ORIGIN AND PATHOGENICITY OF
GM-CSF AUTOANTIBODIES IN PATIENTS WITH
PULMONARY ALVEOLAR PROTEINOSIS

A thesis submitted to attain the degree of
DOCTOR OF SCIENCES of ETH ZURICH
(Dr. sc. ETH Zurich)
presented by

LUCA PICCOLI

2nd Cycle/Graduate Degree in Pharmaceutical and Medical Biotechnologies,
University of Pavia, Italy

born on 12.07.1984
citizen of Italy

accepted on the recommendation of

Prof. Dr. Antonio Lanzavecchia, examiner
Dr. Federica Sallusto, co-examiner
Prof. Dr. Manfred Kopf, co-examiner

2015
# Table of contents

1. Acknowledgments ........................................................................................................................................... 5

2. Abstract .......................................................................................................................................................... 7
   2.1. Summary ................................................................................................................................................... 7
   2.2. Riassunto .................................................................................................................................................. 8

3. General introduction ........................................................................................................................................ 9
   3.1. Generation of antibody diversity ............................................................................................................ 9
       3.1.1. Antibody structure .......................................................................................................................... 10
       3.1.2. V(D)J recombination ....................................................................................................................... 13
       3.1.3. BCR diversity in the pre-immune antibody repertoire ................................................................... 14
   3.2. The antibody response ............................................................................................................................ 15
       3.2.1. T-independent responses ................................................................................................................ 15
       3.2.2. T-dependent responses .................................................................................................................... 16
       3.2.3. The germinal center reaction: somatic mutations and class switch .............................................. 17
       3.2.4. BCR diversity in the post-immune antibody repertoire .................................................................. 20
   3.3. Immunologic tolerance and fate of autoreactive B cells ........................................................................ 21
       3.3.1. Mechanisms of tolerance induction in developing B cells .............................................................. 21
       3.3.2. The fate of autoreactive B cells in germinal centers ...................................................................... 23
   3.4. Autoantibodies and autoimmune diseases .............................................................................................. 26
       3.4.1. Origin of autoantibodies .................................................................................................................. 26
       3.4.2. Mechanisms of induction of autoantibody production .................................................................. 27
       3.4.3. Specificity ......................................................................................................................................... 31
       3.4.4. Pathogenicity .................................................................................................................................... 33
       3.4.5. Cytokine autoantibodies ................................................................................................................ 35
   3.5. GM-CSF autoantibodies .......................................................................................................................... 40
       3.5.1. GM-CSF and GM-CSF receptor ...................................................................................................... 40
       3.5.2. Autoimmune pulmonary alveolar proteinosis ................................................................................. 44
       3.5.3. Pathogenic and non-pathogenic GM-CSF autoantibodies .............................................................. 47
<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>3.6. Autoantibodies to citrullinated protein antigens</td>
<td>49</td>
</tr>
<tr>
<td>3.6.1. Rheumatoid arthritis</td>
<td>49</td>
</tr>
<tr>
<td>3.6.2. PAD enzymes and citrullination</td>
<td>50</td>
</tr>
<tr>
<td>3.6.3. Etiopathogenic model for rheumatoid arthritis</td>
<td>52</td>
</tr>
<tr>
<td>4. Research plan</td>
<td>54</td>
</tr>
<tr>
<td>4.1. Aims of the research</td>
<td>54</td>
</tr>
<tr>
<td>4.2. Experimental approach</td>
<td>55</td>
</tr>
<tr>
<td>5. Results and discussion</td>
<td>56</td>
</tr>
<tr>
<td>5.1. Neutralization and clearance of GM-CSF by autoantibodies in pulmonary alveolar proteinosis</td>
<td>56</td>
</tr>
<tr>
<td>5.1.1. Abstract</td>
<td>57</td>
</tr>
<tr>
<td>5.1.2. Introduction</td>
<td>58</td>
</tr>
<tr>
<td>5.1.3. Methods</td>
<td>60</td>
</tr>
<tr>
<td>5.1.4. Results</td>
<td>64</td>
</tr>
<tr>
<td>5.1.5. Figures and tables</td>
<td>69</td>
</tr>
<tr>
<td>5.1.6. Discussion</td>
<td>80</td>
</tr>
<tr>
<td>5.2. Somatic mutations play a crucial role in the generation of autoantibodies binding to GM-CSF and citrullinated vimentin</td>
<td>83</td>
</tr>
<tr>
<td>5.2.1. Abstract</td>
<td>83</td>
</tr>
<tr>
<td>5.2.2. Introduction</td>
<td>83</td>
</tr>
<tr>
<td>5.2.3. Methods</td>
<td>85</td>
</tr>
<tr>
<td>5.2.4. Results</td>
<td>86</td>
</tr>
<tr>
<td>5.2.5. Figures and tables</td>
<td>88</td>
</tr>
<tr>
<td>5.2.6. Discussion</td>
<td>94</td>
</tr>
<tr>
<td>6. Concluding remarks and future perspectives</td>
<td>97</td>
</tr>
<tr>
<td>7. References</td>
<td>99</td>
</tr>
<tr>
<td>8. Appendix</td>
<td>122</td>
</tr>
<tr>
<td>8.1. List of abbreviations</td>
<td>122</td>
</tr>
<tr>
<td>8.2. Curriculum vitae</td>
<td>126</td>
</tr>
<tr>
<td>8.3. List of relevant publications</td>
<td>127</td>
</tr>
</tbody>
</table>
1. Acknowledgments

