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Chronic rejection of a lung transplant is characterized by a profile of specific autoantibodies

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Summary

Obliterative bronchiolitis (OB) continues to be the major limitation to long-term survival after lung transplantation. The specific aetiology and pathogenesis of OB are not well understood. To explore the role of autoreactivity in OB, we spotted 751 different self molecules onto glass slides, and used these antigen microarrays to profile 48 human serum samples for immunoglobulin G (IgG) and IgM autoantibodies; 27 patients showed no or mild bronchiolitis obliterans syndrome (BOS; a clinical correlate of OB) and 15 patients showed medium to severe BOS. We now report that these BOS grades could be differentiated by a profile of autoantibodies binding to 28 proteins or their peptides. The informative autoantibody profile included down-regulation as well as up-regulation of both IgM and IgG specific reactivities. This profile was evaluated for robustness using a panel of six independent test patients. Analysis of the functions of the 28 informative self antigens showed that eight of them are connected in an interaction network involved in apoptosis and protein metabolism. Thus, a profile of autoantibodies may reflect pathological processes in the lung allograft, suggesting a role for autoimmunity in chronic rejection leading to OB.

Keywords: antigen microarray; apoptosis; autoantibodies; bronchiolitis obliterans syndrome; lung transplantation

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Introduction

Bronchiolitis obliterans syndrome (BOS) in lung transplant recipients is defined by progressive airflow obstruction. Its pathological correlate is inflammation and obliteration in small bronchioles, referred to as obliterative bronchiolitis (OB).[1] BOS is the most important determinant of long-term survival in lung transplant recipients[2,3] and develops in nearly all patients to varying degrees.[4] This progressive obstruction of airways results in decreasing lung function and may lead to complete respiratory failure. Currently, there is no well-documented

and established treatment to prevent or arrest the development of OB[5], and the 5-year survival rate is only around 50%.[4]

The specific aetiology and pathogenesis of OB are not well understood.[6] OB is thought to arise from repeated injury to the graft caused by, for example, acute rejection and lymphocytic bronchitis, leading to airway epithelial tissue damage and loss,[7,8] followed by an exaggerated healing response.[9] Little is known about the specific immune effectors or target molecules. A working hypothesis is that OB is an inflammatory process in which airway epithelial cells in the transplanted lung are activated by T cells and antibodies, up-regulating expression of adhesion, costimulatory, and major histocompatibility complex (MHC) class II molecules. The activated epithelial cells secrete various growth factors, chemokines and cytokines, which activate fibroproliferation and recruit inflammatory lymphocytes and granolycytes, resulting in tissue damage and repair as observed histologically.[10]

Autoimmunity is considered to be a potential mechanism in the development of BOS.[11,12] T-cell reactivity to type V collagen (COL5) located within the lung interstitium,[13,14] and antibodies to the epithelial-specific protein K-α1 tubulin (TUBA1B),[15] are present in a significant number of BOS patients. Furthermore, development of an alloimmune response in the lung has been shown to promote autoreactivity against COL5 and TUBA1B.[16]

Currently, the diagnosis of BOS is made by serial measurements of lung function; the demonstration of OB lesions by transbronchial biopsy is invasive, variable and insensitive. Thus, diagnosis, monitoring and treatment would be enhanced by the availability of a simple, non-invasive means to detect and follow BOS. Here we describe the use of an antigen microarray device coupled with an informatics analysis to detect an antibody profile that characterizes OB. We have applied to BOS an approach found to be useful in detecting informative profiles of autoantibodies in diabetes,[17] cancer[18,19] and multiple sclerosis.[20]

We covalently spotted 751 different self molecules onto coated glass slides, incubated these antigen microarrays with sera obtained from 48 patients at various stages of BOS, and detected the amounts of antibodies binding to the different antigen spots by laser illumination. We now report that a repertoire of autoantibodies can discriminate patients with no or low BOS from patients with medium or severe BOS. Finally, we demonstrate that many of the proteins bound by informative antibodies are organized in interaction networks with distinctive functional identities.

