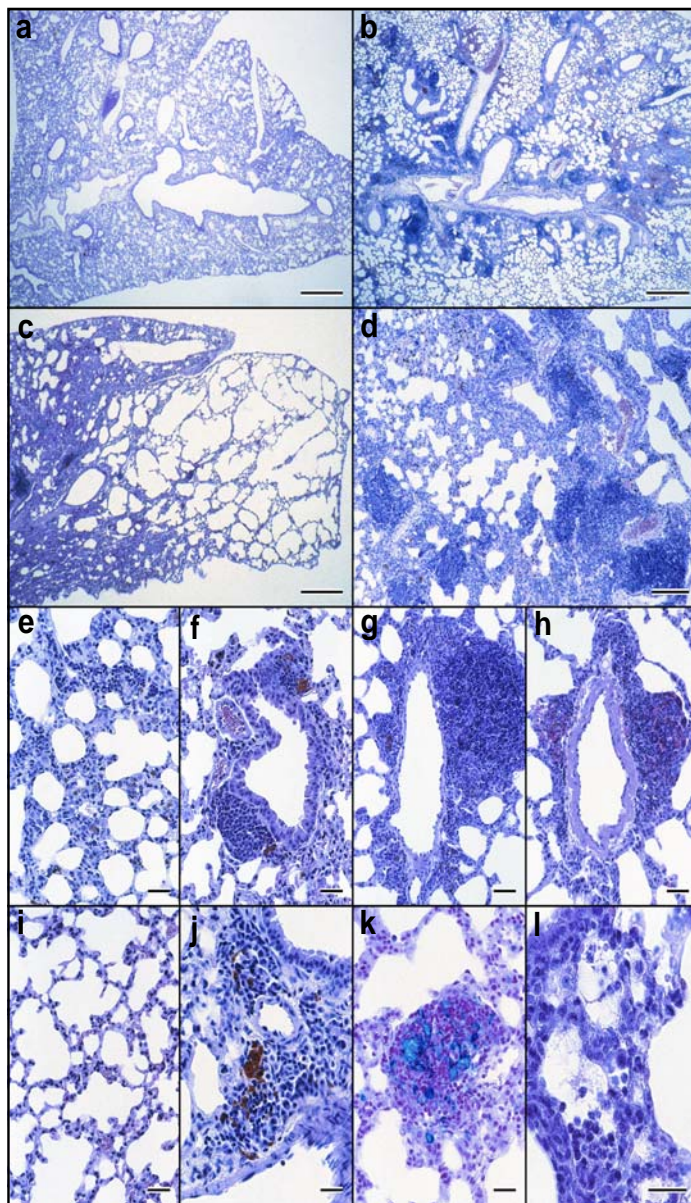


Figure 8: Immunohistology analysis of lung from SPC-HA x TCR-HA double transgenic mouse compared to SPC-HA transgenic mouse. Lung of a healthy SPC-HA transgenic mouse (a) compared with a SPC-HA x TCR-HA double transgenic mouse (b). Alveolar emphysema in a SPC-HA x TCR-HA mouse (c). Lymphocytic infiltration in a double transgenic mouse (d) distinct lymphocytic infiltration patterns in the interalveolar (e), around bronchi (f), veins (g) and arteries (h). Normal interalveolar septa of a SPC-HA control mouse (i; compare with e). Lymphocytic aggregates containing brownish pigment-loaded macrophages (j) suggestive of hemosiderin. Turnbull blue stain for iron revealed significant iron deposition (k; blue color). Lung of a 9-days-old double transgenic mouse (l) with acute tissue damage. H&E stain (a-j, l) and Turnbull blue stain for the detection of iron (k). Bars = 500µm (a-c), 200µm (d) 100µm (e-i) and 50µm (j-l) (adapted from Bruder et al., 2004).

Despite the fact that HA is expressed not only in the lung but also to some extent in the thymus of SPC-HA mice, no reduction in the frequency of HA-specific $6.5^+ CD4^+$ autoreactive T cells was observed in the periphery of SPC-HA x TCR-HA double transgenic mice. These findings are indicative for inefficient thymic deletion of potentially autoreactive T cells. In addition, the proliferative capacity of $CD4^+$ T cells

from double transgenic mice in response to *in vitro* antigenic stimulation was comparable to that of CD4⁺ T cells from TCR-HA single transgenic T cells, suggesting that peripheral autoreactive T cells in double transgenic SPC-HA x TCR-HA mice are fully functional, thus having the potential to cause lung inflammation.

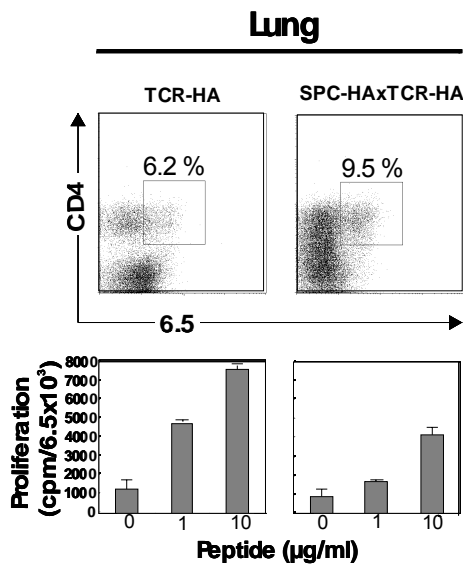


Figure 9: Increased number of HA-specific CD4⁺ T cells in the lung of SPC-HA x TCR-HA mice. Percentages of 6.5⁺CD4⁺ T cells isolated from the lung were determined by fluorescence-activated cell sorter (FACS) analysis. Proliferative capacity of lung lymphocytes from single and double transgenic mice was compared in an *in vitro* stimulation assay; cpm = counts per minute (adapted from Bruder et al., 2005).

The lung inflammation observed in SPC-HA x TCR-HA double transgenic mice did not progress in an uncontrolled way, i.e. inflammation did not result in complete tissue destruction or death. Thus, although multifocal acute alveolar necrosis occur with hemorrhage, intraalveolar fibrin deposition, consistent with acute tissue damage, which could be observed in 9-day-old SPC-HA x TCR-HA double transgenic mice, inflammation reached a plateau, which was characterized by primarily diffuse to follicular lymphocytic infiltration in elder mice. These findings suggested a regulatory mechanism that may exist and/or develop under autoimmune conditions to counteract the progression of inflammatory processes in the lung, thus preventing uncontrolled tissue destruction and lethal physiological derangement.

Detailed analysis of HA-specific 6.5⁺CD4⁺ lung lymphocytes obtained from diseased SPC-HA x TCR-HA double transgenic mice exhibited a regulatory phenotype. It could be shown that the proliferative capacity of lung lymphocytes from SPC-HA x TCR-HA mice was reduced by approximately 50% when compared with lung T cells from healthy TCR-HA control mice (Bruder et al., 2004).

Moreover, cytokine and gene expression profiling on isolated 6.5⁺CD4⁺ autoreactive T cell from diseased mice demonstrated a changed phenotype compared to cells derived from TCR-HA control mice. This phenotype resembles a T_R1 regulatory

phenotype and suggested the *in vivo* induction of these suppressor T cells during the progress of lung inflammation in SPC-HA x TCR-HA mice.



Figure 10: Cluster analysis of genes differentially expressed in 6.5^+CD4^+ T cells isolated from lungs and spleens of diseased SPC-HA x TCR-HA as well as healthy TCR-HA mice. Red indicates induction of gene expression, green indicates repression (+3: bright red; -3: bright green). Black indicates no changes. Genes in which expression was at least twofold increased were considered to be regulated. Lu (infl.), lung inflamed, genes differentially expressed in 6.5^+CD4^+ T cell from the inflamed lung of SPC-HA x TCR-HA mice compared with the lung of healthy TCR-HA donors. Sp (infl.), spleen inflamed, represents genes differentially expressed in 6.5^+CD4^+ T cells from spleen of SPC-HA x TCR-HA mice compared with TCR-HA. Lu versus Sp, lung versus spleen, defines basal expression level of genes in the lung. The left lanes represent an overview and the middle and right lanes are an enlarged view outlining clusters of special interest. **Cluster A:** genes downregulated in the lung of SPC-HA x TCR-HA mice upon airway inflammation, although being, in part, higher expressed in the lung than periphery in healthy mice. **Cluster B:** genes with high basal expression level in the lung of healthy donors, being upregulated during pulmonary disease. **Cluster C:** genes being expressed at lower levels in the lung than in the spleen under normal conditions, which are upregulated in lung $CD4^+$ T cells, but downregulated in self-reactive $CD4^+$ T cells in the inflamed lung, but partially downregulated in the periphery (adapted from Bruder et al., 2004).

The SPC-HA x TCR-HA double transgenic mouse model combines important features of a variety of lung diseases and will contribute to a better understanding of the requirements for, and consequences of chronic T cell-mediated lung injury. Further analysis will clarify the mechanisms for the induction of peripheral tolerance and the development of adaptive regulatory T cells. Additionally, the role of the self-antigen expressing type II alveolar epithelial cells in the progression of disease in SPC-HA x TCR-HA double transgenic mice and the induction of a tolerogenic environment have to be studied.

CHAPTER II

Results

Part I:

Characterization of autoreactive CD4⁺ T cells from SPC-HA x TCR-HA mice

1 Aims of the study

To dissect the immunologic and molecular mechanisms of autoantigen-specific CD4⁺ T cell dysregulation, a transgenic mouse expressing hemagglutinin A/PR8/34 (HA) under the control of the lung-specific surfactant protein C promoter in type II alveolar epithelial cells (AEC II) was generated (Bruder et al., 2004). Breedings of these transgenic mice with TCR-HA mice bearing a major histocompatibility complex class-II-restricted T cell receptor specific for the peptide HA110-120 (Kirberg et al., 1994) results in the development of a progressive interstitial pneumonitis characterized by a massive lymphocytic and plasmacytic infiltration of interalveolar septa. Pulmonary inflammation reached a plateau state in older mice with prominent formation of lymphoid follicles, but reduced interstitial infiltration. Extensive immunologic characterization and molecular analysis of self-reactive CD4⁺ T cells isolated from the inflamed lung suggested the induction of regulatory T cells in the site of inflammation. To study the impact of naturally occurring and induced regulatory T cells on disease progression and prevention of autoimmunity in more detail, the following studies should be performed:

- Isolation of peripheral self-reactive T cells from SPC-HA x TCR-HA double transgenic mice and TCR-HA control mice to characterize their activation status in different compartments.
- Analysis of the gene expression pattern of lung lymphocytes derived from SPC-HA x TCR-HA double transgenic and TCR-HA control mice with focus on marker molecules specific for regulatory T cells.
- Analyzing the impact of naturally occurring regulatory T cells on the outcome of disease in an adoptive transfer system based on the transfer of HA-specific CD4⁺ T cells into SPC-HA single transgenic mice.
- Examinations regarding the effects of *in vivo* induced regulatory T cells on the proliferation of autoreactive T cells in an adoptive transfer system based on the transfer of HA-specific CD4⁺ T cells into SPC-HA x TCR-HA double transgenic mice.

2 Results

2.1 HA-specific CD4⁺ T cells are present in the periphery of SPC-HA x TCR-HA mice

The prerequisite for the development of autoimmune diseases is inefficient deletion of autoreactive T cells during the process of T cell maturation in the thymus. Self-antigen specific T cells that escaped thymic deletion are present in the periphery of every individual and therefore represent a potential risk for the organism.

In the above described SPC-HA x TCR-HA mouse model the HA-specific (6.5⁺) CD4⁺ T cells play a key role for the establishment of autoimmunity. Despite the fact that HA is expressed not only in the lung epithelium but also to some extent in the thymus of SPC-HA transgenic mice, autoreactive CD4⁺ T cells were detectable in the spleen and mesenteric lymph nodes (MLN) of SPC-HA x TCR-HA double transgenic mice (Bruder et al, 2004).

To analyze the presence of 6.5⁺CD4⁺ T cells in the periphery of SPC-HA x TCR-HA double transgenic mice in more detail, T cells from lung, spleen, MLN and in addition from bronchial lymph nodes (BLN), inguinal lymph nodes (INLN), cervical lymph nodes (CVLN) and axillary lymph nodes (AXLN) of SPC-HA x TCR-HA and TCR-HA control mice were isolated and analyzed by flow cytometry for the expression of the transgenic T cell receptor (figure 11).

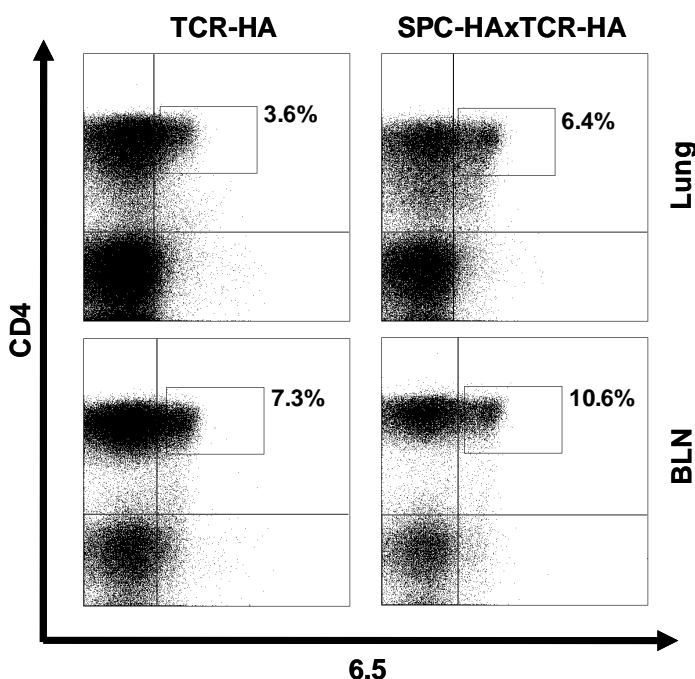


Figure 11: HA-specific CD4⁺ T cells are present in periphery of SPC-HA x TCR-HA mice. SPC-HA x TCR-HA and TCR-HA control mice were sacrificed, lung and BLN, MLN, Spleen, AXLN, INLN, CVLN were isolated and stained for CD4 and 6.5 expression to measure the percentage of transgenic T cells in the different compartments. These results are a representative of two similar experiments.

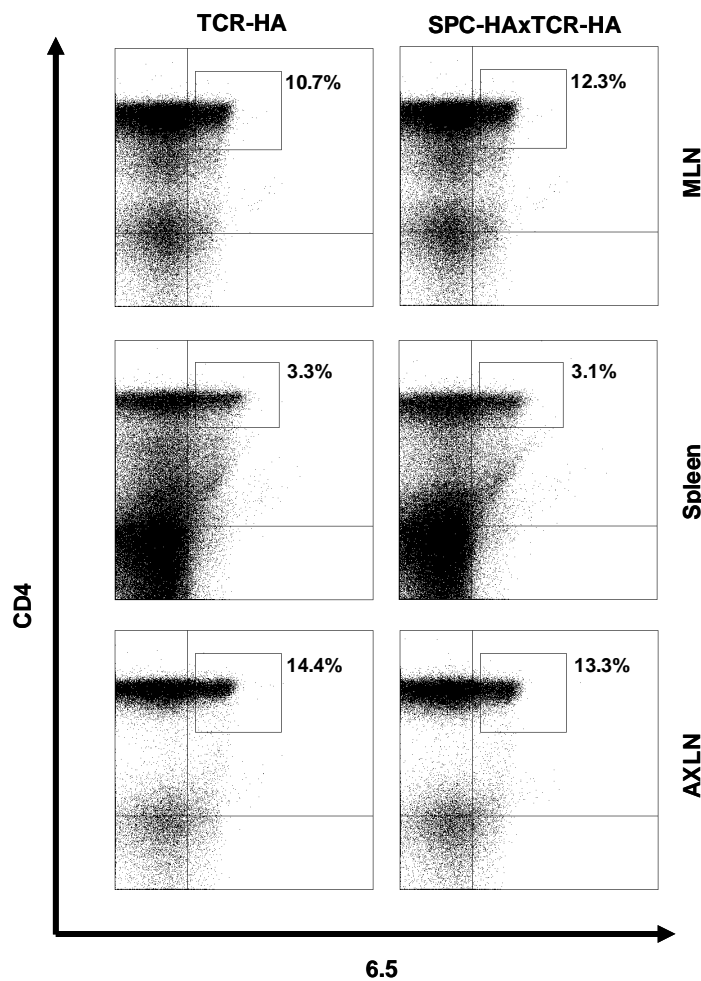


Figure 11 (continued): HA-specific CD4⁺ T cells are present in the periphery of SPC-HA x TCR-HA mice.

In any case no reduction in the frequency of 6.5⁺CD4⁺ T cells could be observed in peripheral lymphoid tissues of double transgenic SPC-HA x TCR-HA mice compared to TCR-HA control mice, thus indicating inefficient thymic deletion of potentially autoreactive T cells.

The percentage of 6.5⁺CD4⁺ T cells in the lung of SPC-HA x TCR-HA mice was even found to be increased. This is directly linked to infiltration of antigen-specific T cells to this tissue which was confirmed by morphologic changes observed in the lungs of double transgenic mice (Bruder et al., 2004). Also, the BLN draining the lung contained elevated numbers of 6.5⁺CD4⁺ T cells in double transgenic mice.

In the other compartments analyzed no remarkable increase of transgenic T cells could be detected. Therefore, spleen, MLN, AXLN, CVLN and INLN from SPC-HA x TCR-HA mice exhibit comparable amounts of 6.5⁺CD4⁺ T cells like TCR-HA control mice.

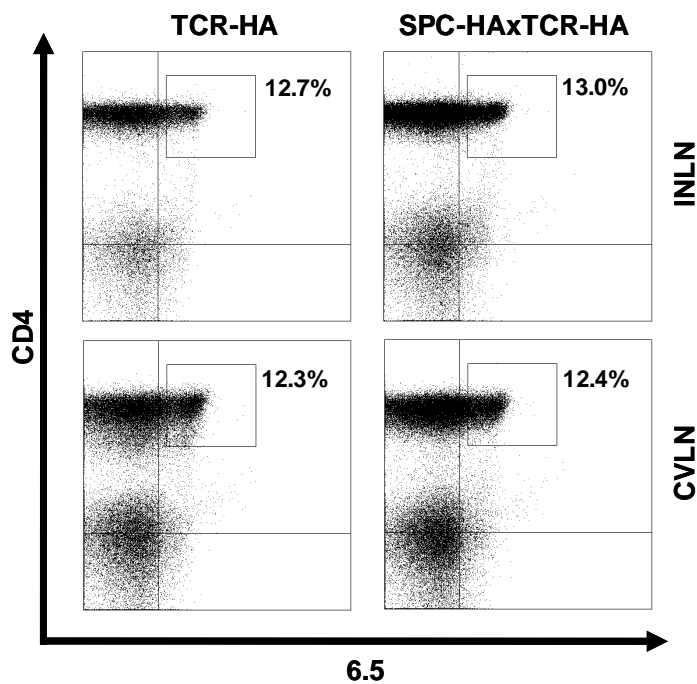


Figure 11 (continued): HA-specific CD4⁺ T cells are present in the periphery of SPC-HA x TCR-HA mice.

2.2 HA-specific CD4⁺ T lymphocytes from SPC-HA x TCR-HA mice have an activated phenotype

Antigen encounter by specific T cells should result in changes of the expression pattern of certain activation and/or memory markers. To characterize HA-specific 6.5⁺CD4⁺ T cells from the inflamed lung and surrounding lymphoid organs of SPC-HA x TCR-HA mice in more detail, these cells were analyzed for the expression of the activation and memory markers CD69, CD25, CD45RB and CD62L by flow cytometry (figure 12). As already shown before, comparing the expression patterns on 6.5⁺CD4⁺T cells isolated from the inflamed lung of SPC-HA x TCR-HA mice and healthy lungs of TCR-HA control mice, autoreactive CD4⁺ T cells from SPC-HA x TCR-HA mice exhibit an activated and memory phenotype. The activation markers CD25 and CD69 on the surface of 6.5⁺CD4⁺ T cells are upregulated and accordingly the memory makers CD45RB and CD62L on the surface of autoreactive T cells are downregulated. Surprisingly, 6.5⁺CD4⁺ T cells isolated from the BLN draining the inflamed lung show no activation/memory phenotype (figure 12).

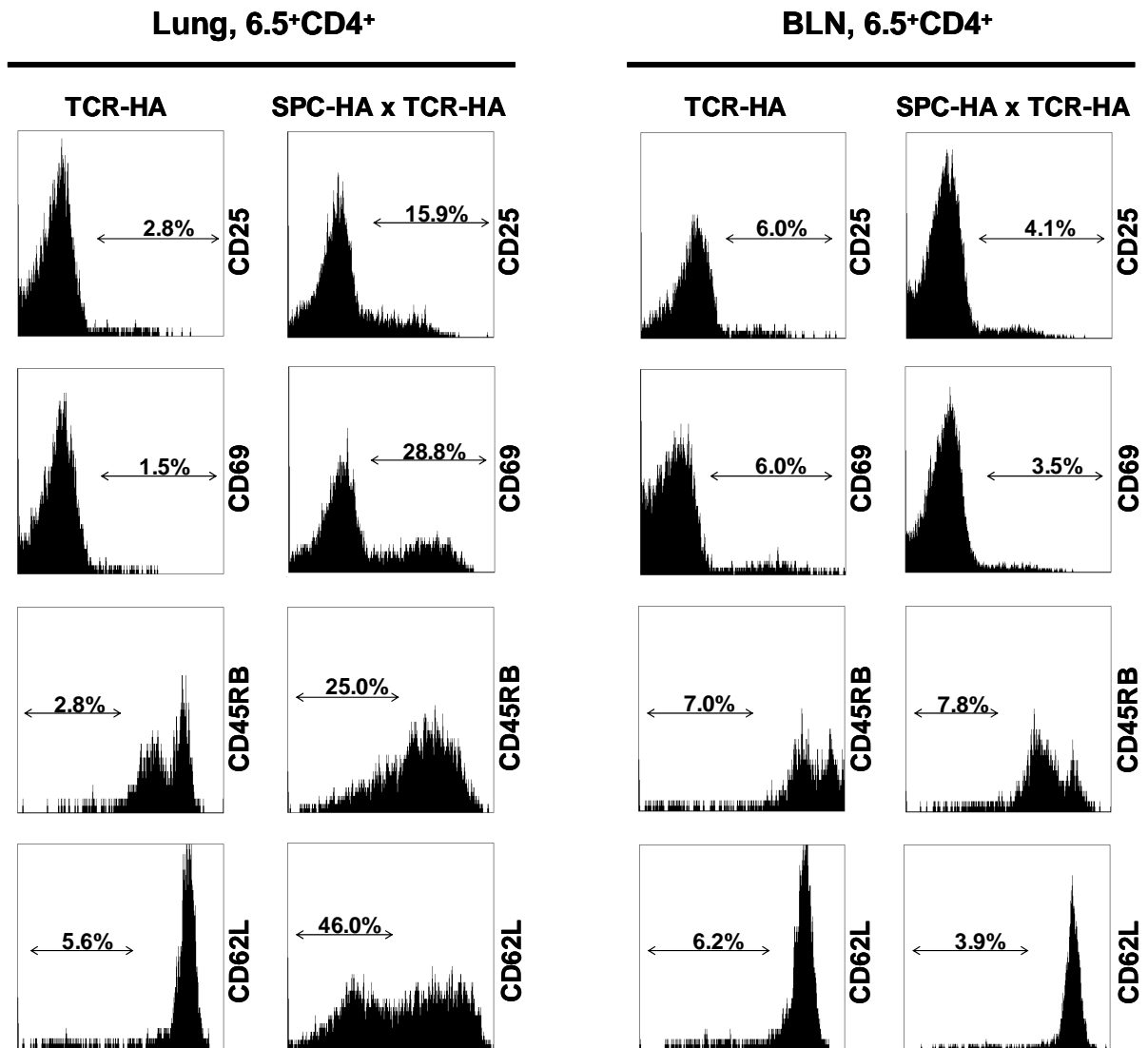


Figure 12: Activation/memory phenotype of HA-specific CD4⁺ T cells from double transgenic SPC-HA x TCR-HA mice compared to healthy TCR-HA control mice. T cells were isolated from lung and BLN, spleen, MLN, AXLN, INLN and CVLN of SPC-HA x TCR-HA and TCR-HA mice, respectively. Lymphocytes were stained with 6.5 and CD4 antibodies as well as CD25, CD69, CD45RB and CD62L antibodies. 6.5⁺CD4⁺ T cells were gated and analyzed regarding the expression of CD25, CD69, CD45RB and CD62L by FACS. These results are a representative of two similar experiments.

