Immunological risk stratification of the bronchiolitis obliterans syndrome after lung transplantation

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Immunological risk stratification of the bronchiolitis obliterans syndrome after lung transplantation

Immunologische risico stratificatie van het bronichiolitis obliterans syndroom na longtransplantatie

(met een samenvatting in het Nederlands)

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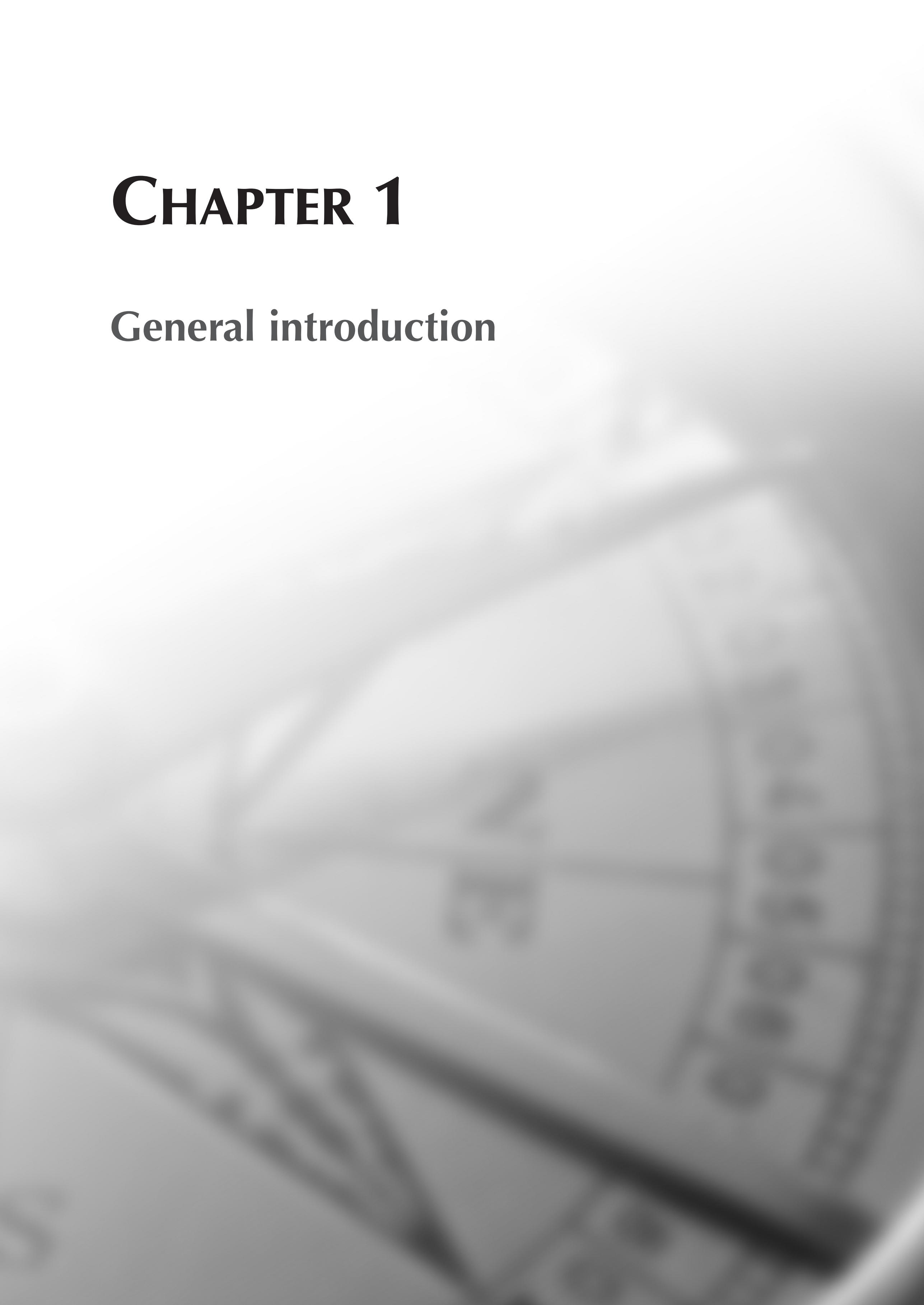

CHAPTER 1

General introduction

General Introduction

Lung transplantation is the ultimate treatment for end-stage lung disease and is currently a widely accepted therapy. Since 1988, 5-year survival rates have increased significantly from 47% to 54%, largely due to improving 1-year survival rates (74% in 1988 and 81% in 2006)[1]. Nevertheless, the survival half-life of 1-year survivors has not changed significantly (6.9 vs. 7.1 years). This lack of improvement in long-term survival is probably caused by the development of chronic allograft rejection or malignancies and the side effects of immunosuppressive therapy. Although 17% of transplant recipients develop a malignancy within 5 years, the development of chronic allograft rejection is by far the strongest contributing factor to poor long-term survival because almost 50% of transplant recipients fulfill the criteria of chronic rejection 5 years after lung transplantation[1].

Histopathological view of chronic allograft rejection

Chronic allograft rejection after lung transplantation is histopathologically characterized by a fibrous scarring of the lung that primarily affects the bronchioles and results in a partial or complete luminal obstruction: obliterative bronchiolitis (OB)[2]. At times (in up to 1.4% of cases), chronic allograft rejection is associated with accelerated fibrointimal changes that affect pulmonary arteries and veins[2,3]. In the early phases, there are perivascular or monovascular infiltrates in the submucosa of the bronchioles, and occasionally, eosinophils may be seen in the submucosa, a condition referred to as lymphocytic bronchiolitis (LB)[2]. Eventually, this condition leads to epithelial damage with necrosis, metaplasia and ulceration with exudates, cellular debris and neutrophil infiltration. Because of the fibrosis that obstructs the bronchial lumen, there is an accumulation of mucus and foamy histiocytes that can lead to accompanying inflammation. Because OB is characterized by a patchy distribution, transbronchial biopsies without histopathological signs of rejection do not exclude this diagnosis.

BOS

Histopathological confirmation of OB is hampered by the need for transbronchial biopsies taken at the right place at the right time. Therefore, a surrogate marker

based on a decline in lung function, bronchiolitis obliterans syndrome (BOS), is currently the gold standard. BOS is defined as a permanent decline of 20% in expiratory flow for three weeks or more in the absence of infection, acute rejection, anastomotic stenosis, bronchospasms and native disease recurrence[4] (see Table 1). Baseline FEV1 (forced expiratory volume in 1 second) and FEF25-75 are defined as the average of the two highest measurements at an interval of at least three weeks.

Table 1. BOS Classification

2002 Classification	
BOS 0	FEV1 > 90% of baseline and FEF_{25-75} > 75% baseline
BOS 0-p	FEV1 81-90% of baseline and/or FEF_{25-75} ≤ 75% baseline
BOS 1	FEV1 66%-80% of baseline
BOS 2	FEV1 51%-65% of baseline
BOS 3	FEV1 < 50% of baseline

Legend for Table 1: 2002 revised BOS criteria.
FEV1 = forced expiratory volume in 1 second. FEF25-75 = maximal mid-expiratory flow rate

Potential biomarkers

The hallmark in the diagnosis of BOS is a permanent decline in FEV1, and it is likely that certain markers or parameters are elevated or reduced before this measurable reduction is observed. Perhaps even patients at risk for developing chronic allograft rejection could be identified. By identifying such biomarkers or patients prone to developing BOS, it is possible that immune suppression can be adapted and BOS may be prevented. Prevention of BOS would enable an intervention early in the development of chronic rejection, hopefully leading to better long-term survival in the future.

Chronic allograft rejection in transplanted lungs is associated with immune (antigen dependent) and non-immune factors[5-10]. Although the exact mechanism of BOS has not yet been revealed, the hallmark of chronic allograft rejection seems to be repeated injury and inflammation of epithelial and subepithelial cells leading

to an immunological response resulting in the obliteration of the bronchioles and fibrosis of the parenchyma. Many articles have shown associations between biomarkers and the development of BOS, and an overview of the available literature for humans is illustrated in a table, with the presumed mechanisms explained in the text.

HLA mismatches and HLA and non-HLA antibodies

Antibodies against HLA (human leukocyte antigen) are formed when non-self HLA is encountered. HLA-encoding genes are located on chromosome 6, and the antigens can be discriminated into HLA Class I and Class II. HLA Class I antigens (HLA-A, HLA-B and HLA-C) are found on all nucleated cells and present peptides primarily derived from intracellular sources. HLA Class II antigens (HLA-DP, HLA-DQ and HLA-DR) are found on antigen-presenting cells (APCs) and primarily present peptides derived from extracellular antigens. In addition, non-HLA antibodies have been detected. Obviously, these antibodies may pose a risk for the transplanted organ, as both HLA and non-HLA antibodies can be directed against antigens expressed on the donor allograft. The risk of HLA mismatches, the transfer of preformed antibodies and de novo antibodies must also be taken into account.

Table 2 is an overview of the literature describing the effect of HLA mismatches on the occurrence of BOS. An increased risk for the development of BOS was detected if 1-2 HLA Class I mismatches were present[11-13]. Additionally, an association between HLA Class II mismatches (2 HLA-DR mismatches) and the development of BOS was observed[14,19]. T-cells from patients with a diagnosis of BOS showed a proliferative alloreactivity against donor HLA Class I and II peptides[16-18]. All of these associations were detected during treatment with an immunosuppressive regimen consisting of cyclosporine (CsA), azathioprine (AZT) and prednisone, with the exception of the study by Hodge and coworkers[19]. In that study, a limited number of BOS patients (6) were included, and it is unclear what type of immunosuppression the BOS patients received. It was also unclear whether this regimen was changed after the diagnosis of BOS because a transbronchial biopsy was taken for only 1 patient after diagnosis with BOS. Therefore, it is still likely that the introduction of new immunosuppressive drugs such as tacrolimus

Table 2. Effects of HLA mismatch on the occurrence of bronchiolitis obliterans syndrome after lung transplantation

Technique used for HLA mismatch determination	Mismatch	LTx (N)	BOS/ non-BOS	Therapy	Association with BOS	Ref.
Typing	0-1 HLA-A	134 + 50 HLTx	?	Unknown Probably 1	↓ BOS $p=0.03$	11
	1-2 HLA-A	126	60/66	1	↑ BOS ns	12
	1-2 HLA-A	94	40/54	1	↑ BOS $p=0.031$	13
	2 HLA-DR	94	66/28	1	↑ BOS $p<0.01$	14
	2 HLA-DR	277		1 + 2	↑ BOS $p=0.61$	15
Functional Test	HLA Class I	9 + 3 HC	5/4	1	proliferative response BOS 2-3x higher $p<0.001$	16
	HLA (A/B) or HLA-DR	13	7/6	Unknown Probably 1	Alloreactive T-cell Class I ↑ $p=0.025$ Class II ↑ $p=0.033$	17
	HLA-DR	18	9/9	1	lymphocyte proliferation HLA-DR ↑ BOS $p<0.001$	18
	HLA-DR Expression *	22	6/16	3	↑ BOS $p<0.05$	19

Legend for Table 2: HLA mismatches were determined by HLA typing of patients and donors or by functional testing including T-cell proliferation assays or measurement of HLA-DR expression on epithelial cells (Hodge et al.). Associations between BOS and HLA mismatch were mainly found for HLA-A or HLA-DR. HLTx means combined heart and lung transplantation. HC means healthy controls and ns means non significant. The symbol * means on airway epithelial cells. For therapy, the numbers mean: 1) CsA/AZT/prednisone, 2) Tac/AZT/prednisone and 3) CsA or TAC/AZT or MMF/prednisone.

(Tac) has led to the disappearance of the association between HLA mismatches and BOS, as confirmed in a study by Munster et al.[15]. That study was performed in Groningen, and it is worth mentioning that the cohort reported by van den Berg et al. (for which an association between HLA-DR mismatches and the development of BOS was observed) was included in the study by Munster and coworkers[14,15]. The lack of an association between HLA mismatches and the development of BOS

may be explained by a decreased incidence of BOS in patients treated with new immunosuppressive drugs and the lack of a crucial role for HLA mismatches in the development of BOS.

Table 3 provides an overview of the literature that describes the effects of donor-specific antibodies on the occurrence of BOS. After lung transplantation, antibodies against mismatched HLA can be produced, which has been associated with rejection of the graft. Jaramillo has shown that 67% of BOS patients develop anti-HLA antibodies, compared to none of the BOS-negative patients, and that the detection of HLA antibodies precedes the diagnosis of BOS by 20 months ($p=0.005$)[7]. No anti-HLA activity was found against Class II molecules, but Palmer and coworkers have shown that unsensitized lung transplant recipients can develop anti-HLA Class II antibodies[7,9]. Associations between BOS and HLA Class I and/or II antibodies were found during treatment with an immunosuppressive regimen consisting of cyclosporine, azathioprine and prednisone[7,9,20,23,27]. A study by Girnita et al. has also shown an association between HLA antibodies and the development of BOS, using an immunosuppressive regimen of Tac/AZT and prednisone[24]. However, a study performed using an immunosuppressive regimen of tacrolimus, mycophenolate mofetil (MMF) and prednisone did not detect any association between HLA antibodies (Class I, II or MICA) and BOS in 49 patients[25].

Approximately one third of BOS patients develop antibodies against airway epithelial cells (AECs)[22]. Increased expression of growth factors accelerates fibroproliferation. Binding of antiepithelial antibodies to the epithelial cells results in a significant increase in the expression of VEGF (6-fold, 13 vs. 79 ng/ml, $p<0.05$), heparin-binding epidermal growth factor-like growth factor (HB-EGF; 2-fold, 27 vs. 56 ng/ml, $p<0.05$) and TGF-β (2.5-fold, 12 vs. 32 ng/ml, $p<0.05$) compared with the expression levels observed after exposure to sera with no epithelial-specific reactivity[26]. Antibodies that recognize the K-α1 tubulin antigen expressed on airway epithelial cells have been detected in BOS patients approximately 3-15 months before the clinical diagnosis of BOS[26].

In conclusion, relationships between BOS and HLA antibodies have been found only during treatment with a regimen that included AZT. No associations between HLA antibodies and BOS have been found with MMF treatment. This

Table 3. Effect of donor-specific antibodies on the occurrence of bronchiolitis obliterans syndrome after lung transplantation

Effects of donor-specific antibodies

	Typing	LTx (N)	BOS/ non-BOS	Therapy	Association with BOS	Ref.
HLA	Class I	27	15/12	Unknown Probably 1	BOS p<0.001	7
	Class I or II	90	38/52	1	BOS p=0.05	9
	Class I or II	80	50/30	1	BOS p<0.01 BOS MICA p<0.01	20
	IgG or IgM antibodies	48	25/23	1†	BOS IgG Ag p<0.001 BOS IgM Ag p<0.001	21
	Class I and/or II	27	16/11	Unknown	BOS anti-AEC p=0.05	22
	and non-HLA			Probably 1	BOS anti-HLA p=0.001	
	HLA Class II #	62	31/31 matched	1	BOS p=0.04	23
	Class I and/or II	51	19/32	2	BOS p<0.001	24
	Class I and/or II and MICA	49	9/40	4	BOS ns	25
Non-HLA	Anti-K- 1 tubulin*	72	36//36 + 10 HC	1	Anti-AEC p<0.0001	26
	*	27	16/11	Unknown Probably 1	BOS p=0.05	22
	pre-LTx **	133	48/85	1	BOS p<0.05	27

Legend for Table 3: HLA and non-HLA antibodies directed against antigens expressed on the donor allograft were found during treatment with a regimen that included AZT but not MMF. HC means healthy controls and ns means non significant. AEC means airway epithelia cells. For therapy, the numbers mean: 1) CsA/AZT/prednisone, 2) Tac/AZT/prednisone, 3) CsA or TAC/AZT or MMF/prednisone and 4) Tac/MMF/prednisone. Symbols mean: † some MMF, # 1-2 months after transplantation, *anti-epithelial antibodies, **collagen V or k-alpha 1 tubulin.

finding may imply that MMF reduces the development of antibodies. Also, non-HLA antibodies directed against antigens presented on AECs have been associated with the development of BOS, but thus far, associations have only been found with an immunosuppressive regimen consisting of CsA/AZT and prednisone[22,26]. Because HLA antibodies are not associated with the development of BOS during a therapeutic regimen that contains MMF, it is important to determine the influence of non-HLA antibodies during MMF treatment.

Epithelial-mesenchymal transformation and matrix metalloproteinases

The processes by which epithelial cells are remodeled by fibrotic scar tissue are the missing piece of the puzzle. Epithelial-mesenchymal transformation (EMT) has been implicated in the process of pulmonary fibrosis[28,29]. Fibrosis is the excessive deposition of extracellular matrix (ECM). The ECM is controlled by ECM production and degradation by matrix metalloproteinases (MMPs) and plasminogen. MMPs are proteolytic enzymes that are produced as inactive precursors. Degradation of the ECM is controlled by the balance of plasminogen activators, plasminogen activator inhibitors, MMP expression and the inhibition of active MMP by tissue inhibitor of metalloproteinases (TIMP). Therefore, an imbalance in the MMP/TIMP ratio can be critical in bronchial tissue formation and repair.

Table 4 presents an overview of the literature regarding the effect of epithelial remodeling and the occurrence of BOS. In one study, 3 mesenchymal markers (α-smooth muscle actin (αSMA), S100A4 and a splice variant of fibronectin) were found to be significantly increased in BOS patients compared to non-BOS patients[19]. Recently, growth of more than 10 mesenchymal colony forming units (CFUs) in BALF has been associated with the development of BOS, and an increased fibroproliferative response has been observed in patients with BOS[30].

Neutrophils contain large amounts of proMMP-9, and activated MMP-9 can degrade type IV collagen, fibronectin, elastin and other connective tissue matrices, affecting chemotaxis and the activation of growth factors. Therefore, many studies have been performed to analyze the effect of MMPs and the development of BOS[39]. In an experimental model of airway remodeling, simvastatin can reduce MMPs[40]. In contrast to tacrolimus, glucocorticoids and CsA inhibit the expression

Table 4. Effects of epithelial remodeling on the occurrence of bronchiolitis obliterans syndrome after lung transplantation.

Effects of epithelial remodeling and matrix metalloproteinases on BOS

	Marker	LTx (N)	BOS/ non-BOS	Therapy	Compartment	Association with BOS	Ref.
EMT	Expression of mesenchymal protein	22	6/16	3	brush	↑ expression p<0.05	19
	Fibroblastoid CFUs >10 *	162	33/129	Unknown Probably 1	BALF	↑ CFU p<0.0001	30
	Fibroblast proliferative response	77	30/47	3	BALF	↑ response p<0.001	31
MMP		24	12/12 matched	1	BALF	Gelatinase ↑ p<0.005 MMP-9 ↑ p<0.05 MMP-2 ↑ p>0.05	32
		30 + 15 HC	12/18	3	sputum	MMP-9 ↑ p=0.03 ↓ BOS TIMP-1 p=0.09	33
		20	8/12	3	BALF	↓ BOS TIMP p=0.03 MMP-9 ↑ p=0.04 MMP-9:TIMP-1 p<0.0001	34
		44	13/21	6	BALF	MMP-9 ↑ p<0.005 Fibronectin promoter activity ↑ p=0.026	35
		72	23/49	5	Serum	MMP-2 ns MMP-8 ns MMP-9 ns TIMP-1↑ p=0.04 TIMP-2 ns	36
					BALF	MMP-2 ns MMP-8 ↑ p=0.003 MMP-9 ↑ p=0.001 TIMP-1↑ p<0.001 TIMP-2 ns	
		22	7/7	4	BALF	MMP-8↑ p<0.05) MMP-9↑ p<0.05	37
		110	21/89	4	Serum and genotyping 9 matched BOS	MMP-7↓ p=0.023	38

Legend for Table 4: Aspects of epithelial remodeling and the development of BOS.
HC means healthy controls and ns means non significant. BALF means bronchoalveolar lung fluid. For therapy, the numbers mean: 1) CsA/AZT/prednisone, 2) Tac/AZT/prednisone, 3) CsA or TAC/AZT or MMF /prednisone, 4) Tac/MMF/prednisone, 5) CsA or Tac/MMF/prednisone, 6) CsA or Tac/AZT/prednisone and 7) CsA/AZT or MMF. Symbols mean: * 6 months after LTx. EMT: Epithelial-mesenchymal transformation, CFU: colony-forming unit, MMP: matrix metalloproteinase, TIMP: tissue inhibitor of metalloproteinases

of MMP-9 in rat mesangial kidney cells by inhibiting the proinflammatory transcription factors nuclear factor κB (NF-κB) and activator protein-1 (AP-1)[41,42]. The mTOR inhibitor rapamycin causes an increase in MMP-9 promoter activity and an increase in AP-1 and NF-κB, which leads to MMP-9 transcription. However, this response is counteracted in the posttranscriptional regulation of MMP-9 mRNA stability by targeting destabilizing elements in the 3′-UTR of the MMP-9 gene[43].

There is a discrepancy in the literature regarding the inhibitor of MMP-9 (TIMP-1) and its association with BOS[33,34,36]. Beeh and Hubner have shown a decrease in TIMP-1 levels in sputum and BALF from BOS patients, whereas using a larger cohort, Taghavi has shown an association between BOS and an increase in TIMP-1 in BALF and serum[33,34,36].

MMP-7 (matrilysin) levels in serum samples from 9 matched BOS patients were found to be lower in the BOS patients compared to the non-BOS patients, but longitudinal analysis did not show a different pattern compared to non-BOS patients[38]. For MMP-8 (collagenase) levels, an association with BOS has been shown in BALF but not in serum[36,37].

In conclusion, there is an association between MMP-9 levels in BALF and sputum, but not in serum, and the development of BOS, independent of most immunosuppressive regimens used in clinical lung transplantation programs[32-37]. Regarding other metalloproteinases, few studies have been conducted on the influence of MMP-2 (gelatinase), MMP-7 and MMP-8 on the development of BOS, so no conclusions concerning possible biomarkers or speculations about the influence of immunosuppressive drugs can be made. Additionally, no conclusions can be drawn for mesenchymal markers, mesenchymal CFUs or increased fibroproliferative responses due to the limited number of studies.

Innate immunity

The innate immune system is stimulated by the recognition of pathogen-associated molecular patterns (PAMPs), which are microbe-specific molecules, by pattern recognition receptors (PRRs) on immune cells. The complement system plays an important role in the innate immune system and can be activated by three different pathways: the classical, alternative and lectin pathways. This system opsonizes pathogens, induces inflammatory responses and can attack some pathogens directly.

In Table 5, an overview of the literature regarding associations between several aspects of innate immunity and the occurrence of BOS is presented. Magro and coworkers found that C4d, C5-9 and C1q deposition along the bronchial epithelium and bronchial wall microvasculature is associated with the development of BOS during an immunosuppressive regimen of CsA/AZT and prednisone[44,45]. Although C4d deposition in the peritubular capillary has been regarded as antibody-mediated complement activation, Imai and coworkers have demonstrated in kidney transplant patients that complement activation by mannose-binding lectin (MBL) can also result in C4d deposition[52]. Concerning lung transplantation, a positive association between the donor MBL promoter LXPA haplotype and survival was found, but no association with the development of BOS was demonstrated[15].

CD14 is a PRR found on macrophages, dendritic cells (DCs) and activated neutrophils and serves as the cellular receptor for lipopolysaccharide (LPS). CD14 can indirectly trigger IL-1, IL-6, IL-8 and TNF-α production and promotes signaling through Toll-like receptor-4 (TLR-4)[53,54]. Patients with the TT genotype at CD14-159 C/T have elevated levels of CD14 in their bloodstream[55]. Polymorphic variation in the promoter region at position -159 (CD14-159 C/T) also influences the development of BOS[46]. Increased sCD14 levels ($p=0.05$), IFN-γ levels ($p=0.03$) and TNF-levels ($p=0.04$) have been detected in patients homozygous for this CD14 promoter polymorphism, suggesting increased activation of the innate immunity[46]. In contrast, it has been demonstrated that patients homozygous for the major alleles of rs1898830 (in intron 1 of the gene encoding TLR-2), rs352162 (in TLR-9) and rs187084 (in the TLR-4 promoter) have an increased risk of developing BOS compared with carriers of the minor alleles. Homozygotes for the minor alleles of rs7656411 (TLR2) and rs1927911 (intron 1 of the TLR4) have an increased risk of developing BOS compared with carriers of the major alleles[56].