Materials and methods

Patient selection and study design

Patients were selected from the out-patient clinic at the Danish National Lung Transplant Programme and were in a stable clinical condition for at least 3 months before blood sampling. The transplant programme has been described in detail previously.[21] After approval by the Copenhagen Ethics Committee for Scientific Research, blood was sampled from 48 lung transplant patients who arrived on scheduled visits in the out-patient clinic during a half-year period. Blood samples were allowed to clot at room temperature and centrifuged, and the serum was stored at −20°. Clinical characteristics of the patient cohort are presented in Table S1. Nearly all patients (90%) received antihypertensive treatment during the study and most patients (78%) had received treatment for acute rejection during the first 2 years post transplant [A2 or higher according to the International Society for Heart and Lung Transplantation (ISHLT) grade]. All patients had experienced at least one episode of mild acute rejection grade A1 during the first 2 years. All patients had received at least one course of broad-spectrum antibiotics at some time after transplantation. Based on spirometry measurements of forced expiratory volume in 1 second (FEV1), each patient was regularly assigned a BOS grade (0, 0-p, 1, 2 or 3) ranging from no BOS (grade 0) to severe BOS (grade 3).[1]

Autoantibody profiling

Antigen microarrays were prepared as described previously.[17] Specifically, 751 different antigens, covering 272 recombinant proteins and synthetic peptides from the sequences of key proteins, as well as nucleotides, phospholipids and other self and non-self molecules, were spotted in replicates of three or four, and the microarrays blocked for 1 hr at 37° with 1% bovine serum albumin and incubated under a coverslip overnight at 4° with a 1 : 5 dilution of the test serum in blocking buffer – a volume of < 10 μl. The arrays were then washed and incubated for 1 hr at 37° with a 1 : 500 dilution of detection antibodies. Two detection antibodies were used: a goat anti-human IgG Cy3-conjugated antibody and a goat anti-human IgM Cy5-conjugated antibody (all purchased from Jackson ImmunoResearch Laboratories Inc., West Grove, PA, USA). Image acquisition by laser and storing were performed as previously described.[17] Antibody binding on the microarray was shown to be specific and significantly more sensitive than standard enzyme-linked immunosorbent assays (ELISAs).[20] Data preprocessing and quality control are described in detail in Data S1 and S2.

Identification and analysis of differentially reactive proteins

A group of six patients were set aside, to be used for later evaluation of the robustness of the results. For each antigen and detection antibody, differential reactivity between patients with BOS 0, 0-p or 1 (n = 27) and patients with BOS 2 or 3 (n = 15) was evaluated by calculating t-statistics

and *P*-values. Multiple testing was controlled using the false discovery rate (FDR) as implemented in the Benjamini and Hochberg step-up procedure.[22] Repertoires of antigens showing collective reactivity effects between groups of patients were evaluated using principal component analysis (PCA). Over-representation of concordant IgM and IgG reactivity changes (either both up or both down) among groups of antigens were identified by binomial testing.

Constructing a high-confidence network of human protein interactions

As described previously,[23] a human protein interaction network was created by pooling human interaction data from several of the largest databases and the coverage increased further by transferring data from model organisms. A network-wide confidence score for all interactions, based on network topology, experimental type and interaction reproducibility, was then established. The reliability of this score as a measure of interaction confidence was confirmed by fitting a calibration curve of the score against a high-confidence set of about 35 000 human interactions. All interactions with a confidence score above 0·154 were included, resulting in a network containing approximately 154 000 unique interactions between approximately 12 500 human proteins. Of the 272 proteins on the antigen microarray, 260 (96%) were among these 12 500 human proteins.

Significance, expansion and biological themes of networks

The statistical significance of the number of proteins in a network (the size) extracted from a given larger set of proteins was estimated by randomly selecting sets of proteins of the same size, each time recording the size of the largest network that it was possible to extract. For 10^6 such randomizations, the proportion of random sets of proteins for which equally sized or larger networks could be extracted establishes the *P*-value of the network extracted from the original protein set. For a given set *S* of proteins, any protein *p* not in this set is identified as preferentially interacting with proteins in *S*, if *p* has two or more interaction partners in *S*, and these constitute 15% or more of all its interaction partners. A network is expanded by including all preferentially interacting proteins. For all networks containing three or more proteins, over-represented biological processes were identified by hypergeometric testing of gene ontology terms.

Results

BOS development in patients

We collected sera from 48 patients at various stages of BOS and used antigen microarrays to profile IgG and IgM autoantibodies binding to the array of self antigens. Reactivity data from six patients were set aside as a test set and inspected only after analysis of the data from the other 42 patients had been completed. Fig. 1 shows the development of BOS in these 42 patients, from the time of transplantation to the time of serum sampling.