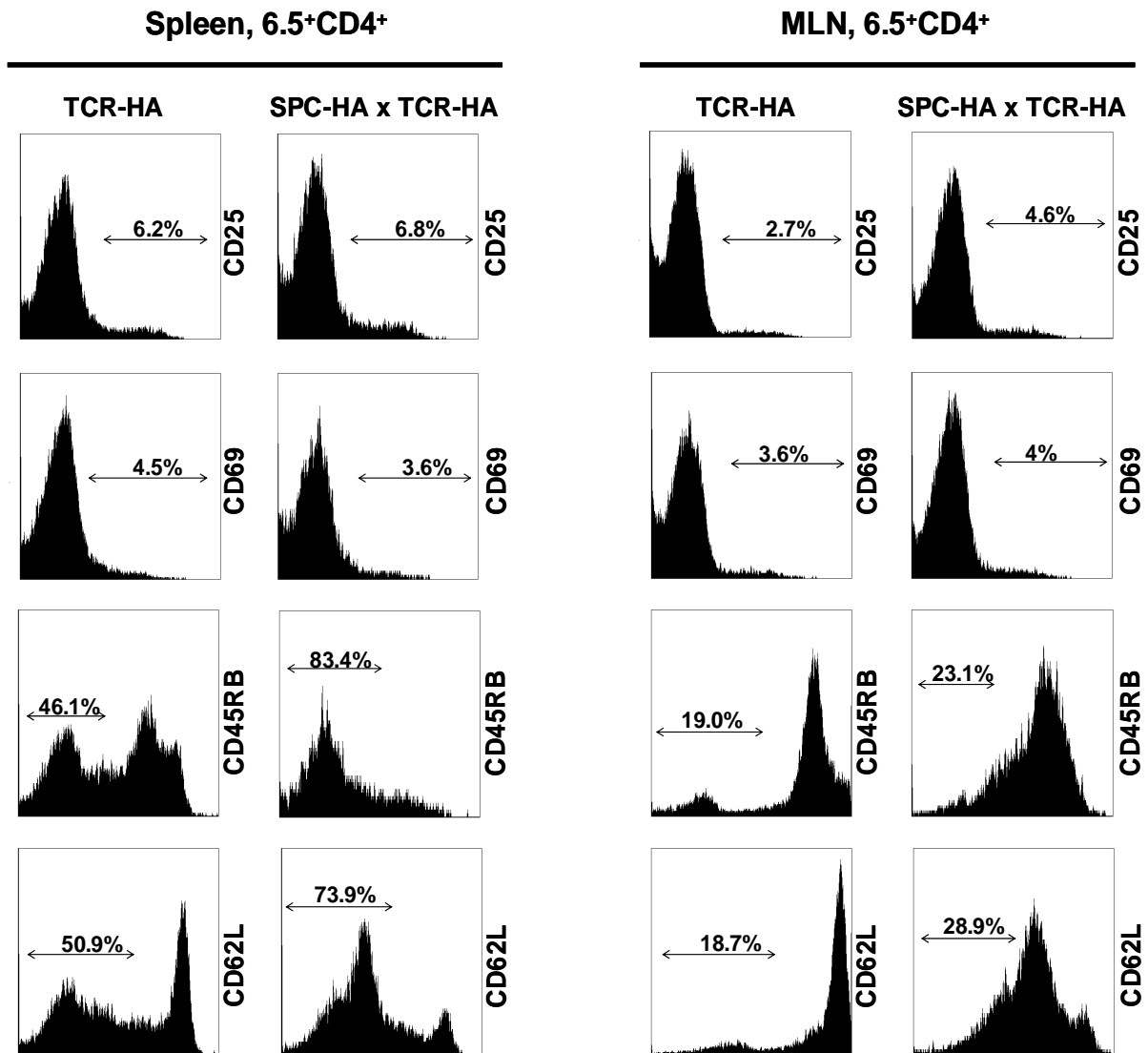


Figure 12 (continued): Activation/memory phenotype of HA-specific CD4⁺ T cells from double transgenic SPC-HA x TCR-HA mice compared to healthy TCR-HA control mice.

Also, in contrast to the lung the expression of CD25 and CD69 was not increased when the peripheral compartments spleen and MLN from SPC-HA x TCR-HA mice and control mice were compared. However, the expression of the memory markers CD45RB and CD62L was downregulated in these compartments on 6.5⁺CD4⁺ T cells from double transgenic mice (figure 12).

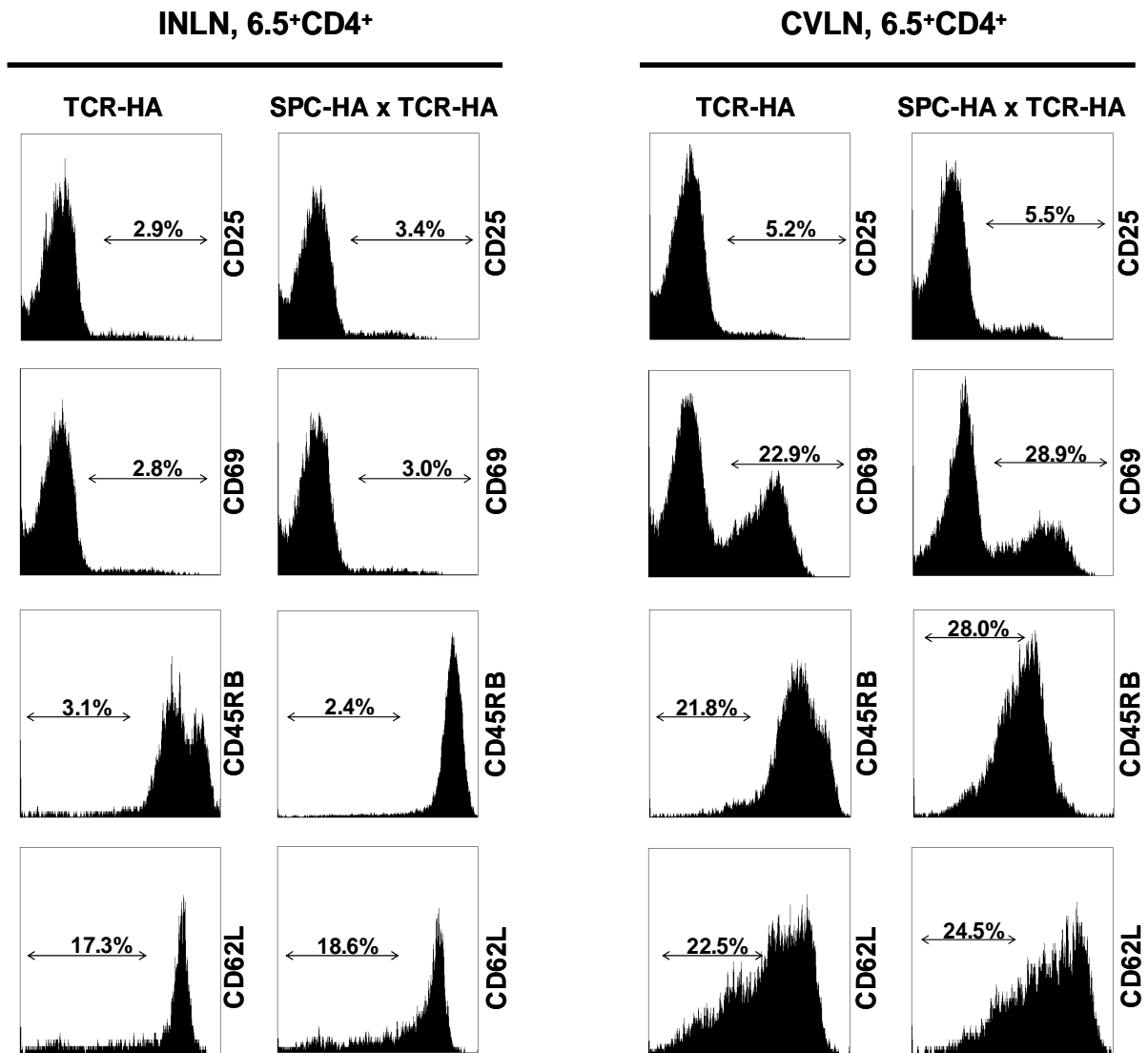


Figure 12 (continued): Activation/memory phenotype of HA-specific CD4⁺ T cells from double transgenic SPC-HA x TCR-HA mice compared to healthy TCR-HA control mice.

In INLN, CVLN and AXLN no obvious changes in the expression level of CD25, CD69, CD45RB and CD62L could be detected on autoreactive T cells from SPC-HA x TCR-HA mice when compared to TCR-HA controls.

AXLN, 6.5⁺CD4⁺

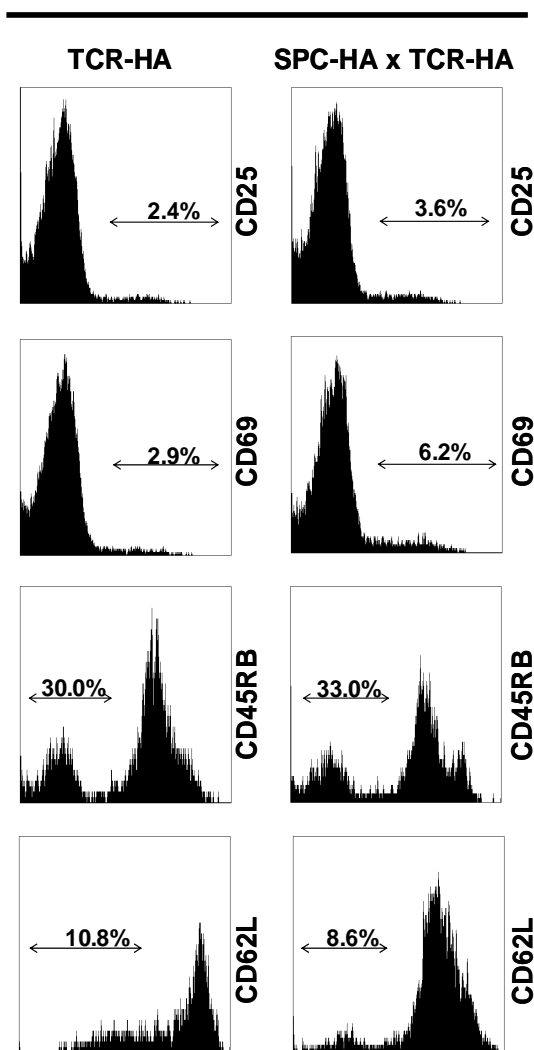


Figure 12 (continued): Activation/memory pattern of HA-specific CD4⁺ T cells from double transgenic SPC-HA x TCR-HA mice compared to healthy TCR-HA control mice.

In summary, FACS data suggest that in SPC-HA x TCR-HA mice in which the lung epithelium expresses the self-antigen hemagglutinin, the lung is the place where the inflammation starts and extends out in the lung tissue due to autoreactive CD4⁺ T cell antigen recognition. The autoreactive 6.5⁺CD4⁺ T cells proliferate in and/or infiltrate the lung and exhibit a strongly activated phenotype in the presence of the self-antigen HA.

2.3 Marker genes for regulatory T cells are expressed in 6.5⁺CD4⁺ lung lymphocytes isolated from SPC-HA x TCR-HA double transgenic mice

It has been shown previously that expression of HA under the control of the Ig κ promoter by hemopoietic cells resulting in permanent antigen expression both in thymus and in the periphery leads to tolerance rather than inflammation (Buer et al. 1998). Therefore, it was reasonable to analyze whether mature 6.5⁺CD4⁺ T cells found in peripheral lymphoid organs of SPC-HA x TCR-HA double transgenic mice are still functional with respect to their proliferative capacity upon antigen encounter. In previous works it could be shown that the proliferative capacity of lung lymphocytes from SPC-HA x TCR-HA mice was reduced by approximately 50% when compared with lung T cells from TCR-HA control mice (Bruder et al., 2004). Moreover, data from this study suggested an important role of T_{reg} cells for the development and progression of CD4⁺ T cell mediated lung disease in the SPC-HA x TCR-HA mouse model. Cytokine and gene expression profiling on isolated 6.5⁺CD4⁺ autoreactive T cells from diseased mice demonstrated a changed phenotype compared to cells derived from TCR-HA control mice. This phenotype resembled a T_{reg1} regulatory phenotype and suggested the *in vivo* induction of these suppressor T cells during the progress of lung inflammation in SPC-HA x TCR-HA mice. To corroborate and extend the observed differences of the gene expression pattern between naïve 6.5⁺CD4⁺ lung lymphocytes and primed 6.5⁺CD4⁺ lung lymphocytes from SPC-HA x TCR-HA, autoreactive T cells were isolated from lung tissues of diseased and healthy mice for semi-quantitative analysis by PCR.

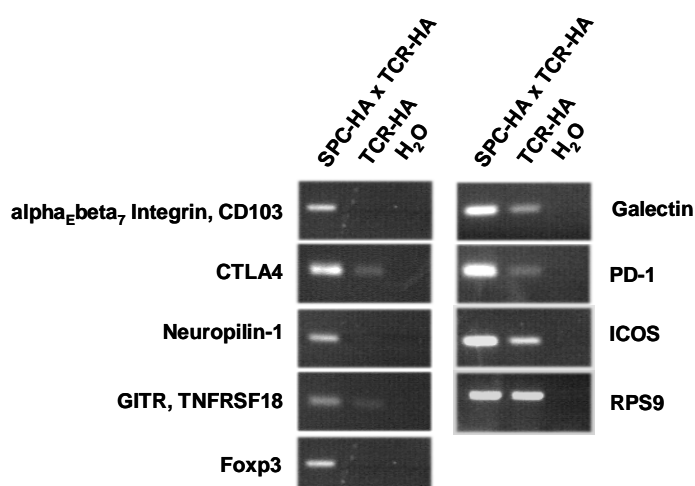


Figure 13: Semi-quantitative RT-PCR analysis of ex vivo isolated 6.5⁺CD4⁺ lung lymphocytes from SPC-HA x TCR-HA mice and TCR-HA control mice. The expression of different molecular marker genes for regulatory T cells as CD103 (alpha_Ebeta₇), CTLA4, Neuropilin-1, GITR (TNFRSF18), Foxp3, Galectin, PD-1 and ICOS was analyzed. RPS9 was used as a housekeeping gene.

In line with results obtained by Affymetrix Arrays, PCR analysis revealed elevated expression of CTLA4, PD-1, ICOS, GITR and $\alpha\text{E}\beta\text{7}$ (CD103) in autoreactive T cells from double transgenic mice compared to naïve T cells from the TCR-HA mice (figure 13). Whereas these genes are known to be expressed not only on regulatory but in addition on activated T cells, also strong upregulation of Neuropilin-1 and Foxp3 was detectable. These genes are activation independent and exclusively linked to regulatory T cells, thus clearly indicating the induction of self-antigen specific regulatory T cells in the lung of diseased SPC-HA x TCR-HA mice.

2.4 Progressive infiltration of HA-specific CD4⁺ T cells in the lung of SPC-HA mice after adoptive transfer

As mentioned above, HA expression in the lung of SPC-HA mice leads, despite the fact of HA expression in the thymus, to strong inflammatory reactions in the lung of double transgenic SPC-HA x TCR-HA mice. Consistent with the FACS data shown in figure 12, the inflammation is restricted exclusively to the lung. Importantly, although the early inflammatory lesions observed in the lungs of newborn and young SPC-HA x TCR-HA mice were quite severe, there was no evidence for uncontrolled progression of the inflammation leading to complete tissue destruction or death in elder mice (Bruder et al., 2004), but data suggest the induction of regulatory mechanisms counter-regulating disease progression. With intent to analyze the impact of regulatory T cells on the outcome of autoimmunity and to clarify whether the small proportion of naturally occurring HA-specific T_{reg} cells is able to suppress the proliferative and inflammatory capacity of 6.5⁺CD4⁺CD25⁻ T cells *in vivo*, adoptive transfer experiments were performed.

To this end, first of all SPC-HA and BALB/c control mice were adoptively transferred with autoreactive CD4⁺ T cells. 1 x 10⁷ CD4⁺ T cells from TCR-HA mice prior activated *in vitro* were injected i.v. in transgenic SPC-HA mice and BALB/c control mice. At days 1, 3 and 7 mice from each group were sacrificed and the lungs were dissected for histological examination (figure 14).

This analysis revealed rapid and distinct lymphocytic infiltration, thus clearly demonstrating the strong impact of autoreactive CD4⁺ T cells on disease development and progression. After establishing the conditions for the adoptive

transfer, the transfer model was used for further analyzing the influence of antigen-specific naturally occurring T cells and *in vivo* induced antigen specific regulatory T cells.

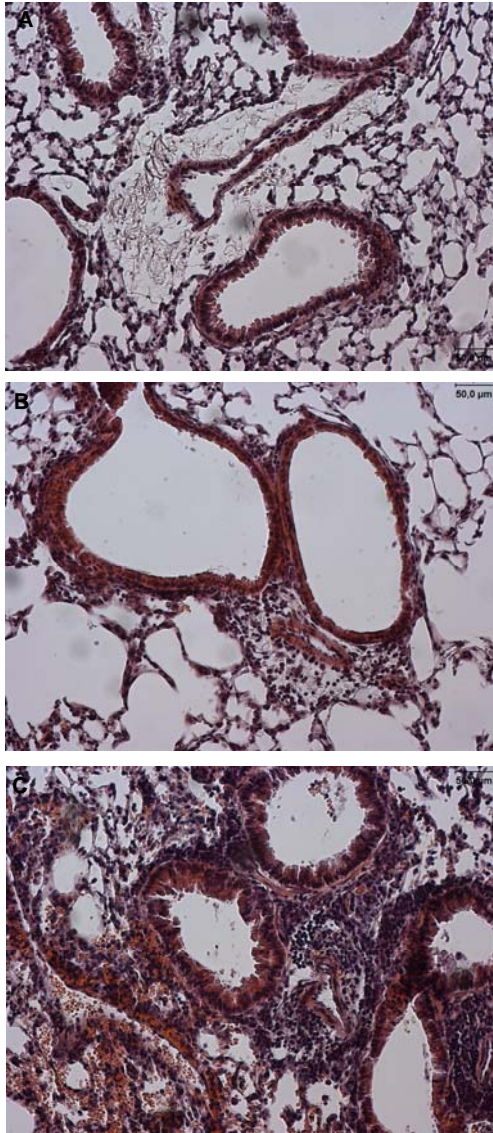


Figure 14: Progressive infiltration of HA-specific CD4⁺ T cells in the lung of SPC-HA mice after adoptive transfer. 1×10^7 CD4⁺ T cells from TCR-HA transgenic mice prior activated *in vitro* were injected i.v. in transgenic SPC-HA or BALB/c mice, respectively. At day 1 (A), 3 (B) and 7 (C) the mice were sacrificed and lungs were dissected to perform histological analysis. From day 1 to day 7 a rapid progressive infiltration of lymphocytes was documented as shown here for arteries. Hematoxylin and eosin (H&E) stain, bar = 50.0 μ m. These results are a representative of two similar experiments.

2.5 Adoptive transfer of 6.5⁺CD4⁺ and 6.5⁺CD4⁺ depleted of CD25⁺ regulatory T cells into SPC-HA transgenic mice

HA-specific lung lymphocytes from double transgenic SPC-HA x TCR-HA mice have an activation/memory phenotype and show a reduced proliferative capacity when stimulated *in vitro* with the corresponding peptide. The autoreactive 6.5⁺CD4⁺ T cells from the lung of SPC-HA x TCR-HA mice secreted drastically reduced amounts of the proinflammatory cytokines interferon (INF)- γ and IL-2, but an increased level of IL-5 upon *in vitro* stimulation.

Elevated levels of IL-10 and no detectable IL-4 production and moreover the global gene expression profiling of alveolar HA-specific CD4⁺ T cells from diseased mice, which revealed many genes associated with regulatory CD4⁺ T cells like the surface markers CTLA-4, PD-1, Nrp1 and $\alpha\text{E}\beta_7$ suggested a T_R1 regulatory T cell phenotype. Data were underlined by histological evidence in elder diseased mice which showed a controlled inflammation in the lung of double transgenic SPC-HA x TCR-HA mice (Bruder et al., 2004).

CD4⁺CD25⁺ T cells derived in the thymus constitute a major population of regulatory T cells that are able to suppress T cell responses *in vitro* (Read et al., 1998; Thornton & Sevaach, 1998) as well as *in vivo* (Read et al., 2000; Suri-Payer et al., 1998).

To clarify whether the small proportion of naturally occurring HA-specific 6.5⁺CD4⁺CD25⁺ regulatory T cells is able to suppress the proliferative and inflammatory capacity of 6.5⁺CD4⁺CD25⁻ T cells *in vivo*, adoptive transfer experiments were performed following the conditions described above. Naïve CD4⁺ and CD4⁺CD25⁻ T cells were isolated from spleen and lymph nodes of TCR-HA transgenic mice by negative selection using AutoMACS. The percentages of 6.5⁺CD4⁺ T cells were analyzed by FACS and CFSE labelling was performed. 2×10^6 6.5⁺CD4⁺ T cells or 6.5⁺CD4⁺ depleted from CD25⁺ T_{reg} cells (6.5⁺CD4⁺CD25⁻) were injected i.p. into SPC-HA transgenic mice. 7 days after adoptive transfer the *in vivo* proliferation of 6.5⁺CD4⁺ transgenic T cells was investigated by estimating the loss of CFSE dye in proliferating HA-specific CD4⁺ T cells from lung, BLN and spleen (figure 15).

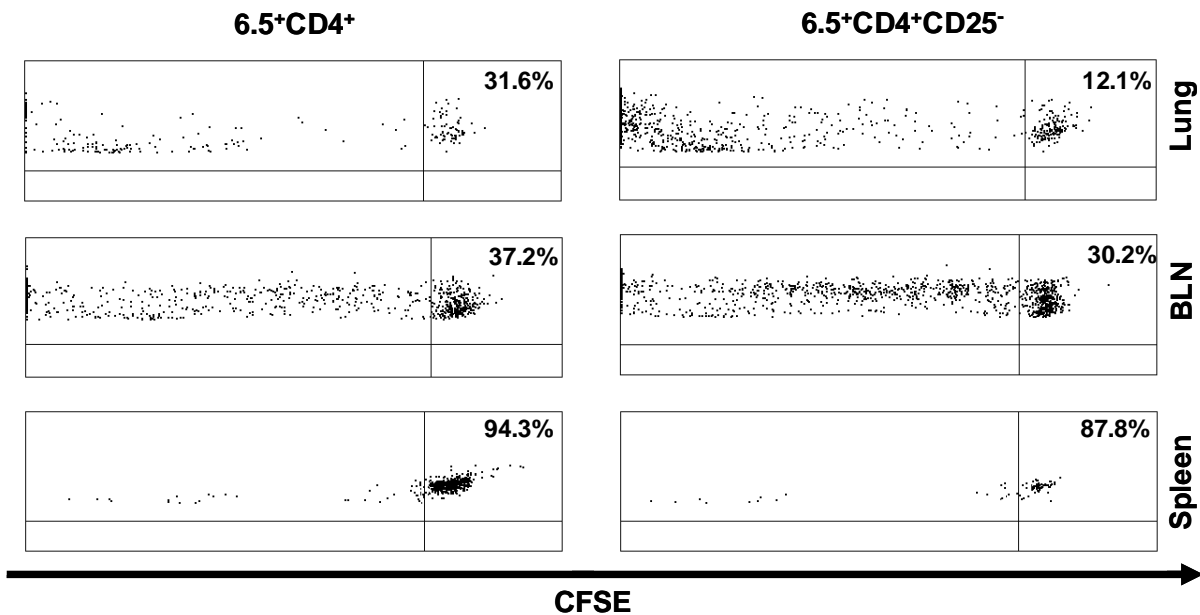


Figure 15: Proliferative response of 6.5⁺CD4⁺ T cells *in vivo* to tissue derived antigen after adoptive transfer into SPC-HA recipients. 2 × 10⁶ CFSE labelled 6.5⁺CD4⁺ or 6.5⁺CD4⁺CD25⁻ T cells were adoptively transferred into SPC-HA recipients. 7 days later cells from lung, BLN and spleen were isolated and stained for 6.5 and CD4 expression. CFSE profiles gated for 6.5⁺CD4⁺ T cells were estimated. These results are a representative of two similar experiments.