Defensins are small, arginine-rich, cationic antimicrobial peptides with antibiotic activity against bacteria, virus, fungi and protozoa. Defensins function by binding to the microbial cell membrane and forming “ion pores” in those cell membranes. Some defensins act as proinflammatory mediators by stimulating the expression of interleukins (IL-6) and chemokines, whereas others act as chemo-attractants for DCs, neutrophils, memory T-cells and mast cells[57]. In humans, α-

Table 5. Effects of innate immune components on the occurrence of bronchiolitis obliterans syndrome after lung transplantation.

Markers of innate immunity

	Typing	LTx (N)	BOS/ non-BOS	Therapy	Compartment	Association with BOS	Ref.
C4d		23	7/16	Unknown Probably 1	Biopsy, serum	↑BOS C4d p=0.04 ↑BOS C5-9 p=0.03 ↑BOS C1q p=0.004	44
		13	13	Unknown Probably 1	Biopsy, serum	↑BOS C4d ↑BOS C5-9 ↑BOS C1q	45
		133	48/85	1	BALF	↑BOS C4d↑ P<0.001	27
MBL	donor LXPA	277 (154 BOS evaluation)		1	DNA	P=0.006	15
PRR	CD14	193	69/124	Unknown Probably 1	DNA, serum,	TT genotype ↑ p=0.006	46
TLR	TLR-4	193	69/124	Unknown Probably 1	DNA, serum,	↓ BOS TLR-4 p=0.04 TT genotype more BOS p=0.006	46
	TLR-4	170		Unknown Probably 1	DNA	↓ BOS in TLR-4 heterozygous p=0.08	47
	TLR-2 TLR-4 TLR-9	110	20/90	4	DNA	TLR-2 p=0.03 ↓ BOS TLR4 p=0.05 TLR-9 p=0.04	38
defensins	HBD2	15	6/9	Unknown	BALF	↑ BOS p<0.001	48
CCSP		17+ 5HLTx	6/16	1	BALF serum	↑ BOS CCSP ↓ p<0.001 ↑ BOS CSSP ↓ p<0.001	49
		27	11/16	4		CCSP ↓ ns SP-D ns	50
Heat shock protein		16	8/8	4	BALF, plasma	plasma Hsp 27 ↑ p=0.042	51

Legend for Table 5: Aspects of innate immunity are presented. AR means acute rejection. HLTx means combined heart and lung transplantation and ns means non significant. BALF means bronchoalveolar lung fluid. For therapy, the numbers mean: 1) CsA/AZT/prednisone, 2) Tac/AZT/prednisone, 3) CsA or TAC/AZT or MMF/prednisone, 4) Tac/MMF/prednisone, 5) CsA or Tac/MMF/prednisone, 6) CsA or Tac/AZT/prednisone and 7) CsA/AZT or MMF. MBL: mannose-binding lectin; PRR: pattern recognition receptor; TLR: toll-like receptor; HBD2: human β-defensin type 2; CCSP: Clara cell secretory protein; SP: surfactant protein; Hsp: heat shock protein

defensins are produced in the granules of neutrophils and in intestinal Paneth cells, and these defensins have microbicidal activity against Gram-negative and Gram-positive bacteria, fungi and enveloped viruses. β-defensins are produced in the epithelial cells of the skin and the gastrointestinal, urogenital and pulmonary tracts and have activity against Gram-negative bacteria and yeast. Human β-defensin type 2 (HBD2) levels are increased in patients with BOS (1270 pg/ml) compared to non-BOS patients (82 pg/ml)[58]. However, these results come from one study with a limited number of patients, an unknown immunosuppressive regimen and an unclear sampling timepoint; therefore, replication of these results is needed to support this association.

Clara cell secretory protein (CCSP or CC16) is produced by nonciliated bronchiolar Clara cells and can inhibit the activity of phospholipase A2 and the production and activity IFN-γ[59]. One study has shown that a decrease in CCSP in BALF and serum precedes diagnosis with BOS[49]. However, this study was performed using an immunosuppressive regimen consisting of CsA/AZT and prednisone, and a study performed using an immunosuppressive regimen of Tac, MMF and prednisone indicated that CCSP could not be used as a predictive biomarker for BOS[49,50].

A study by Wood and coworkers found an association between the level of heat shock protein 27 (Hsp27) in serum, but not in BALF, during treatment with Tac, MMF and prednisone in 8 matched BOS patients[51]. Heat shock proteins (Hsps) are thought to play a role in auto- and alloimmunity, although their specific role remains unclear. They prevent rejection and cell injury (including oxidative stress) and induce tolerance[60].

In conclusion, regarding innate immunity, an association between molecular variants of TLR4 and the development of BOS has been demonstrated using different immunosuppressive regimens; heterozygous recipients have a reduced risk of developing BOS[47,56]. An association between BOS and C4d was detected, but only with an immunosuppressive regimen consisting of CsA/AZT and prednisone. Because HLA antibodies are not associated with the development of BOS during a therapeutic regimen that contains MMF, it would be interesting to know whether C4d is associated with the development of BOS on a regimen that contains MMF. Although C4d deposition has been regarded a form of an antibody-

mediated complement activation, it has been shown in kidney transplant patients that complement activation by MBL can also result in C4d deposition[52]. Because only one study of mannose-binding lectin and its effect on lung transplantation has been published, no general statements concerning MBL and the development of BOS can be made. CCSP levels in BALF and serum precede diagnosis with BOS[49]. However, the study responsible for this observation was performed using an immunosuppressive regimen consisting of CsA/AZT and prednisone, and a study performed with an immunosuppressive regimen consisting of Tac, MMF and prednisone indicated that CCSP could not be used as a predictive biomarker for BOS[49,50]. Therefore, no conclusions concerning the influence of CCSP or CC16 on the development of BOS can be drawn. Also, due to the limited number of studies, no conclusion concerning the influence of heat shock proteins on the occurrence of BOS can be made.

Oxidative stress

On the one hand, oxidative stress represents an imbalance between the production and distribution of reactive oxygen species (ROS) and reactive nitrogen species (RNS); on the other hand, it represents the detoxification of the reactive intermediates by an antioxidant defense system. This antioxidant defense system includes enzymes like superoxide dismutase, catalase and glutathione (GSH) peroxidase as well as nonenzymatic antioxidants like ascorbate (vitamin C), α-tocopherol (vitamin E) and retinol (vitamin A). Chemically reactive iron and nitric oxide (NO) may contribute to the generation of ROS. ROS can cause toxic effects through the production of peroxide and free radicals, which damage all components of the cell, including proteins, lipids and DNA and, as a consequence, inhibit normal function and sometimes lead to inflammation and fibrosis. Oxidized glutathione (GSSG) and lipid peroxides (LPOs) are regarded as biomarkers of oxidative stress.

An overview of the literature regarding markers of oxidative stress and the occurrence of BOS is presented in Table 6. Increased levels of myeloperoxidase (MPO), a neutrophil oxidative enzyme, are elevated in BALF from BOS patients compared to that from non-BOS patients, independent of the immunosuppressive

Table 6. Markers of oxidative stress and associations with Bronchiolitis Obliterans Syndrome after Lung Transplantation.

Markers of oxidative stress

LTx (N)	BOS/ non-BOS	Therapy	Compartment	Association with BOS	Ref.
26	13/13 matched	1	BALF	MPO ↑ $p<0.001$ ECP ↑ $p=0.003$ GSSG ↑ ns	61
33 (72 samples)	16/32 samples + 24 samples 3 mo after LTx	1	BALF serum	API ns NE-API↑ $p<0.001$ SLPI ↓ $p<0.05$ API ↑ ns	62
16 + 6 HLTx	9/13	1	BALF	GSHt ↓ $p<0.05$ GSH ↓ $p<0.005$ GSSG ↑ $p<0.001$ MPO ↑ $p<0.005$ Met O%↑ $p<0.001$	63
13 + 8	7/14	1	BALF	Albumin ↑ $p=0.05$ HS↓ ns Ferritin ns NO2 ns	64
20 + 5 HLTx	5/20	1	BALF	MPO↑ $p<0.001$ ECP↑ $p<0.001$ Albumin ns	65
58	17 BOS 2-3 18 BOS 0p-1	Unknown	BALF serum	LPO↑ $p=0.001$ GSSG↑ $p=0.007$ Vitamin E ns Vitamin A ns Vitamin C ns	66
63	47/16	4	BALF	SLPI ↓ $p<0.01$	67
24	12/12	1	BALF	NE↑ $p<0.01$ SLPI ns	32
22	7/7 8 AR	4	BALF	MPO↑ $p<0.05$	37

Legend for Table 6: Aspects of oxidative stress are presented. AR means acute rejection. HLTx means combined heart and lung transplantation and ns means non significant. For therapy, the numbers mean: 1) CsA/AZT/prednisone, 2) Tac/AZT prednisone, 3) CsA or TAC/AZT or MMF/prednisone, 4) Tac/MMF/prednisone, 5) CsA or Tac/MMF/prednisone, 6) CsA or Tac/AZT/prednisone and 7) CsA/ AZT or MMF. MPO: myeloperoxidase; ECP: eosinophil cationic protein; GSSG: glutathione disulfide; API: α1-protease inhibitor; NE: neutrophil elastase; SLPI: secretory leukocyte protease inhibitor; GSHt: total glutathione; LPO: lipid peroxidase

regimen used[37,61,63,65]. Additionally, the eosinophil cationic protein (ECP) from activated eosinophils is elevated in BALF from BOS patients[61,65]. Oxidized glutathione (GSSG), a marker of stress, is often found to be elevated in BOS patients, except in one study[61,63,66]. Perhaps this study did not find a significant difference because of interindividual variability in measurements and the relatively small study size[61]. The levels of the antioxidants ascorbate, urate and GSH are lowered in BOS patients compared to non-BOS patients, but the difference is not statistically significant[61]. Neutrophil elastase (NE) is the most important neutrophil protease and can cause emphysematous changes in the lung, and the principal antiprotease of the human lung is α1-protease inhibitor (API). In BOS patients, NE levels are elevated in BALF during treatment with a regimen of CsA, AZT and prednisone[32,62]. However, although there is an increase in NE levels in BOS patients in addition to unaltered levels of secretory leukocyte protease inhibitor (SLPI), which is an antiprotease, serine protease levels are unaltered, suggesting that NE does not play a role in the development of BOS[32]. API is not elevated in BALF nor serum, but the NE:API ratio is elevated[62].

Because the number of patients in studies attempting to elucidate the effects of oxidative stress on the occurrence of BOS is often small, conclusions that draw associations between aspects of oxidative stress and BOS are hampered. Nevertheless, studies have demonstrated a rise in MPO in BALF from BOS patients, independent of the immunosuppressive regimen used. A rise in the levels of the markers GSSG and NE is associated with BOS, and although a decrease in the antioxidant defense system was expected, such a decrease was not found to be statistically significant[61-64,66].

Cells in innate immunity

Pattern recognition is essential in priming the innate immune system. Key players in innate immunity include neutrophils, macrophages, eosinophils, basophils, natural killer cells and dendritic cells, and these cells move freely throughout the body and interact with foreign particles or invading microorganisms.

An overview of the literature regarding cells of the innate immune system and the occurrence of BOS is presented in Table 7. An association between BOS and

Table 7. Associations between cells of the innate immunity and the occurrence of Bronchiolitis Obliterans Syndrome after Lung Transplantation.

Innate immune cells

LTx (N)	BOS/ non-BOS	Therapy	Compartment	Association with BOS	Ref.
26	13/13	1	BALF	Neutrophils ↑ p<0.002 Eosinophils ns Lymphocytes ns Macrophages ns	61
33 (72 samples)	16/32 samples	1	BALF	Neutrophils ↑ p<0.001 Monocytes ns Lymphocytes ns	62
16 + 6 HLTx	9/13	1	BALF	Neutrophils ↑ p<0.001 Eosinophils ns Lymphocytes ns Macrophages ns	63
21 + 21 HC	7/14	1	BALF	Neutrophils ↑ p<0.001	64
19 + 10 HLTx	10/19	1	BALF	Neutrophils ↑ p<0.01 BALF return ↓ p<0.05	68
20 + 5 HLTx	5/20	1	BALF	Neutrophils ↑ p<0.01 Eosinophils ns Lymphocytes ns Macrophages ns	65
25	8/17	1	BALF	Neutrophils ns Eosinophils ns Lymphocytes ns	69
45	22/23	1	BALF	Neutrophils ↑ p<0.00001	70
24	5/19	1	BALF	Neutrophils ↑ p<0.05 Eosinophils ns Lymphocytes ns Macrophages ns BALF return ↓ p<0.05	71
21	8/13	1	BALF	Neutrophils ↑ p<0.0001 Macrophages ↓ p<0.0001	72
17 + 5HLTx	6/16	1	BALF	Neutrophils ↑ p<0.001	49
34	12/20	1	BALF	Neutrophils ↑ p=0.0002 Macrophages ↓ p<0.0005 Lymphocytes ↓ p<0.05	73

Table 7. Associations between cells of the innate immunity and the occurrence of Bronchiolitis Obliterans Syndrome after Lung Transplantation.

Innate immune cells

	LTx (N)	BOS/ non-BOS	Therapy	Compartment	Association with BOS	Ref.
	132	36/96	3	BALF	Neutrophils ↑ $p<0.0001$ Macrophages ↓ $p<0.0001$ Eosinophils ns Lymphocytes ↑ $p<0.05$	74
	115 9 infection	31/52 + 23 AR +	3	BALF	BALF return ↓ $p<0.0001$ Neutrophils ↑ $p<0.0001$ Macrophages ↓ $p<0.0001$ Eosinophils ns	75
	63	47/16	4	BALF	Neutrophils ↑ $p<0.05$	67
	70	19/51	3	blood	$CD56^+CD16^+$NK ↑ $p<0.05$	76
DCs	90	48/42	3	serum	mDCs ↑ $p<0.05$ pDCs ↓ <0.05	77
	30	8/22	3	EBB, TBB	$CD1a^+$ ↑ $p<0.02$ High Class II expression ↑ $p<0.00001$ RFD1 macrophage correlated with $CD1a^+$ $r=0.57$ $p=0.003$	78

Legend for Table 7: Aspects of innate immunity are presented. HLTx means combined heart and lung transplantation and ns means non significant. For therapy, the numbers mean: 1) CsA/AZT/prednisone, 2) Tac/AZT/prednisone, 3) CsA or TAC/AZT or MMF/prednisone, 4) Tac/MMF/prednisone, 5) CsA or Tac/MMF/prednisone, 6) CsA or Tac/AZT/prednisone and 7) CsA/AZT or MMF. EBB: endobronchial biopsy, TBB: transbronchial biopsy

increased MMP-9 and MPO levels has been demonstrated. Because neutrophils contain large amounts of pro-MMP9 and because MPO is a neutrophil oxidative enzyme, it was expected that neutrophils would be associated with BOS. Neutrophils are one of the first responders during inflammation, and all studies of BOS have shown an increase in neutrophils in BALF from BOS patients, independent of the immunosuppressive regimen used[49,61-65,68,70-72]. One study did not show a significant increase in neutrophils, but this result is likely due to the performance of bronchoscopy in only 6 of the 8 BOS patients because of oxygen desaturation[69].

No association between eosinophils or lymphocytes and the development of BOS has been found. There is some discrepancy about the relationship between BOS and macrophages, but concentrating on the larger studies (of 34 or more patients) reveals a decrease in macrophages in BOS patients[73-75].

BALF return volume as well as macrophages have been shown to be significantly reduced, but these results were detected during a regimen of CsA, AZT or MMF and prednisone. These results have not been repeated in a regimen consisting only of Tac, MMF and prednisone[68,71,75].

Although more DCs, specifically those expressing Class II MHC, have been detected in BOS patients, these results are in contrast with another study in which hardly any CD1a+ DCs were seen[78,79]. Another study showed that in BOS patients stage II and III, myeloid DCs (mDCs) are elevated and plasmocytoid DCs (pDCs) are decreased compared to non-BOS patients[77]. Nevertheless, because there are limited studies on DCs and lung transplantation, no conclusions can be drawn concerning the influence of DCs on the development of BOS.

In conclusion, all studies have shown an increase in neutrophils in BALF from BOS patients. Interestingly, this increase in neutrophils in the BALF has been observed using different treatment modalities. No associations between eosinophils or lymphocytes and the development of BOS have been found. There is some discrepancy regarding BOS and macrophages, but concentrating on the larger studies (of 34 or more patients), a decrease in macrophages in BOS patients is revealed[73-75]. However, because none of these studies was performed using an immunosuppressive regimen consisting of Tac/MMF and prednisone, no conclusions can be made regarding the influence of immunosuppressive drugs. BALF return volume is significantly reduced, as well as macrophages, but these results were detected with a regimen of CsA, AZT or MMF and prednisone, and the results have not been repeated using a regimen consisting only of Tac, MMF and prednisone[68,71,75].

Cells in adaptive immunity

Adaptive immunity involves highly specialized cells, T- and B-cells, that recognize and remember specific pathogens. T-cells play a role in the cellular immune response, and B-cells act in the humoral response.

An overview of the literature regarding cells of the adaptive immune response

Table 8. Associations between cells involved in adaptive immunity and the occurrence of Bronchiolitis Obliterans Syndrome after Lung Transplantation.

Adaptive immune cells

LTx (N)	BOS/ non-BOS	Therapy	Compartment	Association with BOS	Ref.
25	8/17	1	BALF, EBB,TBB	CD4 ns CD8 ns CD25 ns	69
29	17/12	1	EBB	$CD8^+$ T-cell infiltration ↑ $p=0.008$ $CD4^+/CD8^+$ ↓ $p=0.02$	80
26	17/10?	1	BALF	$CD4^+$ ↑ $P<0.0001$ $CD8^+$ ↓ $P<0.05$ $CD4^+/CD8^+$ ↑ $P<0.0001$	81
70	19/51	3	Blood	$CD4^+CD25^{high}CD69-$ ↓ $p<0.05$	76
90	48/42	3	Serum	$CD4^+CD25^{high}FoxP3^+$ ↓ $p<0.05$	77
16	9/7	1	Blood	IL-10 producing $CD4^+$ T-cells ↓ $p<0.05$ IL-5 producing $CD4^+$ T-cells ↓ $p<0.05$	82
22	6/14	8	BALF blood	$FoxP3^+$ ↓ $p<0.001$ $CD4^+FOXp3^+$ <3.2% $P<0.001$ $FoxP3^+$ ↓ ns	83
47	13/34	4	BALF	$CD3^+CD4^+CD25^{hi}Foxp3^+$ p=n.s. $CCR4^+$ ns $CCR7^+$ ↓ $p=0.04$	84
38	4/34	4	Serum	sCD30 pre-LTx ↑ $p=0.04$	85
40	20/20	1	Serum	sCD30↑ post-LTx $p<0.001$	86
18	9/9 matched	Probably 1	Serum	sCD30↑ post-LTx $p<0.05$	87

Legend for Table 8: Aspects of adaptive cells are presented. BALF means bronchoalveolar lung fluid, EBB means endobronchial biopsy, TBB means transbronchial biopsy and ns means non significant. For therapy, the numbers mean: 1) CsA/AZT/prednisone, 2) Tac/AZT/prednisone, 3) CsA or TAC/AZT or MMF /prednisone, 4) Tac/MMF/prednisone, 5) CsA or Tac/MMF/prednisone, 6) CsA or Tac/AZT/prednisone and 7) CsA/ AZT or MMF.

and the occurrence of BOS is presented in Table 8. Using an immunosuppressive regimen of CsA/AZT and prednisone, the influence of CD4+ and CD8+ T-cells on the development of BOS is unclear. However, Treg cells are decreased in BOS patients regardless of the immunosuppressive regimen used, although one study performed using an immunosuppressive regimen consisting of Tac/MMF/prednisone did not find a decrease in Tregs in BOS patients[76,77,83]. However, that study showed that Tregs expressing CCR7 are associated with a lower incidence of BOS and may play a role in tolerance[84]. Levels of 6Ckine/CCL21 in BALF correlate with CCR7+ Treg subsets and appear to be protective against BOS.

Activated Th2 cells express CD30, which is a member of the TNF family, and interestingly, patients with high sCD30 levels before transplantation are prone to developing BOS[85]. After transplantation, using a regimen of CsA/AZT and prednisone, there is an increase in sCD30 levels during BOS development (7.57± 2.63 months before the clinical diagnosis of BOS)[86,87]. These studies were performed using a regimen of CsA/AZT and prednisone so no conclusions regarding other immunosuppressive regimens can be made.

Cytokines and chemokines

Cytokines are small proteins involved in immunosurveillance. Chemokines are chemoattractants and direct leukocyte trafficking to places of injury. Chemokines are classified by structure, and 4 groups have been identified: C, CC, CXC and CX3C. The chemokine receptors are XCR, CCR, CXCR and CX3CR.

An overview of the literature elucidating the influence of cytokines and chemokines on the occurrence of BOS is presented in Table 9. IL-8 is a proinflammatory cytokine and plays an important role in the recruitment and activation of neutrophils. In the paragraph concerning cells in the innate immunity, it was previously mentioned that an increase in neutrophils in BALF is observed independent of the immunosuppressive regimen used. An increase in IL-8 is observed in BALF and serum regardless of the immunosuppressive regimen[37,65,67,68,72,73,75,77]. One study did not find a significant increase in IL-8 levels in serum, but these results may be due to the small size of the cohort (n=20)[23].

Table 9. Associations between cytokines and chemokines and the occurrence of Bronchiolitis Obliterans Syndrome after Lung Transplantation.

Cytokines and chemokines

LTx (N)	BOS/ non-BOS	Therapy	Compartment	Outcome	Ref.
19 + 10 HTx	10/19	1	BALF	IL-8↑ $p<0.01$	68
20) + 5 HLTx	5/20	1	BALF	IL-8 ↑ $p<0.01$	65
21	8/13	1	BALF	IL-8 ↑ $p=0.006$ RANTES/CCL5 ↑ $p=0.05$ MCP-1/CCL2 ↑ $p=0.05$ sICAM-1 ns sVCAM ns	72
34	12/20	1	BALF	RANTES/CCL5 ns IL-8 ↑ $p<0.005$	73
20	10/10 matched	1	Serum	IFN-α ns IFN-γ ns IL-1β ↑ $p<0.05$ IL-2 ↑ $p<0.05$ IL-4 ns IL-5 ns IL-6 ns IL-7 ns IL-8 ns IL-10 ↓ $p<0.05$ IL-12↑ $p<0.05$ IL-13 ns IL-15 ↑ $p<0.05$ IL-17 ns IL-1Rα ns IL-2R ns Eotaxin/CCL11 ns GM-CSF ns MIP-1α/CCL3 ns MCP-1/CCL2 ↑ $p<0.05$ MIP-1β/CCL4 ns MIG/CXCL9 ns RANTES/CCL5 ns TNF-α ns	23

Table 9. Associations between cytokines and chemokines and the occurrence of Bronchiolitis Obliterans Syndrome after Lung Transplantation.

Cytokines and chemokines

LTx (N)	BOS/ non-BOS	Therapy	Compartment	Outcome	Ref.
16	8/8	1	BALF	MIP-1α/CCL3 ns MIP-1β/CCL4 ns TARC/CCL17 ns MIP-3β/CCL19 ↓ $p<0.01$ MIP-3α/ CCL20 ↑ ns MDC/CCL22 ↑ $p=0.02$ Eotaxin/CCL11 ns $CD3^+CCR7^+$ ns $CD3^+CCR6^+$ ns $CD3^+CCR4^+$ ns $CD68^+CCR7^+$ ns $CD68^+CCR6^+$ $p<0.01$ $CD68^+CCR4^+$ $p<0.05$	88
77	30/47	3	BALF	IL-13 ↑ $p<0.05$ IL-4 ns TGF-β↑ ns	31
115	31/52 + 23 AR + 9 inf	3	BALF	IL-8 ↑ $p<0.05$	75
22	7/7 8 AR	4	BALF	IL-8 ↑ $p<0.05$ PGP ↑ $p<0.05$	37
63	47/16	4	BALF	IL-8 ↑ $p<0.01$	67
132	36/42 + 43 AR + 11 inf	3	BALF	IL-1β ↑ $p<0.01$ IL-2 ↓ $p<0.05$ IL-6 ↑ $p<0.05$ TGF-β↑ $p<0.05$ mRNA IL-17 ↑ $p<0.05$ mRNA IL-23 ↑ $p<0.05$	74
1) 78 2) 198		1 and 2	DNA	In the first cohort, IL-6 polymorphism $p<0.05$	89
90	48/42	3	Serum	IL-8 ↑ $p<0.05$ TNF-α ↑ $p<0.05$	77

Table 9. Associations between cytokines and chemokines and the occurrence of Bronchiolitis Obliterans Syndrome after Lung Transplantation.