Reaching the completion of my PhD thesis would have not been possible without the support of my colleagues, sponsor, family and friends. Therefore, I would like to thank the people that have helped me during my doctoral studies.

First, I want to thank Antonio Lanzavecchia, my supervisor, for giving me the opportunity to do my PhD in his research group, for trusting in my capabilities and letting me work on very interesting projects. His passion for science is an inspiration for any student or scientist and I want to thank him for the great scientific discussions that helped me to grow as a researcher and to become more independent.

I wish to thank Federica Sallusto and Manfred Kopf for being part of my thesis committee and positively supporting the completion of my doctoral studies.

I’d like to give special thanks to Davide Corti who has helped me a lot during my PhD by always providing good suggestions for my experiments and constructively discussing my ideas and results.

I want to thank Cem Gabay from the Division of Rheumatology of the University Hospitals of Geneva and Maurizio Luisetti and Ilaria Campo from the Respiratory Disease Unit of the IRCCS San Matteo Hospital Foundation of Pavia for providing blood samples for my research.

I am thankful to Fondazione Aduvare for funding my research in these years, thus making it possible for me to complete my studies.

I’d like to thank my lab mates, in particular, Chiara Silacci Fregni, Blanca Maria Fernandez Rodriguez and Andrea Minola for patiently training me to the good lab practice in the first months after my arrival at IRB and for contributing to the results of my thesis project. Thanks also to the other members of our lab, Isabella Giacchetto-Sasselli, Debora Pinna, Sandra Jovic and Valentina Gilardi for their help and support.
A special thanks goes to Kathrin Pieper for the constructive discussions and suggestions during my thesis writing. I want also to thank Gloria Agatic, Laurent Perez, Luca Varani and Mattia Pedotti for their theoretical and technical support and Leontios Pappas for his collaboration that helped the both of us to obtain useful results for our PhD theses.

Finally, I want to thank my family and friends:

Vorrei fare un ringraziamento speciale ai miei genitori Mariella e Cesare, a mia sorella Silvia e ai miei nonni Gianna e Tino per aver sempre creduto nelle mie capacità e per avermi sostenuto anche in quest’ultimo percorso di studi.

Un particolare grazie va a Daniel, Elena, Vittorio, Gloria, Edoardo, Caterina, Christian, Daniele, Elisabetta, Massimiliano e Marco per avermi sempre incoraggiato e aver apprezzato i risultati che ho raggiunto in questi anni.

Last but not least, I want to thank my friends Julia, Daniela and Lorenzo for the great company and all the fun moments we have spent together in Bellinzona.

Bellinzona, January 2015

Luca Piccoli
2. Abstract

2.1. Summary

A fundamental property of the adaptive immune system is the ability to generate an extraordinarily large number of different receptors. In particular, antibody diversity is generated at two distinct stages of B cell development: in bone marrow by rearrangement of the VDJ genes and in germinal centers by somatic mutations. Both processes have the potential to generate self-reactive B cells that, when activated, can produce autoantibodies and, depending on their specificity, cause an autoimmune disease.

The aim of my thesis is to understand the pathogenesis of autoimmune pulmonary alveolar proteinosis (PAP) and the origin of autoantibodies to granulocyte-macrophage colony stimulating factor (GM-CSF) that cause this severe autoimmune disease. By immortalizing memory B cells, I isolated a panel of anti-GM-CSF monoclonal autoantibodies from PAP patients and identified the epitopes recognized. By measuring GM-CSF neutralization, I found that three non-cross-competing autoantibodies sequester GM-CSF in stable immune complexes and completely neutralize the cytokine activity in vitro, while in vivo the same immune complexes are rapidly cleared in a Fc-dependent fashion. In contrast, single autoantibodies could only partially neutralize GM-CSF activity in vitro, depending on the stoichiometry of the assay, and enhanced the levels of bioavailable GM-CSF in vivo. These findings provide a plausible explanation for the severe phenotype of PAP patients that developed high levels of GM-CSF autoantibodies to multiple epitopes of the molecule forming immune complexes that mediate GM-CSF sequestration and degradation.