Antibody reactivities reflect BOS grade

Of the 751 antigens on the microarray, all antigens with IgG and/or IgM reactivity above noise level in at least four patients (504 and 610 antigens, respectively) were judged positive for antibody binding (see Data S2). Most of the positive antigens were detected by both IgG and IgM (473 out of the 504 and 610). Each detected antigen was tested for differential reactivity between patients with no or mild BOS (FEV1 not < 66% of baseline which equals grade 0, 0-p and 1) and patients with moderate or severe BOS (grade 2 and 3) using *t*-testing. This division of patients is clinically relevant as most patients develop BOS grade 1 within 10 years.[3]

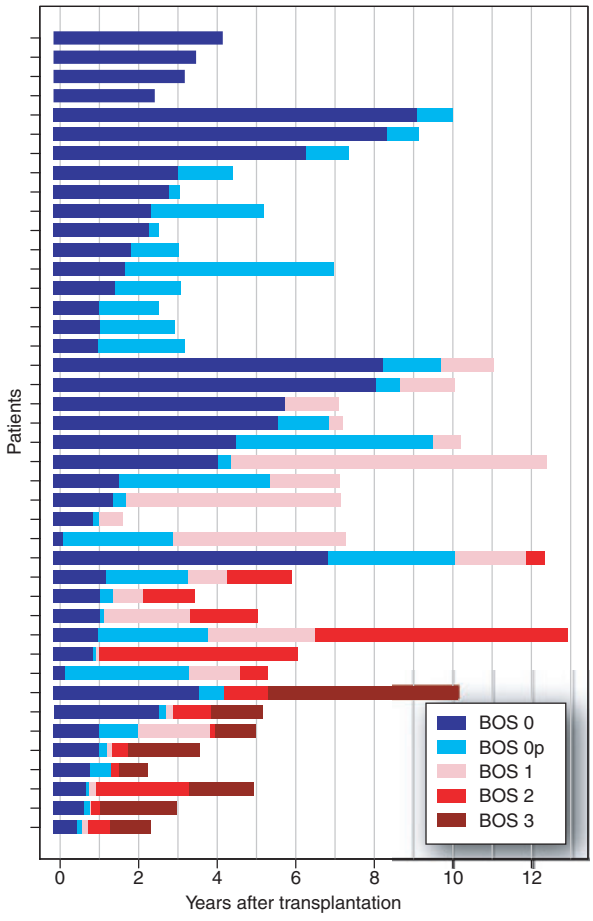

Figure 1. Bronchiolitis obliterans syndrome (BOS) progression. For each patient is shown the progression of BOS, from the time of transplantation to the time of blood sampling.

At a significance threshold of $P < 0{\cdot}001$ (corresponding to FDR < 0·05), we identified three antigens that bound IgG and 15 that bound IgM. All of these detected antigens are derived from different proteins, except two IgM detected antigens, which are different peptide sequences both from T-cell receptor gamma alternative reading frame protein (TARP). This selection is represented by the left arrow in Fig. 2. At this significance cut-off, none of the three antigens binding IgG were among those that bound IgM. In general, however, for those antigens that were bound by both IgG and IgM, the lower the *P*-values, the more likely it was that the IgG and IgM reactivities changed in the same direction (see Fig. S1). For $P < 0{\cdot}05$, 31 antigens, corresponding to 27 different proteins (as several peptides from the same protein were detected) were reactive with IgG and 140 antigens, corresponding to 78 different proteins, were reactive with IgM. Of the 27 proteins with significant IgG reactivities, 17 (63%) were also among the IgM reactive proteins, and 14 of these changed reactivity in the same direction, which is a significant concordance ($P < 0{\cdot}014$ by binomial testing). The significant concordance was observed for a wide range of *P*-value cut-offs (see Table S2), and therefore did not result from specifically choosing $P < 0{\cdot}05$. This observation indicates that there are small but widespread and mostly similar changes in both IgG and IgM reactivities that correlate with the progression of BOS. The 17 proteins with $P < 0{\cdot}05$ for both IgG and IgM reactivities were also selected for further analysis (Fig. 2, left arrow). As indicated in Fig. 2, six proteins were identified by both criteria. The 17 + 17 − 6 = 28 proteins identified in total are listed in Table 1. For each protein, the values listed are for the most significant antigen identified.