Cytokines and chemokines LTx (N)	BOS/ non-BOS	Therapy	Compartment	Outcome	Ref.
47	13/34	4	BALF	MIP3β/CCL19 no correlation with CCR7+ Treg ns 6ckine/CCL21 correlated with CCR7+Treg p=0.03	84
22	6/14	8	BALF	MDC/CCL22 ↓ p=0.04 TARC/CCL17 ns	83

Legend for Table 9: Associations between cytokines and chemokines and the occurrence of Bronchiolitis Obliterans Syndrome after Lung Transplantation. For therapy, the numbers mean: 1) CsA/AZT/prednisone, 2) Tac/AZT/prednisone, 3) CsA or TAC/AZT or MMF/prednisone, 4) Tac/MMF/prednisone, 5) CsA or Tac/MMF/prednisone, 6) CsA or Tac/AZT/prednisone, 7) CsA/AZT or MMF and 8) Tac or CsA/AZT or MMF or Sir/prednisone. IL: interleukin; RANTES: regulated upon activation, normal T-cell expressed and secreted; MCP-1: monocyte chemotactic protein-1; sICAM-1: soluble intercellular adhesion molecule-1; sVCAM: soluble vascular cell adhesion molecule; IFN: interferon; GM-CSF: granulocyte-macrophage colony-stimulating factor; MIP: macrophage inflammatory proteins; MCP: monocyte chemotactic protein-1; TGF: transforming growth factor; PGP: phagocyte glycoprotein-1; MDC: macrophage-derived chemokine; TARC: thymus and activation regulated Chemokine. Inf means recipients were diagnosed with an infection.

Although many studies have been performed to evaluate cytokine levels in BOS patients, few reproducible outcomes (except for IL-8, as mentioned above) can be discerned if the influence of immunosuppressives is taken into account. IL-1β levels are elevated in patients with BOS according to a study performed using a regimen of CsA/AZT and prednisone and also a study using a regimen consisting of Tac or CsA/AZT or MMF/prednisone[23,74]. Two studies analyzing IL-2 levels and the development of BOS have been published, with conflicting results. Using a regimen consisting of CsA/AZT and prednisone, an increase in IL-2 levels is observed in BOS patients, whereas a decline in IL-2 levels is observed in a regimen consisting of Tac/MMF and prednisone[23,74]. Similar results have been described for MDC/CLL22: using CsA/AZT and prednisone, MDC/CLL22 levels are elevated, whereas decreased MDC/CCL22 levels are observed in BOS patients using Tac or CsA/AZT or MMF or Sir/prednisone.

In one study using CsA/AZT and prednisone, the chemokine RANTES/CCL5 has been found to be elevated in BOS patients, whereas in two other studies using the same immunosuppressive regimen, RANTES/CCL5 was not associated with allograft rejection. Using an immunosuppressive regimen of CsA/AZT and prednisone, there is a correlation between IL-6 -174 G/C and the development of BOS, but this correlation vanishes when the immunosuppressive regimen consists of Tac/AZT and prednisone[89]. Elevated IL-6 levels have also been observed in a study were the immunosuppressive regimen consisted of Tac or CsA/AZT or MMF/prednisone[74]. IL-13 levels were elevated in one study performed using a regimen of CsA or Tac/AZT or MMF/prednisone but not in a study with CsA/AZT and prednisone[23,31].

Levels of IL-10 and MIP-3β/CCL19 are decreased, and the levels of the cytokines IL-12, IL-13, IL-15, IL-17, IL-23 and PGP are increased in BOS patients, but these results have not yet been reproduced[23,31,37,74,88]. MCP-1/CCL2 levels are elevated during immunosuppressive therapy with CsA/AZT and prednisone. No information about the influence of MCP-1/CCL2 on the development of BOS after lung transplantation has been published, although Tac/AZT and prednisone are used to prevent allograft rejection[23,72]. TARC/CCL17 has not been associated with the development of BOS in a regimen consisting of CsA/AZT and prednisone or Tac or CsA/AZT or MMF or Sir/prednisone, but results with a regimen consisting of Tac/MMF and prednisone have not been generated yet[83,88].

In conclusion, an increase in IL-8 and IL-1β levels is associated with the occurrence of BOS under different immunosuppressive regimens. There are conflicting results regarding BOS and IL-2, IL-6, IL-13, MDC/CLL 22 or RANTES/CCL5 levels. For IL-10, MIP-3β/CCL19, IL-12, IL-13, IL-15, IL-17, IL-23, MCP-1/CCL2 and PGP, no reproducible outcomes have yet been published.

Outline of this thesis

Long-term survival after lung transplantation is compromised by the development of bronchiolitis obliterans syndrome (BOS). According to standard ISHLT criteria, BOS is defined as a permanent decline of more than 20% in FEV1 from the post-operative baseline at 2 distinctive time-points in the absence of infection or other etiology 4. It is likely that, before this measurable reduction in FEV1 is achieved,

certain markers or parameters are elevated or reduced, and hopefully, patients at risk for developing BOS can be identified. By identifying patients at risk to develop BOS, we hope to intervene early in the process of chronic rejection, before lung function is permanently lost. Such intervention will hopefully lead to better long-term survival in future.

In chapter 2, we study soluble CD30 (sCD30), a member of the tumor necrosis factor superfamily and a marker for Th2 activation, as a biomarker for allograft rejection after lung transplantation. Before transplantation, low sCD30 levels are correlated with better survival outcomes after heart, lung and kidney transplantation.

In chapter 3, we evaluate the chemokine thymus and activation regulated chemokine (TARC/CCL17) as a marker for the development of BOS after lung transplantation. TARC/CCL17 is a chemokine involved in the recruitment of Th2 cells.

In chapter 4, we study the applicability of sCD30 as a potential biomarker to evaluate disease severity in patients with atopic dermatitis treated with calcineurin inhibitors or myfortic (a combination of these therapies is used after lung transplantation).

In chapter 5, we study the influence of MBL levels on the development of BOS and the (re)occurrence of CMV infection. MBL is involved in infections, and there is a correlation between the development of BOS and viral infections.

In chapter 6, we evaluate whether killer immunoglobulin-like receptor (KIR) ligand incompatibility and the individual KIRs of transplant recipients influences the development of BOS. The development of BOS is associated with viral infections, and natural killer (NK) cells are involved in the lysis of virally infected cells. The activation of NK cells is controlled by activating and inhibitory KIRs.

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Chapter 2

Soluble CD30 measured after lung transplantation does not predict the Bronchiolitis Obliterans Syndrome in a tacrolimus/ mycophenolate mofetil based immunosuppressive regimen

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Abstract

Background

The purpose of this study was to determine the usability of post transplant serum sCD30 levels as a biomarker for the development of the bronchiolitis obliterans syndrome (BOS) after lung transplantation during a tacrolimus/ mycophenolate mofetil based regimen

Methods

Soluble CD30 concentrations were measured prior to transplantation and in 175 samples taken after transplantation in 7 patients developing BOS and 7 non-BOS patients closely matched for age, underlying diseases, follow up and gender.

Results

High pre transplant sCD30 levels dropped significantly after lung transplantation, but in the post transplant samples no differences could be detected between patients developing BOS or not, and no changes were found prior or during the development of BOS.

Conclusions

After transplantation sCD30 levels are consistently suppressed but BOS is not prevented, indicating that sCD30 can not be used as a biomarker to predict BOS after transplantation in the regimen employed.

Introduction

The bronchiolitis obliterans syndrome (BOS) is the main cause of long-term morbidity and mortality after lung transplantation, and is considered to be the consequence of chronic lung allograft rejection[1,2]. The specific etiology and pathogenesis of BOS are largely unknown but risk factors like acute rejection, viral infections and HLA mismatches have been associated with the development of BOS[1,2]. When BOS is diagnosed it generally does not respond to augmentation of immune suppression[3]. Possibly BOS may be prevented by higher levels of immune suppression in patients at risk of chronic rejection. Therefore, reliable parameters capable of discriminating and monitoring these patients should be identified.

CD30 is a member of the tumor necrosis factor super family and is expressed on eosinophils, NK-cells, T- and B-cells[4-7]. After CD30+ T-cell activation, the metalloproteinase TNFα converting enzyme cleaves the extracellular domain of CD30 and a soluble form of CD30 (sCD30) is released into the blood stream[8,9]. In transplantation medicine, a relation between low pre transplant sCD30 and better survival outcome was found in kidney, heart and lung transplantation[10-12]. Post transplant sCD30 levels are a risk factor for acute rejections and a lower graft survival in renal transplantations[13,14]. Also in lung transplantations a similar observation was made: high post transplant sCD30 concentrations correlated well with the development of BOS[15,16]. In those studies a cyclosporine based immunosuppressive regimen was used. As sCD30 levels are known to be influenced by the type of immunosuppression employed, our objective was to test the usability of post transplant sCD30 as a biomarker for BOS during a tacrolimus based regimen.

Patients and Methods

Patients who underwent lung transplantation at the Heart Lung Center in Utrecht between September 2001 and January 2005 were included in this study. Several hours prior to transplantation and every month during follow-up, serum was collected and stored at -800C. To avoid the bias of postoperative complications, we excluded patients who died during the period from transplantation till three

months after transplantation. According to the standard ISHLT criteria BOS was defined as a decline of the FEV1 of more than 20% in the absence of infection or other etiology[3]. From 68 patients, 7 patients developing BOS could be closely matched to 7 patients without BOS for follow up time after lung transplantation, underlying disease, age and gender (Table 1). All selected patients received bilateral lung transplantations. Standard immunosuppressive therapy consisted of basiliximab, tacrolimus, mycophenolate mofetil and prednisone. The study design was approved by our Medical Ethical Committee (METC) and informed consent was obtained from each patient.

Soluble CD30 was measured prior to transplantation and monthly after transplantation as described previously[10].

Data are presented as mean value ± SEM. The Wilcoxon signed rank test was used to calculate differences between pre and post transplant sCD30 levels in BOS and non BOS patients respectively. The Fisher's exact test was used to compare frequencies. $P < 0.05$ was considered statistical significant.

Results

Seven patients developing BOS were selected for this study as each of these patients could be closely matched for follow up time, underlying disease, age and gender to 7 patients not developing BOS, resulting in a total of 14 patients investigated. Follow up time ranged equally from 9-64 months for the patients with BOS and their matched non-BOS counterparts (Table 1). No significant differences were present with regard to the number of HLA class-I or –II mismatches and none of the patients had pre transplant anti-HLA class-I or –II antibodies. However, the graft survival time significantly differed between patients developing BOS (22.3 ± 9.7 months) versus those remaining BOS-free (38.7 ± 9.3 months) (p=0.02; Wilcoxon signed rank test).

To examine whether the lung transplantation procedure causes a change in circulating levels of sCD30, sera were analyzed taken pre- and post lung transplantation. Post transplant sera were obtained at the onset of BOS or a similar time point after transplantation in the matched non-BOS patients. In sera from the 14 patients investigated, no significant differences were found between pre- (27.7

Table 1. Characteristics of Lung Transplant Recipients

	All	BOS	Non-BOS
Age in years	50.6 ± 7.2	50.7 ± 12	50.6 ± 12
gender			
male	4	2	2
female	10	5	5
Underlying disease			
CF	2	1	1
COPD/ emphysema	8	4	4
Interstitial lung disease	4	2	2
Graft survival (months)	30.5 ± 7.6 months	22.3 ± 9.7 months	38.7 ± 9.3 months
De novo HLA antibodies	0	0	0
HLA mismatch			
Class I	3.2 ± 0.7	3.5 ± 0.8	3.0 ± 0.5
Class II	1.7 ± 0.4	1.5 ± 0.5	1.9 ± 0.3

CF: cystic fibrosis, COPD: chronic obstructive pulmonary disease, HLA- antibodies: antibodies were measured against class I and/or class II, HLA-mismatches: HLA-A , -B and –DR mismatches were taken into account

± 14 U/ml) versus post transplant (19.3 ± 6.7 U/ml) sCD30 levels. However, we noticed that especially the high pre transplant values dropped after transplantation and reanalyzed the data by discriminating high (>20 U/ml) vs. low (≤ 20 U/ml) pre transplant values[10]. In patients with high pre transplant sCD30 levels (n=6), sCD30 levels dropped significantly after transplantation (Wilcoxon signed rank test; p=0.03), whereas no significant decline could be observed in patients with low pre transplant sCD30 values (Figure 1). These data, combined with the observation that median sCD30 levels after transplantation were similar for both patients with high respectively low pre transplant sCD30 levels, indicate that the immunosuppressive regimen employed suppresses sCD30 production (Figure 1).

Post transplant changes in sCD30 levels were examined in sera taken every month during the first year after transplantation and every 3 months thereafter, thus encompassing the period of BOS development and diagnosis. This resulted in

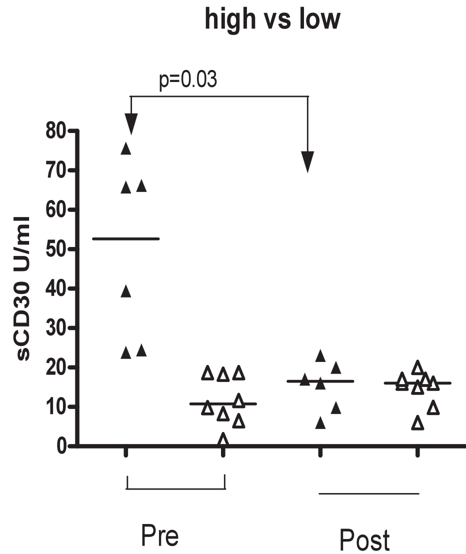

Figure 1. Suppression of sCD30 levels after transplantation
In 14 lung transplant recipients pre and post transplant sCD30 levels were measured by ELISA. High pre transplant levels of sCD30 (> 20 U/ml) were compared to post transplant sCD30 levels at the clinical diagnosis of BOS or the corresponding time visit in non BOS patients. The medians are illustrated with a horizontal line. In 6 patients with high pre transplant sCD30 levels the decrease of sCD30 levels after transplantation was significant (Wilcoxon signed rank test p=0.03) whereas it was not significant in patients with low pre transplant sCD30 levels (≤20U/ml).

analysis of 175 serum samples showing post transplant sCD30 levels of 17.2 ± 12 U/ml (Figure 2). Patients developing BOS had sCD30 levels of 17.4 ± 11.8 U/ml which was almost identical to those detected in patients not developing BOS (17 ± 17.3 U/ml). Furthermore, no difference was found between patients suffering from CF, ILD or COPD with regard to post transplant sCD30 levels (Figure 2). Focusing on the usability of sCD30 as a biomarker, we examined whether changes in sCD30 levels were related to clinical manifestations. No relation could be found between changes in sCD30 and infection or decrease of FEV1, including the 2 disproportional high sCD30 values 160 U/ml in a non-BOS patient and 80 U/ml in a patient eventually developing BOS (Figure 2). Moreover, in none of the patients developing BOS the clinical diagnosis of BOS was heralded by an increase in sCD30 levels of which examples of 3 patients are shown in Figure 3.

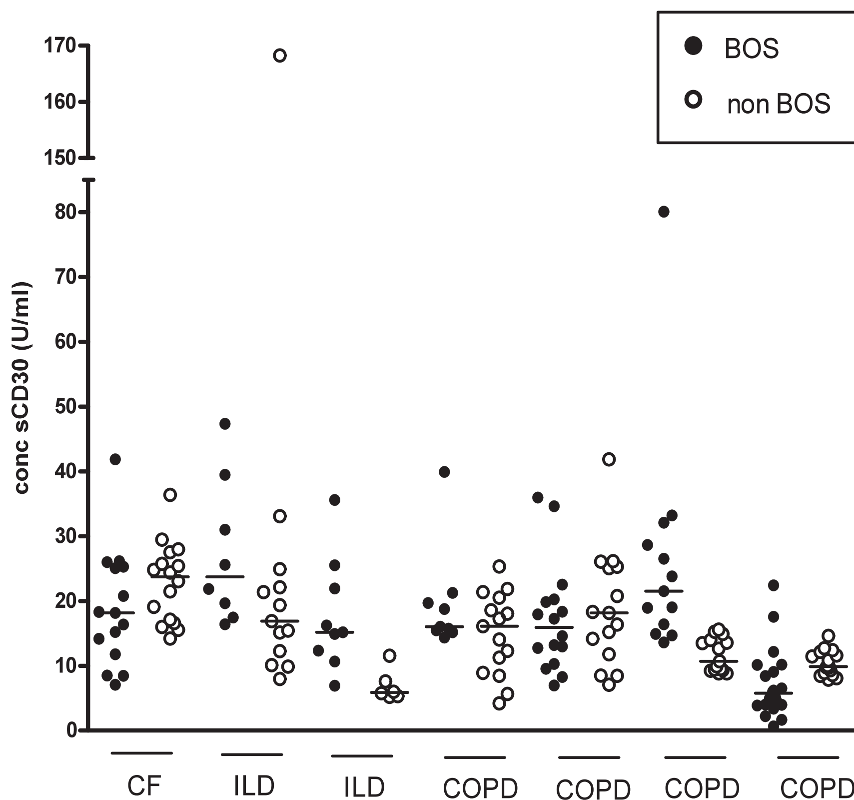

Figure 2. Post transplant sCD30 levels do not correlate with underlying diseases nor with the development of BOS.

Monthly after transplantation serum was collected and sCD30 levels were measured in 14 lung transplant recipients (7 patients developing BOS closely matched for follow up time, age, gender and underlying disease to 7 non-BOS patients). Samples ranged from 6-25 measurements per lung transplant recipient and the sCD30 measurements are depicted as dots. Underlying diseases are depicted in the figure and represent a patient with BOS (a black dot) and its closely matched counter partner without BOS.

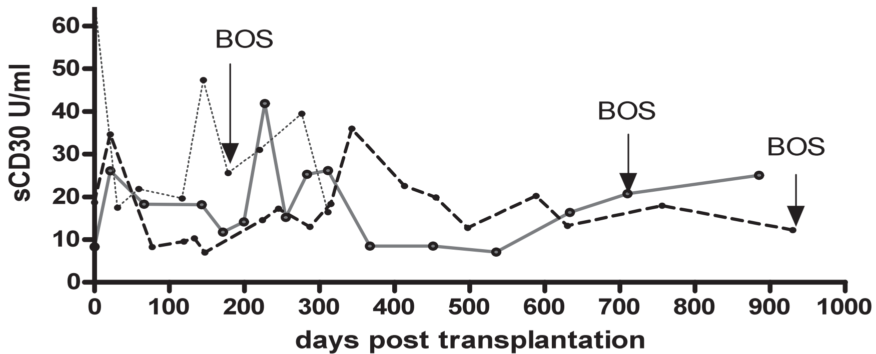

Figure 3. The clinical diagnosis of BOS is not heralded by an increase in sCD30 levels.
Post transplant sCD30 levels were measured in serial serum samples of 14 lung transplants (7 patients with BOS and 7 non-BOS patients). Three out of seven patients developing BOS are depicted and are representative for patients developing BOS. Every line represents sCD30 measurements of a single lung transplant recipient eventually developing BOS and time points are indicated when BOS was clinically diagnosed. Immunosuppressive regimens were not altered prior, during or after the diagnosis of BOS.

Discussion

Parameters to predict the development of BOS or to identify patients at risk are beneficial in managing lung transplant recipients. Both pre-transplant and post transplant soluble CD30 levels have been reported to be associated with the development of BOS. Pre transplant sCD30 levels were associated with the development of BOS in a tacrolimus based regimen but not in a cyclosporine based regimen and the association between post transplant sCD30 levels and the development of BOS was observed only in a cyclosporin based immuno-suppressive regimen[10,15,16]. This study investigated the usability of sCD30 as a biomarker for the development of BOS in a tacrolimus based regimen.

Studies in kidney transplantation demonstrated that the suppression of sCD30 after transplantation is influenced by the type of immunosuppressive regimens: tacrolimus probably in combination with mycophenolate mofetil is a more potent suppressor of sCD30 than cyclosporine[14,17]. The exact mechanism of sCD30

suppression is unknown but clinical studies showed less acute rejections and a trend to a lower incidence of BOS in a tacrolimus based regimen compared to cyclosporine's and less malignancies were observed in mycophenolate mofetil compared to azathioprine[18,19]. During the development of BOS Fields and colleagues reported a 10 fold rise in sCD30 levels, absent in the matched non-BOS patients and therefore concluded that sCD30 could be used as a biomarker for the development of BOS[15]. They also observed development of HLA antibodies in 35% of the patients. However, after altering their standard immune suppression to a regimen with tacrolimus, mycophenolate mofetil and prednisone in patients developing BOS, they noticed a decrease in sCD30 levels. In our study patients with or without developing BOS, had comparable post transplant sCD30 levels and we could not reproduce high levels of sCD30 during the development of BOS. Furthermore, post transplant anti-HLA antibodies could not be detected. However, we observed a significant decline in sCD30 levels after transplantation in patients with high pre transplant sCD30 levels. These data suggest that both sCD30 and the production of anti-HLA antibodies are effectively suppressed by, our standard immune suppression consisting of tacrolimus and mycophenolate. Therefore, post transplant sCD30 measurements are unable to identify patients developing BOS under the immunosuppressive regimen currently used.

After lung transplantation BOS is not prevented in a tacrolimus/ mycophenolate mofetil based immune suppressive regimen and is not heralded by an increase in sCD30. Despite low post transplant levels of sCD30, BOS still develops and therefore we presume that sCD30 is an epiphenomenon and does not have a causative relationship with the development of BOS.

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Chapter 3

Serum TARC levels post lung transplantation as a predictor for the Bronchiolitis Obliterans Syndrome

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Abstract

The main reason for mortality after lung transplantation is the bronchiolitis obliterans syndrome, which represents chronic rejection. As soluble CD30 which is mainly produced by activated Th2 cells, was shown to be related to development of BOS, we aimed to investigate the relation between development of BOS and Th2 chemoattractant thymus and activation regulated chemokine (TARC/CCL17). In 54 patients we measured serum TARC levels prior to transplantation by ELISA and in 44 of them, sera were analyzed at month 1, 2 and 3 after LTx. In addition, longitudinal measurements were performed in sera from 8 healthy controls and 14 patients; the latter taken over a period of 2 years post transplantation from 7 patients developing BOS plus 7 clinically matched BOS-free patients. Median serum TARC levels post transplantation of patients who developed BOS were significantly lower than those of the matched BOS-free patients (p=0.05). A ROC analysis (AUC 0.77) together with a Kaplan-Meier analysis showed that serum TARC levels below 325 pg/ml in the first month post transplantation can predict development of BOS post transplantation (p=0.001). In contrast, pre transplant serum TARC levels were not significantly different between patients developing BOS, BOS-free patients or healthy controls. In conclusion, pre transplantation serum TARC levels do not predict the development of BOS post transplantation but measurement of the serum TARC levels in the first month directly after transplantation can provide us with a tool to identify the group at risk of developing BOS.

INTRODUCTION

Lung transplantation (LTx) is the final treatment option in end stage lung disease. The proportion of patients living 5 years after LTx is limited to approximately 50% and the main cause of long-term morbidity and mortality is the bronchiolitis obliterans syndrome (BOS), which represents chronic lung allograft rejection[1-3]. Data have shown that 58% of the recipients are diagnosed with BOS within 5 years post LTx with a median of diagnosis between 16-20 months, and it is generally considered that most recipients that survive operative and infectious complications will ultimately develop BOS[4,5].