In lung and BLN it could be shown that in the absence of naturally occurring regulatory T cells the proportion of 6.5⁺CD4⁺ transgenic T cells underwent a strong proliferative activity in response to the lung-derived self-antigen HA. The proliferation of 6.5⁺CD4⁺ T cells containing the regulatory CD25⁺ HA-specific T cell population was less prominent suggesting that naturally occurring CD25⁺ T cells suppress antigen specific proliferation of lung-specific CD4⁺ T cells.

The percentages of 6.5⁺CD4⁺ transgenic T cells obtained after re-isolation from the recipient mice underline this hypothesis. As summarized in Figure 16 the percentage of 6.5⁺CD4⁺ transgenic T cells is increased in the lungs of SPC-HA mice that received 6.5⁺CD4⁺ depleted from naturally occurring CD25⁺ T cells. Together, these data suggest the active suppression of proliferation of autoreactive T cells in the lung after antigen encounter by HA specific 6.5⁺CD4⁺CD25⁺ regulatory T cells, pointing out the importance of T_{reg} cells for the control of autoimmune mediated T cell responses in the lung.

Antigen specificity of T cell proliferation was underlined by the fact that no proliferation was observed in transferred cells re-isolated from the spleen of SPC-HA mice, which does not express the HA antigen.

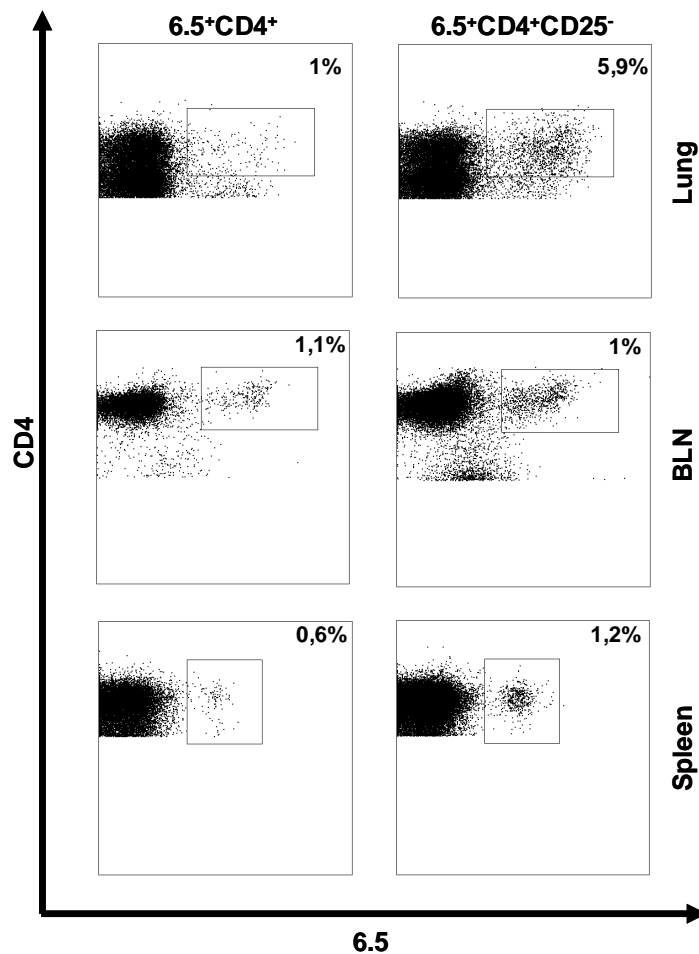


Figure 16: Clonal expansion in response to self-antigen. 2×10^6 6.5⁺CD4⁺ or 6.5⁺CD4⁺CD25⁻ T cells were adoptively transferred i.p. into SPC-HA recipients. 7 days later T cells from lung, BLN and spleen were isolated and stained for 6.5 and CD4 expression to measure the percentage of transgenic T cells in the different compartments. These results are a representative of three similar experiments.

2.6 Adoptive transfer of 6.5⁺CD4⁺ T cells in SPC-HA x TCR-HA double transgenic mice

To analyze whether in addition to naturally occurring regulatory T cells also the proportion of *in vivo* induced HA-specific regulatory T cells has the potential to suppress antigen specific expansion of adoptively transferred 6.5⁺CD4⁺ T cells, an adoptive transfer of 6.5⁺CD4⁺ T cells was performed into SPC-HA x TCR-HA double transgenic mice as well as into SPC-HA transgenic mice as control. It could be hypothesized, that only in SPC-HA x TCR-HA mice regulatory T cells were induced upon chronic self-antigen stimulation in the lung, whereas this *in vivo* induced HA-specific T_{reg} should be absent in the lung of SPC-HA single transgenic mice. T cells were isolated from spleen of TCR-HA transgenic mice and were activated *in vitro*

with the corresponding peptide. The proportion of 6.5^+CD4^+ T cells was analyzed by FACS and CFSE labelling was performed. 2.5×10^6 6.5^+CD4^+ transgenic T cells were injected i.p. into SPC-HA x TCR-HA double transgenic and SPC-HA transgenic control mice. 10 days after adoptive transfer the *in vivo* proliferation of 6.5^+CD4^+ transgenic T cells was investigated by analyzing the loss of CFSE dye in proliferating HA-specific $CD4^+$ T cells from lung, BLN and spleen (figure 17).

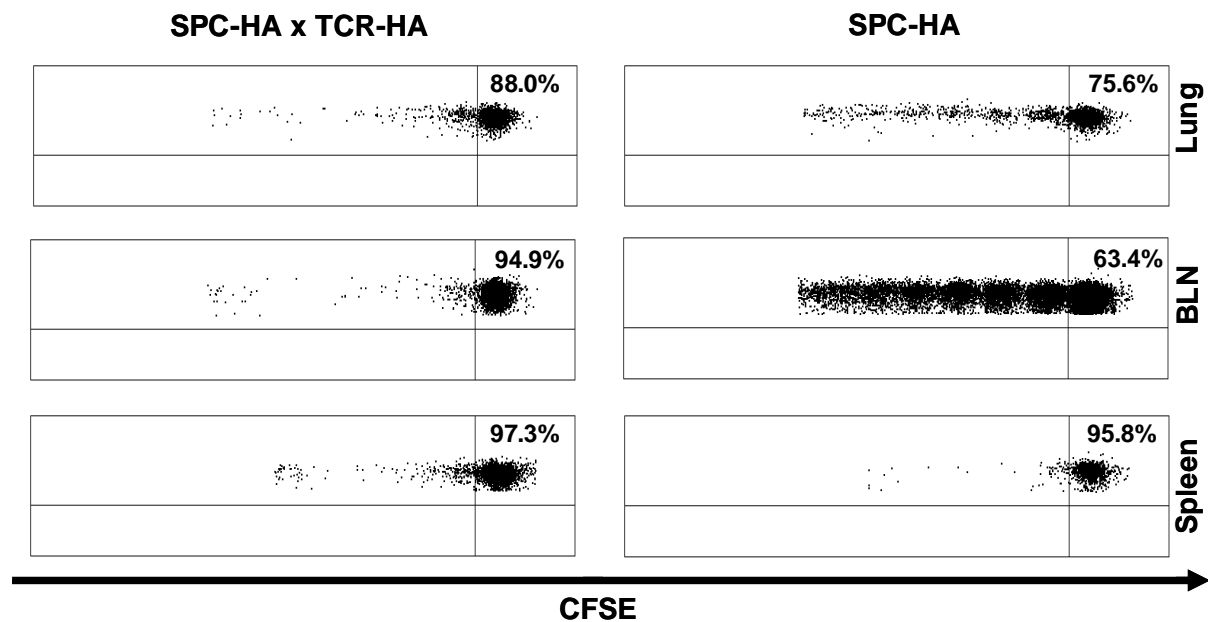


Figure 17: Proliferative response of 6.5^+CD4^+ T cells *in vivo* to tissue derived antigen after adoptive transfer in SPC-HA x TCR-HA and SPC-HA recipients. 2.5×10^6 CFSE labelled 6.5^+CD4^+ T cells were adoptively transferred into SPC-HA x TCR-HA and SPC-HA recipients. 10 days later cells from lung, BLN and spleen were isolated and stained for 6.5 and CD4 expression. CFSE profile gated for 6.5^+CD4^+ T cells were estimated. These results are a representative of three similar experiments.

The data summarized in figure 17 clearly indicate a strong inhibition/suppression of the proliferation of adoptively transferred 6.5^+CD4^+ T cells in lung and BLN of SPC-HA x TCR-HA double transgenic mice. In contrast, 6.5^+CD4^+ T cells adoptively transferred into SPC-HA transgenic control mice underwent a strong proliferation in these compartments. These data strongly suggest the capability of *in vivo* induced regulatory T cells to suppress the proliferation of autoreactive T cells *in vivo*.

CHAPTER II

Results

Part II:

Characterization of self-antigen expressing alveolar type II epithelial cell from SPC-HA x TCR-HA mice

3 Aims of the study

Immune regulation is a dynamic process based on many already known as well as undefined factors. Many cells are involved in triggering or regulation of immune responses and more and more details about the mechanisms underlying inflammation and tolerance are known. However, less is known about the function of cells expressing self-antigens and being the target for autoreactive T cells in the progress of initiation and/or regulation of autoimmunity.

The SPC-HA x TCR-HA double transgenic mouse represents an autoimmune disease model that offers the possibilities to examine both sides of the immune response, i.e. the autoreactive effector cells on the one hand as well as the self-antigen expressing cells on the other hand. In the SPC-HA transgenic mouse the alveolar type II epithelial cells do express the self-antigen hemagglutinin and represent the target for autoreactive T cells. Already 1977 Mason and Williams developed the concept of the alveolar type II epithelial cell (AECII) as a defender of the alveolus (Mason and Williams, 1977). AECII may act as immunoregulatory cells and can interact with resident and mobile cells, either directly by membrane contact or indirectly via cytokines/growth factors and their receptors. Thus alveolar type II epithelial cells represent an integrative unit of immune responses within the alveolus. To investigate the role of hemagglutinin expressing AECII in SPC-HA x TCR-HA double transgenic mice on the outcome of autoimmunity, extensive analysis should be performed including:

- Establishing a protocol to isolate murine type II alveolar epithelial cells exhibiting a high purity and vitality.
- Gene expressing profiling of type II alveolar epithelial cells from diseased double transgenic SPC-HA x TCR-HA mice compared to healthy SPC-HA control mice.
- Characterization of the antigen presenting capacity of AECII from healthy SPC-HA and diseased SPC-HA x TCR-HA mice to rule out their possible role in the initiation and regulation of autoreactive T cell responses leading to lung inflammation.

- Characterization of the impact of AECII on the induction of regulatory T cells.

4 Results

4.1 Isolation of murine alveolar type II epithelial cells

Alveolar type II epithelial cells (AECII) are critical for normal lung development, homeostasis, and repair after injury. AECII produce pulmonary surfactant lipids and proteins required for reducing alveolar surface tension (Finkelstein et al., 1983; Shannon et al., 2001). As essential progenitors for type I epithelial cells, they are also critical for normal alveolar development and tissue remodelling after injury (Adamson and Bowden, 1974; Adamson and Bowden, 1975). The ability to investigate organogenesis and disease progression by overexpressing and deleting genes in mice, particularly genes expressed by alveolar type II epithelial cells, has recently favoured the use of mouse models in pulmonary research.

Although mice are advantageous for manipulating genes, they have not been useful for isolating alveolar type II epithelial cells for *ex vivo* study so far. In contrast, rat and rabbit AECII have successfully been isolated using velocity centrifugation through a gradient of albumin (Dobbs and Mason, 1979 and Finkelstein et al., 1983). Isolation of mouse AECII by this method has been less successful. In order to isolate highly pure and vital AECII, a fluorescence-activated cell sorting protocol was established based on the use of anti-CD32, anti-CD16, anti-CD45, anti-CD11b and anti-F4/80 antibodies. Using this antibody cocktail, all hemopoietic cell types were labelled. Moreover, further cell populations could be distinguished by cell granularity (sideward scatter, SSC) and size (forward scatter, FSC). Using a MoFlow cell sorter (Cytomation, Fort Collins, CO) a cell population could be purified (figure 18) that was identified by immune fluorescence staining as alveolar type II epithelial cells (figure 19).

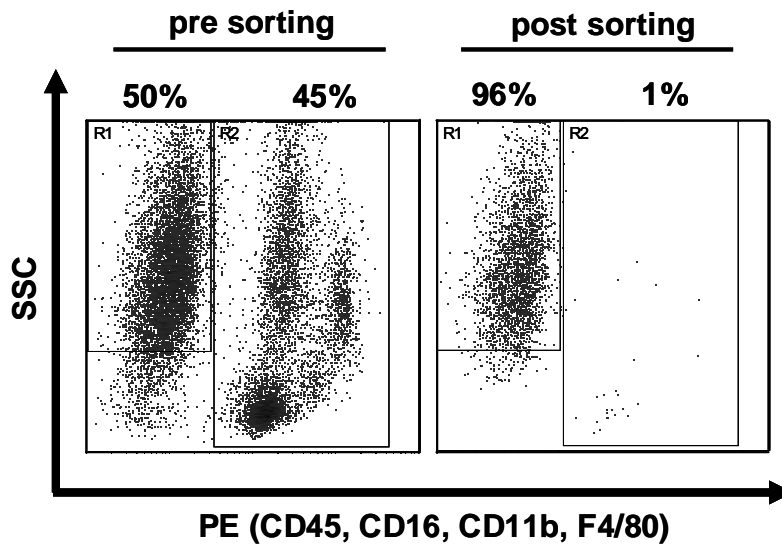


Figure 18: Purification of alveolar type II epithelial cells by fluorescence-activated cell sorting. Cells obtained after enzymatic lung digestion were labelled using a CD45, CD16, CD32, CD11b, and F4/80 antibody cocktail. After exclusion of antibody negative cells, AECII were identified by size and granularity. Reanalysis of sorted cells have shown a contamination of as few as 1% other cells.

The reanalysis of the sorted cells demonstrated an extremely high purity of an unlabelled AECII population. Positive immunostaining for the surfactant proteins A, B, C and D verified that the sorted cells were alveolar type II epithelial cells.

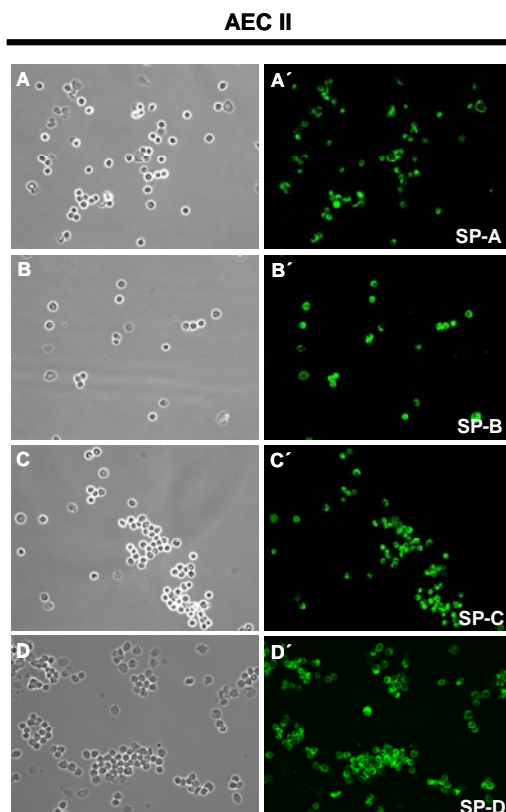


Figure 19: Identification of sorted cells as alveolar type II epithelial cells by immunofluorescence staining for surfactant proteins A, B, C and D. Cytospins of post-sorted cells were stained for the surfactant proteins A, B, C and D. Almost 100% of fixed post-sorted cells were positive for the surfactant protein staining. Picture A, B, C and D represent the phase contrast of fixed cells. Pictures A', B', C', and D' represent the corresponding surfactant protein immunostainings.

Tissue disintegration and FACS-sorting represents physical stress for the cells which could lead to apoptosis and cell death during the purification procedure. To assure vitality of sorted cells, trypan blue staining and propidium iodide staining were performed. Visually counting of trypan blue positive AECII resulted in a proportion of only 10% dead cells. The fluorescence-activated measurement of propidium iodide positive AECII resulted in a vitality of 90% (figure 20). Thus, both methods demonstrated that only a small portion of AECII cells did not survive the isolation procedure.

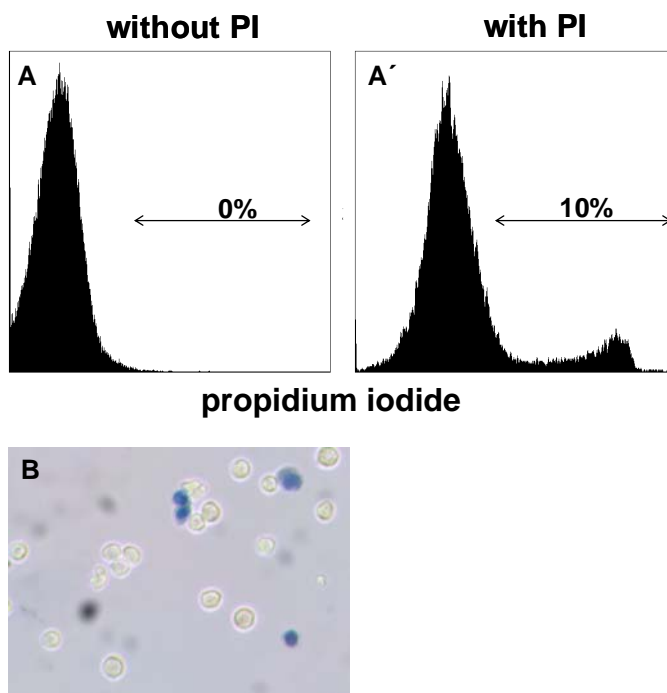


Figure 20: Determination of vitality of sorted alveolar type II epithelial cells. Vitality of freshly isolated type II alveolar epithelial cells was determined by FACS analysis by using propidium iodide (A → A') and visually counting of trypan blue (B) staining. Both methods indicated a vitality of 90%.

Reanalysis of the sorted alveolar type II epithelial cells showed a minor contamination (1%) with $CD45^+/CD32^+/CD16^+/CD11b^+/F4/80^+$ cells. To determine in more detail which hemopoietic cell type was present within the alveolar type II cell preparation, PCR analysis were performed using CD3 (T cells), CD19 (B cells) and CD14 (macrophages) specific primers. Furthermore the sorted cells were tested by PCR analysis for the presence of AECII specific marker RNA (surfactant protein A, D and C and Alkaline phosphatase) (figure 21).

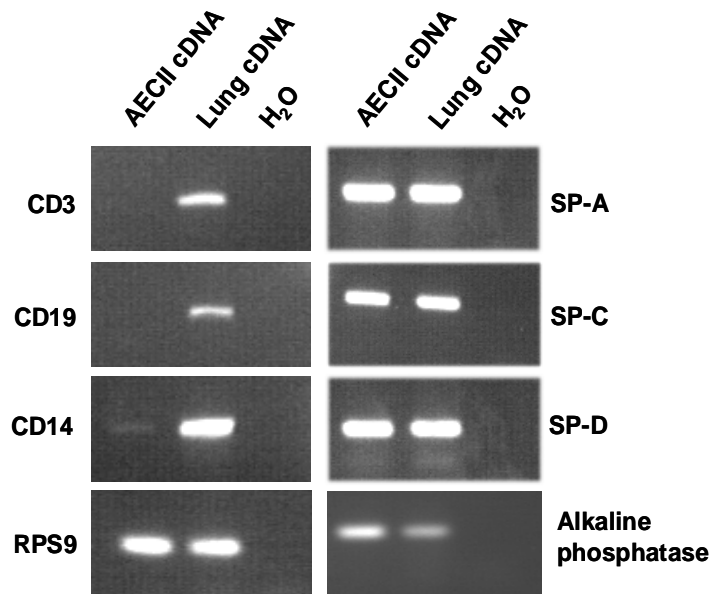


Figure 21: Analysis of hemopoetic cell contamination of post-sorted AECII population by PCR. RNA from freshly isolated alveolar type II epithelial cells was compared with RNA obtained from complete lung tissue. Primer pairs CD3, CD19 and CD14 were chosen to test for hemopoetic cells in the type II cell preparation. Primer pairs SP-A, SP-C, SP-D and alkaline phosphatase were chosen to test the RNA for specific AECII expressed genes. RPS9 represents a housekeeping gene expression.

As shown before by (figure 19) immunofluorescence stainings for surfactant proteins, PCR analysis verified a highly pure population of alveolar type II epithelial cells (Figure 21). In addition, the AECII marker gene alkaline phosphatase (Edelson et al., 1988) could be detected. Only PCR analysis using CD14 specific primers resulted in a weak PCR amplification product in the purified AECII cDNA sample, but no products were obtained with CD19 or CD3 specific primer pairs. Thus, the contamination by hemopoetic cells was neglected for subsequent experiments and the AECII cells were considered to be highly pure.

As expected, the expression analysis for type II epithelial cell specific proteins was positive for every gene analyzed (figure 21).

Together these data show that the newly established protocol represents a highly efficient method to obtain pure and vital murine alveolar type II cells.

4.2 Global gene expression profiling of murine alveolar type II epithelial cells

In the SPC-HA transgenic mouse the alveolar type II epithelial cells are the cells expressing the self-antigen and therefore represent the target for HA-specific T cell reactivities. As previous data describe, the 6.5^+CD4^+ T cells in elder SPC-HA x TCR-HA mice have a T_R1 phenotype. The questions raised by this finding are: a) how do activated autoreactive T cells change their features to a more regulatory phenotype and b) which role do the self-antigen expressing AECII play in this process? To shed more light into the role of AECII in the induction of T cell tolerance in the lung, comparative global gene expressing profiling of alveolar type II epithelial cells from diseased SPC-HA x TCR-HA double transgenic mice and healthy SPC-HA mice was performed. Therefore, RNA was prepared from AECII and subjected to differential gene expression analysis using Affimetrix MG U74Av2 oligonucleotide arrays. The advantage of this technology is that every analyzed gene is represented by sixteen independent probe pairs which together establish the basis for statistical evaluations of the respective signals. Therefore, only those genes that are reproducibly regulated are included in the analysis. For each gene fulfilling these criteria, the average fold change in expression for AECII from SPC-HA x TCR-HA and SPC-HA mice was calculated and the ratio was depicted on a base-2 logarithmic scale. To get an impression of basal expression level of analyzed genes in AECII under normal conditions, an alignment of AECII derived from SPC-HA single transgenic and diseased SPC-HA x TCR-HA double transgenic mice was necessary to include. This approach led to a comprehensive overview about the functional gene classes that are found to be regulated in type II pneumocytes upon inflammation including for example regulators of transcription and translation, secreted or signalling molecules, genes involved in cell cycle, apoptosis and survival.