To investigate the origin of autoantibodies, I removed somatic mutations from seven anti-GM-CSF antibodies to produce the “unmutated common ancestor” (UCA), i.e. the antibody produced by the naïve B cell that gave origin to the autoantibody. In 5 out 7 cases, the UCA of anti-GM-CSF antibodies were not able to bind to GM-CSF, while in the remaining 2 cases there was a very low but still detectable binding. Similarly, the UCA of a high-affinity autoantibody isolated from a patient with rheumatoid arthritis and specific for citrullinated vimentin failed to bind to the self antigen. In contrast, the UCA of anti-influenza hemagglutinin (HA) antibodies bound HA with similar or only slightly reduced affinity. Taken together, these findings are consistent with a model where self-reactivity of B cells is acquired through somatic mutations during the response to a foreign antigen.
2.2. Riassunto

Una caratteristica fondamentale del sistema immunitario adattivo è la sua capacità di generare un grandissimo numero di diversi recettori. In particolare, la diversità anticorpale è generata in due distinti stadi dello sviluppo dei linfociti B: nel midollo osseo tramite il riarrangiamento dei geni VDJ e nei centri germinativi tramite mutazioni somatiche. Entrambi i processi possono generare cellule B autoreattive che, se attivate, producono autoanticorpi e, a seconda della loro specificità, causano una malattia autoimmune.


Per studiare l’origine degli autoanticorpi, ho rimosso le mutazioni somatiche da sette anticorpi anti-GM-CSF per produrre il cosiddetto “unmutated common ancestor” (UCA), cioè l’anticorpo prodotto dalla cellula B naïve che ha dato origine all’autoanticorpo. In 5 casi su 7, l’UCA degli anticorpi anti-GM-CSF non era in grado di legare il GM-CSF, mentre negli altri 2 casi c’era ancora un legame molto ridotto, ma misurabile. Analogamente, l’UCA di un autoanticorpo ad alta affinità isolato da un paziente con artrite reumatoide e specifico per la vimentina citrullinata non è riuscito a legare l’autoantigene. Invece, gli UCA degli anticorpi contro l’emagglutinina (HA) del virus dell’influenza legavano l’HA con affinità simile o solo leggermente ridotta. Nel loro insieme, questi risultati sono in accordo con un modello nel quale le cellule B diventano autoreattive acquisendo mutazioni somatiche durante la risposta ad un antigene estraneo.
3. General introduction

In the next paragraphs I will introduce the principles of immunology and the main findings related to the issue and the aims of my thesis that deals with the origin and the pathogenicity of autoantibodies to GM-CSF and to citrullinated proteins. I will start by introducing the adaptive immune system and its peculiarity to generate a high antibody diversity. Then I will describe the antibody response and the role of somatic mutations to increase the antibody diversity. I will continue by introducing the problem of self-reactivity arisen from antibody diversification analyzing the mechanisms that induce self tolerance in B cells and the fate of autoreactive B cells. Furthermore, I will analyze the origin of autoantibodies and the mechanisms that induce their production, followed by a description of the specificity and of the pathogenicity of autoantibodies, especially of cytokine autoantibodies. Finally I will describe in depth autoantibodies to GM-CSF and to citrullinated proteins.

3.1. Generation of antibody diversity

One of the main properties of the adaptive immune system is the ability to generate an extraordinarily large number of different receptors, named B-cell receptors (BCRs) on B lymphocytes and T-cell receptors (TCRs) on T lymphocytes. According to the theory of clonal selection, an individual lymphocyte expresses membrane receptors that are specific for a distinct antigen and differentiated effector cells derived from an activated lymphocyte will bear receptors of identical specificity as the parental cell (Burnet 1976). In addition, the activated B lymphocytes are able to produce a soluble form of the BCR, named antibody.

The unique receptor specificity is determined before the lymphocyte is exposed to the antigen through a process of gene recombination, called V(D)J rearrangement (Brack 1978; Bernard 1978).

In the next paragraphs I will first describe the antibody structure followed by the mechanisms that generate diversity in the antibody repertoire.
3. General introduction