Due to airflow obstruction and decline of graft function, BOS manifests as the development in a progressive deterioration in forced expiratory volume in 1s (FEV1) and it can be diagnosed by the definitive decline of 20% in FEV1 of the baseline value with no indication for other complications including infections, AR, and suture problems among others[6-8]. Although the pathogenesis of BOS is unclear, the disease has a patchy character of fibroproliferation and obliteration of the small airways[9]. Several risk factors are identified including acute rejection, primary graft dysfunction, ischeamic time of the graft during transplantation, viral infections like CMV, gastro oesophageal reflux disease (GERD) and HLA mismatches[3,10-13]. None of these factors however can be used as clinical markers for the early onset of the disease.

High sCD30 levels prior to LTx were also identified as a risk factor for the development of BOS[14-16]. CD30 is expressed on the surface of Th2 cells and secreted in the bloodstream as a soluble form (sCD30) upon activation[17]. The relation between sCD30 and BOS led us to speculate that chemokines involved in recruitment of Th2 cells might also be associated with the development of BOS. The thymus and activation regulated chemokine (TARC/CCL17) can act as a chemoattractant for T helper cells type 2 by binding to the chemokine receptor CCR4 on the surface of these cells[18]. TARC induces recruitment and migration of Th2 cells[18,19]. The chemokine is expressed by various cells including endothelial cells, dendritic cells, epidermal keratinocytes, fibroblasts, platelets and activated

bronchial epithelial cells and can be up regulated by pro-inflammatory cytokines like TNF-α, IL-1 and IFN-γ[20-23].

The objective of this study was to investigate whether TARC levels prior to and post lung transplantation can predict the onset and development of the bronchiolitis obliterans syndrome.

Material and methods

Patients

A total of 57 patients (M/F 28/29, average age 46 years, range 18-61) who underwent lung transplantation at the Heart Lung Center in Utrecht, The Netherlands, between October 2001 and July 2007 and survived more than three months were included in this study.

BOS was defined as a decline of the FEV1 from the post-operative baseline at two distinctive time-points of more than 20% in the absence of infection or other etiology[7].

Standard immunosuppressive therapy consisted of basiliximab, tacrolimus, mycophenolate mofetil and prednisone for all patients. No surveillance bronchoscopies were performed. In patients who had a decline in lung function infections were diagnosed by cultures of BALF and PCR for CMV and EBV. When infections were excluded as the cause of FEV1 decline, the patients were treated with corticosteroids and azithromycine. When no increase in long function was observed the diagnosis BOS was made.

Patient follow up started in September 2004, after approval by the medical-ethical committee and informed consent was obtained from each patient. Forty-four patients donated blood every month in the first year post transplantation and once every three months in the following years. Sera stored for diagnostic purposes from 13 other patients were also included in this study, although they were either transplanted before this date or the serum sampling was not performed systematically as described above. From 54 out of 57 patients pre-transplant serum was present and from 44 out 57 patients sera were available taken monthly after transplantation up to month 3. TARC levels were determined in these sera and also in sera collected

longitudinally up to 25 months post transplantation in a group of 14 patients consisting of 7 patients who developed BOS, which could be closely matched for gender, age, primary disease and follow up to 7 patients who did not develop BOS. Three patients that developed BOS were not included in this longitudinal analysis; due to lack of follow up time, lack of available serum samples or no clinical match to a non BOS patient.

Eight healthy (M/F=5/3, mean age 35 years (range 26-46)) non allergic and non smoking controls donated blood every two weeks for six months and once five years later. In total, 442 samples were measured for serum TARC levels.

ELISA

Serum TARC levels were measured in duplo as described before[24] 96-well ELISA-plates (Becton Dickinson, Franklin Lakes, NJ) were coated with a murine monoclonal capturing antibody directed against anti-human TARC (MAB364, R&D Systems, Abingdon, United Kingdom). Human serum diluted (1/2) was added and standard concentrations (range: 4000 pg/ml – 16 pg/ml) were prepared with recombinant human TARC (364-DN, R7D Systems) in PBS containing 1% BSA. Goat polyclonal biotinylated anti-human TARC antibody (BAF364, R&D Systems) was used as detecting antibody. HRP-Streptavidin Conjugate (Zymed, San Fransisco, CA) and substrate (TMB substrate, Pierce, Rockford, IL) were used according the manual of the manufacturer. Optical densities were measured at 450 nm with a Thermo labsystems Multiskan RC plate reader. The minimal concentration of TARC that could be detected was 16 pg/ml.

Statistical analysis

To compare the healthy controls with the group of patients for the data prior to transplantation the Mann-Whitney rank-sum test was used. In order to evaluate the median of the non BOS versus BOS group post transplantation, or the patients between before and after transplantation the Wilcoxon signed rank test was performed. To asses whether serum TARC levels post transplantation can serve as a BOS predicting factor a receiver operating curve and Kaplan-Meier curve with a Logrank test were used.

Results

In order to study the relation between serum TARC levels in LTx patients and the development of BOS, 57 patients and 8 healthy controls were included in this study. The median follow up time of the patients after transplantation was 11 months with a range from 4 till 75 months. Ten patients (19%) developed BOS and five patients died during the course of the study, three of which were associated

Table 1. Characteristics of study group

	Matched patient Group		Other LTx Patients	
	BOS	non BOS	BOS	non BOS
Total number	7	7	3	40
BOS grade I	0	N.A.	0	N.A.
II	3	N.A.	0	N.A.
III	4	N.A.	3	N.A.
Mean follow-up	38 (69-9)	39 (65-32)	21 (17-33)	17 (76-6)
Mean age	51 (24-61)	50 (22-61)	39 (23-58)	41 (17-64)
Primary disease	1 (14%)	1 (14%)	2 (67%)	16 (40%)
CF	4 (57%)	4 (57%)	0 (0%)	14 (35%)
Emphysema	2 (29%)	2 (29%)	1 (33%)	10 (25%)
Fibrotic disease				
Infections				
CMV	0 (0%)	1 (14%)	0 (0%)	2 (5%)
EBV	0 (0%)	0 (0%)	0 (0%)	1 (2.5%)
Pseudomonas	3 (42%)	1 (14%)	2 (67%)	18 (45%)

Characteristics of 14 matched patients for longitudinal study, and the 43 other LTx patients included in this study. A division is made between the patients that developed BOS and the patients that have not developed BOS. 3 Patients had a CMV infection; 2 patients were CMV negative and received lungs from a CMV positive donor, 1 CMV positive patient received lungs from a CMV negative donor. One patient had a EBV reactivation.

with BOS. The median age of the patient population was 50 years (range 17-64 years), their gender was equally divided (M/F=27/27) and 35%, 36% and 29% suffered from cystic fibrosis, emphysema or fibrotic diseases (fibrosis, sarcoidosis and connective tissue diseases), respectively.

Pre transplant serum TARC and BOS

Analysis of TARC concentrations showed no difference between amounts of TARC present in serum taken prior to transplantation (605 pg/ml ± 380) compared to those in healthy controls (685 pg/ml ± 430). No associations were found between serum TARC concentrations, prior or post transplantation, and age, gender or primary disease. Furthermore, the 10 patients eventually developing BOS had the same amounts of pre-transplant serum TARC levels as those not developing BOS. These data indicate that pre-transplant serum TARC levels were not associated with any of the clinical parameters investigated.

Effect of transplantation and immune suppression on serum TARC levels

To determine whether the transplantation procedure in combination with immunosuppressive therapy had an effect on the serum TARC levels of LTx patients,

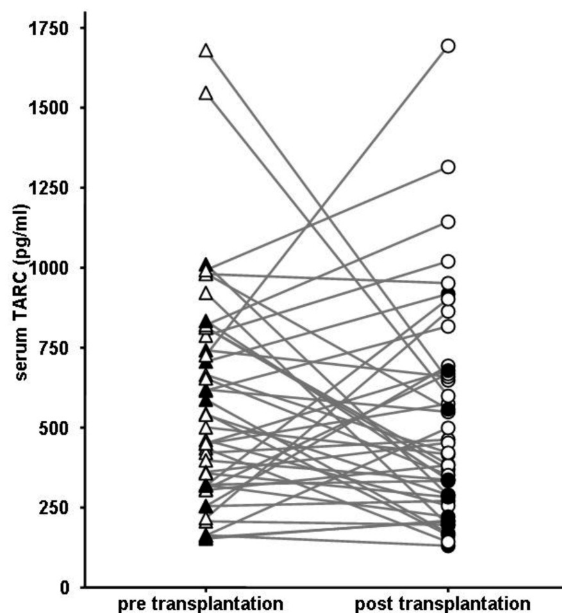

Figure 1.
The transplantation procedure does not influence serum TARC levels. Serum TARC levels were measured through ELISA for 44 LTx patients pre transplantation (triangles) and 1 month post transplantation (cirkels). 10 Patients that eventually developed BOS are indicated with the filled triangles and cirkels.

44 patients were selected in whom serum TARC was measured prior to and 1 month post transplantation. As shown in Figure 1, serum TARC levels decreased in 15 patients; in 14 patients it remained constant whereas in 15 patients an increase was found in serum TARC levels. Overall, no significant difference was found between pre and 1 month post transplantation TARC levels. The patients that developed BOS are marked by the closed symbols. For this group also no differences were found prior to and 1 month post transplantation as 5 patients had a decrease, 2 patients remained and 3 patients had an increase in serum TARC levels. Apparently, the transplantation procedure did not have an effect on serum TARC levels.

14 Patients were followed over time and donated blood once every month in the first year and once every three months in the following years post transplantation. The median follow up time was 40.5 months (range 9-74 months).

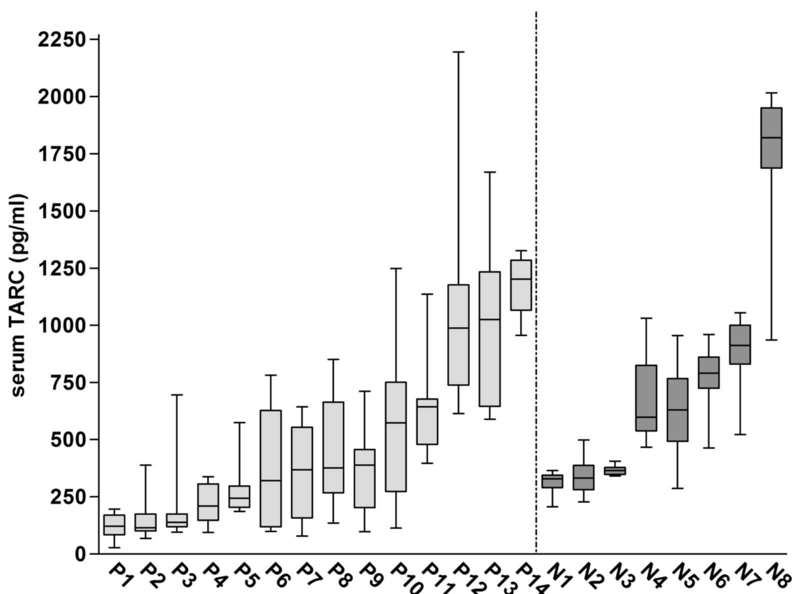

Figure 2.

The serum TARC levels of LTx patients do not differ from those of healthy controls over time. Longitudinally, average of 13 (9-17) measurements, ELISA for serum TARC determined the bandwidth for 22 persons. Patients (P1 to P14) and healthy controls (N1 to N8; non smoking non allergic) were ordered by increasing median of serum TARC.

Seven patients who developed BOS and could be followed longitudinally were closely matched for underlying disease (CF 14%, Fibrotic disease 29% and emphysema 57%), age (median age 49, range 22-61), gender (Male 29%) and follow up time to 7 BOS-free patients.

Serum TARC levels were determined up to 25 months post transplantation and for comparison, levels were also measured in 8 non allergic non smoking healthy controls every two weeks for six months and once five years later. This resulted in an average of 13 (9-17) measurements per individual, providing a band width of serum TARC concentration from 22 persons which are depicted in Figure 2. The values of serum TARC levels post transplantation in patients (P1 to P14) are in the same range of the healthy controls (N1 to N8), indicating that the transplantation plus immune suppression employed did not cause the patient's serum TARC levels to differ from that in healthy individuals.

Post transplant serum TARC and BOS

We next examined whether the course of serum TARC levels is associated with development of BOS, CMV or EBV reactivation or colonization with Pseudomonas. No change was found between the serum TARC levels post transplantation prior, during or after onset of BOS or CMV reactivation or Pseudomonas colonization, in the group of 14 patients followed longitudinally. A relation with EBV appearance could not be investigated as none of the 14 patients experienced a primary EBV infection or reactivation (data not shown). Patients experiencing a decline in FEV1, which were treated with corticosteroids and azithromycine, showed no alteration in serum TARC levels. Also no difference in TARC levels could be found between the 3 patients that developed BOS grade II versus the 4 patients that developed BOS grade III.

To study whether there was a difference in serum TARC levels post transplantation between the group of 7 patients who did develop BOS versus those 7 who did not develop BOS, the median of all measurements per person post transplantation was calculated and the result of the matched patient pairs is displayed in Figure 3. As shown, for 6 out of 7 fully matched patient pairs, the median serum TARC levels of the patients who developed BOS were lower compared to the serum TARC levels of the patients who did not develop BOS.

Statistical analysis indicated that median levels of TARC were significantly lower in the patients eventually developing BOS compared to the BOS-free patients (p=0.05, Wilcoxon rank sum test), indicating that low levels of TARC post transplant are a risk factor for BOS.

Serum TARC level as a BOS predicting factor

A receiver operating characteristics (ROC) curve was used to asses the possibility of predicting BOS by serum TARC levels prior to or in the first 3 months post transplantation. Measurements of serum TARC at the fixed time points month 0, 1, 2 or 3 of 44 patients that survived at least 6 months post transplantation were included.

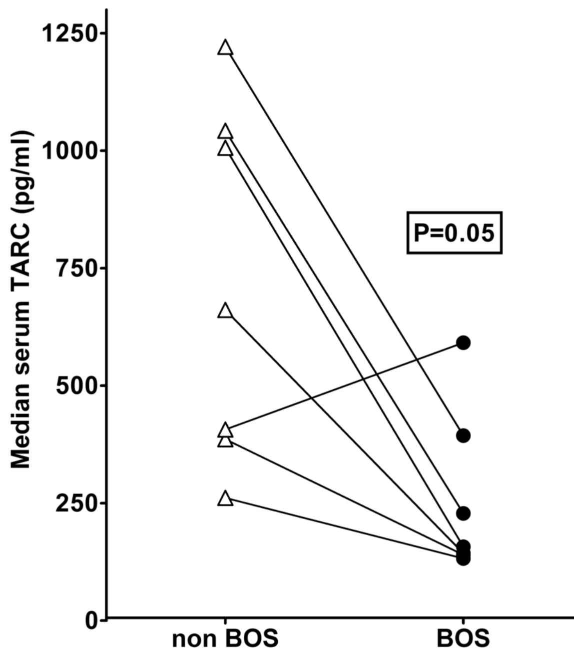

Figure 3.

Median serum TARC level is higher post transplantation in patients without BOS (open triangle) compared to patients that developed BOS (closed round). Patient pairs are connected. 14 Patients, that were closely matched, were followed longitudinally post transplantation. An average of 13 samples per patient was used to calculate the median serum TARC levels. 6 Out of 7 stable LTx patients have a higher median TARC post transplantation than their matched patient with BOS.

Prior to tranplanstation the ROC curve showed an AUC of 0.53, with a cut-off value of 590 pg/ml. This however did not result in a significant difference between the high or low serum TARC group prior to transplantation, neither did any other cut-off value. (figure 4a)

For the 1 month post transplantation time point the curve resulted in an AUC of 0.77 (0.59-0.94), which indicates a good predicting factor for the development of BOS within 5 years post transplantation. The cut-off value defining whether a patient is at risk of developing BOS in the first years post transplantation with highest specificity as well as sensitivity was found at approximately 325 pg/ml serum (range 290- 326 pg/ml) TARC showing a specificity of 71% and a sensitivity of 80%.

This value was used for a Kaplan Meyer analysis, which is shown in figure 4b. The difference between the group with high serum TARC level in the first month post transplantation from those with low serum TARC levels at this time point was significant. ($p=0.001$, Logrank test) The group with low serum TARC levels in the first month post transplantation show a higher incidence of developing BOS within the first five years post transplantation. However analysis at the time points 2 or 3 months post transplantation did not reveal significant differences between the high and low serum TARC groups. Therefore low serum TARC level, <325 pg/ml, in the first month post transplantation is a risk factor for developing BOS.

Discussion

As patients with BOS generally respond poorly to augmented immunosuppressive therapy, a need for markers that predict the decline in graft performance is clearly present, allowing development of a strategy for treatment of patients at risk before onset of BOS. The object of this study was to investigate whether serum TARC levels are associated with the onset and development of BOS. This is the first study showing that measurement of serum TARC levels after lung transplantation has a predictive value for the development of BOS.

Although the immunosuppressive regimen consisting of tacrolimus and mycophenolate mofetil used in this study is known to suppress cellular (allo) immune responses efficiently, their influence on TARC production is not well

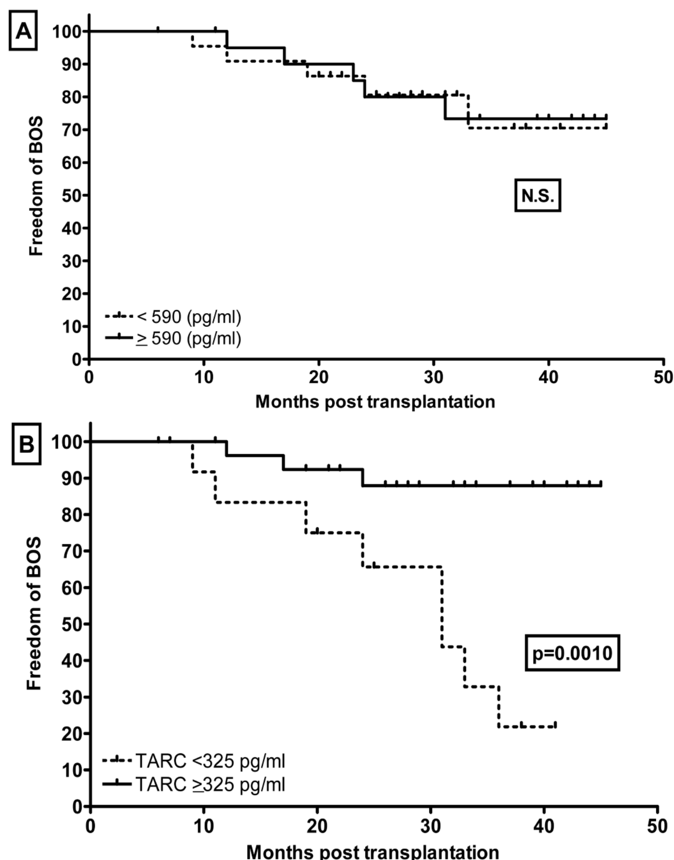

Figure 4.
TARC is a predicting factor for development of BOS. (a) Prior to transplantation no significant differences can be seen with regard to freedom from BOS between, respectively, the 21 vs 23 patients with serum TARC levels above vs below 590 pg/ml. (b) The 30 patients with serum TARC levels above 325pg/ml in the first month post transplantation show a significant higher freedom of BOS (P-0.001) compared to the 14 patients with serum TARC levels below this concentration.

known. In studies with AD and allergic asthma patients it was shown that TARC protein and mRNA levels decreased upon treatment with either cyclosporin A, tacrolimus and dexamethasone, or in combination[24-27]. It is unknown however, whether this decrease in TARC levels was due to a direct effect on TARC production or an indirect effect caused by diminishment of disease activity. Furthermore, TARC can be produced by endothelial cells, dendritic cells, fibroblasts, epidermal keratinocytes and activated bronchial epithelial cells, all which can be differentially affected by immunosuppressives. The main source of TARC in atopic dermatitis seems to be keratinocytes in skin lesions whereas in allergic asthma it appears to be mainly produced by lung macrophages, indicating that the source of circulating TARC could be actually dependent on clinical conditions.

In our study we did not see a difference in serum TARC levels measured in a period without or with immune suppression c.q. prior versus 1 month post transplantation. Moreover, the levels of serum TARC of LTx patients measured longitudinally after transplantation were comparable to those found in healthy controls. This is an unexpected finding, as up regulation shortly after organ transplantation has been shown for many other cyto- and chemokines including IP-10, MCP1, IL-1β, IL-2, IL-12p40, IL-15, IL-2R, IL-6, IL-8 and IL-1Rα, although IL10 was found to be decreased[28-31]. We assume that TARC production after transplantation is upregulated by vigorous allogeneic responses leading to production of known TARC-stimulatory cytokines like IL-1, INF-γ and TNFα[20-23] but inhibited by the immune suppression employed, resulting in serum levels similar to those found in healthy controls. The actual reason for the low serum TARC levels directly after transplantation in patients, who will eventually develop BOS, remains unknown. It has been suggested that pre-existing subclinical inflammation - with its associated chemokine production - present in the donor lungs prior to transplantation, is associated with graft dysfunction and poorer prognosis after transplantation[31]. Alternatively, lowered serum TARC levels also could be due to functional polymorphisms in the promoter region, such as found previously in Japanese individuals[32].

The relation found between low levels of circulating TARC and the development of BOS may be explained by its role as chemoattractant. Recently, it was shown that a subpopulation of Th2 cells expressing CCR4, the receptor for TARC, is characterized by CD4+CD25+, Treg cells and it was postulated that antigen presenting cells in the lungs and activated bronchial epithelium cells can recruit Treg cells towards a site of inflammation through the secretion of TARC[33]. Recruitment of Tregs down regulates inflammatory responses limits tissue damage or autoimmunity. A lowered local production of TARC in the lung after transplantation might lead, according to the model described above, to an insufficient recruitment of Treg cells to the sites of ongoing inflammation, which would result in a deficient clearing of the chronic inflammatory responses in BOS. The role of Tregs in allograft rejection was also supported in a mice model using cardiac allografts. In this model, up regulation of Foxp3 expression was shown in the allografts displaying donor specific tolerance combined with recruitment of

Tregs to the allografts through action of CCR4 and its ligands[34]. Interestingly, both the Th2 cytokine IL-10 known to suppress inflammatory responses and IL-12 were also found to be decreased in the broncho-alveolar lavage of patients with BOS[30,35]. This Treg-hypothesis may not seem to fit with published data showing up regulation of sCD30 prior to BOS[14], However, in our patient cohort we were not able to reproduce this finding and found instead unaltered sCD30 levels prior to BOS under the current immune suppressive regiment.[36] Moreover, shedding of CD30 from Tregs resulting in increased serum sCD30 levels has not been reported yet.

As TARC is a small molecule of 10.5kD and leaks to the circulation without restriction, it can be expected that serum levels measured after lung transplantation reflect quantities locally produced in the lung e.g. by mature dendritic cells, monocytes and activated macrophages. This notion is supported by a recent study, showing that TARC levels in serum correlate well with those in broncho-alveolar lavage in acute eosinophilic pneumonia[37]. A small-scale study showing up regulation of CCL 19, CCL20 and CCL22 in patients developing BOS did not show a indication TARC levels in BAL predictive for BOS at month 3 and 6 after transplantation[38]. These data are in line with our results showing no predictive value for serum TARC levels 3 months after transplantation. We conclude that median serum TARC levels post transplantation in LTx patients without BOS is significantly higher that in those who developed BOS within 5 years after transplantation and that low serum TARC levels in the first month after lung transplantation is a predicting factor for the development of BOS. These data need to be confirmed in a larger cohort of patients, and the cut-off value of 325 pg/ml with a range of 290-326 pg/ml should be set more precisely in such a study.

Measurement of serum TARC levels in combination with other known risk factors may allow identification LTx patients at risk for development of BOS.

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Chapter 4

Differential usefulness of biomarkers thymus and activation-regulated chemokine and soluble CD30 during enteric coated mycophenolate sodium and cyclosporine therapy in atopic dermatitis

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Abstract

Background

Clinical scoring systems evaluating disease activity in patients with atopic dermatitis (AD) are hampered by observer differences.