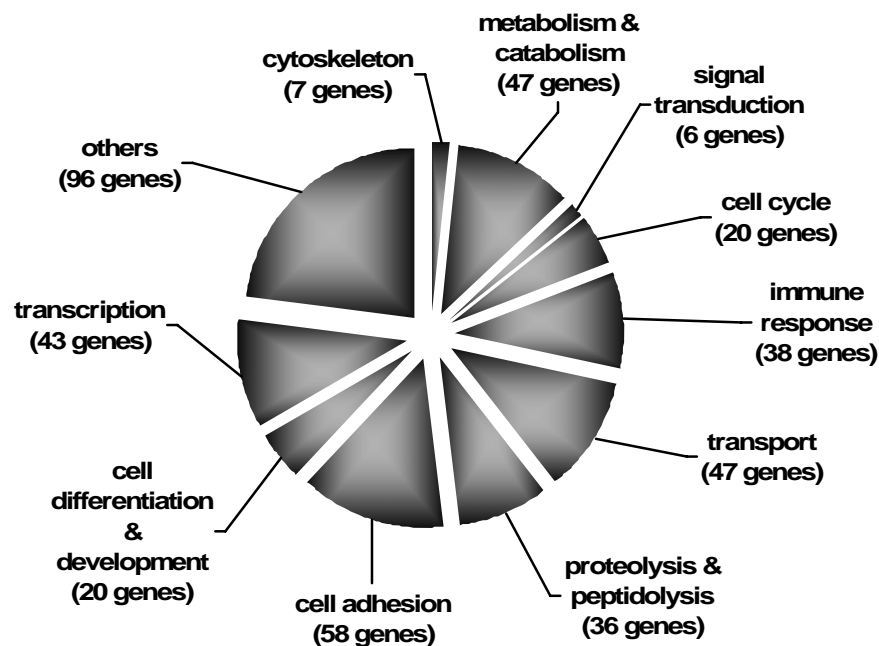


Figure 22: Genes differentially expressed in type II alveolar epithelial cells isolated from diseased SPC-HA x TCR-HA compared to cells from healthy SPC-HA mice. 418 genes were identified as differentially expressed in AECII and regarding their function divided into eleven major groups.

Table 4: Gene expression levels of selected genes from different major groups.

Gene	Functional Group	Fold Induction	Reference
CCL11 (Eotaxin)	immune response	-8.48 -4.06	- Matthews et al., 2005 - Sebastiani et al., 2001
CCL20 (MIP-3 α)		17.12 11.53	- Dieu-Nosjean et al., 2000
CCL9/10 (MIP-1 γ)		4.09 2.53	- Maurer and Stebut, 2004
CXCL13		-4.52 -5.88	- Ebert et al., 2004
CXCL12		-6.71 -7.40	- Balabanian et al., 2005
CXCL2 (MIP-2 α)		3.08 2.37	- Matzer et al., 2004
TGF-b2		-3.00 -2.21	- Buckley et al., 1996
TGF-b3		-2.40 -2.75	- Buckley et al., 1996
Psmb8 Proteasome subunit beta type 8		2.15 4.51	- Niedermann et al., 1999
Psmb9 Proteasome subunit beta type 9		2.78 2.53	- Niedermann et al., 1999

Results were obtained by two independent experiments. Basal gene expression level of AECII derived from healthy SPC-HA mice were compared with gene expression levels of AECII from diseased SPC-HA x TCR-HA mice.

Table 4 (continued): Gene expression levels of selected genes from different major groups.

Gene	Functional Group	Fold Induction	Reference
H2-Ea (major histocompatibility complex, class II, DR alpha)	immune response	2.17	- Krawczyk et al., 2004
H2-Ab1		2.39	
H2-Ab1 (HLA class II histocompatibility antigen, DQ beta 1)		2.05	- Larhammar et al., 1983
TAP1		2.91	
		2.37	- Lankat-Buttgereit and Tampe, 1999
		2.95	
Cyclin A2	cell cycle	1.88	- Bui et al., 1993
		3.42	
Cyclin D2		-2.12	- Wu et al., 1995
		-2.06	
MMP-2 (matrix metalloproteinase 2)	proteolysis & peptidolysis	-10.79	- Chakrabarti and Patel, 2005
		-10.34	
			- Hayashi et al., 1996
			- Xu et al., 2002
			- Corry, et al., 2004
MMP-11 (matrix metalloproteinase 11)		-2.76	- Hayashi et al., 1996
		-1.67	
MMP-23 (matrix metalloproteinase 23)		-6.24	- Hayashi et al., 1996
		-3.78	
MMP-3 (matrix metalloproteinase 3)		-11.59	- Hayashi et al., 1996
		-10.88	
			- Atkinson et al., 2005
MMP-14 (matrix metalloproteinase 14)		-2.51	- Hayashi et al., 1996
		-2.45	
TIMP1 (Tissue inhibitor of metalloproteinase 1)		-11.06	- Hayashi et al., 1996
		-8.62	
TIMP2 (Tissue inhibitor of metalloproteinase 2)		-5.33	- Hayashi et al., 1996
		-9.50	
TIMP3 (Tissue inhibitor of metalloproteinase 3)		-4.03	- Hayashi et al., 1996
		-4.43	
Aqp1 (Aquaporin 1)	transport	-5.99	- Effros et al., 1997
		-4.26	
Egfr (Epidermal growth factor receptor)	cell differentiation & development	-2.06	- Klein et al., 1995
		-2.61	
Fibronectin 1	cell adhesion	-9.88	- Crouch and Longmore, 1987
		-8.60	
			- Maniscalco et al., 1994

Results were obtained by two independent experiments. Basal gene expression level of AECII derived from healthy SPC-HA mice were compared with gene expression levels of AECII from diseased SPC-HA x TCR-HA mice.

Data from figure 22 and table 4 demonstrate broad changes in the gene expression profile within alveolar type II epithelial cells upon T cell mediated airway inflammation. 418 genes could be identified as being differentially expressed in AECII from diseased SPC-HA x TCR-HA double transgenic mice. The classification of the regulated genes in functional groups demonstrate, that many genes that are described to be part of the immune response, cell cycle and proteolysis & peptidolysis appear to be functionally involved in the progression and/or regulation of lung diseases.

Data summarized in table 5 underline the purity of the isolated AECII population, as signal intensities obtained for AECII specific genes were extraordinary high. The signal intensities for hemapoetic cell specific genes are declared as absent or were considerable lower than the signal intensities for AECII specific genes. This is line with the PCR results to analyze the purity of isolated AECII (figure 21).

Table 5: Gene expression levels of selected AECII and hemapoetic cell specific genes to control purity of isolated AECII.

Cell type/gene	Control expression SPC-HA Array1/Array2	present or absent classified*
AECII		
Surfactant associated protein A (Sftpa)	7034/7152	PP
Surfactant associated protein B (Sftpb)	2106/8597	PP
Surfactant associated protein C (Sftpc)	450/1208	PP
Surfactant associated protein D (Sftpd)	13297/16424	PP
Hemapoetic cells		
CD3	15/3	AA
CD11b (ITGAM)	4/22	AA
CD45 (PRPTC)	183/87	PP
CD11c (ITGAX)	44/55	AA

Represented is the signal intensity of indicated genes specific for AECII or hemapoetic cells on Affymetrix MG U74Av2 oligonucleotide array. Results are from pooled individuals (n>3). Present or absent is defined by Affymetrix software logarithm.

4.3 Analysis of genes differentially expressed in AECII from SPC-HA x TCR-HA mice double transgenic mice and SPC-HA transgenic mice.

The global gene expression profiling of murine alveolar type II epithelial cells from SPC-HA x TCR-HA double transgenic mice and SPC-HA control mice resulted in identification of numerous regulated genes. To confirm these results for selected genes the expression levels of platelet factor 4 (PF-4), CCL11 and CCL20 were analysed by real time RT-PCR. These genes have all been discussed before in the context of regulation or modulation of immune responses. It was shown, that platelet factor 4 has suppressor function by downregulating the proliferation and cytokine release of activated T cells (Fleischer et al., 2002). For CCL20 (MIP-3 α) and CCL11 (eotaxin), which are both members of the chemokine ligand family, it is described, that CCL20 plays a critical role for attracting immature dendritic cells to the airway (Reibman et al., 2003) and CCL11 is responsible for eosinophil recruitment (Chvatchko et al., 2003).

To assess the expression level of platelet factor 4 (PF-4), CCL11 and CCL20 total RNA was purified from sorted AECII isolated from SPC-HA x TCR-HA double transgenic mice and SPC-HA mice. Real-time RT-PCR analyses were performed with specific primer pairs for RPS9, PF-4, CCL11 and CCL20 to determine the level of gene expression in comparison with the housekeeping gene expression RPS9 (figure 23).

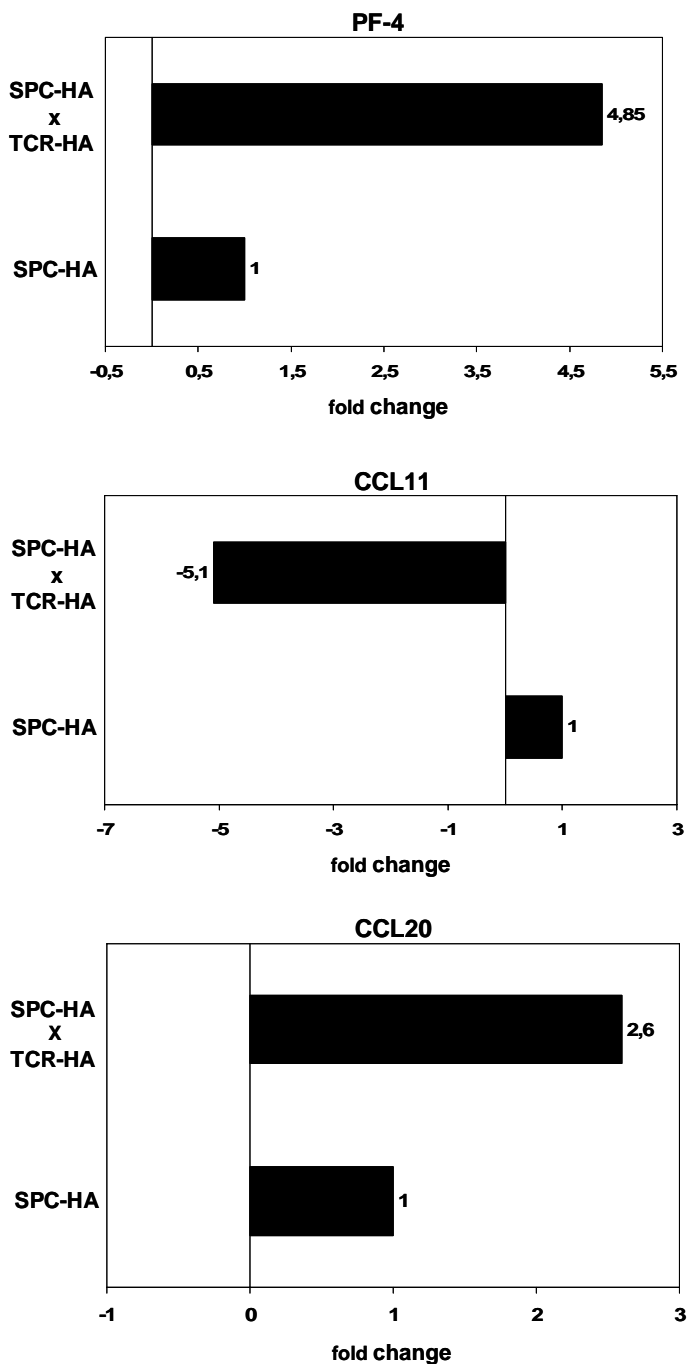


Figure 23: Gene expression level of CCL11, PF-4 and CCL20 in AECII derived from SPC-HA x TCR-HA double transgenic mice and SPC-HA transgenic mice. Real-time RT-PCR analyses for CCL11, PF-4 and CCL20 expression in sorted AECII from SPC-HA x TCR-HA double transgenic mice and SPC-HA transgenic mice. Relative mRNA amounts were normalized with respect to expression levels in AECII derived from SPC-HA transgenic mice (fold change = 1). The mean regulation is indicated.

In line with the results obtained with cDNA arrays the real-time RT-PCR analyses demonstrate an increased mRNA expression for CCL20 and PF-4 in AECII from SPC-HA x TCR-HA double transgenic mice compared to AECII from SPC-HA transgenic mice. Also, analysis of the relative mRNA expression level for CCL11 indicates a significant downregulation in AECII of SPC-HA x TCR-HA double transgenic mice when diseased and healthy mice were compared.

These data suggest that during the inflammatory process in the lung of SPC-HA x TCR-HA double transgenic mice the alveolar type II epithelial cells may have an active immune modulating role which is indicated by increased expression of CCL20 and PF-4 and decreased CCL11 expression.

To further complement the results of the gene expression profile obtained by cDNA array analysis, several other genes of interest were selected and tested by semi-quantitative RT-PCR (figure 24).

For costimulatory or regulatory function of antigen presenting cells the expression of MHC class-II, CD80 (B7.1), CD86 (B7.2), PD1-L (B7-H1) and ICOS-L is necessary. MHC class-II molecules present antigens to effector cells whereas CD80 and CD86 have a costimulatory function and are needed to induce the complete activation of effector cells by binding to their ligand CD28 (Greenwald et al., 2005). PD1-L and ICOS-L are ligands for the receptors PD1 and ICOS expressed on T cells. Binding of ICOS-L or PD1-L by T cells are discussed to be involved in the suppressor function of T_{reg} cells, T cell tolerance, and autoimmunity (Greenwald et al., 2005).

Matrix metalloproteinases (MMP) are a major group of proteinases known to regulate the extracellular matrix (ECM) remodelling and thus they are hypothesized to be important in the process of lung fibrosis or tissue damage in the lung (Corbel et al., 2001).

TGF- β and MCP-1 (CCL2) are important immune regulatory molecules, which are known to be expressed by AECII (Zissel et al., 2000; Paine et al., 1993).

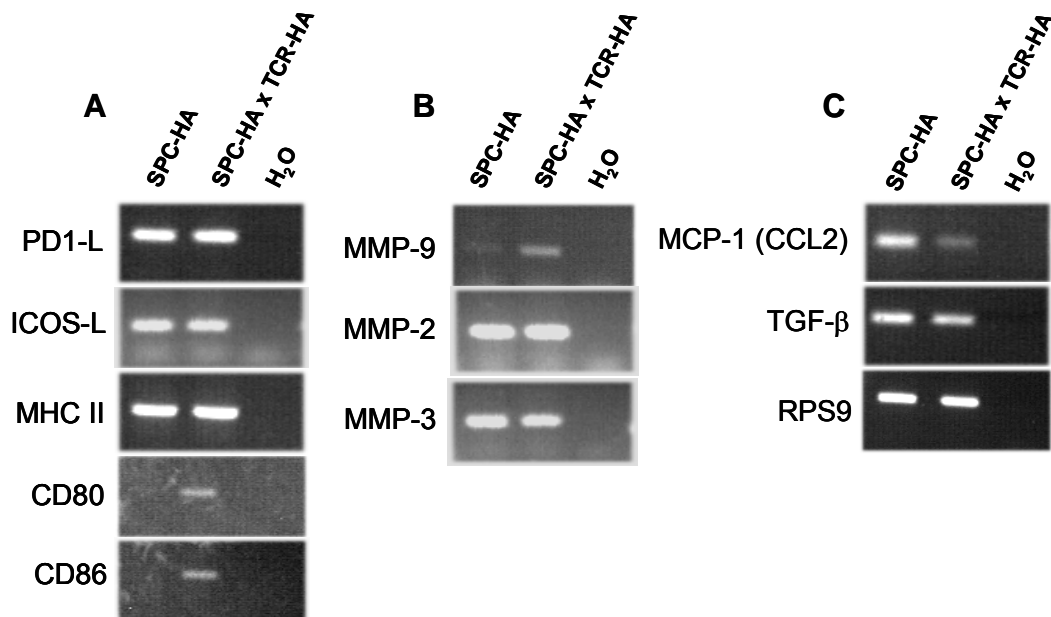


Figure 24: Gene expression in AECII derived from SPC-HA x TCR-HA double transgenic mice and SPC-HA transgenic mice. Different genes were selected to analyze their expression level in AECII by RT-PCR. (A) Genes encoding costimulatory molecules (PD1-L, ICOS-L, MHC II, CD80 and CD86) were used to determine the stimulatory capacity of AECII on the molecular level. (B) represents selected genes for Matrix metalloproteinase (MMP-9, MMP-2 and MMP-3) and (C) MCP-1 and TGF- β . The housekeeping gene RPS9 was used to estimate the quality and quantity of used cDNA.

RT-PCR results clearly indicate a potentially costimulatory function of AECII derived from SPC-HA x TCR-HA double transgenic mice. MHC class-II molecules were constitutively expressed on AECII, whereas CD80 and CD86 were exclusively expressed on AECII from diseased mice. These data suggest that under inflammatory conditions found in SPC-HA x TCR-HA double transgenic mice the expression of costimulatory molecules is upregulated. Furthermore, ICOS-L and PD1-L were both expressed in AECII from healthy and diseased mice, suggesting the putative ability of AECII to regulate T cell responses by binding to their receptors. Regarding RT-PCR data for Matrix metalloproteinases-2 (MMP-2) and MMP-3 both genes were equally present in AECII from SPC-HA x TCR-HA and SPC-HA mice whereas for MMP-9 an upregulation could be detected in AECII isolated from SPC-HA x TCR-HA mice. These data suggest that under inflammatory conditions AECII actively counteract tissue destruction by remodelling the airways at least in part in a MMP dependent process.

As described for AECII they are able to express and release TGF- β to control their accessory cell function (Zissel et al., 2000) and RT-PCR data confirm TGF- β expression by AECII. However, no clear difference in the TGF- β expression level was observed when comparing AECII from diseased and healthy mice.

RT-PCR results for the macrophage inflammatory protein-1 (MCP-1) indicated a decreased expression of MCP-1 in SPC-HA x TCR-HA double transgenic mice. The data were confirmed on protein level by analyzing the culture supernatants of AECII by cytokine bead array (CBA) (data not shown).

4.4 Antigen presentation in the lung

The epithelial surfaces of the lungs and conducting airways are continuously exposed to mixtures of antigens present in ambient air. The adaptive immune system in the lung is faced with the task of accurately categorizing these stimuli, such that T cell responses that are qualitatively appropriate for neutralization of each agent are selected. Secondly, it must tightly control the intensity and duration of these responses, in order to preserve the integrity of the fragile, highly vascularized epithelial surfaces in the organ, particularly those at which gas exchange occurs.

The epithelial surfaces within the major conducting airways in which the majority of inhaled antigen is deposited are protected via the scrubbing action of the overlying mucociliary escalator, and the small proportion of inhaled antigen that escapes this mechanism and penetrates into the underlying epithelial layer is then dealt with via specialized antigen-presenting cells (APC), in particular Dendritic cell (DC) populations, within and below the epithelium. The alveolar surfaces in the deep lung are policed instead by macrophage populations, again backed up to APC population below the alveolar epithelium.

The mechanism for the uptake or presentation of endogenous or self-antigens like the HA in the SPC-HA mouse model is still unclear. Dendritic cells and macrophages are discussed to act as professional APC which are able to take up and cross-present self-antigens from surrounding cells to other immune cells. The interesting question was now, which kind of APC are able and/or essential to present the

hemagglutinin to autoreactive CD4⁺ T cells in SPC-HA x TCR-HA double transgenic mouse and importantly, whether the antigen expressing AECII themselves are able to present the self antigen to the 6.5⁺CD4⁺ T cells. To test whether AECII cells directly present antigen to specific CD4⁺ T cells, or whether AECII derived HA has to be taken up by DC or macrophages to be cross-presented to specific T cells, different types of professional antigen-presenting cells were generated or isolated and cocultured together with freshly isolated antigen expressing AECII derived from SPC-HA mice.

4.5 Presentation of AECII expressed self-antigen mediated by Dendritic cells

To investigate whether Dendritic cells do crosspresent AECII expressed hemagglutinin to autoreactive CD4⁺ T cells, 2.5 x 10⁴ immature bone marrow derived Dendritic cells (BMDC) were cocultured with 1 x 10⁵ freshly isolated type II epithelial cells from the lung of SPC-HA transgenic mice and BALB/c control mice. After 48h 2.5 x 10⁵ CD4⁺ T cells isolated from TCR-HA mice were added as responder cells. The proliferation of 6.5⁺CD4⁺ T cells was measured by ³[H]-thymidine incorporation after further 48h coculture with AECII and BMDC (figure 25). Alternatively, the assay was performed in the presence of LPS to initiate the maturation of BMDC. In this case, 24h after coculture of immature DC with AECII LPS was supplemented.

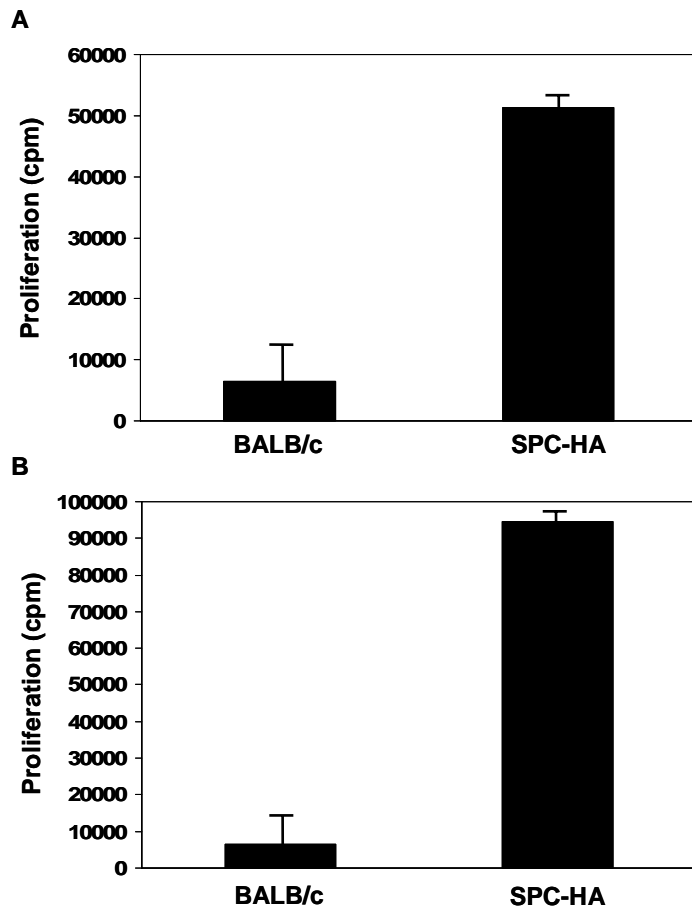


Figure 25: Endogenous self-antigen presentation to autoreactive CD4⁺ T cells mediated by DC. 1 x 10⁵ freshly isolated AECII from SPC-HA or BALB/C mice were cocultured with 2.5 x 10⁴ immature bone marrow derived DC for 48h. After 48h 2.5 x 10⁵ CD4⁺ T cells from TCR-HA mice were added and incubated for further 48h. The proliferation of responder cells was determined by ³[H]-thymidine incorporation. (A) DC-AECII coculture without LPS treatment. (B) After 24h of DC-AECII coculture, LPS was added to initiate the maturation of BM DC. Results are expressed as mean counts per minute + SD (error bars) of triplicate wells and are representative of two independent experiments.

As indicated in figure 25, coculture of AECII with immature and mature Dendritic cells and HA-specific T cells resulted in antigen-specific T cell proliferation, suggesting that Dendritic cells were able to take up and cross present the self-antigen hemagglutinin expressed by AECII from SPC-HA transgenic mice to autoreactive CD4⁺ T cells. LPS treatment during the coculture experiment lead to even stronger proliferation of responder T cells, probably because DC maturation induced by LPS results in upregulation of costimulatory molecules on DC and thus in enhanced T cell activation.