3.1.1. Antibody structure

An antibody or immunoglobulin (Ig) can be expressed in a secreted form or bound to the membrane of B cells to form the BCR. A functional immunoglobulin consists of two identical heavy (H) chains and two identical light (L) chains. Both H and L chains contain a series of domains that fold independently in a globular motif (Ig domain), consisting of two layers of β-pleated sheets held together by a disulfide bridge. Each chain contains one N-terminal variable (V) domain and one or more C-terminal constant (C) domains (one in the L chain contains and 3 or 4 in the H chain). In the BCR, the C-terminal part of the H chains contains an extra membrane-spanning domain that associates with the Igα/Igβ heterodimer to transduce signal intracellularly (DeFranco 1993). According to common determinants, termed isotypes, specific for the C region, Ig can be divided into distinct classes: 5 isotypes (IgD, IgM, IgG, IgA and IgE) for the H chain and 2 isotypes (κ and λ) for the L chain. H and L chains are covalently linked by disulfide bonds between cystein residues in the C-terminus of the L chain and CH1 domain of the H chain, by non-covalent interactions between VL and VH domains and between CL and CH domains. Disulfide bonds between the two H chains and other non-covalent interactions contribute to H chain pairing. Digestion of an Ig with the proteolytic enzyme papain generates three fragments: two Fab (fragment antigen binding) fragments that have antigen-binding ability and one Fc (fragment, crystallizable) with no antigen-binding ability. The V region is responsible for antigen recognition, while the C region mediates several effector functions of Igs such as activation of the complement system and binding of IgG and IgE to Fc receptors (FcR) (Ravetch 1991). Each Ig has two antigen-binding sites, each formed by the V region of the H chain together with the V region of the L chain. The primary sequence of the V domain is functionally divided into 3 hypervariable intervals termed complementary-determining regions (CDRs) that are situated between 4 regions of stable sequence termed framework regions (FRs). The hypervariable regions form a surface that is complementary to the 3D structure of the bound antigen. The CDR3 is the most variable segment and is responsible for the most extensive contact with a bound antigen (Schroeder H. W., Jr. 2010) (Figure 3.1.1).
The quality of the interaction between an antibody and an antigen can be expressed in terms of **specificity and affinity** of the antibody for the antigen. The specificity of an antibody is its ability to discriminate between two different epitopes, recognizing only one epitope. Nevertheless, one antibody can also react with similar epitopes on different antigens. This antibody property is defined cross-reactivity.

The affinity can be defined as the strength of the noncovalent interactions between a single antigen-binding site on an antibody and a single epitope of the antigen. Low-affinity antibodies bind antigen weakly and tend to dissociate readily, whereas high-affinity antibodies bind antigen more tightly and remain bound longer (Azimzadeh 1990).

The kinetics of the interaction between an antibody (Ab) and an antigen (Ag) is characterized by an association phase, followed by an equilibrium phase and a dissociation phase. The association phase (Ag + Ab → Ag-Ab) is driven by the association rate constant $k_a$, while the dissociation phase (Ag-Ab → Ag + Ab) is driven by the dissociation rate constant $k_d$. The ratio $k_a/k_d$ is the association constant $K_A$ measured at equilibrium of reaction and is a measure of the affinity. The reciprocal of $K_A$ is $K_D (k_d/k_a)$, the equilibrium constant of dissociation and can be used to describe the Ag-Ab interaction. Low-affinity antibodies bind to the antigen slowly (low $k_a$) and dissociate quickly (high $k_d$, very high $K_D$), whereas high-affinity antibodies bind to the antigen quickly (high $k_a$) and dissociate slowly (low $k_d$, very low $K_D$).

Nevertheless, the affinity does not reflect the real strength of the Ag-Ab interaction. Considering that an antibody has more than one antigen-binding site (2 for IgG, IgE and
monomeric IgA, 10 for IgM), in presence of an antigen containing multiple repeating epitopes the interaction of an antibody with an antigen at one antigenic-binding site will increase the probability of reaction between these two molecules at the second antigenic-binding site. The strength of such multiple interactions between a multivalent antibody and an antigen is called avidity (Azimzadeh 1990).

The Ag-Ab interaction can be studied with various techniques, including enzyme-linked immunosorbent assay (ELISA), western blot and immunoprecipitation. In particular, the kinetic constants that characterize the affinity of an antibody can be measured with high accuracy by a sensitive technique, called surface plasmon resonance (SPR) (Malmqvist 1993) (Figure 3.1.2).

![Surface plasmon resonance (SPR)](image)

**Figure 3.1.2.** Analysis of Ag-Ab interaction by SPR. (A) Principle of SPR technique. A beam of polarized light is directed through a prism onto a thin gold film on which an antibody is immobilized and is reflected off the gold film towards a light-collecting sensor. At a unique angle some incident light is absorbed by the gold layer and its energy is transformed into charged waves called surface plasmons. A sharp dip in the reflected light intensity, named resonant angle, can be measured at that angle. (B) Kinetics of Ag-Ab interaction. When the antigen flows on top of the gold layer and binds to the antibody, this produces a detectable change in the resonant angle and the rates of association and dissociation can be measured.
3. General introduction