Objective

We evaluated whether the potential serum biomarkers soluble CD30 (sCD30) and Thymus and Activation regulated Chemokine (TARC) can be used as objective disease severity parameters in AD patients during different treatment modalities.

Methods

We measured TARC and sCD30 by enzyme-linked immunosorbent assay in 45 patients. Patients were treated with cyclosporin 5 mg/kg/day (CsA 5) and after 6 weeks of therapy, 23 patients were randomly allocated to cyclosporine 3 mg/kg/day (CsA 3) and 22 patients to enteric coated mycophenolate sodium (EC-MPS) 720 mg twice a day for a period of 30 weeks. Patients were followed up for a period of 36 weeks.

Results

During CsA 5 treatment both sCD30 and TARC levels decreased significantly ($p<0.001$) and increased slightly during CsA 3 or EC-MPS treatment. Serum levels of TARC showed a clear correlation with SCORAD severity scores during CsA 5, CsA 3 and EC-MPS therapy ($r=0.51$, 0.45 and 0.53 respectively). sCD30 levels also showed a clear correlation with SCORAD severity scores during CsA 3 treatment ($r=0.42$). However, there was no correlation between SCORAD and sCD30 during EC-MPS ($r=0.08$).

Limitations

limited number of patients was a limitation

Conclusion

These data show that sCD30 is not suitable as a biomarker to evaluate disease severity in patients with AD during treatment with EC-MPS, whereas TARC can be used during both cyclosporin and EC-MPS therapy.

Introduction

Atopic dermatitis (AD) is a chronic pruritic, erythematous skin disease with a fluctuating course. At the induction of skin inflammation, antigen-presenting Langerhans cells migrate into the lymph nodes and encounter naive T-cells which are activated and transformed preferentially into TH2 cells expressing the homing cutaneous lymphocyte antigen (CLA). Migration and infiltration of the CLA^+ T-cells into the skin is mediated by CLA-expression itself and chemoattractants, including Thymus and Activation Regulated Chemokine (TARC/CCL17) and cutaneous T cell attracking chemokine (CTACK). The chemokine TARC binds to CCR4 expressed on TH2 cells. CCR4 can also bind the ligand Macrophage Derived Chemokine (MDC/CCL22). Upon infiltration in the skin, the CLA^+ TH2 cells mainly produce cytokines like IL-4, IL-5 and IL-13 by which they orchestrate the local inflammatory response.

Disease activity in AD patients is evaluated using the SCORing Atopic Dermatitis score (SCORAD) or Leicester Sign Score (LSS)[1,2]. Although these scoring systems are at present the gold standard for evaluating systemic therapy, they are intrinsically hampered by inter and intra observer differences. Therefore, biomarkers which predict the course of disease or that monitor disease activity during treatment are clearly needed. A number of objective markers correlating with disease activity in AD patients have been identified. Neurotrophins such as brain-derived neutrotrophic factor (BDNF) and nerve growth factor (NGF) have been associated with AD[3-6]. However, in other studies nor the increased NGF levels in AD patients, nor the correlation between disease activity and NGF levels could be reproduced[4,7]. Also no differences in nucleotide variation could be found between neutrophin genes encoding BDNF or NGF in AD patients versus non-atopic healthy individuals[8].Other markers which have been evaluated are chemokines that attract TH2 cells such as TARC/CCL17, MDC/CCL22 and CTACK/CCL27[9-11]. The serum levels of these chemokines correlate with disease activity in AD. Activated TH2 cells also express CD30, which is a member of the tumor necrosis factor family. Serum levels of soluble CD30 (sCD30) were shown to be related to serum levels of TARC in patients with AD and also to disease severity. In addition, treatment of AD patients with CsA induced a decrease of both serum

sCD30 and TARC levels. Together, these data suggest that sCD30 may be applicable as a biomarker in AD[12]. However, objective biomarkers can only substitute disease severity scoring systems if they have the capacity to monitor disease activity before, during and after treatment and are reproducible. Thus far only sCD30 and TARC have been evaluated in AD patients during systemic therapy[9,13]. The aim of this study was to evaluate sCD30 and TARC as potential biomarkers for disease activity in AD patients during different treatment modalities including cyclosporin and enteric coated mycophenolate sodium (EC-MPS).

Methods

Subjects

Forty six patients diagnosed with Atopic Dermatitis (AD) according to the criteria of Hanifin and Rajka., were included in this study[14]. One patient who developed HIV infection possibly during the study, was excluded since it has been reported that HIV infections are associated with increased sCD30 levels[15,16]. Patients were treated with cyclosporin 5 mg/kg/day for 6 weeks and thereafter they were randomly allocated to cyclosporine 3 mg/kg/day or to EC-MPS 720 mg twice a day for another 30 weeks. One independent observer who was blinded for the treatment allocation evaluated disease activity of AD by using the objective scoring atopic dermatitis score (SCORAD, range 0-67.5) at each visit[17]. Serum was collected prior to treatment and after 3, 6, 9, 12 and every four weeks thereafter with the exception of two serum samples at time point 36. The medical ethical committee of the University Medical Center Utrecht approved the study described and the study was conducted according to the Declaration of Helsinki Principles.

ELISA

Serum TARC and sCD30 levels were measured using an ELISA kit, employed according to the manufacturer's guidelines (TARC: R&D Systems, Minneapolis, Minn) (sCD30: Bender Med Systems, Vienna, Austria). All samples were tested in duplicate and mean values were analysed.

Statistical analysis

The effect of CsA 5 on sCD30 and TARC was assessed using a Wilcoxon signed rank test comparing baseline values with the values at 6 weeks. To show their (dis)agreement, we made graphs showing the trend over time of TARC, sCD30 and SCORAD during CsA 3 treatment and during EC-MPS treatment. Spearman correlation coefficients between SCORAD and TARC, SCORAD and sCD30, and TARC and sCD30 were calculated for each time point during the different treatments (CsA 5, CsA 3, and EC-MPS). The mean, minimum and maximum correlation coefficient over the time points was presented. All analyses were performed using SPSS version 15.1.

Results

During EC-MPS and cyclosporin 3 and 5 mg/kg/day treatment, SCORAD severity scores were lower than that on admission (data not shown). No large differences were present at inclusion between the groups treated with cyclosporin 3 mg/kg/day

Table 1.

Baseline Characteristics and Clinical Parameter at Admission

	All patients N=45	CsA 3 N=23	EC-MPS N=22
Age, mean (SD), y	35.6 (11.7)	34.7 (13.1)	36.5 (10.3)
Female	18 (40%)	8 (35 %)	10 (46 %)
SCORAD, mean (SD)	42.8 (10.3)	42.8 (11.5)	42.9 (9.2)
sCD30 (U/ml), median (interquartile range)	49 (31-71)	52 (32-69)	46 (29-90)
TARC (pg/ml), median (interquartile range)	1489 (818-3323)	1779 (831-4668)	1265 (793-3028)

CsA 3= cyclosporin A 3 mg/kg/day, EC-MPS= mycophenolate sodium, SCORAD = Scoring Atopic Dermatitis, TARC= thymus and activation-regulated chemokine

Notes: Levels of sCD30 and TARC were measured en block at one time moment in the laboratory.

and enteric coated mycophenolate sodium regarding age, gender, sCD30 and TARC levels (see table 1). The clinical results of the study comparing CsA 3 with EC-MPS will be published separately.

To examine whether treatment decreases sCD30 levels, sCD30 concentrations were measured at admission, after 3 and 6 weeks of treatment with cyclosporin 5mg/kg/day and at multiple time points during treatment with cyclosporin 3 mg/kg/day or EC-MPS. During cyclosporin 5 mg/kg/day a significant decline in sCD30 levels was seen (see figure 1A and 1B). When after 6 weeks therapy was

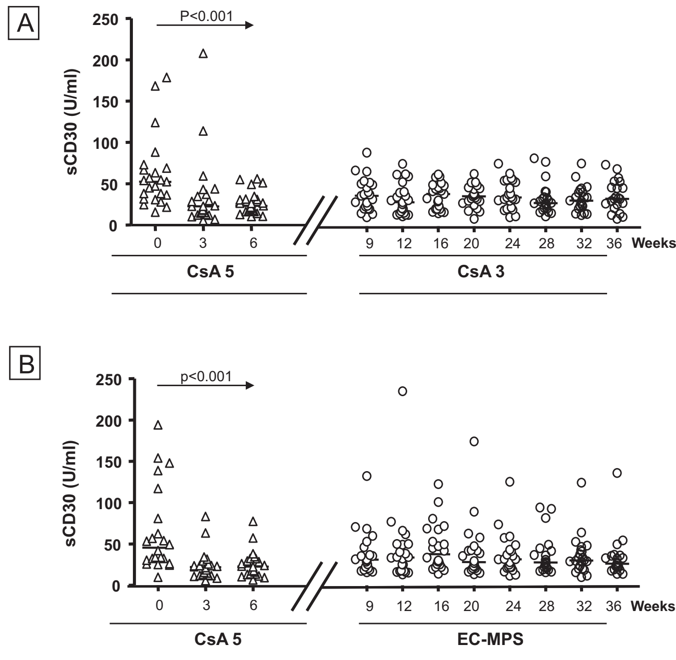

Figure 1. Soluble CD30 levels decrease in a cyclosporin based treatment and this decrease persists after switching to a lower dose of cyclosporin or to enteric coated mycophenolate sodium.
In 45 AD patients sCD30 levels were measured before and during treatment with cyclosporine 5 mg/kg/day (CsA 5) and after switching to cyclosporine 3 mg/kg/day (CsA 3) (A) or enteric coated mycophenolate sodium (EC-MPS) (B). Every measurement is depicted as an open triangle (CsA 5) or dot (CsA 3 or EC-MPS). Means are indicated with horizontal bars. Please note that sCD30 levels are depicted in a linear scale.

switched to cyclosporine 3 mg/kg/day (figure 1A) or to EC-MPS (figure 1B), sCD30 levels showed first a small increase (rebound effect) and were after this rebound effect quite constant over time. The highest sCD30 levels observed during EC-MPS treatment (see figure 1B) belonged to one patient who did not use any co-medication but, despite detectable mycophenolic acid (MPA) blood levels, had severe and therapy resistant atopic dermatitis without symptoms or side-effects of concurrent disease like tuberculosis, morbus Wegener or rheumatic diseases which can increase sCD30 levels[18-20].

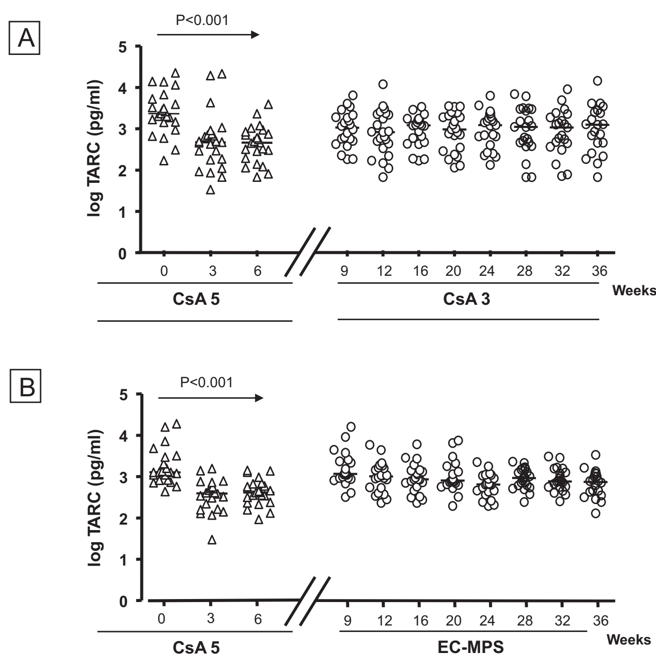

Figure 2. TARC levels decrease in a cyclosporin based treatment and this decrease persists after switching to a lower dose of cyclosporin or to enteric coated mycophenolate sodium.

In 45 AD patients sCD30 levels were measured before and during treatment with cyclosporine 5 mg/kg/day (CsA 5) and after switching to cyclosporine 3 mg/kg/day (CsA 3) (A) or enteric coated mycophenolate natrium EC-MPS (B). Every measurement is depicted as an open triangle (CsA 5) or dot (CsA 3 or EC-MPS). Means are indicated with horizontal bars. Please note that TARC levels are depicted in a logarithmic scale.

The same serum samples were analyzed for TARC levels to determine if TARC levels decrease during treatment with cyclosporin 5 mg/kg/day, and if this decrease would maintain after switching therapy to either cyclosporin 3 mg/kg/day or to EC-MPS. The 23 patients initially treated with cyclosporin 5 mg/kg/day and after 6 weeks switched to a dose of 3 mg/kg/day (figure 2A) showed a significant decline in TARC concentrations during CsA 5 therapy ($p<0.001$). After reducing the dose of cyclosporin, a small increase in TARC levels was observed and after this rebound effect, TARC levels were fairly constant over time (figure 2A). The 22 patients

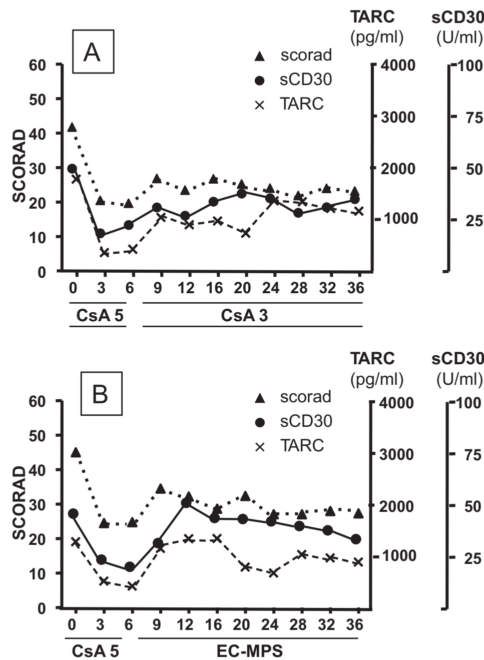

Figure 3. SCORAD, TARC and sCD30 levels decrease during cyclosporin treatment and rebound after switching to a lower dose of cyclosporin or to enteric coated mycophenolate sodium.

In 45 patients SCORAD, TARC and sCD30 levels were measured before and during treatment with cyclosporine 5 mg/kg/day (CsA 5) and after switching to cyclosporine 3 mg/kg/day (CsA 3) (A) or enteric coated mycophenolate natrium EC-MPS (B) as described in methods. During EC-MPS therapy, the rebound of sCD30 levels was pronounced compared to SCORAD and TARc levels, whereas after switching to CsA3 therapy the rebound effects were moderate compared to CsA5 therapy.

initially treated with CsA 5 and then switched to EC-MPS (figure 2B) also showed a significant decrease in TARC levels during CsA5 treatment ($p<0.001$).
Before conducting the analysis to compare the potential biomarkers with the disease severity parameter, we focused on the tendency of sCD30 and TARC levels to follow SCORAD in different treatment modalities. Patients initially treated with CsA5 and then switched to CsA3, showed a minor increase in SCORAD, TARC and sCD30 levels during the first 3 weeks after the switch of treatment (figure 3A). After this rebound effect, SCORAD, TARC and sCD30 levels remained quite constant in time. Also the group of patients initially treated with CsA5 and then switched to EC-MPS (figure 3B), showed a rebound effect of SCORAD, TARC and sCD30 levels which lasted until week 12.

In order to examine whether TARC and sCD30 can be used as biomarkers for disease severity, their serum levels were compared with SCORAD results during different treatment modalities. Serum levels of TARC were correlated to SCORAD severity scores during CsA 5, CsA 3 and EC-MPS therapy (see table 2). Also sCD30 levels were correlated with SCORAD severity scores during CsA 3, but the correlation was lower during CsA 5 and during EC-MPS treatment (see table 2). Excluding the patient with high sCD30 values mentioned in figure 1, lowered the correlation coefficient for EC-MPS even more.

Table 2. Correlation coefficients of potential biomarkers

Correlation coefficients	CsA5 (N = 45)*	CsA3 (N = 23)	EC-MPS (N = 22)
SCORAD-sCD30, mean (range)	0.21 (0.13–0.28)	0.42 (0.22-0.66)	0.20 (0.07–0.37)
SCORAD-TARC, mean (range)	0.51 (0.51–0.51)	0.45 (0.14-0.78)	0.53 (0.34-0.83)
sCD30-TARC, mean (range)	0.49 (0.44–0.54)	0.44 (0.30-0.68)	0.08 (-0.32-0.63)

Notes: Levels of sCD30 and TARC were measured en bloc at one time moment in the laboratory. Spearman correlation coefficients were calculated for each time point during the different treatments. The correlation coefficient for CsA5 is based on two timepoints, whereas correlation coefficients for both CsA3 and EC-MPS are based on eight timepoints.
CsA3 = Cyclosporine A 3 mg/kg/day, CsA5 = cyclosporine A 5 mg/kg/day, EC-MPS = enteric coated mycophenolate sodium, SCORAD = SCORing Atopic Dermatitis (index), TARC = thymus and activation-regulated chemokine.
* All patients.

Discussion

Evaluating disease activity in AD patients using the SCORAD score is hindered by subjectivities and intra-observer variations in time. Therefore, an objective biomarker corresponding to disease severity and not influenced by different therapy modalities, is urgently needed. This study aimed to evaluate the applicability of potential biomarkers such as sCD30 and TARC in patients with AD during different drug treatments.

In the present study, we demonstrated that sCD30 levels correlate with SCORAD scores and that both sCD30 and TARC levels decrease during cyclosporin treatment. The finding that TARC levels correlate with disease severity during systemic treatment with cyclosporin is consistent with the study of Hijnen et al[9]. The strong correlation we found between sCD30 levels and both SCORAD and TARC levels, indicate also the potentiality of sCD30 as a biomarker for AD patients during treatment with CsA. However, during EC-MPS treatment, the correlation between disease severity and sCD30 levels was much lower than the correlation between disease severity and TARC levels. Also the correlation between the biomarkers sCD30 and TARC was absent during EC-MPS treatment while clearly present during CsA5 and CsA3 treatment. The weak correlation (r=0.21) we see between sCD30 levels and disease severity during CsA5 treatment (see table 2) is probably caused by the measurements at only two time points.

In organ transplantation medicine, sCD30 levels have been evaluated as a biomarker for graft survival. Low sCD30 levels before transplantation are correlated with better survival outcomes in heart, lung and kidney transplantation[21-23]. Moreover, after transplantation sCD30 has shown to be a marker for acute allograft rejection in kidney transplantation[24,25]. However these studies evaluated post transplant sCD30 levels for not longer than 19 and 30 days after transplantation. In lung transplantation, the usability of post transplant sCD30 as a biomarker for the development of chronic allograft rejection has shown to be dependent on the immunosuppressive regimen[26-28]. Fields et al. used an immunosuppressive regimen that consisted of cyclosporin, azathioprine and prednisone and they found that sCD30 levels were increased before the clinical diagnosis of bronchiolitis obliterans syndrome (BOS)[26]. However, in a previous study we established that

during a tacrolimus, mycophenolate mofetil and prednisone based regimen sCD30 levels could not predict the development of BOS after lung transplantation[28]. Since the development of BOS is associated with multiple factors, a possible explanation for the discrepancy of the usability of sCD30 levels as a biomarker for the development of BOS could be the effect of the transplantation procedure itself or the immunosuppressive regimen used. Although immune responses in organ transplants are directed against alloantigens, and in AD the response is characterized by a chronic inflammation similar to that seen in bronchial asthma, in both groups treatment modalities contain similar drugs although applied in different dosage. In this study, we demonstrated that sCD30 levels decrease during systemic therapy and that sCD30 levels correlate with disease severity during cyclosporin but not during EC-MPS treatment. This result may explain why the development of BOS is not always preceded by an increase of sCD30 levels.

Both cyclosporin and tacrolimus belong to the family of calcineurin inhibitors and each bind to a different group of immunophilins: cyclosporin binds the cytoplasmic protein cyclophilins and tacrolimus to the FK506-binding protein. The resulting complexes bind and inhibit the Ca^{2+}-activated serine/threonine phosphatase calcineurin, thereby preventing to activate nuclear factor of activated T-cells (NF-ATc). This leads to a reduced transcriptional activation of early cytokines genes for IL-2, tumor necrosis factor alpha, IL-3, IL-4, CD40L, granulocyte-macrophage colony stimulating factor and interferon-gamma. Enteric-coated mycophenolate sodium is a cytotoxic drug and is metabolized to mycophenolic acid (MPA). MPA is an inhibitor of the enzyme inosine 5′-monophosphate dehydrogenase (IMPDH), which is involved in de novo synthesis of guanosine nucleotides. Blocking of IMPDH leads to depletion of guanosine and inhibits T- and B-cell proliferation. The finding that the correlation between clinical parameters evaluating disease activity and potential biomarker sCD30 is lost during MPS treatment may suggest that MPS influence shedding of CD30. Possibly the intracellular downstream cascade following MPS interacts with the metalloproteinase TNFα converting enzyme. This enzyme cleaves the extracellular domain of CD30 whereby a soluble form of CD30 is released in the blood stream[29].

In conclusion we demonstrated that sCD30 in contrast to serum TARC is not applicable as a biomarker to evaluate disease severity in patients with AD upon

treatment with EC-MPS. This study emphasizes the importance of evaluating the influence of systemic therapy on potential biomarkers.

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Chapter 5

Mannose-binding lectin deficiency linked to CMV reactivation and survival in lung transplantation

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Abstract

Introduction

Despite the use of immunosuppressives mainly influencing T- and B-cell responses, the prevalence of the bronchiolitis obliterans syndrome (BOS) after lung transplantation is high. Mannose-binding lectin (MBL) is a pattern recognition molecule of complement and an important component of the innate immunity. MBL is associated with rejection, infection and survival in other solid organ transplantations. In this study the relation between functional MBL levels and CMV reactivations, and the development of BOS and survival after lung transplantation was investigated.

Methods

MBL levels were measured in 85 patients before and in 57 of these patients after lung transplantation. The relation of MBL on survival, CMV reactivation and the development of BOS were investigated with Kaplan Meier (log rank) survival analysis.

Results

MBL levels decreased on average by 20% ($p<0.001$) after transplantation and eventually returned to pre transplant levels. Fourteen of the 85 patients had deficient pre transplant MBL levels and these patients had a tendency towards a better survival compared to those with normal MBL levels ($p=0.08$). Although no correlation was found between MBL deficiency and the development of BOS, more CMV reactivations occurred in recipients with deficient versus normal levels of MBL ($p=0.03$).

Conclusions

Our results suggest that MBL deficiency is associated with CMV reactivations and a longer overall survival but not with the development of BOS.

Introduction

Lung transplantation is an accepted therapy for end stage lung diseases. Although survival after lung transplantation has significantly improved in the last decades, a 5 years survival of approximately 50% remains poor[1]. This is mainly due to the development of the bronchiolitis obliterans syndrome (BOS) which is clinically diagnosed by a decrease in lung function[2]. BOS is acknowledged to be an immune driven response. Nevertheless, despite widely used immunosuppressives like calcineurin inhibitors, prednisone, mycophenolate acid derivates and mTOR inhibitors, all mainly influencing T- and B-cell responses, the occurrence of chronic rejection is high suggesting that the innate immune system may contribute at least in part to the development of BOS.

The complement system plays an important role in the innate immune system and can be activated by three different pathways: the classical, the alternative and the lectin pathway. The initiator of the lectin pathway is the mannose binding lectin (MBL) which binds to carbohydrate residues including mannose, fucose and N-acetylglucosamine on the surfaces of microorganisms such as viruses, bacteria and fungi. After binding to MBL, the MBL-associated serine protease (MASP-2) is activated and leads to cleavage of C4 and C2 followed by formation of C4b2a. The latter acts as a C3 convertase and further on in the cascade the microorganism is opsonized and phagocytosed. MBL deficiencies are associated with an enlarged vulnerability to bacterial infections as seen in allogeneic stem cell transplantation, after liver and simultaneous pancreas-kidney transplantation and in neutropenic patients after chemotherapy[3-6]. In addition, MBL deficiency is a risk factor for cytomegalovirus (CMV) infection after kidney transplantation[7,8].