4.6 Peritoneal exudate cells (PEC) as antigen presenting cells can not mediate self-antigen presentation to autoreactive T cells

Macrophages and B lymphocytes are also professional antigen presenting cells, which are able to take up antigens and present it to responder cells. To clarify whether macrophages and B lymphocytes contribute to the presentation of the hemagglutinin expressed by AECII in SPC-HA x TCR-HA double transgenic mice to autoreactive CD4⁺ T cells, PEC isolated from BALB/c mice were cocultured together with AECII from SPC-HA transgenic mice and BALB/c control mice. Peritoneal exudate cells (PEC) were used as a cellular source for macrophages and B lymphocytes. 2.5 x 10⁴ PEC were cocultured with 1 x 10⁵ freshly isolated AECII from SPC-HA and BALB/c mice. After 48h 2.5 x 10⁵ CD4⁺ T cells from TCR-HA mice were added as responder cells. After further 48h proliferation of the responder cells was measured by ³[H]-thymidine incorporation.

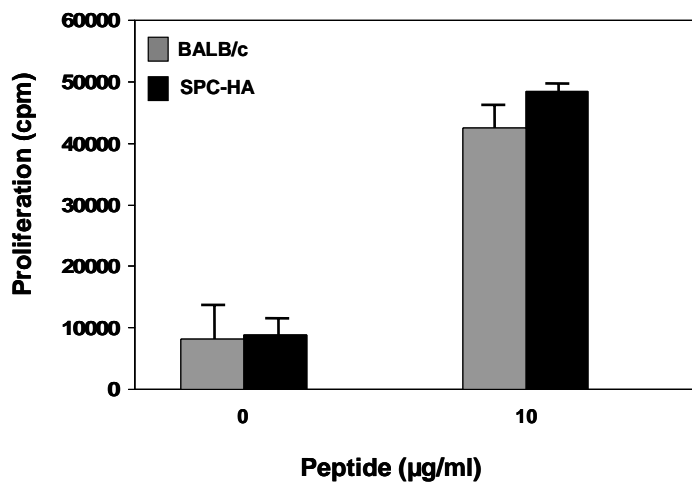


Figure 26: PEC were not able to cross present the AECII expressed hemagglutinin to responder cells. 2.5 x 10⁴ PEC were cocultured together with freshly isolated AECII from SPC-HA transgenic mice and BALB/c mice. After 48h 2.5 x 10⁵ CD4⁺ T cells from TCR-HA mice were supplemented. Corresponding HA-peptide was added as positive control. Proliferation of responder cells was measured by ³[H]-thymidine incorporation. Results are expressed as mean counts per minute + SD (error bars) of triplicate wells and are representative of two independent experiments.

Macrophages as well as B lymphocytes were not able to present the AECII derived hemagglutinin to 6.5⁺CD4⁺ T cells and to activate them (figure 26). No significant antigen specific proliferation was detectable in this coculture assay without addition of exogenous HA-peptide. This could be an indication that macrophages and B

lymphocytes might not be involved in the hemagglutinin antigen presentation and activation of autoreactive T cells in SPC-HA x TCR-HA double transgenic mice.

4.7 The antigen presenting capacity of alveolar type II epithelial cells

Data summarized in figure 25 suggest that the presentation of the self-antigen hemagglutinin in SPC-HA x TCR-HA mice could be mediated by Dendritic cells. It is well established, that epithelial cells themselves have the capacity to function as APC and that they are able to trigger MHC class-I-restricted immune responses (Cunningham et al., 1994; Shao et al., 2005). To demonstrate whether alveolar type II epithelial cells from murine lung are able to present a MHC class-I-restricted peptide to CD8 T cells, 1×10^5 freshly isolated AECII from BALB/c mice were loaded with different concentration of the corresponding HA-peptide and incubated together with 2×10^5 CD8⁺ responder T cells isolated from Clone-4 mice bearing a major histocompatibility complex class-I-restricted T cell receptor specific for an H-2K^d restricted HA peptide (Morgan et al., 1996). T cell proliferation was measured by ³[H]-thymidine incorporation.

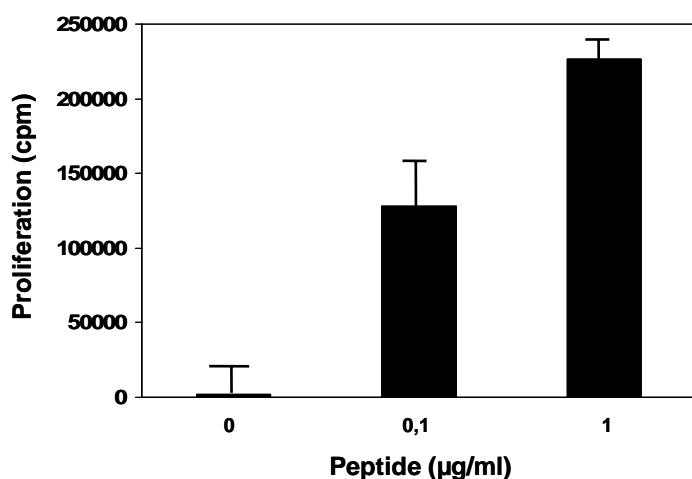


Figure 27: AECII mediate CD8⁺ T cell proliferation. 1×10^5 freshly isolated AECII from BALB/c mice were loaded with different concentrations of the corresponding HA-peptide and co-cultured together with 2×10^5 CD8⁺ T cells from CL4 mice. Proliferation of responder T cells was measured by ³[H]-thymidine incorporation. Results are expressed as mean counts per minute + SD (error bars) of triplicate wells and are representative of two independent experiments.

Clearly, alveolar type II epithelial cells from murine lung are able to present the MHC class-I restricted HA-peptide to CD8⁺ T cells and provoke a dose dependent antigen specific T cell proliferation.

Zissel and colleagues described the upregulation of MHC class-II molecules for AECII under inflammatory settings and as shown in figure 24, MHC II expression is also observed in AECII from healthy mice. To assess whether freshly isolated AECII have the ability to present the MHC class-II restricted peptide HA110-120 to CD4⁺ T cells from TCR-HA mice, 1 x 10⁵ freshly isolated AECII from BALB/c mice were loaded with the corresponding HA-peptide and incubated together with 2 x 10⁵ CD4⁺ responder T cells isolated from TCR-HA mice. T cell proliferation was measured by ³[H]-thymidine incorporation.

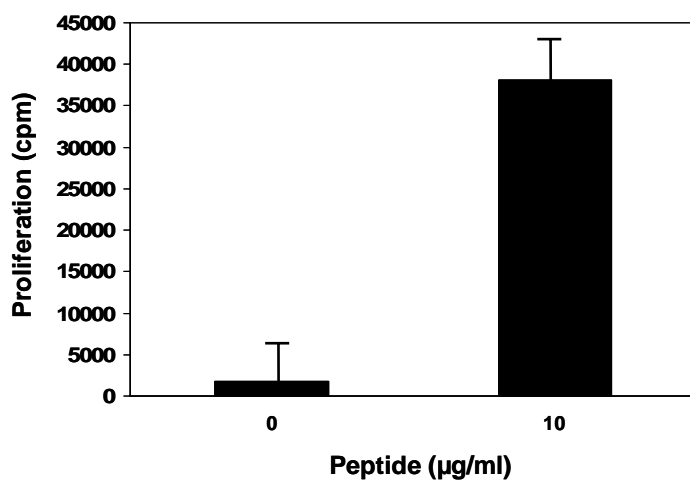


Figure 28: AECII mediate CD4⁺ T cell proliferation. 1 x 10⁵ freshly isolated AECII from BALB/c mice were loaded with the corresponding HA-peptide and cocultured together with 2 x 10⁵ CD4⁺ T cells from TCR-HA mice. Proliferation of responder T cells was measured by ³[H]-thymidine incorporation. Results are expressed as mean counts per minute + SD (error bars) of triplicate wells and are representative of two independent experiments.

Data revealed that not only MHC class-I, but also the MHC class-II restricted peptide HA110-120 was presented by AECII to 6.5⁺CD4⁺ T cells and resulted in the stimulation and proliferation of responder T cells (figure 27, figure 28).

It was interesting to analyze, whether endogenous HA expressed by type II epithelial cells in SPC-HA transgenic mice could also be MHC class-II presented by AECII themselves to stimulate MHCII restricted autoreactive T cells. To test this, alveolar type II epithelial cells from SPC-HA transgenic mice were isolated and used as APC without the addition of further APC to stimulate CD4⁺ T cells isolated from TCR-HA mice. 1 x 10⁵ AECII were cocultured together with 2.5 x 10⁵ naïve CD4⁺ T cells to determine the stimulatory capacity of antigen expressing AECII. AECII from BALB/c mice were used as a negative control. After 48h T cell proliferation was measured by ³[H]-thymidine incorporation. Alternatively, the same assay was performed with T cells previously activated *in vitro* with the corresponding peptide.

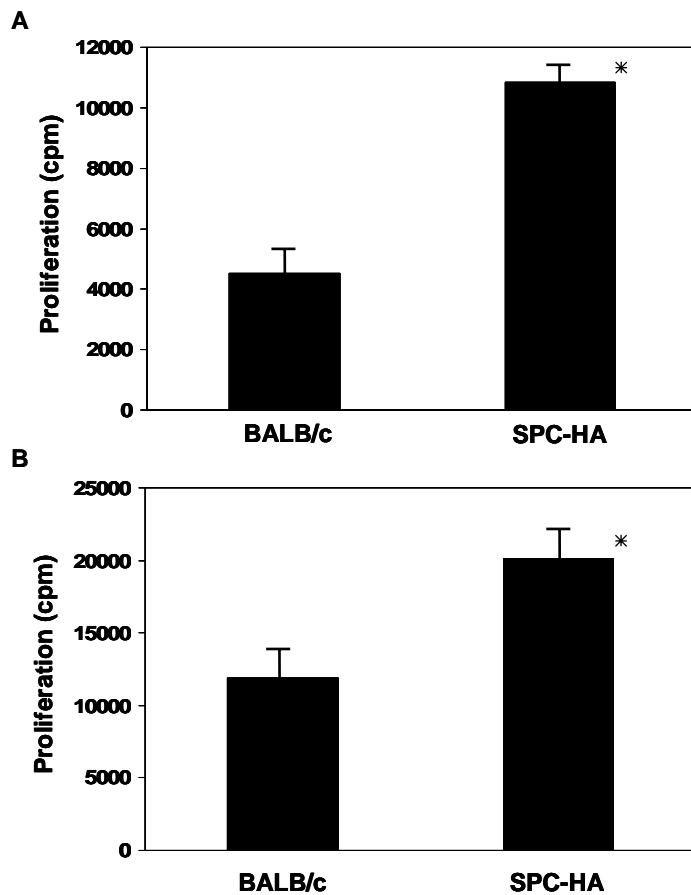


Figure 29: Stimulatory capacity of AECII from SPC-HA transgenic mice. 1×10^5 freshly isolated AECII from SPC-HA transgenic mice were cocultured with 2.5×10^5 naïve CD4⁺ T cells (A) or activated CD4⁺ T cells (B) isolated from TCR-HA mice. After 48h T cell proliferation was determined by ³[H]-thymidine incorporation. Results are expressed as mean counts per minute + SD (error bars) of triplicate wells and are representative of two independent experiments. P-values calculated by unpaired Student's test are indicated (*) < 0.002.

The data obtained demonstrate that alveolar type II epithelial cells have the inherent capacity to present endogenous antigen on MHC class-II molecules and to stimulate naïve as well as activated CD4⁺ T cells (figure 29). These results suggest that self-antigen presentation in the lung of SPC-HA x TCR-HA double transgenic mice may not exclusively be dependent on professional APC like dendritic cells, but that HA expressing AECII might themselves contribute to the stimulation of antigen-specific T cells and thus in the initiation of inflammation. This interesting aspect suggests a key role of the alveolar type II epithelial cells for the development of T cell mediated lung disease in the SPC-HA x TCR-HA mouse model.

4.8 Reduced stimulatory capacity of AECII from SPC-HA x TCR-HA double transgenic mice

To examine the role of AECII for the induction and regulation of autoreactive T cells responses in more detail, further experiments were performed to compare the stimulatory capacity of AECII derived from SPC-HA x TCR-HA double transgenic diseased mice with AECII from SPC-HA transgenic healthy mice. To this end, 1×10^5 freshly isolated AECII from either SPC-HA x TCR-HA double transgenic mice, SPC-HA transgenic mice or BALB/c mice were cocultured together with naïve or activated CD4⁺ T cells from TCR-HA mice. After 48h the proliferation of the responder T cells was measured by ³[H]-thymidine incorporation (figure 30).

Again it was found, that alveolar type II epithelial cells from SPC-HA mice were able to stimulate HA-specific T cells. However, data presented in figure 30 demonstrate a significant difference in the stimulatory capacity of AECII derived from healthy SPC-HA and diseased SPC-HA x TCR-HA. AECII from diseased mice exhibit a significantly reduced stimulatory capacity in comparison to AECII from healthy SPC-HA mice. The proliferation of naïve T cells cocultured together with AECII from SPC-HA x TCR-HA double transgenic mice was decreased to background level of the BALB/c control and even lower, suggesting not only reduced stimulatory capacity, but also inhibition of T cell proliferation by AECII from diseased mice. Similar results were obtained when pre-activated T cells were used as responder cells (figure 30).

Analyses of the activation/memory phenotype of 6.5⁺CD4⁺ T cells re-isolated after coculture with alveolar type II epithelial cells from healthy and diseased mice by FACS underline the proliferation data from coculture experiments. Summarizing these findings, 6.5⁺CD4⁺ T cells cocultured with AECII from SPC-HA transgenic mice have shown a more activation/memory phenotype compared to T cells cocultured with AECII from diseased SPC-HA x TCR-HA mice (data not shown).

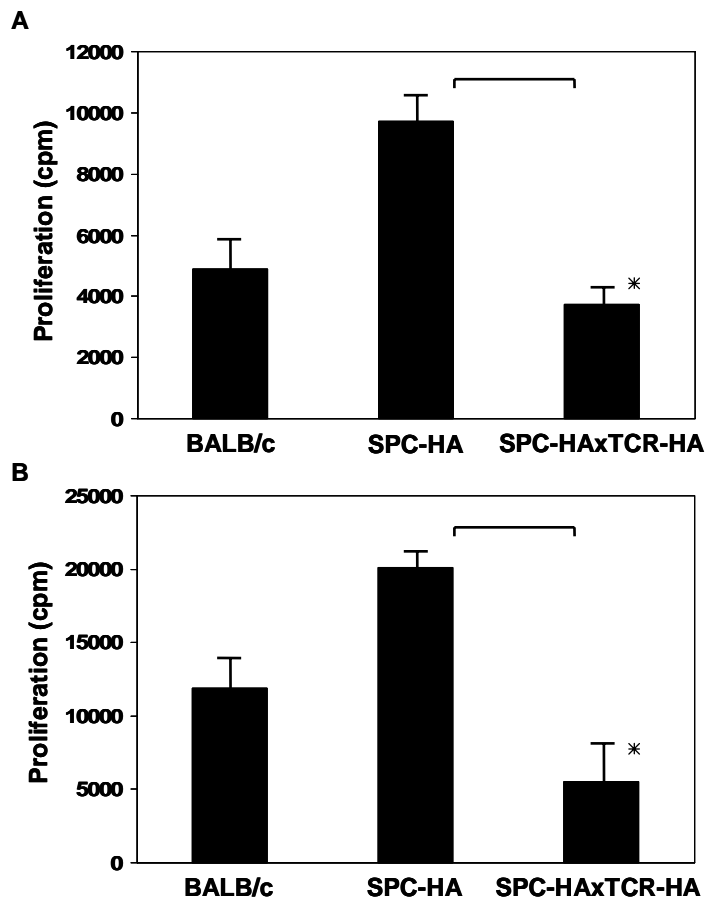


Figure 30: Reduced stimulatory capacity of AECII from SPC-HA x TCR-HA double transgenic mice. 1×10^5 AECII from SPC-HA x TCR-HA double transgenic mice, SPC-HA transgenic mice and BALB/c mice were freshly isolated and cocultured together with naïve (A) or activated (B) $CD4^+$ T cells from TCR-HA mice. After 48h the proliferation of responder T cells was measured by ^3H -thymidine incorporation. Results are expressed as mean counts per minute + SD (error bars) of triplicate wells and are representative of three independent experiments. P-values calculated by unpaired Student's test are indicated (*) < 0.001 .

4.9 Analysis of hemagglutinin expression in AECII SPC-HA x TCR-HA double transgenic mice and SPC-HA transgenic mice

The self-antigen hemagglutinin (HA) is expressed under the surfactant protein C promoter in alveolar type II epithelial cells of SPC-HA transgenic mice. To assess whether the reduced stimulatory capacity was simply the consequence of a loss of HA expression in AECII from SPC-HA x TCR-HA mice, the level of HA expression in AECII under inflamed and noninflamed conditions was determined. Therefore, total mRNA was isolated and analyzed with specific primer pairs by RT-PCR. RPS9 was used as housekeeping gene to test the RNA quality and quantity (figure 31).

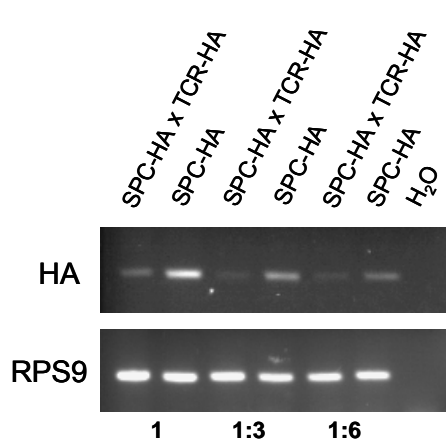


Figure 31: Semi-quantitative RT-PCR analysis of AECII from SPC-HA x TCR-HA mice and SPC-HA control mice to estimate the HA expression. mRNA from AECII derived from SPC-HA x TCR-HA double transgenic mice and SPC-HA transgenic mice was isolated and analyzed for the hemagglutinin (HA) expression by RT-PCR. Different amounts of cDNA were used. (1 = undiluted; 1:3 diluted and 1:6 diluted). RPS9 was used as a housekeeping gene.

Results of the RT-PCR indicates a decreased HA mRNA expression level in SPC-HA x TCR-HA double transgenic mice compared to healthy SPC-HA transgenic mice. However, also in the inflamed environment HA expression was clearly detectable, thus reduced antigen expression alone could not explain the complete loss of HA-specific T cell stimulation.

4.10 The impact of AECII conditioned media on CD4⁺ T cell proliferation

Data presented above demonstrate, that alveolar type II epithelial cells derived from the inflamed lung tissue of SPC-HA x TCR-HA double transgenic mice have a reduced T cell stimulatory capacity. Moreover, cocultured responder T cells exhibited a less activation/memory phenotype compared to autoreactive T cells cocultured together with AECII from healthy SPC-HA transgenic mice.

The reduced stimulatory capacity of AECII or the suppression of T cell proliferation respectively could be the result of cell-cell contact dependent mechanisms or depend on soluble factors, secreted by AECII. To assess the impact of soluble factors derived from AECII on T cell activation and proliferation, freshly isolated AECII from SPC-HA x TCR-HA double transgenic mice, SPC-HA transgenic mice and BALB/c control mice were cultured for 48h. The culture supernatants were collected and used as culture medium for naïve CD4⁺ T cells stimulated with anti-CD3 ϵ antibodies. Proliferation of the anti-CD3 ϵ stimulated CD4⁺ T cell was measured by ³[H]-thymidine incorporation. Fresh IMDM culture medium was used as a control to estimate the proliferation of anti-CD3 ϵ stimulated CD4⁺ T cells under standard conditions.

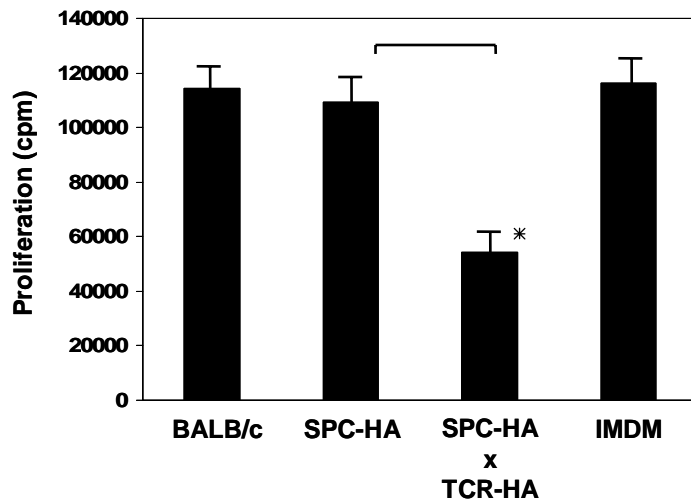


Figure 32: AECII conditioned media suppressed proliferation of naïve T cells. 1×10^5 AECII isolated from BALB/c, SPC-HA and SPC-HA x TCR-HA mice were cultured for 48h. 1×10^5 naïve $CD4^+$ T cell were stimulated *in vitro* with anti-CD3 ϵ antibodies in the presence of conditioned media derived from different AECII cultures. Proliferation was measured by $^3[H]$ -thymidine incorporation after 72h. Results are expressed as mean counts per minute + SD (error bars) of triplicate wells and are representative of three independent experiments. P-values calculated by unpaired Student's test are indicated (*) < 0.001.

As shown in figure 32, T cells cultured in conditioned medium of AECII derived from SPC-HA x TCR-HA exhibit a 50% reduced proliferation rate in comparison to T cells cultured in AECII conditioned medium from SPC-HA, BALB/c control mice or fresh control media.

These results strongly suggest that AECII derived from SPC-HA x TCR-HA diseased mice do not only have a reduced capacity to stimulate $CD4^+$ T cells, but furthermore actively suppress T cell proliferation at least in part by soluble factors secreted in the culture medium.

4.11 Differences in the surfactant protein expression in AECII from SPC-HA x TCR-HA double transgenic mice and SPC-HA transgenic mice.

Pulmonary surfactant was initially identified as a lipoprotein complex that reduces surface tension at the air-liquid interface of the lung (Clements, 1957; Pattle, 1955). This definition has been reassessed in light of recent studies that show that surfactant also functions in pulmonary host defence and that surfactant proteins are expressed also in non-pulmonary sites. The host defence functions of surfactant are primarily mediated by SP-A and SP-D, which are members of the collectin family of

proteins. An emphasis is placed on recent studies showing that, in addition to their well-established role as opsonins, SP-A and SP-D also have novel functions in initiating parturition, facilitating clearance of apoptotic cells and directly killing bacteria. Furthermore, immunoregulatory functions of the surfactant proteins A and D on T cells are discussed (Wright, 2005).

To assess the expression level of the surfactant proteins A, C and D under inflammatory condition RNA was purified from sorted AECII isolated from SPC-HA x TCR-HA double transgenic and SPC-HA transgenic mice. Real-time RT-PCR analyses were performed with specific primer pairs for RPS9, SP-A, SP-C and SP-D to determine the gene expression of the surfactant proteins in comparison with the expression of the housekeeping gene RPS9 (figure 33).