3.1.2. V(D)J recombination

During B-cell development in the bone marrow, the different BCRs are generated by a complex series of gene rearrangement events, named V(D)J recombination, that assembles three different gene segments, the variable (V), the diversity (D) and joining (J), into unique functional BCR gene (Figure 3.1.3). Each V gene segment encodes for the first 3 FRs (FR1, FR2 and FR3), CDR1, CDR2 and the N-terminal portion of CDR3, while the J gene segment encodes for the C-terminal region of CDR3 and the complete FR4. The D gene is present only in the H chain locus end encodes for the middle part of CDR3. The creation of a V domain is directed by recombination signal sequences (RSSs) that flank the rearranging gene segments and the rearrangement is catalyzed by the enzymes encoded by recombination-activating gene 1 (RAG-1) and RAG-2. Both enzymes introduce a DNA double-strand break between the terminus of the rearranging gene segment and its adjacent RSS. These breaks are then repaired by a mechanism of DNA repair known as nonhomologous end-joining (NHEJ). The NHEJ process creates precise joins between the RSS ends and imprecise joins of the coding ends to which non-germline-encoded nucleotides (N nucleotides) are variably added by terminal deoxynucleotidyl transferase (TdT) (Schroeder H. W., Jr. 2010).

Figure 3.1.3. V(D)J recombination of H and L chain loci can generate up to 10^16 different Igs in the pre-immune repertoire.
3. General introduction

V(D)J recombination occurs first in the H chain locus during B cell development at the pre-B cell stage. At this stage the pre-B cell expresses a μ H chain paired with an invariant heterodimer, called surrogate light chain (SLC), forming the pre-BCR. Rearrangement of the L chain locus occurs after complete rearrangement of the H chain locus and the B cells starts expressing either a κ or λ chain that pairs with the μ H chain forming a functional BCR of IgM isotype (Schroeder H. W., Jr. 2010).

3.1.3. BCR diversity in the pre-immune antibody repertoire

It has been estimated that a pre-immune antibody repertoire of greater than $10^{16}$ different Igs can be generated through V(D)J recombination (Figure 3.1.3). The κ locus contains 30-35 functional Vκ genes and 5 Jκ genes, while the λ locus contains 29-33 functional Vλ genes and 4-5 Jλ genes. Any V gene can rearrange to any of the J genes creating up to 185 and 165 potential recombinations to generate both κ and λ L chains respectively. The H chains locus contains 38-46 VH genes, 23 D genes and 6 JH genes that can create up to 6300 potential recombinations during gene rearrangement. Pairing one H chain with one L chain leads to more than $2 \times 10^6$ combinations of different Igs. Nevertheless, this number can be highly increased by other mechanisms that increase variability: i) D gene segments can rearrange by either inversion or deletion and can be translated in each of the 3 possible reading frame, ii) N-nucleotide can be added by TdT, iii) a variable palindromic sequence can be formed by addition of germline-encoded nucleotides to solve the hairpin structure resulting from the gene rearrangement, iv) during the recombination process a loss of 1 to several nucleotides can occur at the terminus of each rearranging gene segment. Furthermore, the antibody repertoire can be broadened in mature B cells that undergo somatic hypermutation, a process that substitutes single nucleotides during the late phase of an immune response in the peripheral lymphoid tissues (Schroeder H. W., Jr. 2010).
3. General introduction

3.2. The antibody response

Mature B cells can leave the bone marrow and reach secondary lymphoid organs in search for antigen. B cells that have not encountered the specific antigen yet are called naïve. When B cells encounter the specific antigen, they can be activated in order to undergo clonal proliferation and terminal differentiation into memory B cells and plasma cells. According to the “two-signal model” the engagement of the BCR by the antigen (signal 1) is not sufficient to activate the B cell, that need also a costimulatory signal (signal 2) to be completely activated (Bretscher 1975). Activation can be either T-cell independent (TI) or T-cell dependent (TD) according to the properties of the stimulating antigen. In the next paragraphs I will describe both TI and TD responses.

3.2.1. T-independent responses

TI antigens are chemically and immunologically heterogeneous and are generally divided into two main categories, TI type 1 (TI-1) and TI type 2 (TI-2) antigens. TI-1 antigens are mitogenic for B cells and bacterial wall components, like lipopolysaccharide (LPS) and DNA. Type 1 T cell-independent activation occurs when a B cell binds to an antigen and receives costimulation by toll-like receptors (TLRs), such as TLR4 for LPS and TLR9 for DNA. The B cell activation is polyclonal because both specific B cells and B cells with a BCR with different specificities can be activated and produce antibodies to TI-1 antigens. TI-2 antigens are large molecular-weight molecules with repeating antigenic epitopes, like polysaccharides and phospholipids (Mond 1995). B cells are activated by TI-2 antigens by a first signal of multivalent cross-linking of the BCR molecules and by a second signal provided by engagement of TLRs (Vos 2000).