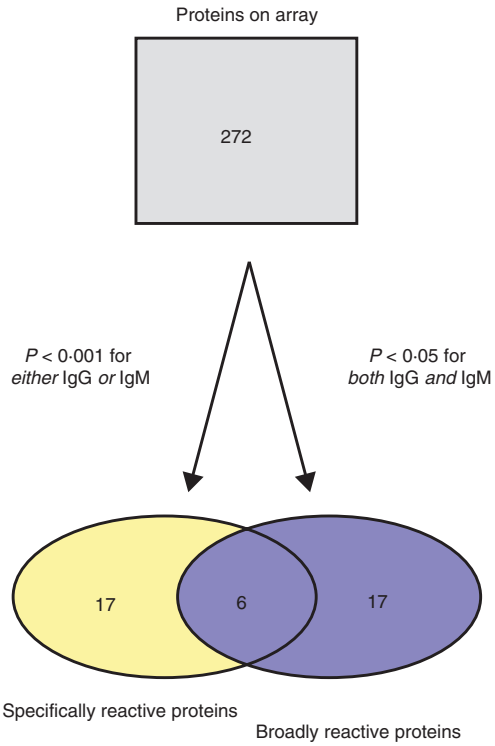

Figure 2. Analysis flowchart. Each of the 272 different proteins included on the array is tested for significant differential reactivity against immunoglobulin G (IgG) and IgM by *t*-testing. When a protein is represented by a series of synthetic peptides, each peptide is tested individually and the peptide achieving the lowest *P*-value is used to represent that protein. Two sets of proteins are selected on this basis: a set of proteins specifically reactive to either IgG or IgM with high significance (left arrow, yellow circle), and a set of proteins broadly reactive to both IgG and IgM although with lower significance (right arrow, blue circle). These two partly overlapping sets together represent the differentially reactive proteins identified by the analysis.

BOS profiles are related to specific protein interaction networks and biological processes

Mindful that most proteins interact in defined networks,[24] we analysed the known interactions among the 28 proteins displaying significant differential autoantibody reactivity (Table 1). This allowed us to examine whether the informative antigens formed networks with specific biological functions. Other large-scale data integrative methods have shown that well-defined interaction networks can often be functionally related to pathological processes and complex diseases.[23,25] For 27 of the 28 proteins, interaction data were available, and, indeed, we identified two interconnected networks consisting of two and eight proteins, respectively, the larger of which was significantly greater than would be expected by chance ($P < 0{\cdot}05$ as determined by randomly selecting 27 proteins out of the 260 proteins on the array where interaction data are available, recording the largest interconnected network that it was possible to construct from these, and repeating this 10^6 times). As observed for significant concordance, the significant network size was also seen for a wide range of *P*-value cut-offs (see Table S2). To expand on these observations, we included, in addition to the 28 differentially reactive molecules, 12 molecules that preferentially interact with these (see Materials and Methods). Only one of the additional molecules identified in this manner, TEP1, which interacts with protein kinase C alpha (PRKCA) and tumour protein p53 (TP53), was present on the antigen microarray; TEP1 bound IgG with threefold increased reactivity at a significance level ($P < 0{\cdot}002$) that was just above the cut-off criterion ($P < 0{\cdot}001$). The other 11 preferentially interacting molecules were not present on the antigen microarray and their reactivities are therefore unknown.

The expanded networks are shown in Fig. 3, and all 40 proteins are listed together in Table S3. As seen in Fig. 3, for the significant network consisting originally of eight proteins, eight preferentially interacting proteins were identified, allowing one additional differentially reactive