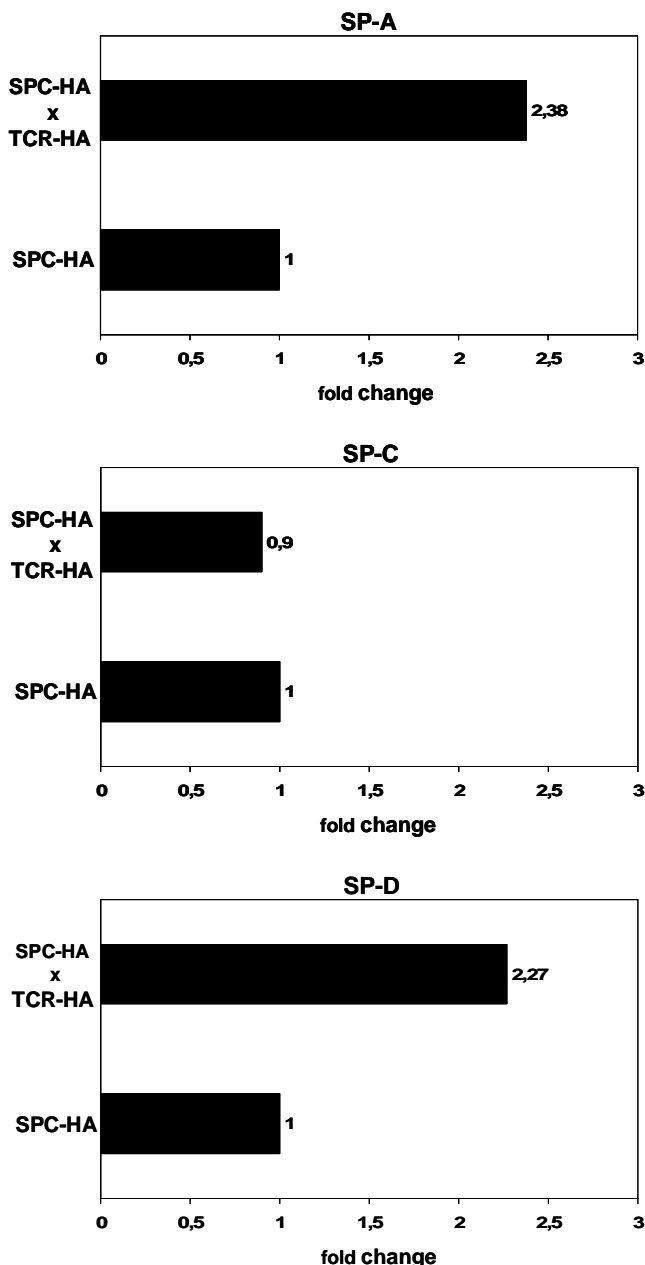


Figure 33: Surfactant protein expression in AECII derived from SPC-HA x TCR-HA double transgenic mice and SPC-HA transgenic mice. Real-time RT-PCR analyses for SP-A, SP-C and SP-D expression in sorted AECII from SPC-HA x TCR-HA and SPC-HA mice. Relative mRNA levels were normalized with respect to expression levels in AECII derived from SPC-HA transgenic mice (fold change = 1). The mean regulation is indicated.

Results of the real-time RT-PCR analysis for the surfactant proteins SP-A, SP-C and SP-D demonstrate an increased mRNA expression for SP-A and SP-D in AECII from SPC-HA x TCR-HA mice compared to AECII from SPC-HA mice. Analysis of the relative mRNA expression level of SP-C indicates no significant regulation when diseased and healthy mice were compared (figure 33).

These data suggested that due to the inflammatory process in the lungs of SPC-HA x TCR-HA transgenic mice expression of SP-A and SP-D were increased in alveolar type II epithelial cells and may contribute to the suppression of T cell proliferation observed after T cell stimulation in AECII conditioned medium (figure 32).

4.12 The effect of AECII/CD4⁺ T cell coculture on the T cell phenotype and function

Data above suggested an immune modulatory role of AECII in SPC-HA x TCR-HA double transgenic mice. The coculture experiments above demonstrated a reduced proliferative capacity of CD4⁺ T cells (figure 30) and the suppression of CD4⁺ T cell proliferation by AECII conditioned medium (figure 32). Furthermore, the *ex vivo* analysis of lung lymphocytes from diseased mice indicates the induction of regulatory T cells with a typical T_{reg}1 phenotype in the lung.

To directly examine the effect of AECII derived from diseased SPC-HA x TCR-HA and healthy SPC-HA on the phenotype and function of autoreactive T cells, 5 x 10⁵ freshly isolated AECII from either SPC-HA x TCR-HA double transgenic mice, SPC-HA transgenic mice or BALB/c mice were cocultured together with 1.4 x 10⁶ naïve CD4⁺ T cells isolated from TCR-HA mice. After 48h the responder T cells were re-isolated by ficoll density gradient centrifugation and total RNA was isolated for gene expression analyses by RT-PCR (figure 34).

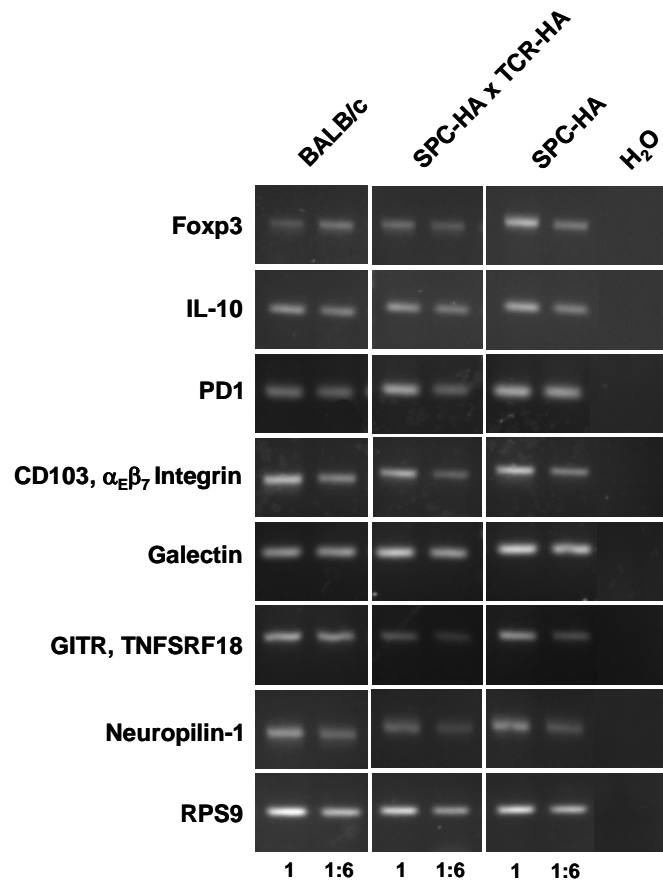


Figure 34: Semi-quantitative RT-PCR analysis of re-isolated CD4⁺ T cells prior cocultured with AECII from SPC-HA x TCR-HA, SPC-HA and BALB/c mice. To assess the effect of AECII derived from SPC-HA x TCR-HA double transgenic mice, SPC-HA transgenic mice and BALB/c control mice on the phenotype of antigen-specific CD4⁺ T cells the expression of different molecular marker genes for regulatory T cells including CD103 ($\alpha_E\beta_7$), Neuropilin-1, GITR (TNFRSF18), Foxp3, Galectin, PD-1 and IL-10 was analyzed. RPS9 was used as a housekeeping gene. Different amounts of cDNA (undiluted (1) and 1:6 diluted) were used for the semi-quantitative RT-PCR analyses.

Alternatively, CD4⁺ T cells were re-isolated after coculture with AECII and used as effector cells in standard inhibition assay. Therefore, 5×10^4 freshly isolated CD4⁺ responder T cells were plated together with the same number of T cells re-isolated after AECII coculture in 96-well flat-bottom plates in a final volume of 200 μ l IMDM in the presence of 0.75 μ g/ml soluble α -CD3 ϵ and 5×10^5 irradiated BALB/c splenocytes. As control, the proliferative capacity of re-isolated CD4⁺ T cell alone was determined by restimulation with soluble α -CD3 ϵ . To this end, 5×10^4 T cells re-isolated after the coculture with AECII were plated in 96-well flat-bottom plates in a final volume of 200 μ l IMDM in the presence of 0.75 μ g/ml soluble α -CD3 ϵ and 5×10^5 irradiated BALB/c splenocytes. Cells were cultured at 37°C for 48h. Over the last 8h of the

experiment ^3H -thymidine incorporation was measured by scintillation counting to assess proliferation (figure 35, figure 36). As a further internal control, 10^5 naïve responder T cells were cultured in the presence of 5×10^5 irradiated BALB/c splenocytes to assess the proliferation of naïve T cells alone.

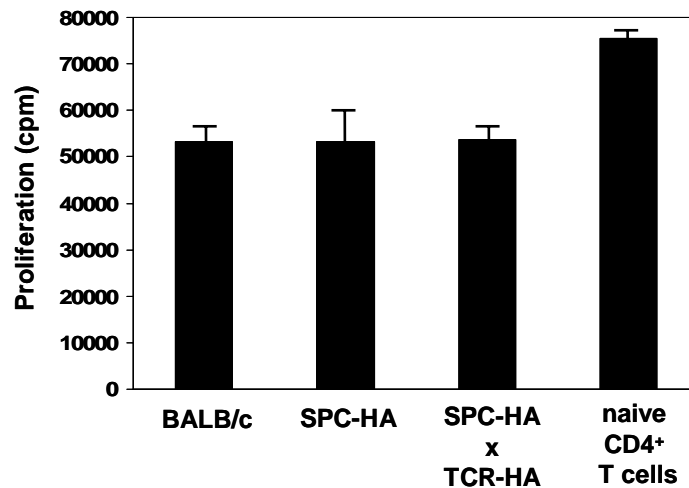


Figure 35: AECII cocultured CD4⁺ T cells show no inhibitory effect on the proliferation of naïve CD4⁺ T cells. 5×10^4 naïve CD4⁺ responder T cells were plated together with the same number of T cells re-isolated after coculture with AECII from BALB/c, SPC-HA and SPC-HA x TCR-HA mice in the presence of $0.75\mu\text{g/ml}$ soluble $\alpha\text{-CD3}\epsilon$ and 5×10^5 irradiated BALB/c splenocytes. As internal control 10^5 naïve responder T cells were cultured in the presence of irradiated BALB/c splenocytes. Cells were cultured at 37°C for 48h. ^3H -thymidine incorporation was measured by scintillation counting to assess the proliferation of responder T cells. Results are expressed as mean counts per minute + SD (error bars) of triplicate wells and are representative of three independent experiments.

The analysis of CD4⁺ T cell re-isolated after coculture with AECII derived from SPC-HA x TCR-HA, SPC-HA and BALB/c mice did not result in significant differences of gene expression pattern (figure 34). Although molecular marker genes specific for regulatory T cells could be detected on mRNA level by RT-PCR, no significant differences regarding their expression level could be observed comparing re-isolated T cells after coculture with AECII from diseased SPC-HA x TCR-HA or healthy SPC-HA or BALB/c control mice. Only the expression of PD-1 was increased in T cells cocultured with AECII from SPC-HA x TCR-HA mice and SPC-HA mice compared to AECII derived from BALB/c mice. Furthermore, increased mRNA expression was found for the transcription factor Foxp3 in T cells re-isolated from cocultures with AECII derived from SPC-HA transgenic mice in comparison to AECII from diseased SPC-HA x TCR-HA or BALB/c control mice.

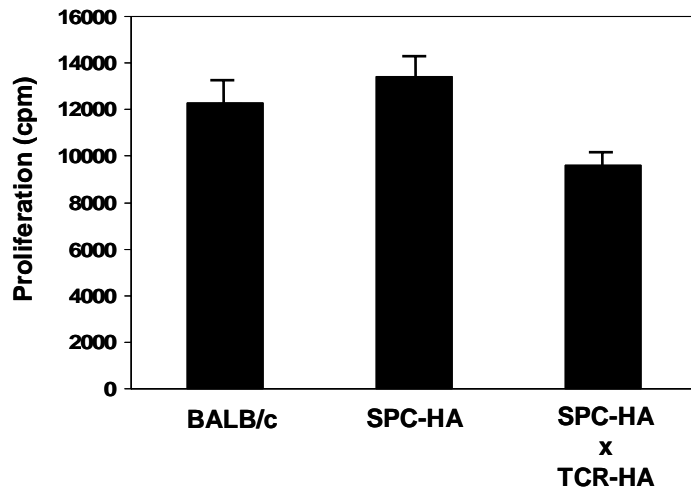


Figure 36: Reduced proliferation of T cells re-isolated after AECII coculture. CD4⁺ T cells re-isolated after coculture with AECII from BALB/c, SPC-HA and SPC-HA x TCR-HA mice were restimulated with 0.75µg/ml soluble α-CD3ε. ³[H]-thymidine incorporation was measured by scintillation counting to assess the proliferation. Results are expressed as mean counts per minute + SD (error bars) of triplicate wells and are representative of three independent experiments.

The functional characterization of re-isolated T cell concerning their putative inhibitory ability after coculture with AECII indicates no suppressive effect of T cells on responder T cells activated *in vitro*. In any case the responder T cells proliferated equally well in the presence of soluble α-CD3ε as freshly isolated CD4⁺ T cells alone. The increased proliferation rate of naïve CD4⁺ T cells alone was caused by the higher numbers of responder T cells used for the assay to get equal cell numbers. Furthermore, a reduced proliferative capacity was observed upon restimulation of T cells prior cocultured with AECII (figure 35).

Nevertheless the proliferative capacity of T cells cocultured with AECII derived from SPC-HA x TCR-HA double transgenic mice was reduced in comparison to T cells cocultured with AECII from SPC-HA transgenic mice and BALB/c control mice (Figure 36).

In conclusion, data obtained from these *in vitro* coculture experiments did not reveal the direct effect of AECII on the induction of a certain T cell phenotype. The analyses of the expression levels of molecular marker genes specific for regulatory T cells did not result in significant differences depending on the AECII used for coculture. Moreover, no suppressor function could be detected for re-isolated T cells. However, T cells re-isolated after coculture with AECII from diseased mice exhibit a reduced proliferative capacity when compared with T cells isolated after SPC-HA or BALB/c AECII coculture, thus suggesting an active role of AECII isolated from an inflammatory environment in the induction of T cell unresponsiveness and anergy.

CHAPTER III

Discussion and Summary

1 Discussion

The respiratory mucosa as well as immunity in the respiratory tract is a wide area for scientific investigations. In comparison to the gastrointestinal tract and its immunological function little is known about the immune system of the respiratory tract. The respiratory tract is a fragile tissue with architecture that is finely designed for gas exchange. Due to this main function the lung is exposed to numerous pathogens and other harmful air pollutions and developed many mechanisms to prevent infectious and inflammations. In the first line of defence are structural mechanisms coming from barriers such as epithelial cell layers, mucus and cilia, which avoid the invasion of pathogens or antigens. A battery of mediators that constitute the innate response including lactoferin, lysozyme, collectins and defensins is followed. Activation of these molecules can lead directly to lysis of pathogens, or to destruction through opsonisation or the recruitment of inflammatory cells (Boyton et al., 2002). A further mechanism for the respiratory defence is the adaptive immune response including the production of neutralising antibodies and the response of T lymphocytes. To guarantee the gas exchange even in situations of infections and inflammations, the lung has to balance immune responses to avoid immunological collapses and/or caused tissue damages. Therefore, the lung creates a tolerogenic and suppressive milieu to prevent consequences of an uncontrolled immune response.

A significant number of lung diseases are presumed to be T cell mediated based in part on the observation of T cell accumulation in sites of disease activity. These accumulations can be mediated by CD8⁺ T cells as well as CD4⁺ T cells like in interstitial lung diseases (ILD). ILD represent a large class of heterogeneous disorders involving the lung parenchyma, that is to say, the alveolar epithelium, the interstitial connective tissue, vasculature, and lymphatic tissue. However, the different aspects influencing the balance of lymphocyte immigration, local proliferation, and apoptosis, and thereby the numbers of lymphocytes in the lung under disease conditions, are only partially understood in interstitial lung disease (Pabst et al., 1999).

Even less understood are the mechanisms regulating CD4⁺ T cell responses directed to lung specific proteins and the regulation of their effector activity, because lung lymphocyte reactions can not be reflected by the situation of lymphocytes in the

blood. Therefore, the well established and described SPC-HA x TCR-HA transgenic mouse model (Bruder et al., 2004) is an important tool to get further insights into the mechanisms underlying the induction and regulation of T cell mediated immune reactions in the lung. The SPC-HA x TCR-HA double transgenic mouse model combines important features of a variety of lung diseases and can be used to get a better understanding of the requirements for, and consequences of chronic T cell-mediated lung injury.

The outcome of autoimmune diseases is caused by an inefficient deletion of self-reactive T cells in the thymus and insufficient self-tolerance to self-antigens in the periphery. In SPC-HA x TCR-HA double transgenic mice, 6.5^+CD4^+ self-reactive T cells could be detected in the peripheral lymphatic organs including spleen, mesenteric lymph nodes (MLN), bronchial lymph nodes (BLN), cervical lymph nodes (CVLN), axillary lymph nodes (AXLN), inguinal lymph nodes (INLN) and the lung as the self-antigen expressing organ (figure 11). These finding was not unexpected, as it has been described previously that expression of the HA-antigen in pancreas (Degermann et al., 1994; Sarukhan et al., 1998) and in hemapoetic cells (Lanoue et al., 1997) of INS-HA x TCR-HA and IgHA x TCR-HA double transgenic mice does not lead to complete deletion of 6.5^+ T cells. A possible explanation for the escape from central tolerance might involve coexpression of two different T cell receptors by the same cell. Due to allelic inclusion of TCR α genes self-reactive T cells may leave the thymus resulting in induction of autoimmunity in the periphery (Sarukhan et al., 1998).

Flow cytometry analysis revealed an increased number of 6.5^+CD4^+ T cells in SPC-HA x TCR-HA double transgenic mice in lung and BLN in comparison to healthy TCR-HA transgenic control mice. Other examined lymphoid organs did not show increased numbers of autoreactive T cells (figure 11). The detailed analysis of 6.5^+CD4^+ T cells in SPC-HA x TCR-HA double transgenic mice by Flow cytometry showed that autoreactive T cells isolated from the lung exhibited an activated phenotype. As shown, before the activation markers CD69 and CD25 were significantly increased as well as the expression of the CD45RB and CD62L memory markers was reduced (figure 12). This is consistent with the hypothesis, that HA-specific $CD4^+$ T cells encounter their specific antigen which is expressed exclusively by alveolar type II epithelial cells (AECII). Surprisingly, no enhanced/reduced

expression of activation/memory markers could be shown for the BLN, which are the lymph nodes draining the lung (figure 12). This finding could be an indication for a locally limited inflammatory response and may indicate that the lung itself and not the draining lymph nodes represent the immunological compartment where autoimmune T cell activation is induced and immune regulation takes place.

Also, in contrast to lung lymphocytes the expression level for CD25 and CD69 of lymphocytes from other peripheral tissues like spleen, MLN, AXLN, CVLN and INLN was not increased when the different compartments from SPC-HA x TCR-HA mice and TCR-HA mice were compared. The same could be observed for the expression of CD45RB and CD62L. Exceptions for the expression of these two memory markers were the lymphocytes from spleen and MLN, which exhibited decreased expression of CD45RB and CD62L in SPC-HA x TCR-HA mice compared to TCR-HA transgenic mice (figure 12).

Taken together, in SPC-HA x TCR-HA double transgenic mice the lung epithelium expresses the self-antigen hemagglutinin and the lung appears to be the place where primary T cell activation occurs. Autoreactive 6.5^+CD4^+ T cells proliferate in and/or infiltrate the lung and exhibit a strongly activated phenotype in the presence of the self-antigen HA. No evidence was found that the inflammation overlaps to other tissues or lymphatic organs, thus in SPC-HA x TCR-HA mice disease is exclusively restricted to the lung.

In SPC-HA x TCR-HA double transgenic mice the lung inflammation did not progress uncontrolled, i.e. inflammation did not result in complete tissue destruction or death. Thus, although multifocal acute alveolar necrosis with hemorrhage, intraalveolar fibrin deposition, consistent with acute tissue damage could be observed in 9-day-old SPC-HA x TCR-HA double transgenic mice, inflammation reached a plateau which was characterized by primarily diffuse to follicular lymphocytic infiltration in elder mice (Bruder et al., 2004). These results suggested a regulatory mechanism that may exist and/or develop under autoimmune conditions to counteract the progression of inflammatory processes in the lung, thus preventing uncontrolled tissue destruction and lethal physiological derangement.

In previous works it could be shown that expression of HA under the control of the Ig κ promoter by hemopoietic cells resulted in tolerance rather than inflammation due to the permanent antigen expression both in thymus and in the periphery (Buer et al.,

1998). Therefore, detailed analysis was reasonable to assess whether mature 6.5^+CD4^+ T cells found in peripheral lymphoid organs of SPC-HA x TCR-HA double transgenic mice are still functional with respect to their proliferative capacity upon antigen encounter. Because of the finding that mature 6.5^+CD4^+ T cells from SPC-HA x TCR-HA double transgenic mice can be activated with the corresponding peptide *in vitro*, Bruder and colleagues could exclude the possibility that the self-reactive peripheral T cells from SPC-HA x TCR-HA double transgenic mice were rendered in an anergic state. However, they could also show that the proliferative capacity of lung lymphocytes from SPC-HA x TCR-HA mice was reduced by approximately 50% when compared with lung T cells from TCR-HA control mice. Further molecular analysis of 6.5^+CD4^+ T cells isolated from inflamed lung of SPC-HA x TCR-HA double transgenic mice resulted in a changed chemokine and gene expression profile compared to autoreactive T cells isolated from TCR-HA control mice. This phenotype resembles that of T_{reg} 1 cells. To verify the results obtained by global gene expression profiling of 6.5^+CD4^+ lung lymphocytes derived from SPC-HA x TCR-HA mice, the expression of specific marker genes for regulatory T cells was tested by RT-PCR (figure 13). RT-PCR results confirmed array data obtained from the autoreactive T cells isolated from diseased mice and demonstrated an elevated expression of CTLA4, PD-1, ICOS, GITR and $\alpha\beta_7$ (CD103) in autoreactive T cells from double transgenic mice compared to naïve T cells from the TCR-HA mice. It is known that these genes are not exclusively expressed on regulatory T cells but on activated T cells, too (O'Garra and Vieira, 2004). Nevertheless a strong upregulation of the activation independent genes Neuropilin-1 and Foxp3 was detectable. These molecular marker genes are discussed to be specific for regulatory T cells (Bruder et al., 2004; O'Garra and Vieira, 2004) thus these data clearly indicate the induction of self-antigen-specific regulatory T cells in the lung of diseased SPC-HA x TCR-HA mice. Thus the SPC-HA x TCR-HA double transgenic mouse model represents an important tool to examine the mechanisms underlying the induction and the function of regulatory T cells induced *in vivo* in the respiratory tract.

The adoptive transfer experiments that were performed in this study demonstrated the enormous impact of naturally occurring regulatory T cells and/or *in vivo* induced regulatory T cells on the proliferation of autoreactive T cells in the lung. The

immunohistochemistry of the lung (figure 14) shows the fast progress of the infiltration of autoreactive T cells in SPC-HA transgenic mice after adoptive transfer of CD4⁺ T cells from TCR-HA transgenic mice. Within seven days a rapid and distinct lymphocyte infiltration took place and revealed the strong impact of autoreactive CD4⁺ T cells on disease development and progression. The infiltration was antigen-specific because the documented lymphocytic invasion was completely absent in BALB/c control mice (data not shown). Using this adoptive transfer system, further analysis to study the influence of antigen specific naturally occurring T cells and *in vivo* induced regulatory T cells could be performed.

Adoptive transfer experiments into SPC-HA transgenic mice demonstrated that the small proportion of naturally occurring regulatory T cells was sufficient to efficiently reduce the proliferation of naïve transgenic T cells *in vivo* (figure 15). In most of the published transfer experiments which were performed to characterize the properties of regulatory T cells, the hosts are lymphopenic and the transferred T cell subsets are polyclonal with unknown antigen specificity (Asseman et al., 2003; Maloy et al., 2003). Therefore, physiological regulatory functions cannot be distinguished easily from effects that are caused by homeostatic proliferation and clonal expansion of transferred cells (Bach, 2003; Barthlott et al., 2003). Transfer experiments into SPC-HA mice are based on the use of animals with an intact immune cell repertoire. Thus, the results summarized in figure 16 demonstrate that the effect of transferred T cells on antigen specific proliferation and clonal expansion is due to suppressor function of T_{reg} cells and not a result of homeostatic proliferation in a lymphopenic host. Due to the fact, that no proliferation of re-isolated transferred T cell could detect in other organs (figure 15, figure 16) the observed T cell proliferation in lung and BLN is based on the antigen specificity of the transferred T cells. Summarizing, the data obtained from this adoptive transfer suggest the active suppression of proliferation of autoreactive T cells in the lung after antigen encounter by HA specific 6.5⁺CD4⁺CD25⁺ regulatory T cells and underlines the importance of T_{reg} cells for the control of autoimmune mediated T cell responses in the lung.