TI responses generally result in rapid antibody production by short-lived plasma cells that derive from B-1 cells located in serous cavities and marginal zone B cells of the spleen. In the last years it has been demonstrated that both populations can give rise to memory B cells and long-lived plasma cells (Defrance 2011; Alugupalli 2004; Weller 2004). Differently from the TD counterpart, memory B cells generated in the response to TI antigens do not show an enhanced sensitivity to antigen restimulation nor an extend lifespan (Obukhanych 2006), and the long-lived plasma cells derived from TI responses have a lower secretion capacity and do not undergo a contraction phase during the first two months after immunization compared to plasma cells generated from TD responses (Taillardet 2009).
3. General introduction

3.2.2. T-dependent responses

TD antigens consist of soluble proteins or peptides. Differently from TI antigens, TD antigens can be processed and associated with MHC class II molecules on the surface of antigen-presenting cells (APCs), like dendritic cells (DCs) or macrophages. APCs can present the antigen peptides to T cells present in secondary lymphoid organs by interacting with their TCR and through binding of accessory and adhesion molecules. Specific T cells are activated and interact with specific B cells forming immunological synapses. B cells receive help from cognate T cells to proliferate and form germinal centers where they undergo affinity maturation through somatic hypermutation and class-switching, finally generating a repertoire of long-lived memory B cells and plasma cells (Zubler 2001).

Activated follicular helper T cells (T<sub>FH</sub>) express CXC-chemokine receptor 5 (CXCR5) and home to follicles where B cells are localized. They also express the activation markers CD69 and ICOS (inducible T-cell costimulator), and secrete interleukin-10 (IL-10) to support GC B-cell growth and differentiation. After stimulation T<sub>FH</sub> cells also upregulate expression of OX40 (CD134), CD40 ligand (CD40L), IL-21, BCL-6 and SAP (signaling lymphocytic activation molecule (SLAM)-associated protein). OX40 is required for follicular T–cell homing and for memory B cell responses. CD40L is bound by CD40 expressed on the surface of activated B cells and, together with secreted IL-21, CD40-CD40L interaction induces GC B cell proliferation, class-switch recombination and differentiation into memory B cells. BCL-6 is a transcription factor that leads the differentiation of both naïve T and naïve B cells into follicular T and follicular B cells. SAP is an adaptor molecule that binds CD84 expressed by B cells and modulated T<sub>FH</sub>-cell signaling (Vinuesa 2005).

Follicular B cells are recirculating mature naïve B cells that, like T<sub>FH</sub> cells, express CXCR5 to home to follicles in secondary lymphoid organs. The ligand of CXCR5 is the CXC-chemokine ligand 13 (CXCL13) that is secreted by stromal cells and by follicular dendritic cells (fDCs) that capture antigen bound to antibody or to complement C3d. Once naïve B cells encounter and bind protein antigens, they increase the expression of CC-chemokine receptor 7 (CCR7) and home to the outer T-cell zone of secondary lymphoid tissues following the gradient of the CC-chemokine ligand 21 (CCL21) (Vinuesa 2005). Follicular B cells bind the antigen by recognizing conformational or accessible linear epitopes, and after internalizing and processing the antigen they present a series of peptides to T<sub>FH</sub> cells that recognize the same peptide presented by the same MHC class II
molecule that they had seen on a DC or a macrophage (Lanzavecchia 1985). In addition to the follicular pathway, B cells can follow an extrafollicular pathway in which they move from the T-cell zone to the splenic bridging channels or the lymph-node medullary cords where they form extrafollicular foci and differentiate into short-lived plasma cells that produce low-affinity antibodies (Vinuesa 2005).