Interestingly, MBL levels have been correlated with graft survival. In a follow up of more than 10 years, MBL deficiency was correlated with an improved graft survival in 266 kidney respectively 99 pancreas-kidney transplant recipients[9,10]. In heart transplantation MBL deficiency was associated with chronic rejection in a study following 38 heart transplant recipients but these results could not be confirmed in 90 heart transplant recipients[11,12]. Maybe the permissive cold ischemia time is of influence, which is much longer in kidney and pancreas-kidney transplantation compared to heart transplantation. MBL deficiency prevents

complement activation and it has been suggested that MBL deficiency may be beneficial in ischemic injury[13,14]. In lung transplantation an association was found between donor lungs with the MBL-encoding haplotype LXPA and BOS[15]. This haplotype is associated with MBL deficiency[16-18]. The influence of MBL genotype from donor lungs on the development of BOS, instead the MBL genotype of the recipients, is remarkable since MBL is mainly produced in the liver. This finding suggests that in lung transplant recipients, production of extrahepatic MBL production is relevant to bronchiolar damage after lung transplantation

The relation between circulating levels of MBL and clinical outcome after lung transplantation has not been studied yet. Therefore, the aim of our study was to examine the relation between functional MBL levels in recipients and the development of BOS and/or (re)occurrence of CMV-infection.

Materials and Methods

Subjects

Patients who underwent lung transplantation at the Heart Lung Center in Utrecht between September 2001 and November 2008 were included in this study. Since September 2003 all patients were included into a research protocol whereby sera and clinical information were collected regularly. Several hours prior to transplantation serum was collected and stored at -80°C. According to the standard ISHLT criteria BOS was defined as a decline of the FEV1 of more than 20% in the absence of infection or other etiology[2]. To avoid the bias of postoperative complications in the diagnosis of BOS, we excluded patients who died during the period from transplantation until three months after transplantation. The immunosuppressive regimen consisted of anti-CD25 induction, tacrolimus, mycophenolate mofetil and prednisone. Patients at risk for CMV reactivation, defined as a CMV seropositive donor or recipient, were treated with valganciclovir for 6 months post transplantation. The study was approved by the medical ethical committee and informed consent was obtained from each patient.

MBL analysis

Serum was collected prior to transplantation, stored at -80°C and at a later time point thawed for quantitative determination of functional MBL levels performed by a commercially available enzyme immunoassay (WieslabTM COMPL MP320, Land, Sweden)[19,20]. This assay evaluates all three pathways of complement activation. In brief for MBL, wells were precoated with mannan. Sera were diluted and pipetted in these wells..After washing, the wells were incubated with antibodies against C9 and further processed according the manufactures instructions. Absorbance values were read at 405 nm. The negative control is given the value of 0%, the positive control is given the value of 100%, and subsequently, the values of the sera were expressed as a percentage of the positive control. This assay was standardized and validated in sera from 120 healthy donors in 3 laboratories. In this standardization and validation study, the mean value of complement activity was calculated from these 120 donors. There is a large variation of MBL levels with a distribution skewed to the left. The threshold level for MBL deficiency was arbitrarily set at 10% of signal in the ELISA corresponding to MBL levels below 300 ng/ml.Samples were not frozen and thawed more than once. No MBL levels were measured in the donor.

CMV (re)activation

CMV serostatus of patient and donor were determined by enzyme-linked immunoassay (ELISA; VIDAS-biomerieux, Marcy L'Etoile, France). Post transplant monitoring for CMV (re)activation was performed by PCR. We used the PCR (Taqman) whereby a serological (re)activation was defined as a successive assay detecting more than 400 copies/ml in serum. After October 2003 we increased our volume and used 3 ml serum in stead of 1 ml and therefore our threshold for a successive assay could be lowered to 50 CMV copies/ml in stead of 400 copies/ml. Not all of our CMV reactivations did need treatment with valganciclovir. Patients with a clinical CMV (re)activation, defined as more than 1000 copies/ml were treated with valganciclovir and reducing immune suppression. Duration and intensity of valganciclovir depended on clinic and viral load. None of the MBL deficient patients were on immunosuppressives.

Statistical analysis

Analysis was based on the availability of sera. A power analysis was performed to detect the number of transplant procedures needed to be included to detect a difference in 5-year graft survival of 30% between MBL deficient recipients vs. MBL sufficient recipients. Based on literature about both CMV infections and MBL values in kidney transplantation and graft survival in lung transplantation, 5-year graft survival was estimated between 45-50%[1,22]. With a two-side risk of 5%, a power of 80% and the estimation that 33% of the population had low MBL values, we needed 82 transplant procedures. Kruskal-Wallis test was used to compare MBL levels between native lung diseases. In order to evaluate MBL levels before and after lung transplantation the Wilcoxon signed-rank test was performed. Post MBL values were compared by a multivariate analysis of covariance (ANCOVA), adjusted for gender, type of transplantation, underlying disease and the development of BOS. Statistical significance of MBL levels in relation to survival, CMV reactivation and the BOS-free period was analyzed with a log rank test in the Kaplan-Meier curve. The Fisher's exact test was used to compare frequencies. $P < 0.05$ was considered statistical significant.

Results

In the period from September 2001 to November 2008, 133 lung transplant procedures were performed. Thirty two patients were transplanted before September 2003 and from 13 of these patients' pre transplantation sera were available, no post transplantation sera. Since September 2003 101 patients were transplanted and in this group 17 patients died within 3 months after transplantation and 3 patients were transferred to other transplantation centers and therefore excluded. From 9 patients pre and post transplantation sera are missing. Six patients died before the second serum sample and from 6 patients post transplantation sera were missing. Sera from 85 patients were collected prior to transplantation from 72 patients we collected serum after transplantation and from 57 patients we have collected both prior and after transplantation sera. Twenty one (25%) of the 85 patients included in the study developed BOS during their follow-up. Two patients underwent a re-transplantation due to graft failure. The characteristics of this study cohort are shown in table 1.

Table 1. Characteristics of study group

	Pre transplantation N=85	MBL < 10% N=14	MBL ≥ 10% N=71
MBL values (in %)	95% CI (63.5-84.7)		
Mean follow up (in months)	55 (5-102)	55 (5-102)	51 (30-102)
Age (in years)	45 (16-64)	48 (17-62)	50 (16-64)
Gender m/f	42/43	8/6	34/37
Bilateral/unilateral	69/16	9/5	60/11
Native disease			
CF	23 (27 %)	4 (29%)	21 (30%)
Emphysema	38 (45 %)	9 (64%)	29 (40%)
Fibrotic disease	24 (28 %)	1 (7%)	21 (30%)
CMV IgG status			
Donor+/recipient-		1	19
Donor+/recipient+		3	7
Donor-/recipient+		8	16
Donor-/recipient-		2	17
Donor?/recipient +		-	12
Copies in CMV reactivation Mean (range)		2651 (range 50-2651)	2558 (range 0-10724)
HLA Class I mismatch		3.1 ± 0.8	3.2 ± 0.8
HLA Class II mismatch Mean (SD)		1.6 ± 0.5	1.7 ± 0.5
BOS	21	6	15

MBL: Mannose Binding Lectin; CF: Cystic Fibrosis; CMV: cytomegalovirus; IgG: immunoglobulin G; Donor?: means that the CMV status of the donor was not determined ; CMV reactivation: detecting more than 50 copies/ml in serum; HLA: Human Leucocyte Antigen; BOS: Bronchiolitis Obliterans Syndrome

MBL values before and after lung transplantation

Prior to lung transplantation 14 out of 85 patients had MBL values less than 10% (MBL deficient), corresponding with MBL levels below 300 ng/ml. No significant differences in recipients' age, HLA-mismatches, sputum cultures shortly before transplantation, CRP or gender distribution were observed between the two groups with sufficient and MBL deficient values. Of the 85 patients, 24 patients suffered from fibrotic disease with mean MBL values of 92.5% (95% CI 71.6- 113.4%), 38 from emphysema with mean MBL values of 63.5 % (95% CI 47.2-79.7%) and 23 from CF with mean MBL values of 73.8% (95% CI 54.5-93.3%). Analysis of MBL values showed no association between pre transplant MBL values and native lung diseases.

To analyze whether the transplantation procedure in combination with immunosuppressive therapy had an effect on MBL levels, in 57 patients MBL values were measured before and ± 20 months after transplantation. As shown in figure 1A, MBL values decreased significantly after transplantation ($p<0.0001$)

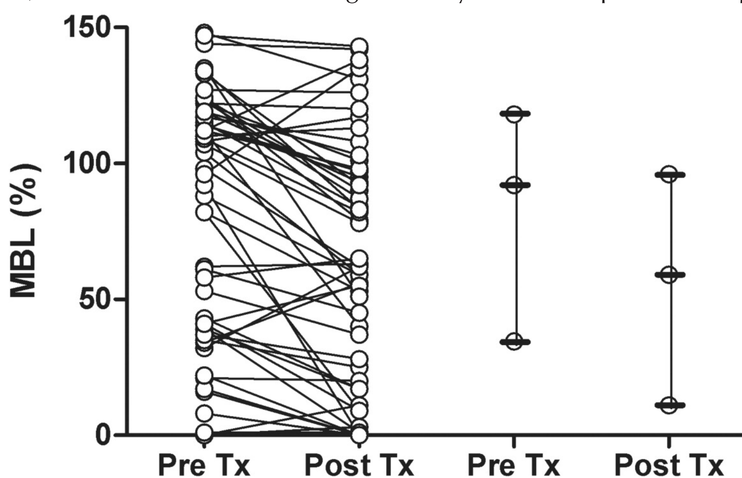

Figure 1.

In 57 lung transplant recipients serum MBL values were measured before and 20 months after lung transplantation. Each circle represents the MBL measured in one serum. The decrease in MBL values was significant and medians and 25%-75% intervals are depicted prior and post lung transplantation. In the 45 patients that showed a decrease in MBL values after transplantation, no differences in native lung diseases were detected. Twelve patients (6 CF and 6 emphysema) demonstrated an increase in MBL values after transplantation.

(95% CI 9.9-23.8). On average MBL levels were reduced by 20% after transplantation. Although in most patients MBL values decreased after transplantation, in 12 patients an increase in MBL values was detected after transplantation. No correlation was found between an increase in MBL values and sputum cultures before transplantations, native disease or CMV copies after transplantation.

MBL values and CMV reactivation

In 24 of the 71 patients with normal pre transplant MBL values (33%), CMV copies were detected during follow up, which was not significantly different compared to 7 of the 14 patients with low MBL values (50%) (p=0.30). None of the CMV reactivations occurred while patients received valganciclovir prophylaxes. It is remarkable that in 50% of patients with low pre transplant MBL levels CMV copies were detected since only one patient was considered a high risk patient for CMV

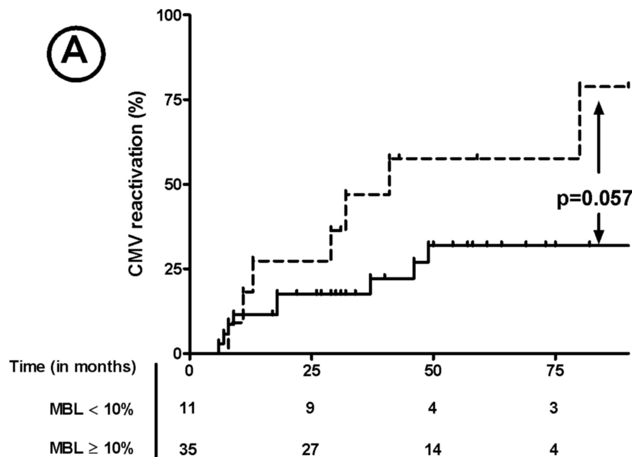

Figure 2A.

Kaplan Meier analysis of cytomegalovirus (CMV) reactivation showed a trend towards more CMV reactivations in recipients with deficient pre transplant MBL values (solid line). Patients with normal pre transplant MBL values are depicted with a dashed line. CMV reactivation was defined as a successive assay in the polymerase chain reaction after transplantation in those patients who had a seropositive CMV status before transplantation.

reactivation (CMV serostatus donor+/recipient-). This may suggest that patients with pre transplant MBL deficiency have a high risk of CMV reactivation. Therefore we analyzed CMV reactivation in recipients with a CMV IgG positive status. Prior to transplantation 35 out of 71 patients (49%) with normal MBL levels and 11 out of 14 patients (79%) with MBL deficient levels had IgG antibodies against CMV (p=ns). In the CMV seropositive patients, CMV reactivation after transplantation was detected in 9 of 35 recipients with normal pre transplant MBL levels (26%), compared to 7 of the 11 with MBL deficient levels (64%) (p=0.03). Kaplan-Meier analysis showed a non-significant tendency towards more CMV reactivations in patients with pre transplant MBL deficient levels and a positive IgG status (figure 2A) (p=0.06; HR 0.3 (95% CI 0.1-1.0)).

Post transplant sera were available in 72 patients but no influence of post transplantation MBL on CMV reactivation was detected (p=0.4).

MBL values, BOS and survival

During the study 13 patients died of whom 6 were diagnosed with BOS. Fourteen of the 71 patients with normal pre transplant MBL values (20%) developed BOS, compared to 7 of the 14 patients in the group with MBL deficient values (50%) (p=0.02). In a Kaplan-Meier analysis no correlation was found between pre transplant MBL values and the development of BOS (Figure 2B) (p=0.13; HR 0.4 (95% CI 0.1-1.3)). However, there was a non-significant tendency towards a better survival in patients with MBL deficient levels prior to transplantation (Figure 2C) (p=0.08; HR 3.4 (95% CI 0.9-4.0)).

From 72 patients post transplant sera were available but no influence of post-transplant MBL values on survival (p=0.82; HR 1.32 (95% CI 0.3-5.6)) nor BOS was detected (p=0.17; HR 0.4 (95% CI 0.1-1.4)).

Analyzing the 57 patients for whom pre- and post transplantation sera were available showed that no patients with MBL deficiency died whereas 83% of patients with normal MBL survived (p=0.25; HR 0.4 (95% CI 0.4-24.9)). In this group MBL deficient patients had a freedom from BOS of 53% vs. 78% in normal MBL patients (p=0.32; HR 2.2 (95%CI 0.5-11.0)).

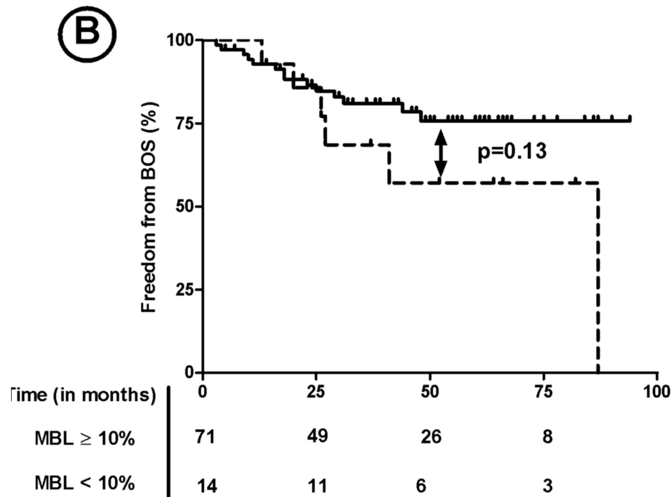

Figure 2B.

Kaplan Meier analysis of freedom from BOS showed no significant difference in recipients with deficient pre transplant MBL values (solid line) compared to normal pre transplant MBL values (dashed line).

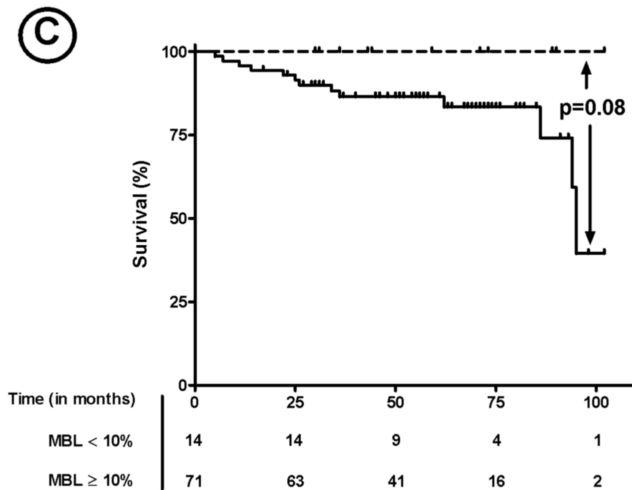

Figure 2C.

Kaplan Meier analysis of survival showed a trend towards a better survival in recipients with deficient pre transplant MBL values (solid line) compared to normal pre transplant MBL values (dashed line).

Discussion

Long term survival after lung transplantation is poor, mainly due to the development of BOS. Although BOS is caused largely by alloimmunity, there is hardly any clinical improvement to augmented therapy compromising the adaptive immune system. Therefore we presume that the innate immunity also plays a role in the development of BOS. Earlier studies suggested an influence of MBL levels on infections and survival in solid organ transplants[8-10,15]. In this study we examined the relation between functional MBL values and clinical outcome (CMV reactivation and (graft) survival) after lung transplantation.

Since immunosuppressive therapy was tapered in the first year after transplantation and maintained thereafter, we presumed that MBL levels would be stable 20 months after transplantation. However, MBL levels dropped in this time frame after transplantation by 20% and later on, MBL-values increased and reached values comparable to those before transplantation. Recently, in accordance with our results, reduced MBL levels in both serum and bronchoalveolar lung fluid have been demonstrated in lung transplant recipients[23]. Because the transplanted lung is continuously exposed to pathogens in the air (e.g. pollution, viruses) there is a persistent epithelial injury and repair, and an increased apoptosis in airway epithelial[24]. Apoptotic epithelial cells are removed by phagocytotic cells and this process is called efferocytosis. MBL influences efferocytosis[25]. Possibly, MBL levels in lung transplant recipients are reduced because of consumption due to persistent efferocytosis. In addition, we can not exclude the influence of drugs. Possibly, due to the immunosuppressives which are mostly cleared by the liver, the MBL synthesis by the liver is compromised. For liver transplantation it was demonstrated that MBL levels increase after transplantation and are stable for more than one year[4,26]. The immunosuppressive regimen in liver transplantation is mild compared to lung transplantation, which may explain the differences in post transplant MBL levels found between these types of organ transplantation. In the aforementioned studies it was also shown that MBL is mainly produced by the transplanted liver (4). Analysis on extra-hepatic production of MBL showed mRNA expression in the small intestine and testis, but not in the lung[27]. Munster et al demonstrated that donor haplotype LXPA correlated

with a superior lung transplant outcome but only during an immunosuppressive regimen of cyclosporine, azathioprine and prednisone. After introducing the new immunosuppressive protocol the influence of MBL haplotype on graft survival disappeared. Since the study of Munster et al. was performed in the Netherlands and the majority of recipients and donors in our cohort are from the Netherlands and matched according the same Eurotransplant algorithms, we presume that the distribution of the MBL-haplotypes is similar to the cohort of Munster et al. In addition their new immunosuppressive protocol is similar to ours with the exception that we use mycophenolate mofetil instead of azathioprine. Nevertheless, MBL has been found in bronchoalveolar lavage (BAL) fluid, although only during inflammation, which may be caused by local production of activated alveolar cells or caused by "leakage" from the serum[28]. Nevertheless, it seems unlikely that small amounts of MBL locally produced in the lung contribute significantly to the amount present in the circulation. Until today no (functional) RNA fragments in the lung have been described in the database of Online Mendelian Inheritance in Man (OMIM)[29].

MBL deficiency is associated with viral infections[30-32]. The results of our study demonstrated more CMV reactivations in patients with pre transplant MBL deficient values. This is in line with the results of Manuel et al. who studied plasma MBL levels in 16 kidney transplant patients with a high risk CMV status (donor-positive/receptor negative) and reported an association with MBL status and CMV reactivation[7]. Also in stem cell transplantation an association was found between MBL levels and CMV infection/reactivation in 131 patients[33]. Although we observed a constantly higher amount of CMV reactivations in the group with MBL deficient levels, this did not reach statistical significance in a Kaplan-Meier analysis (p=0.06). In our daily clinical practice we use CMV reactivation as a tool to detect an over suppressed immune system and if CMV copies are detected, we reduce immunosuppression. During CMV prophylaxis no CMV reactivations occurred. In addition, during the study 13 patients died of which 6 had BOS, indicating that survival and BOS are not linked in this study. This might explain why low MBL values could be associated with a better survival, but not with BOS. Our results on the non-significant correlation between MBL levels and CMV reactivation/ development of BOS should be interpreted with caution, since we have a relatively small number of patients.

Not only MBL deficiency but also the development of BOS and survival are associated with viral, bacterial and fungal infections[34-36]. Unfortunately, in our follow up of lung transplant recipients no surveillance bronchoscopies, sputum cultures nor surveillance viral swabs are implemented and therefore we could not explore the association of MBL values and fungal, mycobacterium and common viral infections. The association between CMV infections and BOS is disputed. Some studies showed an association but others do not[37-39]. We found an association between MBL deficiency and the development of BOS (p=0.02), although this did not reach statistical significance in a Kaplan-Meier analysis. This is in line with the results of Munster et al. who found no longer a correlation between MBL and the development of BOS after switching to an immunosuppressive regimen consisting of tacrolimus, mycophenolate mofetil and prednisone[15]. Since the use of azithromycin some patients with BOS have a reversible or a less decreasing lung function and this may have an impact on survival after lung transplantation[40-42]. Patients with normal MBL values before transplantation had a non-significant tendency towards a worse survival, suggesting that the contribution of MBL in local complement activation is related to graft survival.

A limitation of our study may be that in our study only 14 out of 85 patients had MBL deficient levels (in stead of the expected one third). This could have led to the non-significant result, and a type II error.

In conclusion, the findings of the present study suggest that MBL deficiency is associated with CMV reactivations and a longer survival but not with the development of BOS. Larger studies are needed to further confirm these findings.

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Chapter 6

The killer immunoglobulin-like Receptor (KIR) group A haplotype is associated with the Bronchiolitis Obliterans Syndrome after lung transplantation

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Abstract

Background

The development of the Bronchiolitis Obliterans Syndrome (BOS) after lung transplantation is associated with viral infections. Natural Killer (NK) cells are involved in the lysis of viral infected cells and their activation is largely controlled by activating and inhibitory Killer Immunoglobulin-like receptors (KIRs). We hypothesized that KIR ligand incompatibility and recipients' individual KIRs could influence the development of BOS and the incidence of CMV reactivation after lung transplantation.

Methods

The KIR gene contents were determined in 48 patients who received a lung transplant and HLA-Cw and –Bw4 typing was performed on their respective donors.

Results

Seven patients developed BOS and in 16 patients CMV reactivation occurred. Five out of 19 patients homozygous for KIR haplotype A developed BOS, compared to 2 out of 27 patients with KIR haplotype AB and B (homozygous) ($p=0.03$; log rank test). In none of the patients with BOS the activating KIR2DS5 was detected whereas it was present in 35% of patients without BOS ($p=0.04$; log rank test). No correlation was found between KIR gene content and CMV reactivation.

Conclusion

Our results suggest that the lack of activating KIRs may play an important role in the development of BOS but not in the control of CMV reactivation after lung transplantation.

INTRODUCTION

Lung transplantation is an ultimate therapy for end stage lung diseases, applied to patients who have no other therapeutic options left. Although survival rates have improved over the years, they are still restricted by the development of the bronchiolitis obliterans syndrome (BOS)[1]. At present the exact mechanism of this chronic allograft rejection is not clear, but it has become evident that both innate and adaptive immune responses play an important role in the onset and pathogenesis of BOS[2-5].

Natural Killer (NK) cells are one of the main cellular components of the innate immune system and play an important role in the lysis of virally infected cells and tumors. A multitude of intracellular and extracellular proteins are expressed by NK cells in order to recognize and eliminate target cells. Recognition of target cells occur through integration of different signals derived from cell-surface receptors, including MHC class-I receptors (Killer Immunoglobulin-like Receptors (KIRs), CD94/NKG2A, LILRB1), adhesion molecules (CD2, CD11b/CD18, CD11c/CD18, CD31, CD96, CD49A/CD29, CD226) and costimulatory receptors (CD244 and NKG2D)[6-8]. Elimination of target cells expressing inadequate or no MHC class-I molecules on their cell surface is regulated by opposite signals delivered by inhibitory and activating KIRs, of which the expression may be regulated depending on the type of NK-cell stimulus[9].