Similar to naturally occurring regulatory T cells adoptive or induced T_{reg} cells are discussed to have suppressive functions *in vivo* and *in vitro* (Maloy and Powrie, 2001; Wraith et al., 2004). Adoptive transfer of autoreactive CD4⁺ T cells in SPC-HA x TCR-HA double transgenic mice demonstrated the impact of induced regulatory T cells on

autoreactive T cell proliferation. Data shown in figure 17 clearly indicate a strong inhibition/suppression of the proliferation of adoptively transferred 6.5^+CD4^+ T cells in lung and BLN of SPC-HA x TCR-HA double transgenic mice, whereas transferred autoreactive T cells in SPC-HA transgenic mice underwent a strong proliferation. Thus it could be shown, that the lung specific 6.5^+CD4^+ T cells from SPC-HA x TCR-HA double transgenic mice not only correspond to the phenotype of T_{reg} 1 cells, but that these *in vivo* induced HA-specific T_{reg} cells have the capacity to suppress the proliferation of autoreactive T cells *in vivo*. This small proportion of induced regulatory T cells was able to control the proliferation of autoreactive T cells *in vivo* like natural T_{reg} cells (figure 15).

The SPC-HA x TCR-HA double transgenic mouse exhibits as an autoimmune disease model the possibility to examine immune responses and immune regulation. Furthermore, this model provides insights into antigen-specific AECII/ $CD4^+$ T cell crosstalk to clarify the role of the antigen expressing alveolar type II epithelial cells in disease induction and regulation. Therefore, it was necessary to establish a new protocol to isolate these cells from mice for *ex vivo* studies. Several isolation protocols are already published for AECII for other organisms, which can not be used for the isolation of murine AECII. Despite the lack of suitable surface molecules it was possible to obtain a highly pure and vital AECII population from mice using the newly established protocol. FACS, immunofluorescence, and PCR analysis revealed that purified AECII exhibited only a minimal contamination of $CD45^+$ cells. Isolated cells were positive for AECII specific gene expression on mRNA and protein level (figure 19, figure 21). Furthermore, the vitality of the isolated AECII was high, despite physical stress caused by tissue disintegration and FACS-sorting (figure 20). The minor contamination of hemopoietic $CD45^+$ cells was classified as a small macrophage population by RT-PCR (figure 21) and visually observations by microscopy (data not shown). The data revealed that the newly established protocol for isolation of AECII from mice is an efficient method to obtain a pure and vital population of murine alveolar type II epithelial cells with the required features for *ex vivo* analysis.

After establishing the AECII isolation protocol comparative transcriptional analysis of type II alveolar epithelial cells were performed to bring further insights into the molecular mechanism of type II pneumocyte- $CD4^+$ T cell interaction. Thereby, a heterogeneous set of genes differentially expressed in AECII under inflammatory

conditions was identified. More than 400 genes were regulated in AECII from diseased mice compared the healthy control, which could be classified in different groups (figure 22). Interestingly, the expression levels for molecules involved in antigen presentation were upregulated in AECII obtained from SPC-HA x TCR-HA double transgenic mice. Especially, increased expression of molecules needed for the MHC class-II restricted antigen presentation, like H2-Ea and H2-Ab1 (table 4) were of interest and could be confirmed by RT-PCR analysis (figure 24), which in addition to the presence of MHC class-II molecules also demonstrates elevated expression of costimulatory molecules as CD86 and CD80. Furthermore, expression of the genes encoding for TAP1 and proteasomal subunits were increased, thus suggesting an enhanced efficiency of MHC class-I restricted antigen presentation (table 4).

Genes responsible for cell cycle activity like cyclin A2 and cyclin D2 (Wu et al., 1995; Bui et al., 1993) were regulated in AECII derived from SPC-HA x TCR-HA transgenic mice suggesting a controlled proliferation of AECII in inflamed tissue, which could be underlined by the differential expression of matrix metalloproteinases (MMP) and tissue inhibitor metalloproteinases (TIMP). These proteins are linked to airway remodulation in pulmonary pathology and could be detected in AECII of patients with diffuse alveolar damage and idiopathic pulmonary fibrosis (Chakrabarti and Patel, 2005; Hayashi et al., 1996). RT-PCR results (figure 24) revealed the presence of MMP in AECII and demonstrated an increased expression of MMP-9, which was similar to MMP-2 discussed in the context of pathogenesis of inflammatory, infectious and neoplastic diseases in many organs including the lung (Chakrabarti and Patel, 2005).

The differential expression of different chemokines and other immune modulating molecules like TGF- β 3 and TGF- β 2 (table 4) underlines, that AECII interact with resident and mobile neighbour cells via secreted and diffusible signals (Fehrenbach, 2001). Members of the transforming growth factor-beta family are linked to proliferation or secretion activities of AECII. For TGF- β 3 it could be shown that the production of TGF- β 3 by AECII is dynamically downregulated during the proliferative phase of recovery from acute hyperoxid injury (Buckley et al., 1996). Similar findings could be shown for the TGF- β 3 and TGF- β 2 expression in AECII from SPC-HA x TCR-HA double transgenic mice. Both molecules were downregulated and suggested an enhanced proliferative activity of AECII controlled by TGF- β derivatives.

All these data linked to proliferation of AECII and remodulation of lung tissue correspond to the fact, that AECII are the stem cells for alveolar type I epithelial cells (AECI). To compensate the loss of AECI caused by lung injury through inflammation or tissue damage, AECII have to proliferate and differentiate into AECI suggesting that the observed tissue damage in SPC-HA x TCR-HA mice could be the reason for an enhanced proliferative activity demonstrated by the increased or decreased expression level of genes involved in proliferation of AECII.

Additionally, the CXC chemokines like CXCL2, CXCL13 and CXCL12 were regulated in AECII from SPC-HA x TCR-HA double transgenic mice compared to AECII from SPC-HA transgenic mice. The main function of these chemokines is the attraction of different cell types. For CXCL12 and CXCL13 it is described that these ligands bind to CXCR4 and CXCR5, which are mainly expressed on T lymphocytes or fibrocytes (Ebert et al., 2004). The downregulation of CXCL12 in AECII of SPC-HA x TCR-HA mice could be an indicator of a controlled immune response. CXCL12 is an attractant molecule for T cells and signals through the orphan receptor RDC1 in T lymphocytes (Balabanian et al., 2005). Downregulation of T cell attractant substances could minimize the infiltration of newly developed autoreactive T cells. According to this it is shown, that CXCL13 has a key role in the development of inducible bronchus associated lymphoid tissue (iBALT) in respiratory immunity (Moyron-Quiroz et al., 2004) by attracting T lymphocytes. The role of BALT in mouse and humans are controversially discussed and it is reported that infection or inflammation triggers the organization of lymphoid structures in the lung of both species (Chvatchko et al., 1996; Delventhal et al., 1992; Tschernig and Pabst, 2000). These structures do not fit the classical definition of BALT, as they are not formed independently of antigen (Bienenstock and Johnston, 1976; Plesch et al., 1983). Because the inducible BALT (iBALT) appears in the lung only after infection or inflammation, it is generally assumed that iBALT is simply an accumulation of effector cells that were initially primed in conventional lymphoid organs. The neo-formation of iBALT is caused by inflammatory responses, which directly promote the recruitment, priming and expansion of antigen-specific lymphocytes (Moyron-Quiroz et al., 2004). Concerning these aspects it could be possible that AECII in SPC-HA x TCR-HA double transgenic mice, after the first inflammatory responses, downregulate CXCL13 expression to counteract new formation of iBALT and T cell infiltrations of naïve autoreactive T cells.

The chemokine CXCL2 is involved in attraction of polymorphonuclear granulocytes to sites of infection (Matzer et al., 2004). These neutrophils play an important role as regulators of immune responses through release of cytokines such as IL-1, IL-3, IL-6, IL-12, tumor necrosis factor- α (TNF- α) or TGF- β as well as chemokines such as CCL2 (MCP-1) or CCL20 (MIP-3 α) (Cassatella, 1995; Galligan and Yoshimura, 2003). Thus, increased expression of CXCL2 in AECII of diseased mice could attract neutrophils that are known to act as a kind of control unit of inflammation.

Elevated expression of CCL20 and CCL9/10 by AECII has been shown to attract other proinflammatory cells (Maurer and Stebut, 2004). Both chemokines were found to be upregulated in AECII derived from diseased SPC-HA x TCR-HA mice (table 4). In case of CCL20 it could be shown that AECII constitutively produce this chemokine that can attract immature dendritic cells (imDC) to the lungs (Dieu-Nosjean et al., 2000; Reibman et al., 2003). These immature dendritic cells might be involved in the induction of regulatory T cells (Jonuleit et al., 2000) and provoke a controlled immune response in SPC-HA x TCR-HA double transgenic mice. CCL11 (eotaxin) was drastically downregulated in AECII from diseased mice. CCL11 is an attractant molecule for eosinophils. Blocking experiments with anti-CCL11 revealed a reduced eosinophils infiltration into the lung of respiratory syncytial virus (RSV) infected mice. In addition, anti-CCL11 caused an inhibition of CD4-T-cell influx (Matthews et al., 2004). These experiments suggested that the decreased CCL11 transcription in AECII from diseased mice is a further step to regulate the inflammatory process in SPC-HA x TCR-HA double transgenic mice. Summarizing these facts, data suggest an active immune regulatory role of AECII in SPC-HA x TCR-HA transgenic mice by regulating the expression and secretion of certain chemokines, that may affect other immune cells, which could establish a tolerogenic environment in the lung, with regulatory features to control the inflammatory response.

Further RT-PCR data (figure 24) demonstrate a constitutive expression of ICOS-L and PD1-L in AECII. These molecules are ligands for ICOS and PD-1 expressed on T cells and, which showed elevated expression on autoreactive T cells isolated from diseased lungs of SPC-HA x TCR-HA double transgenic mice (figure 13). The ICOS:ICOS-L pathway appears to be particularly important for stimulating effector T cell responses and T cell tolerance. In addition, the PD1:PD1-L pathway plays a critical role in regulating T cell activation and tolerance. The CD28 homolog ICOS is upregulated on T cells after activation (Yoshinaga et al., 1999; Coyle et al., 2000).

Additionally, in recent studies it could be shown, that ICOS regulates the outcome of autoimmune diseases in the murine model of Multiple Sclerosis. However, ICOS appears to have distinct roles at different times during the pathogenesis of experimental allergic encephalomyelitis (EAE) (Chapoval et al., 2001; Rottman et al., 2001; Sporici et al., 2001). Moreover, ICOS also may control T_{reg} function in autoimmune disease, particularly IL-10-producing T_{reg} cells. In a murine model of type 1 diabetes, T_{reg} played a critical role in regulating the progression of diabetes from prediabetic lesion (Herman et al., 2004). Blockade of ICOS alters the balance between T effector and T_{reg} cells, resulting in progression from a prediabetic insulinitis to diabetes. These findings suggest that T_{reg} cells regulate autoimmune development in an ICOS-dependent manner. Furthermore, ICOS may also be important in the generation of T_{reg} cells. The development of IL-10-producing T_{reg} cells was dependent on ICOS:ICOS-L interactions. These cells have suppressor function in the development of asthma-associated airway hyperreactivity. Thus, these findings suggest a novel mechanism by which ICOS and IL-10 reduce airway inflammation associated with asthma and implicate ICOS in regulating respiratory tolerance (Greenwald et al., 2004).

PD1 (Programmed death-1) is a member of the Ig superfamily related to CD28 and CTLA-4 (Ishida et al., 1992; Vibhakar et al., 1997). The roles of PD1 and its ligands in regulating autoimmune disease have been investigated in animal models of diabetes, colitis, and Multiple Sclerosis. Thus, it could be shown that the PD1:PD1-L interactions regulate both the initiation and progression of autoimmune diabetes in NOD mice. The expression of PD1-L on islet cells in diabetic NOD mice (Ansari et al., 2003; Liang et al., 2003) suggests that PD1-L may critically control the response of self-reactive T cells in the target organ (Liang et al., 2003) and demonstrates that PD1 and its ligands have an important role in regulating T cell activation and tolerance (Greenwald et al., 2004). Considering these facts, AECII could interfere with inflammatory responses in SPC-HA x TCR-HA double transgenic mice by expressing ICOS-L and PD1-L and could have an important role in controlling immune responses and inducing T cell tolerance in the respiratory tract.

Further data of this study deal with in the antigen presenting capacity of alveolar type II epithelial cells. In the SPC-HA transgenic mouse the AECII express the self-antigen hemagglutinin HA. Therefore, these cells represent the target of self-reactive 6.5^+CD4^+ T cells and could even involved in the induction of $CD4^+$ T cell mediated

inflammation in SPC-HA x TCR-HA double transgenic mice. Antigen presentation in the respiratory tract plays a key role for effective adaptive immune response. The small proportions of inhaled antigens and pathogens that escape the first mechanisms for antigenic clearance in the lung and penetrate into the underlying epithelial layer are then dealt with via specialized antigen-presenting cells (APC). In particular these APC are dendritic cell (DC) populations, within and below the epithelium. The alveolar surfaces in the deep lung are policed instead by macrophage populations, again supported by APC populations below the alveolar epithelium. The mechanism for the uptake or presentation of endogenous or self-antigens like the HA in the SPC-HA mouse is still unclear. Dendritic cells and macrophages are discussed to act as professional APC which are able to take up and cross-present self-antigens from surrounding cells to other immune cells. To this end, the antigen presenting capacity of professional antigen-presenting cells (APC) was examined to clarify which kind of APC have the ability to cross-present the HA expressed by AECII to autoreactive T cells (figure 25, figure 26). Data obtained from these experiments indicate, that obviously only DC have the ability to take up and cross-present HA to 6.5^+CD4^+ T cells (figure 25) *in vitro*, whereas macrophages or B lymphocytes derived from the peritoneum of BALB/c mice could not take over this function *in vitro* (figure 26). Additional data clearly demonstrated that peptide loaded AECII derived from BALB/c mice themselves were able to stimulate antigen specific $CD4^+$ and $CD8^+$ T cells (figure 27, figure 28) suggesting their role as antigen-presenting cells.

The question, whether the self-antigen HA expressed by alveolar type II epithelial cells in SPC-HA transgenic mice could also be presented by AECII themselves via MHC class-II molecules to stimulate MHCII restricted autoreactive T cells could be affirmed. Data in figure 29 demonstrate that the HA expressing alveolar type II epithelial cells have stimulatory capacity and are able to stimulate naïve as well as activated $CD4^+$ T cells. The possibility that the contaminating macrophage population in the AECII preparation could mediate the stimulation of autoreactive T cells could be excluded by the fact, that macrophages were not able to cross-present HA to antigen-specific T cells (figure 26). These results strongly suggest that antigen presentation in the lung of the SPC-HA x TCR-HA double transgenic mice may not exclusively be dependent on professional APC like dendritic cells, but that HA expressing AECII might themselves contribute to the stimulation of antigen-specific T

cells and thus in the initiation of inflammation. This interesting aspect suggests a key role of the alveolar type II epithelial cells for the development of T cell mediated lung disease in the SPC-HA x TCR-HA mouse model. Comparative analysis of the stimulatory capacity of AECII from SPC-HA transgenic mice and AECII from SPC-HA x TCR-HA double transgenic mice revealed a reduced stimulatory capacity of AECII derived from diseased mice for naïve as well as pre-activated autoreactive T cells (figure 30). Although previous data demonstrated an elevated expression of costimulatory molecules on AECII from SPC-HA x TCR-HA double transgenic mice (table 4 and figure 24) these cells were not able to stimulate 6.5^+CD4^+ T cells to induce antigen-specific proliferation, and that so far undefined soluble or cell-cell contact dependent factors could interfere with the stimulatory capacity of AECII or even the proliferative response of the T cells *in vitro*. To assess the impact of AECII conditioned medium on $CD4^+$ T cell proliferation AECII culture supernatants from SPC-HA x TCR-HA double transgenic mice and healthy donor mice were collected and used as culture medium for anti-CD3 ϵ stimulated $CD4^+$ T cells. The results clearly point out that AECII derived from diseased mice secrete factors which suppress the proliferation of activated T cells (figure 32) and suggest that the reduced proliferation of autoreactive T cells *in vitro* is at least in part mediated by soluble factors secreted by AECII.

To exclude that loss of HA expression may be a reason for the reduced stimulatory capacity of AECII in SPC-HA x TCR-HA double transgenic mice the expression of the self-antigen HA by AECII from SPC-HA x TCR-HA double transgenic mice and SPC-HA transgenic mice was analyzed on mRNA level. Results indeed indicate a reduced HA expression in AECII derived from diseased mice (figure 31). Nevertheless, minimal amounts of internalized hemagglutinin are necessary to provoke an efficient antigen presentation by MHC class-II molecules so that even a reduced HA expression should be sufficient to stimulate HA-specific T cells. Furthermore, mRNA does not reflect the real protein expression at the cell surface. Moreover, the data above demonstrate that T cell suppression was at least in part mediated by AECII secreted factors (figure 30), thus a putative decreased HA expression could not explain the suppressed proliferation of T cells cultured in AECII supernatants from diseased mice.

The tolerogenic and suppressive environment of the respiratory tract is widely discussed in the literature and in the meantime different factors were found that may

play a role in establishing this regulatory milieu. Candidates which could interfere with the immune response in respiratory tract and are linked to alveolar type II epithelial cells are surfactant proteins. These components of the surfactant fluid have many regulatory functions by opsonizing pathogens and enhancing their uptake and clearing by macrophages but also affecting immune cells directly (Wright, 2005). The surfactant proteins A and D (SP-A and SP-D) secreted by AECII have a key role by modulating the adaptive immunity in the lung. For both proteins elevated mRNA levels could be detected in AECII from SPC-HA x TCR-HA double transgenic mice compared to control mice (figure 33). These data suggest an increased protein expression and secretion of SP-A and SP-D by AECII from diseased mice and assume a regulatory function. It could be shown that surfactant protein A inhibits T cell proliferation via its collagen-like tail and the 210kDA receptor SPR210 (Borron et al., 1998) and that SP-D have the same feature by inhibiting human T lymphocyte proliferation and IL-2 production (Borron et al., 1998). These studies suggest that increased amounts of SP-A and SP-D secreted by AECII from diseased mice in culture supernatants may be involved in suppression of T cell proliferation (figure 30). Furthermore, Brinker and colleagues could show that SP-A modulates the differentiation of murine bone marrow-derived dendritic cells. DC which are the most potent antigen-presenting cells with the unique capacity to activate naïve T cells and initiate adaptive immunity were affected in their maturation into potent T cell stimulators by SP-A treatment and interfere with T cell activation (Brinker et al., 2003). To this end, these data and studies could be an important indication for a further regulatory role of AECII in the SPC-HA x TCR-HA double transgenic mouse model by modulating DC and T cell functions. The fact that AECII derived from diseased mice produced elevated levels of CCL20 (figure 23), a chemokine which attracts immature DC, and the capacity of SP-A to modulate differentiation of immature DC leads to the assumption that immigrated immature DC in the respiratory tract may be affected by high SP-A concentrations and develop a regulatory phenotype which could influence T cell response or even T cell differentiation into an adaptive T_{reg} phenotype. The fact that stimulation of T cells with immature DC leads to the development of T cells with regulatory property underlines this assumption (Dhodapkar et al., 2001; Jonuleit et al., 2000; Maloy and Powrie, 2001). Another interesting fact is that AECII derived from diseased mice produce elevated mRNA levels of platelet factor 4 (PF-4) (figure 23). Platelet factor 4 is a platelet-

derived CXC chemokine and induces the differentiation of monocytes into a subset of macrophages that lack the expression of HLA-DR which suggests a potential role of PF-4 in the modulation of monocyte-dependent T cell activation. Furthermore, studies revealed that PF-4 inhibits T cell proliferation and IL-2 production after anti-CD3 ϵ mediated T cell stimulation (Fleischer et al., 2002). Summarizing these data, AECII derived from SPC-HA x TCR-HA double transgenic mice produce increased levels of SP-A, SP-D and PF-4 mRNA and all these molecules are discussed in the context of inhibiting T cell proliferation. Together, these data strongly suggest a functional role of AECII in regulating T cell proliferation, thus counteracting uncontrolled disease progression.

To get further insights into the influence of AECII derived from diseased or healthy mice on the phenotype of CD4⁺ T cells functional and molecular analyses were performed on T cells re-isolated after AECII coculture. Re-isolated T cell from cocultures were either used in inhibition assays to assess adaptive suppressive functions to responder cells or these primed T cells were used for RT-PCR analysis to examine a distinct expression pattern for T_{reg} markers. The results did not indicate any differences in gene expression or acquired suppressive function of T cells cocultured with AECII obtained from diseased mice in comparison to healthy donors (figure 34, figure 35). However, the re-stimulation of T cells resulted in a decreased proliferation of CD4⁺ T cells after coculture with AECII from SPC-HA x TCR-HA double transgenic mice (figure 36) suggesting that of AECII coculture may induce long term T cell unresponsiveness or anergy.

2 Summary

The mechanisms underlying the induction and regulation of T cell mediated immune reactions in the respiratory tract are still unclear. To get further insights into the immunological and molecular mechanisms of antigen specific CD4⁺ T cell responses in the lung the SPC-HA transgenic mouse expressing hemagglutinin (HA) in alveolar type II epithelial cells of the lung was used. Concomitant expression of HA and presence of MHC class-II restricted HA-specific autoreactive T cells resulted in an autoimmune mediated progressive lung inflammation in SPC-HA x TCR-HA double transgenic mice. This inflammation was accompanied by a massive infiltration and activation of HA-specific CD4⁺ lymphocytes in the lung tissue. The lung inflammation observed in SPC-HA x TCR-HA double transgenic mice did not progress uncontrolled and reached a plateau in elder mice suggesting regulatory mechanism that may exist and/or develop under autoimmune conditions to counteract the progression of inflammatory process in the lung. This assumption was underlined by gene expression profiling of 6.5⁺CD4⁺ isolated lung lymphocytes from diseased mice which revealed a regulatory phenotype.

Transfer of naïve HA-specific 6.5⁺CD4⁺ and 6.5⁺CD4⁺CD25⁻ transgenic T cells depleted from CD25⁺ T cells into SPC-HA transgenic mice demonstrated the regulatory potential of the small proportion of naturally occurring CD4⁺CD25⁺ T cells by suppressing the antigen specific proliferation of autoreactive T cells *in vivo*. In addition, the transfer of autoreactive T cells in SPC-HA x TCR-HA double transgenic mice, but not in healthy control mice resulted in suppression of HA specific T cell proliferation, suggesting regulatory mechanisms established in the lung of diseased mice counterbalancing disease progression. The observed inhibition of proliferation of autoreactive T cells in diseased mice could be an effect of induced regulatory T cells, soluble mediators secreted by AECII or other so far unknown regulatory mechanisms preventing an uncontrolled inflammation or most probably the synergy between both options.