Table 1. Differentially reactive proteins

Abbreviation	IgG $\log_2$fc	IgM $\log_2$fc	IgG *P*	IgM *P*	IgG *n*	IgM *n*	EntrezID	Name
NTF3	0·45	1·00	0·10	0·00027	0	1	4908	Neurotrophin 3
CCL5	0·47	0·55	0·015	0·0018	1	1	6352	Chemokine (C-C motif) ligand 5
NPPB	0·59	0·76	0·094	0·00011	0	1	4879	Natriuretic peptide precursor B
TBX21	0·64	0·90	0·044	0·00068	2	2	30009	T-box 21
PRKCA	0·65	0·76	0·028	0·040	1	1	5578	Protein kinase C, alpha
JUN	0·74	1·23	0·032	0·0064	1	3	3725	jun oncogene
GATA3	0·77	0·69	0·0005	0·011	1	2	2625	GATA binding protein 3
NPPA	0·88	0·59	0·023	0·050	1	1	4878	Natriuretic peptide precursor A
GCG	1·22	0·70	0·006	0·036	1	1	2641	Glucagon
CXCL10	1·43	0·57	0·023	0·017	1	1	3627	Chemokine (C-X-C motif) ligand 10
IL-11	1·50	0·053	0·000050	0·79	1	0	3589	Interleukin-11
PLD3	1·73	0·31	0·00011	0·29	1	0	23646	Phospholipase D family, member 3
SSB	0·37	−1·03	0·59	0·00038	0	1	6741	Sjogren syndrome antigen B
IGF1R	0·97	−0·70	0·29	0·00029	0	1	3480	Insulin-like growth factor 1 receptor
CNTNAP1	−3·05	0·73	0·045	0·033	1	1	8506	Contactin-associated protein 1
HSPD1	−1·02	1·40	0·0039	0·0010	2	7	3329	Heat shock 60-kDa protein 1
FOXP3	−0·49	1·05	0·042	0·0053	1	6	50943	Forkhead box P3
CRYBB1	−0·69	−0·82	0·012	0·015	1	1	1414	Crystallin, beta B1
TNF	−0·51	−0·78	0·018	0·00066	2	3	7124	Tumour necrosis factor
TP53	−0·50	−0·52	0·035	0·0058	1	6	7157	Tumour protein p53
CASP3	−0·48	−1·27	0·036	0·00020	1	1	836	Caspase 3
TARP	−0·36	−0·87	0·0060	0·000019	1	3	445347	T-cell receptor gamma alternative reading frame protein
CYP3A43	−0·36	−0·87	0·071	0·00065	0	1	64816	Cytochrome P450, family 3A43
GAD2	−0·34	−0·65	0·089	0·00064	0	1	2572	Glutamate decarboxylase 2
CASP8	−0·34	−0·96	0·065	0·00032	0	1	841	Caspase 8
HSP90AA1	−0·31	−0·76	0·041	0·0029	2	7	3320	Heat shock protein 90-kDa AA1
EEF1A1		2·14		0·000023	0	1	1915	Eukaryotic translation elongation factor 1A1
SNCG		2·55		0·00039	0	1	6623	Synuclein gamma

For all significantly differentially reactive antigens identified, their corresponding proteins are listed. When several antigens relating to the same protein are significant [listed in the number of antigens (*n*) columns], the most significant antigen is selected to represent that protein [in the $\log_2$ fold-change (fc) and *P*-value columns]. For those antigens that are not detected by immunoglobulin G (IgG) or IgM, no fold-changes or *P*-values are listed.

protein, jun oncogene (JUN), to be included in the network through its interaction with VRK1. Similarly, chemokine (C-X-C motif) ligand 10 (CXCL10) and chemokine (C-C motif) ligand 5 (CCL5) both preferentially interact with chemokine (C-X-C motif) receptor 3 (CXCR3). And finally, for natriuretic peptide precursors A and B (NPPA and NPPB), we identified three preferentially interacting proteins, natriuretic peptide receptors A, B, and C (NPR1, NPR2 and NPR3), which are the receptors on which NPPA and NPPB act. This network expansion thus allows an identification of the networks through which the differentially reactive proteins exert their function that is not limited to the proteins present on the antigen microarray. A similar approach has been used for investigating dynamic complex formation during the yeast cell cycle, by identifying constitutively expressed proteins that preferentially interact with periodically expressed ones.[26] Also shown in Fig. 3 are the results of hypergeometric testing of the gene ontology biological process terms assigned to the proteins in each network. The largest network is involved in regulation of apoptosis ($P < 810^{-5}$) and more generally cellular protein metabolism ($P < 0{\cdot}0003$). The network with five proteins is concerned with regulation of blood pressure ($P < 310^{-12}$), and finally there is a small chemotaxis-related network ($P < 210^{-6}$).

Autoantibody patterns discriminate between BOS grades

To visualize the relations between patients, PCA was used to identify the three-dimensional subspace that captures most of the variance among the differentially reactive antigens whose proteins are listed in Table 1. Figure 4a shows the 42 patients used to identify these significant antigens, and Fig. 4b show the six patients who were not included in the analysis projected onto the same principal components identified in Fig. 4a. As can be seen, the six patients could be linearly separated into two groups that specifically reflect their BOS grade.