KIRs are named according their structural and functional characteristics: the number of extracellular Ig domains (2D or 3D) and the intracellular tail (short (S) or long (L)). Short tails correlate with activating and long tails with inhibitory receptors. Although ligands for activating KIRs are still unknown, the inhibitory KIRs recognize epitopes on HLA-C and the Bw4. Ligands for KIR2DL1 are HLA-C with a lysine residue at position 80 (group2), for KIR2DL2 and 2DL3 are HLA-C with an asparagine residue at position 80 (group1) and for KIR3DL1 the ligand is HLA-Bw4.

KIR genes are located on chromosome 19q13.4 within the Leukocyte Receptor Complex (LRC). Based on population studies, multiple KIR haplotypes have been defined differing in KIR gene content and associated functional properties[10-12]. KIR haplotype A contains 6 inhibitory and 1 activating KIRs and expression of this haplotype will be associated with functional down regulation of NK

cell activity(13). The variety of KIR haplotypes B all contain a mixture of functional activating and inhibiting KIRs[11,14].

In previous reports on haematopoietic stem cell transplantation, significant associations were found between KIR genes (the presence of specific KIRs, the number of KIRs, mismatching of inhibitory KIRs with their respective HLA-ligands) and clinical key-parameters, including chronic graft-versus-host-disease - what can manifest in the lungs as an bronchiolitis obliterans - and CMV reactivation15-17. Recently it was demonstrated that the number of peripheral blood of NK cells are decreased, while an increased number is present in transplanted lungs from patients suffering from BOS compared to that in stable patients. These data suggest that NK cell are recruited from blood to the lungs during the process of chronic rejection18. However, the role of KIRs responsible for NK cell function after lung transplantation is not elucidated yet. In the present study we examined patients' KIR gene content and hypothesized that KIR ligand incompatibility or KIR haplotypes could be associated with the development of BOS and/ or CMV reactivation.

Patients and Methods

Subjects

A total of 48 patients who underwent lung transplantation at the Heart Lung Center in Utrecht between October 2001 and March 2006 and survived more than three months were included in this study. To eliminate postoperative complications, we excluded patients who died during the period from transplantation until three months after transplantation. According to the standard ISHLT criteria BOS was defined as a decline of the FEV1 from the post-operative baseline at 2 distinctive time-points of more than 20% in the absence of infection or other etiology[19]. Patients were monitored for graft function and CMV infections. Standard immunosuppressive therapy consisted of basiliximab as induction immunosuppression, tacrolimus, mycophenolate mofetil and prednisone. Patients at risk for CMV reactivation, which is defined as a CMV seropositive donor or patient, were treated with valganciclovir for at least 6 months post transplantation. The study design was approved by the medical ethical committee and informed consent was obtained from each patient.

Genotyping

Genomic DNA from patients and donors was isolated from peripheral blood lymphocytes, stored and retrospectively typed. cDNA from donors were typed for HLA-B and HLA-C by polymerase chain reaction (PCR)-specific priming (SSP) (Biotest, Dreieich, Ger). HLA-C and HLA-B were segregated into the epitopes groups HLA-C group 1 (HLA-C Asn 80: HLA-Cw1,3,7,8,13,14 alleles), HLA-C group 2 (HLA-Clys80: HLA-Cw2,4,5,6,12,15,17,18 alleles) and HLA-Bw4[20]. DNA from patients was typed for 6 activating (2DS1, 2DS2, 2DS3, 2DS4, 2DS5 and 3DS1) and for 8 inhibitory (2DL1, 2DL2, 2DL3, 2DL4, 2DL5, 3DL1, 3DL2 and 3DL3) KIR genes by LUMINEX SSO (One Lambda, Inc., USA). A missing KIR ligand was defined as the presence of patients' KIR genes in the absence of their corresponding HLA on the transplanted lungs (ligand). The following KIR genes match with their ligands: 2DL1 corresponds with HLA-C group 2, 2DL2/3 with HLA-C group 1 and 3DL1 with HLA-Bw4.

At the moment there are 42 KIR haplotypes detected based on their gene content. All haplotypes contain the framework genes 3DL3, 2DL4 and 3DL2 and contain either 2DL2 or 2DL3, but not both[10-12]. Haplotype A contains framework genes and 2DL3, 2DL1, 3DL1 and 2DS4. Haplotype B is more variable and contains more than one activating KIR gene. Presence of KIR genes 2DL5, 2DS1, 2DS2, 2DS3 or 2DS5 reflects haplotypes B[14]. The presence of KIR haplotypes A (homozygous), AB and B (homozygous) in patients were deducted from the type and number of KIR genes detected[10,12].

Latent CMV reactivation

CMV serostatus of patient and donor was determined by enzyme-linked immunoassay (ELISA; VIDAS-biomerieux, Marcy L'Etoile, France). We excluded patients with a negative CMV serostatus prior to transplantation and transplanted with lungs from CMV negative donors. Post transplant monitoring for CMV reactivation was performed by PCR. We used the PCR (Taqman) whereby a serological reactivation was defined as a successive assay detecting more than 400 copies/ml serums. After October 2003 we enlarged our input and used 3 ml serum in stead of 1 ml and therefore our threshold for a successive assay could be lowered to 50 CMV copies/ml in stead of 400 copies/ml. In 6 patients CMV copies were initially

measured with a threshold of 400 copies/ml. Four out of these 6 patients developed a CMV reactivation. Patients with a clinical CMV reactivation, defined as more than 1000 copies/ml were treated with valganciclovir and reducing immune suppression. Duration and intensity of valganciclovir depended on clinical status and viral load.

Statistical analysis

The associations between patient characteristics and freedom from BOS and CMV reactivation were analyzed with a log rank test in the Kaplan-Meijer curve. The Fishers' exact test was used to compare frequencies. $P < 0.05$ was considered statistically significant.

Results

The median follow up time of the 48 patients studied was 24 months with a range from 9-60 months. All but 5 patients (all non-BOS) received a bilateral lung transplant. Seven patients developed BOS and two patients died during the course of the study, one of which was associated with BOS. The group of patients with BOS was similar to that without BOS regarding age, gender and underlying disease. Baseline characteristics of the overall study group are depicted in Table 1.

Relationship between KIR-ligand matching and development of BOS

To examine whether absence or presence of KIR ligands on transplanted lungs was associated with the development of BOS, we typed patients' KIR genes and HLA of the donor. KIR genotyping of the 48 patients revealed 98% (n=47) of individuals to be positive for 2DL1 and 92% (n=44) for 3DL1. Eighty five percent of the patients (n=40) were positive for KIR 2DL3 and in 40% of the patients (n=19) 2DL2 was positive. From 2 donors DNA could not be obtained for HLA typing. Eighty three percent (n=38) of the donors which could be analyzed were typed for HLA-C group 1, 70% (n=32) for HLA-C group 2 and 75% (n=36) was HLA-Bw4 positive.

Analyzing patients' KIR genes with the absence of the corresponding HLA-ligands on the transplanted lungs, 58% (n=28) of lung transplants had HLA ligand(s) missing for patients KIRs. Of these patients, 71% (n=20) and 29% (n=8) had 1 or 2

Table 1. Baseline Characteristics of Overall Study Group

Characteristics	All	BOS	Non-BOS
Number of patients	48	7	41
Median age, years (range)	50 (16–64)	56 (24–60)	50 (16–64)
Underlying disease			
- COPD	25 (52%)	3 (43%)	22 (54%)
- CF	13 (27%)	1 (14%)	12 (29%)
- interstitial lung disease	10 (21%)	3 (43%)	7 (17%)
Gender			
M	23 (48%)	2 (29%)	21 (51%)
F	25 (52%)	5 (71%)	20 (49%)
Recipient-KIR-haplotypes			
- KIR haplotype A (homozygous)	19 (40%)	5 (71%)	14 (34%)
- KIR haplotype A and B	14 (29%)	1 (14%)	13 (31%)
- KIR haplotype B (homozygous)	15 (31%)	1 (14%)	14 (34%)
Median number of activating KIR genes (range)	3 (1–6)	1 (1–5)	3 (1–6)
Median number of inhibitory KIR genes (range)	6 (2–8)	6 (6–8)	7 (2–8)
Median KIR mismatches (range)	1 (0–2)	1 (0–2)	1 (0–2)
CMV status recipient/donor (pre transplant)			
- positive/positive	8 (17%)	2 (29%)	6 (15%)
- positive/negative	15 (31%)	0	15 (37%)
- negative/positive	12 (25%)	3 (43%)	9 (22%)
- negative/negative	13 (27%)	2 (29%)	11 (27%)
CMV reactivation	16/36	3/5	13/30
Median HLA mismatches (range)			
Class I	3 (2–4)	3 (2–4)	3 (2–4)
Class II	1 (0–2)	1 (1–2)	1 (0–2)

Definition of abbreviations: COPD, chronic obstructive pulmonary disease; CF, Cystic Fibrosis; M, Male; F, Female; KIR, Killer Immunoglobulin-like Receptors;HLA, Human Leucocytes Antigen; CMV, cytomegalovirus.

missing KIR ligands, respectively. The distribution of patients' KIRs and HLA ligand missing is detailed in Table 2. No significant differences in freedom from BOS could be found between patients with KIR ligands present compared to those without.

Table 2. Characteristics of Ligand Absence Between Transplanted Lungs and Kir Patient

	Ligand absent, number (%)	
Lung (donor)	Recipient	
HLA-C group 1 (HLA-CAsn80)	absent for KIR 2DL2/2DL3 patient	10 (22%)
HLA-C group 2 (HLA-CLys80)	absent for KIR 2DL1 patient	15 (33%)
HLA-Bw4 on	absent for KIR 3DL1 patient	12 (25%)
HLA-Bw4 and HLA-C	absent for patient KIR	8 (17%)
No ligand absence		9 (19%)

Definition of abbreviations: KIR, Killer Immunoglobulin-like Receptors; HLA, Human Leucocytes Antigen.

Relationship between patients' KIRs and development of BOS

We also examined whether a relation exists between BOS and the number of activating or inhibitory KIRs in individual patients. The median number of activating KIRs was 3 (range 1-6) and 27% (n=13) of the patients had 5 or more activating KIRs. The median number of inhibitory KIRs was 6 (range 2-8) and 50% (n=24) of the patients had 7 or more inhibitory KIRs. No significant relation was found between the number of inhibitory or activating KIRs and the absence or presence of BOS.

Although some individual KIRs e.g. 2DL2, 2DL5, 2DS1 and 2DS2 appeared to differ in their frequencies in patients developing BOS compared to non-BOS patients, this did not reach statistical significance (see Figure 1). KIR gene 2DS5 however, was present in 34% (n=14) of non-BOS patients but absent in patients with BOS, which was significantly associated with the development of BOS (HR: 0.00; p=0.04) (see figure 2).

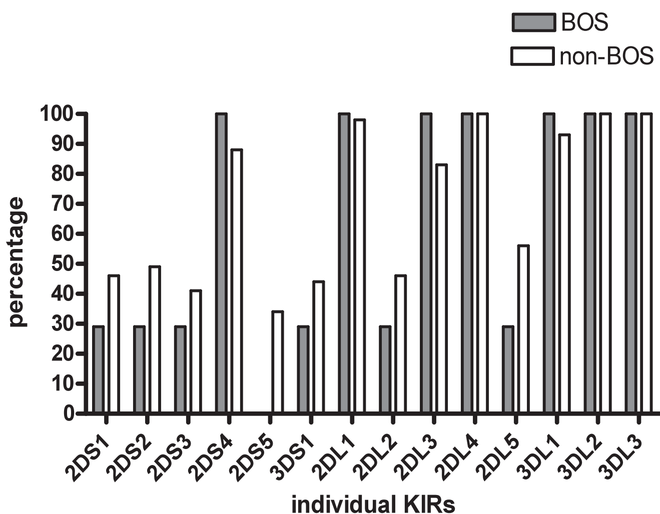

Figure 1. Frequency of individual KIRs in patients with and without BOS.

The presences of KIR genes were determined in patients as described in the Materials and methods section. Shown are the frequencies of individual KIR genes found in patients with or without BOS.

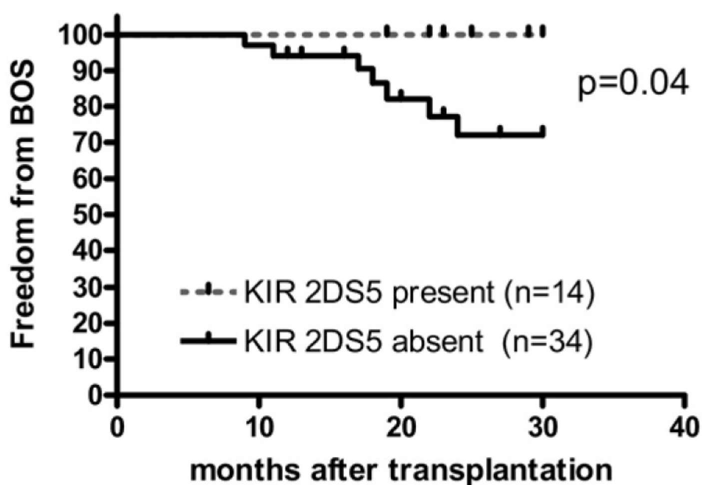

Figure 2. Influence of KIR2DS5 on the freedom from BOS.

Kaplan Meijer analysis of freedom from BOS showed a significant difference between patients (n=34) with KIR-gene (Killer Immunoglobulin-like Receptor) 2DS5 absent and patients (n=14) with KIR-gene 2DS5 present (log rank test, p=0.04)

KIR haplotypes and BOS

Forty percent (n=19) of the patients were homozygous for KIR haplotype A, 31% (n=15) contained a copy of KIR haplotype A and B, whereas 29% (n=14) of the patients were homozygous for KIR haplotype B. No association was found between KIR haplotypes and underlying lung diseases. Upon comparison of KIR haplotypes between BOS and non-BOS patients, it was found that: 71% (n=5), 14% (n=1) and 14% (n=1) of patients with BOS contained KIR haplotypes A, AB or B respectively, whereas 34% (n=14), 34% (n=14) and 32% (n=13) from the non-BOS patients contained these haplotypes. The presence of KIR haplotype A resulted in a significant higher risk of BOS development (HR: 4.9; p=0.03) (see figure 3).

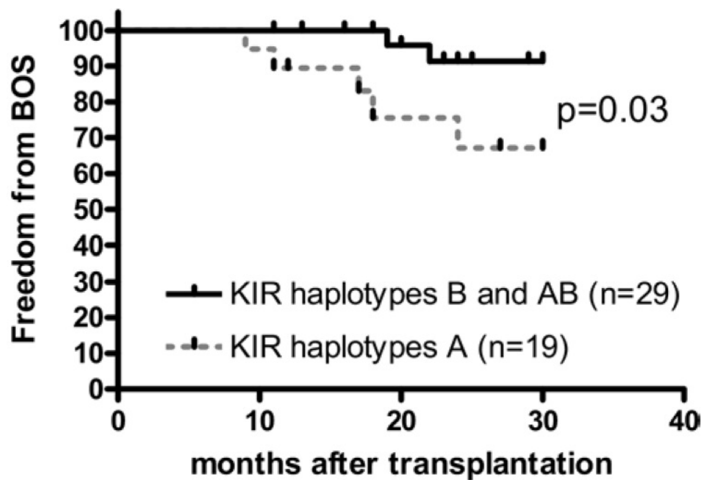

Figure 3. Influence of KIR haplotype A on freedom from BOS.
Kaplan Meijer analysis of freedom from BOS showed a significant difference between patients (n=19) with KIR haplotype A (homozygous) and patients (n=29) with KIR haplotype AB and B (homozygous) (log rank test, p=0.03).

Influences of missing KIR ligands, individual KIRs and haplotypes on CMV reactivation

In order to examine whether CMV reactivation was related to KIR gene content we studied CMV serostatus in donors and patients prior to transplantation and linked them to individual KIRs. This analysis indicated that in 12 transplantations CMV was neither present in donor nor patient. From the 36 remaining patients at risk for

a CMV reactivation 16 patients actually developed a reactivation despite prophylactic use of ganciclovir. CMV reactivation occurred from 4 to 43 months after transplantation with a median from 11 months.

KIR genotyping of the 36 patients at risk for CMV reactivation showed that 97% (n=35) patients were positive for KIR2DL1, 100% for 2DL2 or 2DL3 (53% for KIR2DL2, 86% 2DL3) and 94% (n=34) was positive for 3DL1. Sixty nine percent (n=18) of the transplanted lungs were typed for HLA-C group 1, 75% (n=27) for HLA-C group 2 and 94% for HLA-Bw4. Twenty two patients with CMV reactivation received a lung with KIR ligands missing, whereas in the other 14 patients all ligands were present on the transplanted lungs. Studying the influence of missing KIR ligands on CMV reactivation showed no significant differences in patients with or without a missing KIR ligand. In addition no significant relation could be found between the number of inhibitory or activating KIRs, neither between individual KIR genes nor CMV reactivation. Furthermore, no significant correlation was found between the KIR haplotypes of patients and CMV reactivation (see Figure 4).

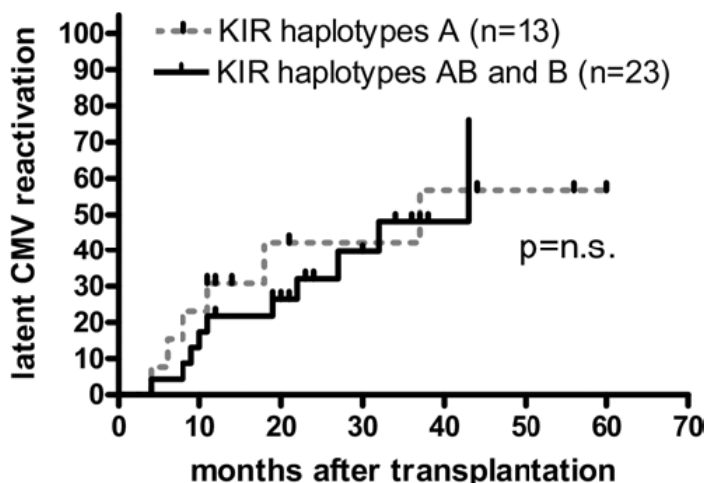

Figure 4. Kaplan-Meier analysis of cytomegalovirus (CMV) reactivation showed no significant difference between the 13 patients with killer immunoglobulin-like receptor (KIR) haplotype A (homozygous, dashed line) and the 23 patients with KIR haplotype AB and B (homozygous, solid line). CMV reaction was defined as a successive assay in the polymerase chain reaction after transplantation in those patients who had a seropositive CMV status before transplantation or who received lungs from a CMV seropositive donor.

Discussion

Stimulated by the reported associations between KIRs and several clinical features after haematopoietic stem cell transplantation, we hypothesized that KIR gene content may be related to chronic allograft rejection and CMV reactivation after lung transplantation. This is the first study analyzing the influence of individual KIR gene contents, their corresponding ligands and haplotypes on the results of lung transplantation. Herein we demonstrate that KIR haplotype A and the absence of KIR2DS5, presumably the lack of activating KIRs, are predictive for development of chronic allograft rejection.

As inhibitory signals derived from KIRs are primarily regulated via ligation with their corresponding HLA-ligands, we first analyzed KIR2DL1-3 and KIR3DL1 from all patients, and HLA-Cw and –Bw4 in the donors. An effect of KIR-ligand matching on chronic rejection after lung transplantation was not detected. These data are in line with a previous report showing no effect of KIR-ligand matching on the 10-years allograft survival in 2,757 patients receiving cadaver kidney transplantation[21]. In a recent study on 69 kidney transplants no association was found between individual KIR genes or KIR haplotypes and the occurrence of acute rejection when tapering their immunosuppressive regimen[22]. However, Kunert et al. demonstrated in 224 kidney transplants that patients without acute rejections had higher frequencies of KIR 2DL2/KIR 2DS2 although no association was found between acute rejections and KIR haplotypes[23].

In the present study we have found that both KIR haplotype A and the absence of KIR2DS5 are significantly associated with BOS. This finding is unexpected when the viewpoint is taken that NK cells are simply killer cells guided by their cell-surface receptors. As NK cells containing the homozygous KIR haplotype A express a phenotype mainly encoded by inhibitory KIRs, this KIR haplotype should be associated with less reactivity against donor cells recognized on lung allografts and thus absence from BOS instead of the association found in the present study. It is possible that the differences observed are a random effect due to a limited sample size. Nevertheless, if these findings are confirmed in a larger study one could speculate that NK cells play a more regulatory role in the alloresponse e.g. by dendritic-cell editing in the period of months/years after transplantation

when allograft damage is persistently induced by the immune system[24]. In the lung, immature dendritic cells (iDC) are located in airway epithelium, alveolar septa and around pulmonary vessels. These iDC are sensitive to killing by NK cells, especially by those expressing activating KIRs and Toll-like receptors. When iDCs become activated and mature, they down regulate secretion of chemokines (CCR1, CCR5 and CCR6) and up regulate expression of CCR7[25,26], and become insensitive to lysis by NK cells[27,28]. The efficiency of iDC killing by activated NK cells may differ between those containing the KIR haplotype A versus AB or B haplotypes, which will downstream result in differences in allo-antigen presentation of DCs to T-cells.

A couple of arguments made the study analyzing the influence of NK cells on CMV reactivation appealing. First of all, NK cells play an important role in control of viral infection and second, the association between viral infections, especially CMV, with the development of BOS[19,29]. Studies in HIV patients have shown that the combination of the activating KIR3DS1 and its presumed ligand HLA-Bw4 is associated with a delayed progression to AIDS, whereas, the presence of 3DS1 in absence of Bw4 is associated with a rapid progression. These data suggest that virus elimination is facilitated upon binding of an activating KIR to its ligand[30]. After haematopoietic stem cell transplantation (SCT) missing ligands for inhibitory KIRs did not influence CMV reactivation whereas in organ transplantation results of such studies have not been reported yet[31]. In our study on lung transplantation, we did not observe any relation between missing KIR ligands, nor the number of activating KIRs or individual KIRs on CMV reactivation. After allogeneic SCT however, patients with KIR haplotypes AB and B – thus with more activating KIRs - showed a lowered incidence of CMV reactivation compared to those with KIR group A haplotypes[16,31]. As CMV reactivation after lung transplantation is associated with BOS and the KIR haplotypes AB and B are associated with lack of BOS, we expected a protective influence of activating KIRs on CMV reactivation, but did not find any. Next to this difference, CMV reactivation occurred 10-20 days post SCT in 55-63% of patients versus 4 months after lung transplantation in 44% (n=16) despite prophylactic use of ganciclovir. Apparently, in the SCT setting where myeloablative therapy was given and the immune sytem was reconstituting under immunosuppression, the influence of KIRs on CMV reactivation differs from that in organ transplantation in which a fully functional immune system is only reduced in activity by immunosuppressive therapy.

Our results show that KIR gene content of recipients, especially activating KIRs, influence chronic allograft rejection after lung transplantation, possibly through editing of DCs. We are aware that our study is hampered by the small patient population, a relatively short follow up, and that our findings need to be confirmed in larger studies elucidating the role of KIR-usage by NK cells in chronic allograft rejection. We propose a detailed analysis of NK cells infiltrating lung allografts including the influence of different immunosuppressives on their function. A challenging issue will be to further explore the mechanism of NK cells activation by characterizing activating ligands and their expression on the lung allograft.

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CHAPTER 7

Summary and future perspectives

Summary and future perspectives

Lung transplantation for end-stage lung diseases, such as cystic fibrosis (CF), chronic obstructive pulmonary disease (COPD) and interstitial lung diseases (ILD), can be considered if no other therapeutic options, such as pharmaceuticals or surgery, are possible. Although the 5-year survival after lung transplantation has improved from 47% in 1988 to 54% in 2006, the mean survival for patients who survive the first year after lung transplantation has only increased from 6.9 years to 7.1 years[1]. The development of chronic allograft rejection after lung transplantation is the most common cause of this poor long-term survival.