A further aspect of this study was to clarify the role of the antigen expressing alveolar type II epithelial cells in disease induction and regulation and to get insights into antigen-specific AECII/CD4⁺ T cell crosstalk. Therefore, the newly established protocol for isolation of murine type II pneumocytes was essential for functional and molecular analyses of AECII in SPC-HA x TCR-HA double transgenic mice. HA

expressing AECII from SPC-HA transgenic mice induced antigen-specific T cell proliferation, whereas the AECII derived from diseased mice had a reduced stimulatory capacity. Additionally, AECII from SPC-HA x TCR-HA secreted soluble factors inhibiting T cell proliferation. Comparative gene expression profiling of AECII from SPC-HA and SPC-HA x TCR-HA mice revealed a heterogeneous set of differentially expressed genes, upon other several molecules linked to regulatory mechanisms that may compensate overwhelming immune responses. Furthermore, analyses by real-time RT-PCR exhibited an elevated expression of surfactant protein A and D as well as platelet factor 4. These gene products can inhibit T cell proliferation and could play an active role in regulating the immune response and may act in concert with induced regulatory T cells observed in diseased SPC-HA x TCR-HA mice.

Together, these findings indicate an active role of antigen expressing alveolar type II epithelial cells not only in the induction but importantly, also in the regulation of CD4⁺ T cell mediated lung inflammation. Therefore, AECII should be considered as potential target cells for strategies aiming to interfere with or modulate the CD4⁺ T cell/AECII crosstalk in order to positively influence the course of such diseases.

CHAPTER IV

Materials and Methods

1 Materials and Methods

1.1 Mice

BALB/c mice were obtained from Harlan (Borchen, Germany). TCR-HA transgenic mice expressing a TCR $\alpha\beta$ specific for the peptide 110-120 from influenza HA presented by I-E^d have been described previously (Kirberg et al., 1994). CL4 transgenic mice expressing a TCR specific for the peptide 512-523 from influenza HA presented by H-2K^d have been described previously (Morgan et al., 1996). SPC-HA transgenic animals were generated using a construct containing the surfactant protein C promoter to achieve lung specific HA expression in alveolar type II epithelial cells and have been described previously (Bruder et al., 2004). Transgene expression was analyzed by PCR screening on genomic tail DNA. PCR was performed using a surfactant protein C promoter specific 5' primer (5'-CTG GAG GGC CAG GAA CAA ACA GGC-3') and a HA specific 3' primer (5'-ATA GTT TTC CGT TGT GGC TGT CTT-3'). TCR-HA and SPC-HA mice were bred in the animal facility at the German Research Centre for Biotechnology. Mice aged 16 to 20 weeks were used for experiments which were all performed according to National and Institutional Guidelines. Extensive microbial and serological studies were performed to exclude the presence of pathogenic bacteria, viruses, fungi and parasites which could potentially cause mucosal inflammation in these mice. No pathogens could be detected in all clinical samples studied.

1.2 Preparation of lymphocyte populations

Spleens were rinsed with erythrocyte lysis buffer (Qiagen, Hilden, Germany). Lymphnodes were disaggregated by passing through a 100 μ m mesh. Cells were washed with FACS buffer (PBS, 2% FCS, 2mM EDTA) and collected by centrifugation. For FACS analysis lung lymphocytes were isolated by passing the lungs through a 100 μ m mesh and subsequent treatment of cells with erythrocyte lysis buffer. For gene expression analysis lung lymphocytes were isolated as described previously (Bruder et al., 2004). Briefly, perfused lungs were excised and finely minced on ice, followed by a

60-90min digestion at 37°C with collagenase/dispase (0.2mg/ml of each) in RPMI medium with 5% fetal calf serum (FCS), in the presence of 0.25µg/ml DNase. To improve tissue disintegration, lungs were pipetted every 5min using a Pasteur pipet. Ethylenediaminetetraacetic acid (EDTA) was added to a final concentration of 5mM, followed by additional 5min incubation at 37°C. Cells were passed through a 70µm cell strainer, washed, and lung lymphocytes were isolated by ficoll density gradient centrifugation (Bruder D. et al., 2004).

1.3 Primary Type II Pneumocytes Preparation

Primary alveolar type II epithelial cells (AECII) were prepared using a modified protocol of a previously published method (Corti et al., 1996). Briefly, mice were killed and exsanguinated by severing the inferior vena cava and left renal artery. The tracheae was exposed and cannulated, and lungs were perfused with 10 to 20ml sterile phosphate buffered saline via the pulmonary artery until visually free of blood. Dispase (BD Biosciences, Heidelberg, Germany) was instilled into lungs via the tracheal catheter, followed by 1% low-melt agarose warmed to 45°C. The lungs were immediately covered with ice and incubated for 2min to gel the agarose. Lungs were then dissected out, put in a culture tube containing an additional 1ml of Disapase, and incubated for 45min at room temperature. Lungs were then transferred to a culture dish containing 7ml serum free DMEM + 25mM HEPES (GIBCO, Eggenstein, Germany) and DNase I (Sigma, Hannover, Germany). The tissue was gently teased away from the airways. The suspension was sequentially filtered and then pelleted. For the sorting of alveolar type II epithelial cells the crude cell suspensions were washed with serum free DMEM + 25mM HEPES and the cells were labeled with anti-CD45, anti-CD32/CD16, anti CD11b and anti-F4/80 antibodies and goat anti rabbit-IgG as secondary antibodies. After staining the cell suspension was washed with PBS containing 2% fetal calf serum and 2mM EDTA for cell sorting. Alveolar type II epithelial cells were separated as CD45⁺/CD32⁺/CD16⁺/CD11b⁻/F4/80⁻ fraction with a MoFLOW and a purity >97% was obtained. Isolated cells were either used for RNA preparation or *in vitro* culture assays. For that purpose cells

were resuspended and cultured in Iscove's Modified Dulbeccos's Medium (IMDM) containing 10% FCS.

1.4 Antibodies and flow cytometry

The monoclonal antibody 6.5 (α -TCR-HA) was purified from hybridoma supernatant and was used in fluorescein isothiocyanate (FITC)-labeled or biotinylated form. Monoclonal antibodies α -CD4 (GK1.5 and L3T4), α -CD25 (PC61), α -CD45RB (16A), α -CD62L (MEL-14), and α -CD69 (H1.2F3) were used as biotin, FITC, allophycocyanin (APC) or phycoerythrin (PE) conjugates. α -CD45 (30-F11), α -CD16/CD32 (2.4G2), α -CD11b (M1/70) and α -F4/80 were used unconjugated or phycoerythrin (PE) conjugates. As secondary antibodies a polyclonal serum including antibodies goat α -rat IgM+IgG+IgA were used as phycoerythrin (PE) conjugates. Monoclonal antibodies α -CD80 (16-10A1), α -CD86 (GL1), α -MHCII and α -MHCI were used as biotin or FITC conjugates. PE-streptavidin- or APC-(Allophycocyanin)-streptavidin-conjugates were used as secondary reagents (BD Bioscience, San Jose, CA). Two- and three colour flow cytometry was performed on a FACSCalibur (BD Bioscience, Heidelberg, Germany). Data were analyzed with CellQuestPro software (BD Biosciences, Heidelberg, Germany). For gene expression profiling and functional assays alveolar type II epithelial cells and 6.5^+CD4^+ lung lymphocytes were sorted with the MoFlow cells sorter (Cytomation, Fort Collins, CO).

1.5 Carboxyfluorescein diacetate, succinimidyl ester (CFSE) labeling of lymphocytes

Cell suspensions were prepared as described above. Cells were washed in RPMI without FCS, resuspended at a concentration of 10^7 lymphocytes/ml and incubated with $2.5\mu\text{M}$ CFSE (Molecular Probes, Göttingen, Germany) for 8min at 37°C . Two volumes FCS were added and cells were incubated for additional 5min at 37°C . After CFSE labeling the cells were washed twice with PBS to remove excess of CFSE and FCS.

1.6 Adoptive transfer

For adoptive transfer experiments in BALB/c, SPC-HA or SPC-HA x TCR-HA mice, red blood cell-depleted splenocytes and/or lymphocytes isolated from mesenteric lymph nodes of TCR-HA mice were enriched by AutoMACS using the CD4⁺ T cell Isolation Kit (MACS, Miltenyi Biotec, Bergisch Gladbach, Germany) following the manufacturers instructions. In case CD25⁺ cells were depleted from the CD4⁺ T cell population, biotinylated α -CD25 antibody was added to the biotin antibody cocktail of the CD4⁺ T cell Isolation Kit. The percentage of 6.5⁺CD4⁺ T cells obtained after MACS separation was determined by flow cytometry analysis as described above. Enriched transgenic cells, either unlabeled or CFSE labeled, were injected into age and sex matched BALB/c, SPC-HA or SPC-HA x TCR-HA mice.

For transfer of activated transgenic T cells splenocytes isolated from TCR-HA mice were stimulated *in vitro* with 10 μ g/ml HA110-120 peptide (SSFERFEIFPK) (Hackett et al., 1983) and cultured for 4 days. Dead cells were removed by ficoll density gradient and viable cells were cultured for an additional day. Flow cytometric analysis were performed to determine the percentage of 6.5⁺CD4⁺ lymphocytes and the activation status of the HA specific CD4⁺T cells was characterized by staining with α -CD25 and α -CD69.

1.7 Histology

Mice were sacrificed and the lungs were immersion fixed in buffered formalin, embedded in paraffin, sectioned at 4 μ m thicknesses and stained with hematoxylin and eosin (H&E). Immunohistochemistry for T lymphocytes was performed using the rat-anti-human-CD3 antibody clone CD3-12 (Serotec Ltd., Kidlington, UK) at 1:1.600 dilutions and the avidin-biotin-complex (ABC) method with diaminobenzidin as chromogen. Immunohisto-chemistry sections were counterstained with hematoxylin.

1.8 Proliferation assay and cocultures experiments

For coculture experiments 1×10^5 AECII were plated in 96-well flat-bottom plates in a final volume of 200 μ l IMDM containing 10% FCS. 2.5×10^5 CD4⁺ T cells from TCR-HA transgenic mice or CD8⁺ T cells from CL4 transgenic mice were added as responder cells and were cultured at 37°C. For standard proliferation assays cells were cultured for 48h and proliferation was determined by ³[H]-thymidine incorporation over the last 15h and was measured by scintillation counting. For CD8⁺ T cell proliferation assay cell suspensions were incubated in the presence or absence of different concentrations of HA512-523 peptide (IYSTVASSLVLL) (Morgan et al., 1996).

In case of coculture assays of AECII and professional APC 1×10^5 AECII were plated together with 2.5×10^4 immature DC or 2×10^4 PEC. After 48h CD4⁺ T cells isolated from TCR-HA transgenic mice were added as responder cells. Therefore the supernatants were discarded and 2.5×10^5 T cells were added with fresh medium to a final volume of 200 μ l and cultured at 37°C for additional 48h.

For proliferation assays of CD4⁺ T cells in AECII conditioned media 1×10^5 CD4⁺ T cells were cultured in 200 μ l AECII conditioned media in the presence of 1 μ g/ml plate-bound α -CD3 ϵ (145-2C11) (BD Bioscience, San Jose, CA) at 37°C for 48h. ³[H]-thymidine was added for the last 15h of culture and incorporation was measured by scintillation counting to assess proliferation. AECII conditioned media were generated by culturing freshly isolated AECII in IMDM with 10% FCS for 48h.

In case T cells were re-isolated after coculture with AECII, cells were harvested after 48h coculture and subjected to ficoll density gradient centrifugation. Re-isolated cells were counted and used either for inhibition assays or were used for RNA isolation and subsequent gene expression profiling.

1.9 Inhibition assay

For standard inhibition assay 5×10^4 CD4⁺ responder T cells were plated together with the same amount of T cells re-isolated after AECII coculture in 96-well flat-bottom plates in a final volume of 200 μ l IMDM (GIBCO, Eggenstein, Germany) in the presence of

0.75µg/ml soluble α-CD3ε (145-2C11) (BD Bioscience, San Jose, CA) and 5×10^5 irradiated BALB/c splenocytes. Cells were cultured at 37°C for 48h. Over the last 8h of the experiment $^3\text{[H]}$ -thymidine incorporation was measured by scintillation counting to assess proliferation.

1.10 Expression analysis by RT-PCR and real time RT-PCR

To analyse expression of different genes in alveolar type II epithelial cells and 6.5^+CD4^+ T cells of TCR-HA transgenic, SPC-HA transgenic and SPC-HAxTCR-HA double transgenic mice, mRNA was isolated from the different cell populations using the RNeasy Kit (Quiagen, Hilden, Germany) following the manufacturer's instructions. cDNA were synthesised using a mixture of oligo-dT primers and oligo-random hexamer primers and SuperScript™ II RNase H-reverse transcriptase (Invitrogen, Karlsruhe, Germany).

PCR was performed using AmpliTaqGold-polymerase (Applied Biosystem, Hamburg, Germany) and gene specific primers (table 6). Amplified PCR products were visualised after electrophoresis in 2% agarose gels containing ethidium bromide. Reactions were run on a PCR thermo cycler (Biorad, München, Germany) using the following conditions: 94°C for 10 minutes followed by amplification. Each cycle of amplification included 45sec denaturation at 94°C, 45sec annealing at 58 or 55°C and 1min synthesis at 72°C. Every PCR was run with 30, 34 or 40 amplification cycles for optimisation of the procedure. Unspecific amplification products were not detected in any of the PCR runs.

Real time RT-PCR for analyzing the expression of SP-A, SP-D, SP-C, CCL20, CCL11 and PF4 were conducted using a Brilliant SYBR Green QPCR Core Reagent Kit (Stratagenene, Heidelberg, Germany) in the GeneAmp 5700 Sequence Detection System (Perkin Elmer, Rodgau-Jügesheim, Germany) according to manufacturer's instructions. The PCR primer sequences are shown in table 6. Results were normalized using the housekeeping gene RPS9.

Tabel 6: RT-PCR and real-time RT-PCR primer sequences.

Primer	Sequence (5' → 3')	Size of PCR product (bp)
SP-A mm 5'	CAC CAA TGG GCA GTC AGT CAA C	198
SP-A mm 3'	ACA GAA GCC CCA TCC AGG TAG TG	
SP-C mm 5'	CGC CTT CTC ATC GTG GTT GTG	150
SP-C mm 3'	CGG GGC TAG GCG TTT CTG AG	
SP-D mm 5'	ATG GCC TGC CTG GTC GTG ATG	159
SP-D mm 3'	GTC CAG GTT CGC CAG CAG AGC	
CD80 mm 5'	AGT GGC TTT TGC TCT TTG GAT A	188
CD80 mm 3'	CAT TTC TTT GGG GCA CAT TGA T	
CD86 mm 5'	AGG GAA AGC AGA AAT CAC AAC ACT	245
CD86 mm 3'	AGC CCT GCC AGC GGA ATA	
MHCII mm 5'	GGC CAC AAT TGG AGC CCT GG	200
MHCII mm 3'	TGG GCT CTC CCA GGT TCA C	
PF4 mm 5'	CAG CGC TGG TCC CGA AGA AA	127
PF4 mm 3'	AAC CGCACA GTG GCG TCC TG	
CCL11 mm 5'	TCCCAACTTCTGCTGCTTTATC	168
CCL11 mm 3'	CATCCTGGACCCACTTCTTCTTG	
CCL20 mm 5'	GTAAGTGGGCTCACCTCTGC	129
CCL20 mm 3'	AGCTTCATCGGCCATCTGTCTT	
Alkal. phosphatase mm 5'	CCC CGG GGC AAC TCC ATC TTT	118
Alkal. phosphatase mm 3'	CCC GTT CAC CGT CCA CCA CCT T	
RPS9 mm 5'	CTGGACGAGGGCAAGATGAAGC	143
RPS9 mm 3'	TGACGTTGGCGGATGAGCACA	
Foxp3 mm 5'	CTGGCGAAGGGCTCGGTAGTCCT	250
Foxp3 mm 3'	CTCCCAGAGCCATGGCAGAAGT	
GITR mm 5'	GAACGCGGGGAGCAGACAGA	222
GITR mm 3'	CATAGGGCCCAATCGTAACTCACC	
Neuropilin-1 mm 5'	GCCTGCTTTCTTCTTGGTTTCA	221
Neuropilin-1 mm 3'	GCTCTGGGCACTGGGCTACA	
ICOS mm 5'	TGC CAG ACT ACA GCC ACA CTT TG	148
ICOS mm 3'	GTA CTT CCC AGA TCC CAG AGA CCA	
PD-1 mm 5'	GGAAATTCGTAGACTGGGGGACTG	125
PD-1 mm 3'	TCACGGAGCTTTTGCCTGGTAA	
Galectin mm 5'	AACCTGCCTTCCCCTTCC	148
Galectin mm 3'	TCCGCCGCCATGTAGTTGAT	
CTLA4 mm 5'	GGGCTGGGTCTTTACACTCATT	230
CTLA4 mm 3'	CTTCCTGTGGCATTAACTTTGTGT	
$\alpha_E\beta_7$ Integrin mm 5'	GAACTGCCGATCCTTGGTGAATA	250
$\alpha_E\beta_7$ Integrin mm 3'	GCTGGGCCCTCCTTGTGCTCT	
CD3 mm 5'	ACACCAGCCTCAAATAAAAACACG	825
CD3 mm 3'	AAGGGGGCAGGGAGGAGGTATGG	
CD19 mm 5'	TGACCCCGCCAGGAGATTC	1121
CD19 mm 3'	GCAGGGTGAGCAGGGATGG	
CD14 mm 5'	CATTTGCATCCTCCTGGTTTCTGA	182
CD14 mm 3'	GAGTGAGTTTTCCCTTCCGTGTG	
MCP-1 mm 5'	CTGTGCTGACCCCAAGAAGG	250
MCP-1 mm 3'	TAAGGCATCACAGTCCGAGTCACA	
MMP-2 mm 5'	TCC CGG GTG CTG CTT CTC CTT CC	206
MMP-2 mm 3'	CAC TGC CCC ATG CCA GGC TGT TA	

Tabel 6 (continued): RT-PCR and real-time RT-PCR primer sequences.

Primer	Sequence (5' → 3')	Size of PCR product (bp)
MMP-3 mm 5'	CTC CCT GCA ACC GTG AAG AAG A	228
MMP-3 mm 3'	CAA CTG CGA AGA TCC ACT GAA GA	
MMP-9 mm 5'	ACT CCG CCT TTG AGG ATC CGC AGA C	147
MMP-9 mm 3'	GAA GCC CGC TGA CGT GGG TTA CCT C	
HA mm 5'	GTCCGGCATCATCACCTCAAAC	221
HA mm 3'	AACCGGCAATGGCTCCAAATAGAC	
ICOS-L mm 5'	TGG GCT CAG GAC TAG GAA GAC C	181
ICOS-L mm 3'	AGC TGC CAA CTC AAG ACC CAT AAC	
PD1-L mm 5'	GTG TCC ACG GTC CTC CTC TTC TTG	182
PD1-L mm 3'	TCC CAT GGG CCC TTT CTT TCA	
IL-10 mm 5'	CTG GAC AAC ATA CTG CTA ACC GAC TC	134
IL-10 mm 3'	ATT TCT GGG CCA TGC TTC TCT GC	
TGF-β mm 5'	ACCTGGGTTGGAAGTGGAT	139
TGF-β mm 3'	GAAGCGCCCGGGTTGTGTTGGTT	
SP-R210 mm 5'	CCG GGT CGA CAG GGT CAA GTC C	209
SP-R210 mm 3'	TGG CCT CTG TGT CGC TGT CAC TCA	

1.11 DNA microarray hybridization and analysis

Total RNA from sorted AECII was isolated using the RNeasy kit (Qiagen, Hilden, Germany). Quality and integrity of total RNA isolated from 2×10^5 sorted AECII cells was assessed by running all samples on an Agilent Technologies 2100 Bioanalyser (Agilent Technologies, Waldbronn, Germany). For RNA amplification the first round was done according to Affymetrix without biotinylated nucleotides using the Promega P1300 RiboMax Kit (Promega, Mannheim, Germany) for T7 amplification. For the second round of amplification the precipitated and purified RNA was converted to cDNA primed with random hexamers (Pharmacia, Freiburg, Germany). Second strand synthesis and probe amplification were done as in the first round with two exceptions: incubation with RNase H preceded the first strand synthesis to digest the aRNA, and the T7T23V oligo for initiation of the second strand synthesis was used. 12.5µg biotinylated cRNA preparation was fragmented and placed in a hybridization cocktail containing four biotinylated hybridization controls (BioB, BioC, BioD, and Cre) as recommended by the manufacturer. Samples were hybridized to an identical lot of Affymetrix MG-U74Av2 chips for 16 hours. After hybridization, GeneChips were washed, stained with streptavidin-PE and read using an Affymetrix GeneChip fluidic station scanner. Analysis

was done with gene expression software (GeneChip, MicroDB, and Data Mining Tool, all Affymetrix).

1.12 Immunofluorescence

For immunofluorescence staining sorted AECII were mounted onto glass cover slips with a density of 2×10^5 cells using a cytospin apparatus, and were fixed with methanol-aceton (1:1) mixture at -20°C for 5min. Rabbit anti SP-A, SP-B, pro-SPC and SP-D antibodies (Chemicon Europe, Hampshire, UK) were all diluted 1:100 and incubated with the fixed cells over night at 4°C . As secondary antibody a FITC coupled goat anti rabbit IgG (Dianova, Hamburg, Germany) was used at a dilution of 1:80 and incubation for 30min at 37°C . All washing steps were performed in PBS and stained cells were embedded in glycerol-PBS.

1.13 Isolation of Dendritic cells (DC)

DC were cultured and purified as described (Brinker et al., 2001). Briefly, bone marrow cells were flushed from the tibiae and femurs of BALB/c mice and differentiated in DC medium (RPMI 1640 containing 5% FCS, 100U/ml penicillin-streptomycin, 20 $\mu\text{g}/\text{ml}$ gentamicin, and 50 μM 2-mercaptoethanol (all GIBCO, Eggenstein, Germany)) in the presence of 25 μM granulocyte macrophage-colony stimulating factor (GM-CSF) (R&D Systems, Wiesbaden-Nordenstadt, Germany). Immature bone marrow-derived DC were collected on day 5 and purified further by positive selection with AutoMACS using the anti-CD11c Isolation Kit (MACS, Miltenyi Biotec, Bergisch Gladbach, Germany). The cells were used for coculture experiments.

CHAPTER V

Abbreviations and References

1 Abbreviations

AECI	Alveolar type I epithelial cell
AECII	Alveolar type II epithelial cell
AM	Alveolar macrophage
APC	Antigen presenting cell
AXLN	Axillary lymph node
BAL	Bronchoalveolar lavage
BALT	Bronchus-associated lymphoid tissue
BLN	Bronchial lymph node
Bp	Base pairs
CCL	Chemokine ligand
CCR	Chemokine receptor
cDNA	copy Desoxyribonucleic acid
CFSE	5'-Carboxyfluorescein diacetat succinimidylester
cpm	Counts per minute
CVLN	Cervical lymph node
CXCL	Chemokine ligand
CXCR	Chemokine receptor
DNA	Desoxyribonucleic acid
DC	Dendritic cell
EDTA	Ethylene diamine tetraacetic acid
FACS	Fluorescence activated cell sorter
FCS	Fetal calf serum
GALT	Gut-associated lymphoid tissue
HA	Hemagglutinin
HEPES	N-2-Hydroxyethylpiperazine-N'-2-ethane sulfonic acid
HEV	High endothelial venules
i.p.	Intraperitoneal
i.v.	Intravenous
IEL	Intraepithelial lymphocyte
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin

ILD	Interstitial lung disease
INLN	Inguinal lymph node
l	Liter
LPL	Lamina propria lymphocyte
LPS	Lipopolysaccharid
μ	Micro
M	Molar
MALT	Mucosa-associated lymphoid tissue
MHC	Major histocompatibility complex
MLN	Mesenteric lymph node
MMP	Matrix metalloproteinase
NO	Nitric oxid
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PEC	Peritoneal excudate cell
RNA	Ribonucleic acid
TCR	T cell receptor
TGF	Tumor growth factor
TH cell	T helper cell
TIMP	Tissue inhibitor metalloproteinase
TNF	Tumor necrosis factor
Tnfrs	Tumor necrosis factor receptor family
T _{reg}	Regulatory T cell

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