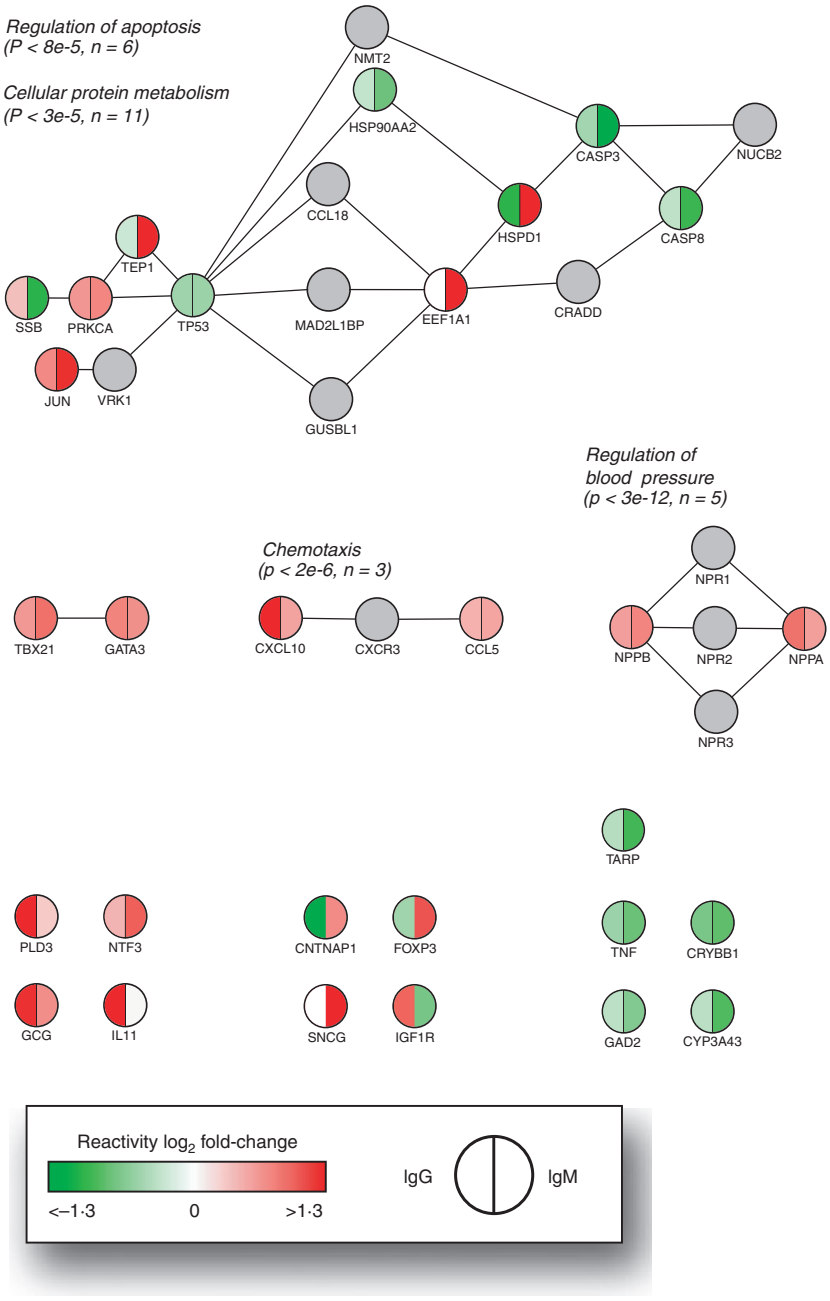

Figure 3. Bronchiolitis obliterans syndrome (BOS) networks. Interaction networks of the differentially reactive proteins and the proteins that preferentially interact with them are shown. Biological themes, summarizing over-represented biological processes, for the three largest networks are indicated. Refer to Table 1 for gene names.

Discussion

This study demonstrates that lung transplant recipients show widespread IgG and IgM autoantibody reactivities and that specific patterns of reactivity to self antigens discriminate between patients with greater and lesser degrees of BOS. Indeed, at our chosen significance cut-off, approximately 10% of all proteins included on the microarray (28 out of 272) displayed significant, usually concordant (21 out of 28) differential autoantibody binding (Table 1). The robustness of these results is further demonstrated by the fact that they can linearly separate a small panel of test patients according to their BOS grade (Fig. 4b).