Chronic allograft rejection in lung transplant patients is characterized by scar tissue formation around bronchioli and vessels and eventually leads to the obliteration of the bronchioli. Because the process of chronic allograft rejection has a patchy distribution, normal lung tissue obtained through a transbronchial biopsy does not exclude the diagnosis of chronic allograft rejection. Therefore, a surrogate marker based on a permanent lung function decline is currently the gold standard: the presence of bronchiolitis obliterans syndrome (BOS). BOS is defined as a permanent lung function decline of 20% in the “forced expiratory volume in 1 second” (FEV1) in the absence of infection, anastomotic stenosis or recurrence of the initial lung disease[2].

The human immune system consists of an innate part and an adaptive part. Although the exact pathogenesis of BOS remains unknown, it seems that repetitive damage to the epithelial and subepithelial cells plays an important role in the development of this syndrome. This repetitive damage leads to a response from the immune system (adaptive and innate) and eventually to inflammation. Due to this response, it is likely that in patients who are going to develop BOS or who are developing BOS, certain parameters or markers are elevated or decreased. In patients who are at risk for the development of BOS, it may be possible that an adaptation of immunosuppression or excluding donor lungs can prevent the future development of BOS. This thesis describes the identification of patients who are at risk for developing BOS using blood samples obtained during regular follow up visits.

In **Chapter 2**, we describe the applicability of CD30 values measured in blood as a biomarker for the development of BOS. CD30 is a member of the tumor

necrosis factor superfamily and is expressed on Th2-cells. If Th2-cells are activated, soluble CD30 is shed into the bloodstream. No differences in soluble CD30 (sCD30) values were detected between patients who developed BOS and those who did not. Therefore, sCD30 values after lung transplantation cannot be used as a biomarker to detect the development of BOS. However, we did observe that high sCD30 values before lung transplantation were suppressed after transplantation. In **Chapter 4**, we report that immunosuppressive drugs suppress sCD30 values. We determined whether Thymus and Activation regulated Chemokine (TARC) and/or sCD30 values in the blood can be used as objective biomarkers in patients with severe atopic dermatitis during the administration of two immunosuppressive drugs that are used to treat lung transplantation patients as well: cyclosporine and enteric coated mycophenolate sodium (EC-MPS). A correlation between the sCD30 values and the severity of atopic dermatitis was observed during the administration of a cyclosporine-based regimen, but this correlation was not observed during EC-MPS treatment. However, a correlation between TARC and clinical parameters that are used to evaluate disease activity in atopic dermatitis was demonstrated during both cyclosporine and EC-MPS treatment. This study emphasizes the importance of evaluating the effect of systemic therapy on potential biomarkers or possible pathogenesis mechanisms in the development of BOS.

In **Chapter 3**, we describe the applicability of TARC values as a possible biomarker for the development of BOS. The chemokine TARC is involved in the recruitment of Th2-cells. No difference in TARC values was observed between patients and healthy controls. We also demonstrated that pre-transplant TARC levels were not associated with the development of BOS. We demonstrated that neither lung transplantation nor immunosuppressive drugs influence TARC values because no differences in the TARC values were observed before and after lung transplantation. If TARC values below the 325 pg/ml are detected at the first month after lung transplantation, there is an increased risk of developing BOS.

In **Chapter 5**, we describe the effect of functional mannose binding lectin (MBL) levels on survival after lung transplantation, the development of BOS and the (re)occurrence of CMV infection. An important part of the innate immune system is the complement system, which consists of different proteins, one of which is MBL. MBL can eliminate bacteria and viruses. We observed a correlation

between low MBL values before transplantation and CMV reactivation after transplantation. There was no association between MBL levels and the development of BOS. A trend between low MBL levels before transplantation and a superior survival rate was found.

In **Chapter 6**, we describe the effect of patients' killer immunoglobulin-like receptor (KIR) gene content with respect to KIR ligands on transplanted lungs, on the development of BOS and/or on CMV reactivation. We showed that the KIR gene content of recipients, especially activating KIRs, influenced chronic allograft rejection after lung transplantation. No correlation was found between KIR gene content and the reactivation of CMV.

Future perspectives.

Unraveling the pathogenesis of BOS will be a challenge for lung transplantation researchers, as well as lung transplant patients and their doctors; the clinical course after BOS is uncertain because the process of rejection may progress, stabilize or regress. Understanding the pathogenesis and prognosis of BOS will hopefully lead to interventions to stop the process of tissue scarring. In **Chapter 1**, an overview of the available articles about BOS in humans is given. We can conclude that various aspects of the immune system influence the development of BOS, and in these studies, various immunosuppressive regimens were used to prevent the development of BOS. In some studies, the immunosuppressive regimen was changed after the diagnosis of BOS, but the influence of this medication switch on BOS was not investigated.

Focusing on our own results from the Heart Lung Center Utrecht, we have a 1-year survival of 82% and a 5-year survival of 72%, and it is very likely that the mean survival after lung transplantation will be 10 years or more (see figure 1).

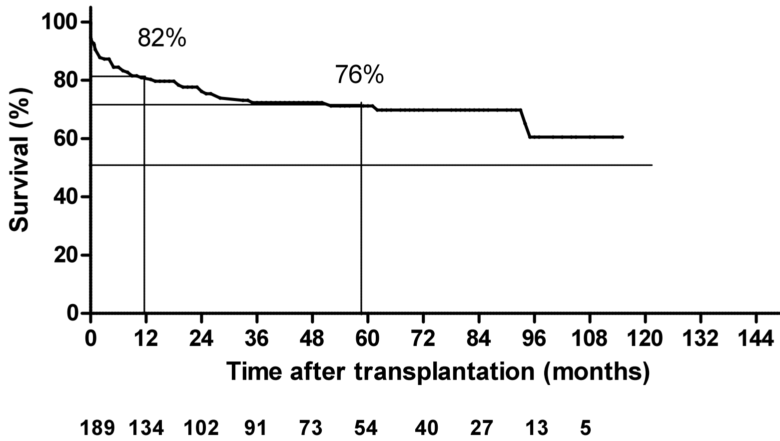

Figure 1. Survival curve for patients treated at the Heart Lung Center Utrecht

Five years after transplantation, the majority of our patients (74%) do not fulfill the criteria of BOS (see Figure 2). These results are excellent compared to the results of the International Society of Heart and Lung Transplantation (ISHLT). It is

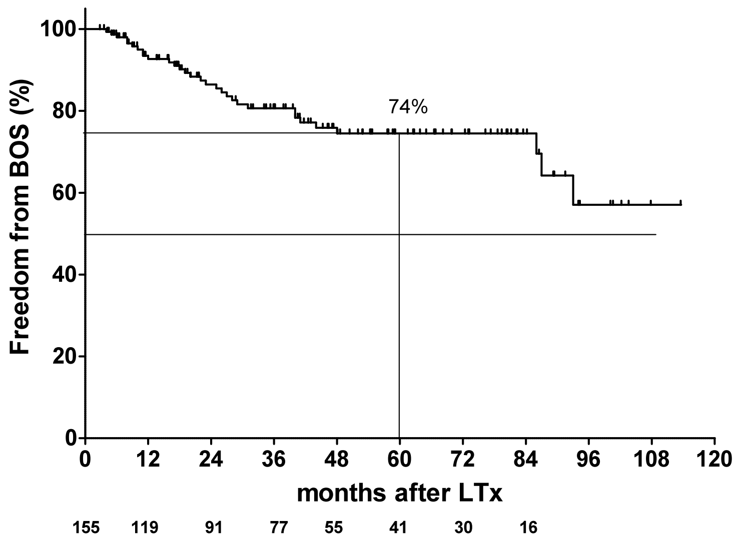

Figure 2. Freedom from BOS at the Heart and Lung Center Utrecht

possible that a different BOS definition is used. Vanaudenaerde demonstrated that in some BOS patients, a neutrophil-initiated pathway is apparent and that these patients respond well to azithromycin with a (partly) reversible FEV1 decline, with BOS resolving completely in some patients[3]. Statins have an anti-inflammatory effect and it is possible that they influence the development of BOS[4]. Recently, it was shown that montelukast, a leukotriene receptor antagonist, was associated with an FEV1 increase in BOS patients[5]. All of these drugs are, according to the best clinical practice, implemented in our patients, which may explain the results of our relatively young lung transplantation center. At our transplantation center, we define BOS as an irreversible lung function decline of 20%.

Medication but also the use of extended donor organs may play a role in the development of BOS. The quality of donor organs is not considered in all research articles. Another confounder may be the use of extended recipients. Due to the shortage of organs, the waiting list for lung transplants is (too) long, and many patients receive transplants from a preferential position (the high urgency list) and therefore may have an altered immune response.

Despite our favorable results, the survival curves for lung transplantation are still poor compared to other organ transplantation outcomes. In addition, the problem of chronic rejection has yet not been solved. Biomarkers that provide insight into the pathogenesis of BOS and offer a therapeutic option, especially in the early and reversible phases of BOS development, are needed. Preferentially, these biomarkers should be detected using procedures that are easily tolerated by patients. The use of such biomarkers will lead to an improvement in patient treatment and as a consequence, will lead to better results and higher patient and doctor satisfaction.

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CHAPTER 8

Samenvatting

Longtransplantatie is een therapeutische optie voor mensen met een eindstadium longlijden. Hoewel de 5-jaars overleving na een longtransplantatie verbeterd is van 47% in 1988 naar 54% in 2006, is de gemiddelde overleving, voor mensen die het eerste jaar na een longtransplantatie hebben overleefd, slechts gestegen van 6.9 naar 7.1 jaar[1]. Het ontstaan van chronische afstoting na een longtransplantatie is de belangrijkste oorzaak van deze matige lange termijnoverleving.

De chronische afstoting van een longtransplantaat kenmerkt zich door litteken vorming rondom de bronchioli (de kleine eindvertakkingen in de long) en leidt uiteindelijk tot een oblitereren (dichtgroeien) van de bronchioli. Omdat het proces van chronische afstoting verspreid door de longen plaatsvindt en normaal longweefsel bij een biopsie, een chronische afstoting niet uitsluit, is een surrogaat marker gebaseerd op blijvend longfunctieverlies nu de gouden standaard: het Bronchiolitis Obliterans Syndroom (BOS). Het Bronchiolitis Obliterans Syndroom is gedefinieerd als een blijven longfunctieverlies van 20% in het geforceerd uitademingvolume geblazen in 1 seconde (FEV1)[2].

Het menselijk afweersysteem bestaat uit een aangeboren deel (innate immunity) en een verworven deel (adaptive immunity). Hoewel het ontstaansmechanisme van BOS nog niet duidelijk is, lijkt herhaalde beschadiging een belangrijke rol te spelen. Deze herhaalde beschadiging leidt tot een respons van het afweersysteem wat uiteindelijk resulteert in obliteratie van de bronchioli en littekenvorming van het longweefsel. Door deze respons van het afweersysteem ontstaat een reactie in het longweefsel (inflammatie) waardoor het zeer voor de hand lijkt te liggen dat in patiënten die BOS (gaan) ontwikkelen, bepaalde markers of parameters verhoogd of verlaagd zullen zijn. Mogelijk kunnen we patiënten die BOS (gaan) ontwikkelen eerder identificeren en een aanpassing in de medicijnen doorvoeren waardoor BOS misschien voorkomen kan worden. Dit proefschrift beschrijft het onderzoek naar het vroegtijdig kunnen identificeren van die patiënten die na een longtransplantatie chronische afstoting (BOS) ontwikkelen door middel van bloedonderzoek wat bij reguliere controles geprikt wordt.

In **hoofdstuk 2** hebben we bestudeerd of CD30 waarden in het bloed als een biomarker gebruikt konden worden voor het ontstaan van BOS. CD30 bevindt zich op bepaalde cellen van het afweersysteem: de Th2 cellen. Als deze Th2 cellen geactiveerd worden, wordt CD30 uitgescheiden in de bloedbaan. Er werd geen

verschil gezien in CD30 waarden na transplantatie tussen de patiënten die wel of geen BOS ontwikkelden. CD30 waarden na longtransplantatie kunnen niet gebruikt worden als marker om BOS te voorspellen. Wel zien we dat de hoge CD30 waarden voor longtransplantatie na een longtransplantatie onderdrukt worden. In **hoofdstuk 4** lieten we zien dat deze onderdrukking van CD30 voornamelijk door het gebruik van medicatie komt. We bestudeerden of TARC en CD30 waarden in het bloed gebruikt kunnen worden als objectieve biomarkers in patiënten met ernstig eczeem tijdens verschillende medicijnen welke ook in de longtransplantatie gebruikt worden: cyclosporine en EC-MPS. Er werd een correlatie gezien tussen sCD30 en ernst van het eczeem tijdens behandeling met cyclosprine maar niet tijdens EC-MPS therapie. TARC waarden daarentegen kunnen wel als marker van ziekteactiviteit van eczeem gebruikt worden tijdens zowel cyclosporine als EC-MPS behandeling.

In **hoofdstuk 3**, bestudeerden we of TARC waarden in het bloed gebruikt kunnen worden als voorspeller van BOS na longtransplantatie. TARC waarden trekken Th2 cellen aan. Er werd geen verschil in TARC waarden gezien tussen gezonden en patiënten. Bovendien zijn TARC waarden in het bloed voor transplantatie geen voorspeller voor het ontwikkelen van BOS na transplantatie. Noch een transplantatieprocedure, noch immuunsuppressiva zijn van invloed op TARC waarden daar er geen verschil tussen TARC waarden voor of na transplantatie werd gezien. Indien patiënten de eerste maand na transplantatie TARC waarden lager dan 325 pg/ml in het bloed hebben, hebben zij een grote kans op het ontwikkelen van BOS en is TARC een zeer vroege marker voor het voorspellen van BOS op de lange termijn.

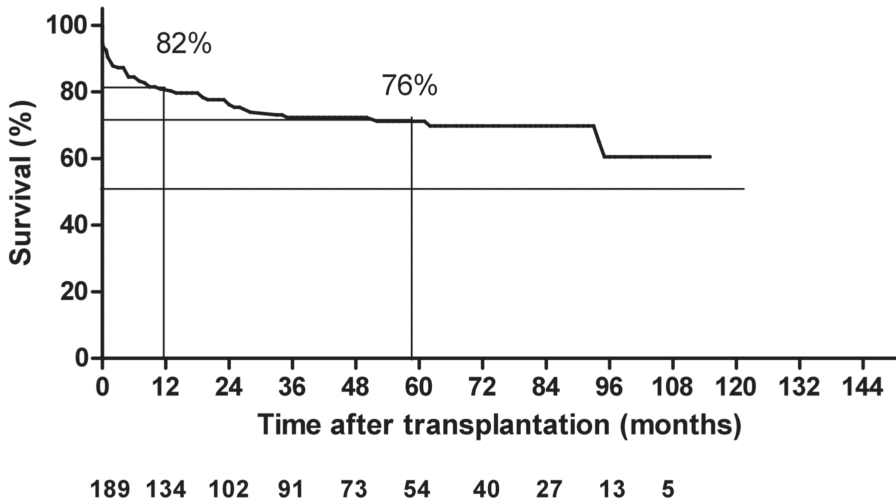
In **hoofdstuk 5**, bestudeerden we het effect van lage MBL waarden in het bloed op overleving na longtransplantatie, het ontwikkelen van BOS en het opkomen (reactiveren) van een CMV-infectie (virus). Een belangrijk deel van het aangeboren immuunsysteem is het complement systeem dat bestaat uit verschillende eiwitten en een daarvan is het Mannose Binding Lectin (MBL). Dit MBL kan lichaamsvreemde eiwitten zoals bacteriën en virussen opruimen. Mensen met een laag MBL voor transplantatie hadden meer CMV reactivaties na transplantaties maar geen grotere kans op het ontwikkelen van BOS. Wel leek er een tendens te bestaan tussen een laag MBL en een betere overleving na longtransplantatie.

In **hoofdstuk 6**, bestudeerden we het effect van het genetisch profiel van de receptoren van NK cellen (onderdeel van het aangeboren afweersysteem) van longtransplantatie patiënten en de liganden (aangrijpingspunten op de donorlongen) op het ontwikkelen van BOS. NK cellen zijn belangrijk in het opruimen van virussen. Op NK cellen zitten receptoren, de Killer Immunoglobulin-like Receptoren (KIR), welke uit activerende en inhiberende receptoren bestaan en die de functie van de NK cellen regelen. Er is een samenhang aangetoond tussen CMV-infecties (virus) en het ontwikkelen van BOS. In deze studie toonden we aan dat het gebrek aan activerende een rol speelt in het ontwikkelen van BOS.

Toekomst perspectieven.

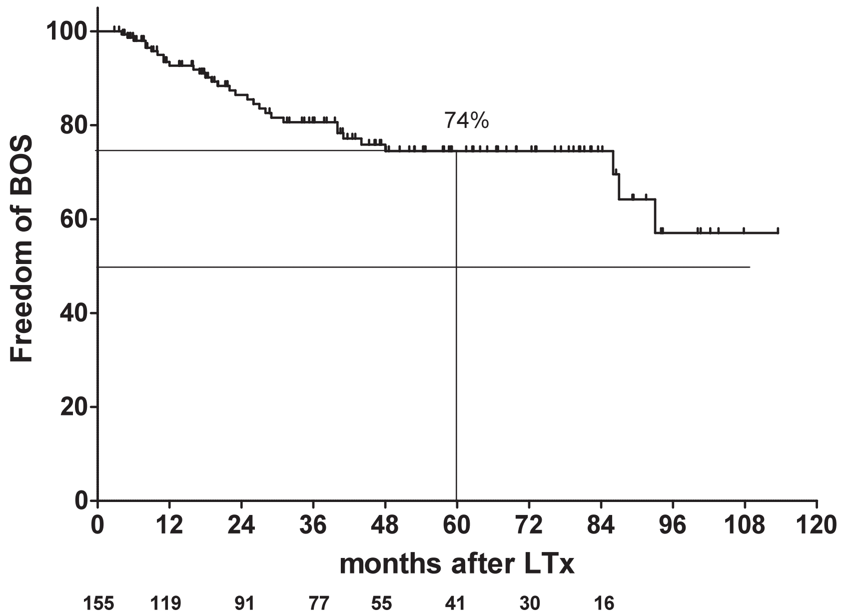
Het ontrafelen van de ontstaanswijze van BOS zal een uitdaging blijven voor iedereen die met longtransplantatie geconfronteerd wordt. Voor patiënten omdat de toekomst na BOS onzeker is: Zal het proces van afstoting doorgaan of stabiliseren? Voor artsen om meer inzicht te krijgen in ontstaanswijze, prognose en hopelijk interventie mogelijkheden. In hoofdstuk 1 is een overzicht gegeven van alle associaties die gepubliceerd zijn in de loop der jaren. Wat opvalt, is dat verschillende aspecten van het immuunsysteem van invloed zijn op de ontwikkeling van BOS en dat in deze studies ook verschillende medicatie regimes zijn gebruikt. In sommige studies werd na het stellen van de diagnose BOS de immuunsuppressieve medicatie aangeapst maar de mogelijke invloed van deze mediactie switch op eventuele otstaansmechanisme van BOS werd niet onderzocht.

Wanneer we kijken naar de eigen resultaten zien we dat onze 1 jaar overleving op 82% ligt, onze 5-jaars overleving op 72% en het zeer aannemelijk is dat de gemiddelde overleving na longtransplantatie 10 jaar of meer is (zie figuur 1). Voor chronische afstoting zien we dat na 5 jaar 74% van de patiënten nog geen aanwijzingen voor chronische afstoting heeft (zie figuur 2). Deze resultaten zijn vergeleken met de resultaten gepresenteerd door Christie en coschrijvers uitmuntend[1]. Mogelijk dat er een verschil in BOS definitie gebruikt is. Vanaudenaerde en coschrijvers heeft eerder aangetoond dat er een neutrofiel pathway is dat leidt tot longfunctiedaling en waarvan een deel van de patiënten een verbetering van de longfunctie laat zien na het starten van azithromycine[3]. Er is geopperd dat mogelijk statinen van invloed kunnen zijn op de ontwikkeling van



Figuur 1.

Overlevingscurve van het Longtransplantatiecentrum Utrecht/ Nieuwegein.



Figuur 2.

Curve die de vrijheid van BOS beschrijft in het Longtransplantatiecentrum Utrecht/Nieuwegein.

BOS[4]. Nieuw onderzoek laat zien dat ook montelukast, een leukotriëne receptor antagonist, een longfunctieverbetering laat zien[5]. Al deze middelen worden ook in onze patiëntencategorie toe gepast en mogelijk verklaart dit de uitstekende resultaten van dit relatief jong transplantatiecentrum. In ons transplantatiecentrum wordt BOS gedefinieerd als er sprake is van een irreversibel longfunctieverlies van 20% is.

Niet alleen een verschillend medicatie beleid maar ook het gebruik van extended donoren kan een rol spelen in de ontwikkeling van BOS. De kwaliteit van donororganen wordt niet meegenomen in onderzoek. Een andere mogelijke confounder is het groot aantal extended donoren. Ten gevolge van een orgaan tekort, is de wachtlijst voor een longtransplantatie (te) lang en worden veel patienten getransplanteerd vanuit een voorrangspositie (the high urgency list) en mogelijk hebben deze patienten een veranderde immuunrespons.

Ondanks deze resultaten blijven de overlevingscurven na longtransplantatie nog achter bij diverse orgaan transplantaties en is het probleem van chronische afstoting nog niet opgelost. Biomarkers die ons inzicht geven in pathogenese van het ontstaan van BOS en ons een behandelingsstrategie geven, bij voorkeur in een vroeg stadium en bij voorkeur patiëntvriendelijk, blijven noodzakelijk. Dit komt de behandeling van de patiënt ten goede, leidt tot betere resultaten en tevredenheid van zowel patiënt als dokter.

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CHAPTER 9

List of publications

List of Publications

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CHAPTER 10

Curriculum Vitae

Curriculum Vitae

De auteur van dit proefschrift werd geboren op 9 juni 1970, in Rosmalen. In 1990 behaalde zij haar eindexamen VWO aan het Maurick College in Vught. Hierna startte zij in 1990 met de studie Geneeskunde aan de Rijksuniversiteit van Groningen. Tijdens haar studie kreeg zij een Erasmusbeurs voor een chirurgische stage in Granada, Spanje en zowel een beurs van de Rijksuniversiteit Groningen als een internationaliseringbeurs voor een wetenschappelijke stage naar Infecties in Neonaten in het Universiteitsziekenhuis van Natal in Durban, Zuid Afrika. In 1998 behaalde zij haar artsexamen, waarna zij als arts-assistent Interne Geneeskunde ging werken in het Franciscus Ziekenhuis in Roosendaal en vervolgens het Groot Ziekengasthuis in Den Bosch. In 2000 startte zij in het Baronieziekenhuis de vooropleiding Interne Geneeskunde (opleider Dr. P.J. Stijnen). Van 2002 tot 2006 werd de opleiding longziekten gevolgd in het Rijnstate Ziekenhuis in Arnhem (opleider Dr. F.J.J. van den Elshout).Tijdens haar opleiding longziekten heeft zij samen met Eric Ullmann, longarts in het Rijnstate Ziekenhuis geparticipeerd in de Christopher studie. Vanaf 2006 werkt zij als longarts in het Universitair Medisch Centrum Utrecht. In deze periode werd gestart met het promotieonderzoek onder leiding van professor J.M.M. van den Bosch en professor J.W.J Lammers wat uiteindelijk heeft geleid tot dit proefschrift Zij is getrouwd met Erik Kwakkel en samen hebben zij twee dochters: Anneroos en Sophie.

CHAPTER 11

Dankwoord

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Hoewel een dankwoord nooit op wetenschappelijke inhoud of kwaliteit beoordeeld zal worden en ook nergens anders gepubliceerd zal worden, het blijft het eerst gelezen deel van een proefschrift. Dit geeft mij de mogelijkheid een aantal mensen in het bijzonder dank te zeggen. Zonder hun hulp was dit proefschrift er nooit gekomen! Dit is voor mij het bewijs van perfecte samenwerking in een goed team met geweldige supporters die met me meeleefden toen het even wat minder ging, me bleven aanmoedigen en nu warm meejuichen met het behaalde resultaat.

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