3.2.3. The germinal center reaction: somatic mutations and class switch

The few antigenic-specific B cells form the GC, a lymphoid structure within the follicles of secondary lymphoid organs organized in a dark zone and a light zone. In the dark zone, GC B cells (named centroblasts) undergo intense proliferation, somatic hypermutation (SHM) and class-switch recombination (CSR). Centroblasts exit the cell cycle, move to the light zone and become centrocytes that compete with one another for binding the antigen that is presented by fDCs in the form of immune complex bound either to complement receptors 1 (CR1/CD35) and 2 (CR2/CD21) or to Fc receptors for IgG (FcγRIIb/CD32b), IgE (FcεR2/CD23), IgA (FcαR/CD89) and IgM (FcμR) immunoglobulins. In addition, since fDCs are source of the B-cell activating factor (BAFF) that is a potent growth factor for B cells, they can maintain the survival of GC B cells (Aguzzi 2014). Centrocytes with a new mutated BCR that has a higher affinity for the antigen are positively selected because they outcompete those with a lower affinity for binding to the antigen, processing and presenting it to T<sub>FH</sub> cells that, in turn, give help for survival and differentiation into memory B cells and long-lived plasma cells. Instead, centrocytes binding self antigens or binding foreign antigens without increased affinity do not receive help form T<sub>FH</sub> cell nor surviving signals from fDCs and are eliminated. In addition, positively selected centrocytes can be induced by T<sub>FH</sub> cell to perpetuate the GC reaction becoming centroblasts again (Vinuesa 2005) (Figure 3.2.1).
Somatic hypermutation is a stochastic process that introduces point mutations in the variable region of both the heavy chain and light chain of an immunoglobulin. SHM are responsible for affinity maturation and changes in fine specificity of the antibodies (Neuberger 2008). During SHM the V(D)J genes undergo somatic mutations at very high rate ($10^{-5}/10^{-3}$ base pair per cell division). Point mutations are found also in the J region introns. It has been proposed that this process is divided into two phases with the first one depending on the mutagenic activity of the enzyme AID (activation-induced deaminase), that is highly expressed in centroblasts, and the second one depending on the error-prone repair of AID-induced mutations (Figure 3.2.2). At the beginning of the process, AID deaminates a cystidine residue in a single-strand DNA bubble that is formed during transcription of both DNA strands. AID preferentially deaminates deoxycytidine (dC) in
WRC hot-spot motifs (W is either of the weakly hydrogen-bound bases adenine (A) or thymidine (T), R is either of the purines guanine (G) or A, C is cytosine), whereas it rarely deamintes dC in SYC cold-spot motifs (S is either of the strongly hydrogen-bound bases G or C, and Y is either of the pyrimidines C or T). The cystidine deamination creates a uridine:guanosine (U:G) mismatch that can be repaired by different mechanisms. In phase 1a, the uracil (U) can be interpreted as T by the replication machinery and one of the daughter cells will acquire a C-to-T transition mutation. During phase 1b, the U can be removed by one of the uracil DNA N-glycosylases (UNG) that leaves an abasic site and trigger short-patch base excision repair (BER) that introduces a single-stranded break by apurinic/pyrimidic endonucleases (APEs) and fills the gap with a error-prone polymerase, which can insert any of the four bases, leading to transitions and transversions at the G:C basepairs. In phase 2, mismatch-repair (MMR) recognizes the U:G mismatch and the U-bearing strand is excised. PCNA (proliferating cell nuclear antigen) recruits error-prone polymerases, like Pol η, to fill the gap, leading to transversion and transition mutations at both A:T bases and neighboring G:C bases. Long-patch BER can compete with MMR to introduce mutations. Phase 1 mutations at G:C basepairs and phase 2 mutations at A:T basepairs are ~40% and ~60% of total mutations, respectively (Neuberger 2008; Peled 2008).

**Figure 3.2.2.** Model of somatic hypermutation. AID deaminates cystidines creating a mismatch that can be processed by different DNA repair mechanisms finally introducing new mutations that increase diversity within the Ig locus. Reprinted from (Peled 2008).
3. General introduction

**Class-switch recombination** is an intrachromosomal deletional recombination event that changes the expression of the constant region of the heavy chain (C_H) of naïve B cells from IgM and IgD isotype to either IgG, IgE or IgA. This process is important because it determines the antibody effector function that is mediated by the constant region of the antibody. CSR can occur very early after antigen exposure and before GC formation. The recombination is possible because of the presence of a switch (S) region located upstream of each C_H genes. Similarly to SHM, CSR is initiated by AID enzyme that deaminates dC at the top and at the bottom strands of transcriptionally active S regions. This process results in double-strand DNA breaks in both S regions followed by recombination of the two S regions by NHEJ and deletion of the intervening C_H genes. The switching to specific isotypes is targeted by specific cytokines that induce transcription of sterile RNAs (germline transcripts) from promoters upstream to each acceptor S region. These transcripts act only in cis and help creating single-strand DNA, that is the substrate of AID, at the transcription bubble (Stavnezer 2008).

3.2.4. BCR diversity in the post-immune antibody repertoire

During an antibody immune response SHM contributes to greatly increase the number of different BCRs in the memory B cell compartment. An example is provided by HIV antibodies that during their affinity maturation can accumulate up to 32% of mutations compared to germline nucleotide sequence of the antibodies produced by the naïve B cell from which the clonally-related memory B cells have differentiated, suggesting that a great number of B cell clones with different BCRs have been generated in the GC (Mascola 2013).

Considering that the estimated number of different BCRs that can be generated in the pre-immune antibody repertoire is greater than \(10^{16}\) different Igs, the additional diversity generated by SHM contributes to further increase the number of unique BCRs that can be potentially generated in the whole life of an individual. It’s not easy to estimate this number, anyway it is extremely high and largely exceed the number of B cells in our body (estimating \(2 \times 10^{12}\) lymphocytes in the human body).
3.3. Immunologic tolerance and fate of autoreactive B cells

Given the extraordinary diversity of receptors that are generated by V(D)J rearrangement and SHM, the immune system is confronted with the problem of avoiding reactivity to self molecules. Paul Erlich at the beginning of the twentieth century defined with the term “horror autotoxicus” the hypothesis that the body must have a system to protect the danger that autoantibodies would pose (Erlich 1902).