Note that IgG and IgM reactivities were often seen to change in the same direction when comparing no or mild BOS with medium or severe BOS (Fig. S1). The natural repertoire of autoantibodies present in healthy newborns and their mothers is also characterized by both IgG and IgM reactivities to common sets of self molecules;[27] thus the development of OB does not appear to perturb this basic relationship between IgG and IgM, although these different antibody isotypes express different biological functions and have markedly different half-lives.

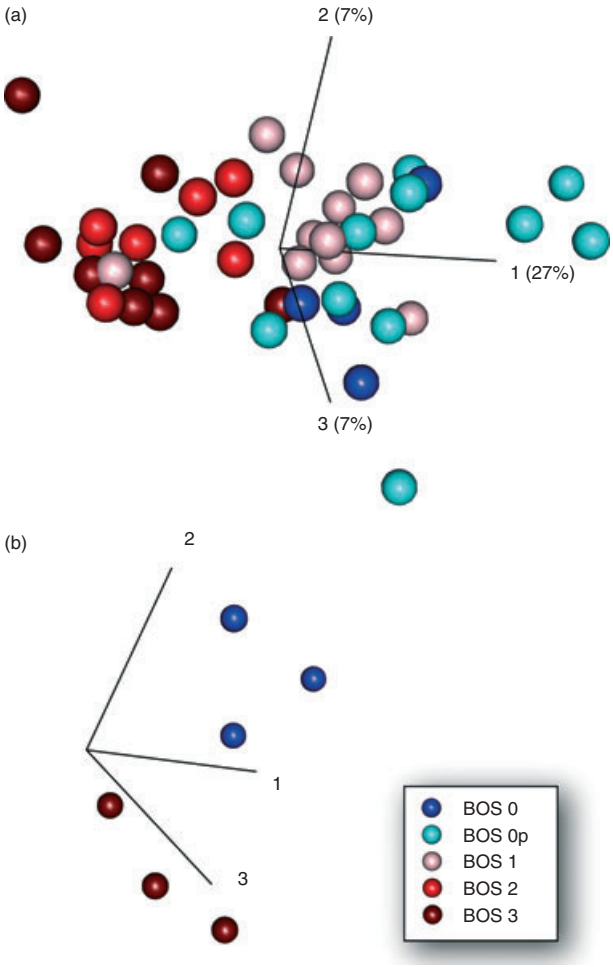

Figure 4. Principal component analysis (PCA) of patients using antigens with significant reactivity patterns. (a) PCA for the 42 patients used to identify differentially reactive antigens. (b) A projection of the six patients set aside to evaluate the robustness of results onto the principal components identified in (a).

We extended the biological meaning of the profile of reactivities by integrating information about interactions among the target antigens with their known functions. We extracted 28 proteins that could be organized into several networks with distinct biological profiles (Fig. 3). The largest of these protein networks is related to regulation of apoptosis and more generally to cellular protein metabolism.

Apoptosis, or programmed cell death, plays a major role in the development and maintenance of multicellular organisms.[28] The apoptotic programme is present in all eukaryotic cells, and is characterized by nuclear DNA degradation, nuclear degeneration and condensation, and overall alteration in cell morphology. Apoptosis can be triggered by an absence of appropriate survival signals as well as by external stimuli.

It has previously been established that apoptosis, detected by DNA breaks *in situ* [TdT-mediated biotin–dUTP nick-end labelling (TUNEL) assay] in transbronchial biopsies, specifically bronchi, bronchioli and alveolar epithelial cells as well as alveolar macrophages, is a feature of the pathophysiology of active OB.[29] Apoptosis has also been observed in mouse heterotopic airway transplant models displaying OB-like symptoms.[30] Finally, suggesting a possible mechanism, apoptosis is observed in mouse models developing OB-like disease as a consequence of administering anti-human leucocyte antigen (HLA) class I antibodies.[31]

All pathways inducing apoptosis activate a caspase cascade, concluding with a caspase-activated DNAse entering the nucleus and cleaving DNA.[32] We observed reduced IgG and IgM reactivity to the initiator caspase, CASP8, as well as the effector caspase, CASP3, which is cleaved and activated by CASP8. Activation of these caspases may be mediated through the activation of FAS and its ligand, which are not present on the antigen microarray, or through activation of tumour necrosis factor (TNF) receptor type 1 by its ligand, TNF, against which we also observed reduced IgG and IgM reactivity (see Fig. 3 and Table 1).

Several other proteins known to be able to regulate and direct the apoptotic response, such as TP53 (p53), heat shock 60-kDa protein 1 (HSPD1; HSP60) and HSP90AA1, are also part of the identified network. Both TP53 and HSP90AA1 display reduced IgG and IgM reactivity. HSPD1 displays reduced IgG reactivity but increased IgM reactivity, perhaps reflecting its complex function. In addition to its role in folding and assembling newly imported proteins, it may function as a signalling molecule in the innate immune system. Depending on the context, HSPD1 has been reported to have either pro-survival or pro-apoptotic functions, which involve differential interactions with CASP3.[33] Interestingly, cellular autoreactivity against HSPD1 has also been demonstrated in peripheral blood and graft-infiltrating lymphocytes following renal transplantation.[34]

Note that the profile of apoptosis molecules was characterized by significant down-regulation of the natural autoantibody reactivities (caspases 3 and 8, TP53 and HSP90AA2) as well as by up-regulated autoantibody reactivities [JUN, PRKCA and eukaryotic translation elongation factor 1A1 (EEF1A1)] and by opposing directions of IgG and IgM reactivity changes [TEP1, HSPD1 and Sjogren syndrome antigen B (SSB)]. Thus, pathological inflammation can be associated with both increased autoreactivity to some self antigens and decreased autoreactivity to other self antigens in the same functional network. Natural autoantibodies to various self antigens are present in healthy newborn infants,[27] and hence are likely to be beneficial.[35] Such natural autoreactivity – the immunological homunculus[36,37] – would appear to be lost in pathological inflammation. Be that as it may, the present study indicates that part of the OB response is manifested as differential B-cell autoreactivity, both up-regulation

and down-regulation, to a specific apoptosis-related network of proteins.

In addition to the apoptosis network, we also identified a network involved in regulation of blood pressure. Two proteins, NPPA and NPPB, displayed increased IgG and IgM reactivity. Paralleling this, it has been reported that antibodies directed against angiotensin II type 1 receptor, mediating the effect of the vasoconstrictor hormone angiotensin II, and also involved in regulation of blood pressure, contribute to vascular rejection of kidney allografts.[38] Angiotensin II is produced from angiotensin I through removal of two terminal residues by the angiotensin-converting enzyme (ACE), which is found predominantly in the capillaries of the lung.[39] Blocking ACE induces potent regulatory T cells and modulates T helper type 1 (Th1)- and Th17-mediated autoimmunity.[40] Although disparate, these different lines of investigation suggest an intimate connection between autoimmunity, components involved in regulation of blood pressure, and organ rejection. As a final example, we also observed increased IgG and IgM reactivity to both T-box 21 (TBX21) and GATA binding protein 3 (GATA3). The differentiation of T helper progenitors to Th1 or Th2 effector cells requires the action of these two opposing transcription factors, TBX21 being the Th1-cell master switch, and GATA3 the Th2-cell master switch. The activity of GATA3 is inhibited by TBX21, which can block the binding region for the GATA3 target DNA30.[41] Changes in the Th1–Th2 balance have been associated with OB,[42] and, as seen here, differential autoreactivity may be involved.

The above conclusions rest on several layers of statistical testing and significance: (1) significance at the level of protein selection, (2) significance at the level of networks, and (3) significance at the level of the biological processes represented in the networks. It is consequently highly unlikely that these or similar conclusions could have been reached by chance ($P < 0{\cdot}036$ for the apoptosis network by 10^4 random samplings). Nevertheless, there are several important limitations to the methodology of this study, which must be addressed in the future. The antigen microarray we used only screened for a small fraction, perhaps as little as 1%, of all the proteins constituting the lung proteome. Therefore, we may have missed a lot of important information. Follow-up, prospective studies are needed to confirm our findings. Moreover, the findings of informative reactivities and networks do not imply causality. Autoreactivity towards apoptosis is involved in BOS, but we cannot say whether it is a casual factor or merely a result of the process. Further studies are needed to elucidate how the reactive proteins themselves behave functionally with respect to the pathogenesis of BOS.

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Disclosures

The authors have no financial conflicts of interest.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. Concordance between immunoglobulin G (IgG) and IgM reactivity changes.

Table S1. Clinical characteristics of patients.

Table S2. Cut-off screening.

Table S3. Expanded protein set.

Data S1. Raw and processed data are available at http://www.nanotech.dtu.dk/Research/Theory/Stochastic.aspx.

Data S2. Data preprocessing protocol and scheme for quality control.

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