It has been estimated that up to 50% of BCRs generated during B cell development in the bone marrow can recognize and bind self antigens (Goodnow 2005; Shlomchik 2008; Wardemann 2003). In addition, SHM in the GC can generate autoreactive B cells that can differentiate into autoantibody-producing plasma cells if they receive proper survival stimuli (Shlomchik, Marshak-Rothstein, 1987). To avoid self reactivity the immune system has developed an important property, named tolerance, that is a specific unresponsiveness to self antigens. Tolerance can be maintained through several mechanisms that eliminate self-reactive clones or rescue them by editing their autoreactive BCR or inducing an unresponsive state, named anergy, before they mature and attack cells and organs (Nemazee 2003). In addition, autoreactive GC B cells can be removed from the secondary repertoire generated by SHM in order to maintain self-tolerance (Brink 2014) (Figure 3.3.1).

We have learnt a lot on the mechanisms that induce tolerance to self antigens by studying self-reactivity in different transgenic mice and analyzing the frequency of self-reactive B cells by expressing a high number of Ig genes from healthy individuals (Goodnow 2005; Nemazee 2003; Tiller 2007). In the next paragraphs I will discuss these mechanisms in more details.

3.3.1. Mechanisms of tolerance induction in developing B cells

During their development in the bone marrow most self-reactive B cells carrying a BCR binding to a self antigen with strong avidity are eliminated by clonal deletion. The binding to the self antigen induces BCR signaling and leads to a rapid internalization of the receptor. As a consequence B cells stop their maturation program and are triggered to die (Goodnow 2005; Nemazee 2003). Deletion results from increase of proapoptotic factors like BIM (BCL-2-interacting mediator of cell death). In addition, homing
receptors, such as CD62 ligand (or L-selectin) that is required to enter secondary lymphoid tissues, are not expressed (Goodnow 2005; Hartley 1993).

Autoreactive B cells can be rescued from deletion by receptor editing, a process in which the self-reactive BCR expressed on immature B cells is edited by further V(D)J rearrangements mediated by RAG1 and RAG2 enzymes to either lose the self-reactivity or reduce the avidity for the self antigen. If the avidity for the self antigen remains high, the cell undergoes clonal deletion (Nemazee 2003; Jankovic 2004) or further rounds of receptor editing (Herzog 2012). In addition, immature B cells undergoing receptor editing can express two or more different light chains and occasionally also more heavy chains. This phenomenon is named allelic inclusion and permits the generation of polyreactive B cells that retain self-reactivity and escape central tolerance through dilution of the aberrant self-reactive receptors (Basten 2010; Wardemann 2007; Shlomchik 2008; Liu S. 2005).

Autoreactive B cells that recognize a self antigen that is not expressed in the bone marrow can continue their maturation program and reach peripheral lymphoid tissues. When autoreactive B cells encounter the self antigen in the periphery, clonal deletion takes place if the BCR have a moderate or high affinity for the self antigen, as in case of cell membrane antigen or soluble antigens with highly repetitive structures. These B cells are rapidly deleted by a BIM-dependent mechanism, unless they receive T-cell help or TLR-dependent costimulatory signals that promote their activation (Basten 2010).

By contrast, low or very-low avidity interactions can induce anergy or ignorance. Using transgenic mice it has been shown that low-valence or weakly cross-linking antigens, as many soluble antigens, can induce anergy (or tuning) of autoreactive B cells. Anergic B cells drastically reduce BCR expression (50-99% reduction) and show decreased responsiveness due to desensitization of BCR signaling, failure of antigen presentation and antibody production, and inability to compete for limiting amounts of BAFF. The remaining BCR molecules only poorly activate tyrosine kinases resulting in weak activation of the transcription factor NF-κB1 (nuclear factor kappa-light-chain-enhancer of activated B cells) with subsequent downregulation of several prosurvival factors in the Bcl-2 family and upregulation of apoptotic factors, like BIM, that eventually limit cell survival (Lesley 2004). The signaling by self-reactive BCRs is further dampened by expression of inhibitory cell-surface proteins like cluster of differentiation 5 (CD5) which harbor an intracellular inhibitory motif (ITIM) recruiting proteins that increase the threshold of B-cell activation, such as the tyrosine phosphatase SHP1 (SH2-domain-
containing protein tyrosine phosphatase 1) and the lipid phosphatase SHIP (SH2-domain-containing inostil-5-phosphatase) (Goodnow 2005; Ravetch 2000; Hippen 2000). An anergic B-cell subset (CD93+ IgMlo termed An1) has been identified in the normal murine B-cell repertoire (Merrell 2006). A similar subset has been described also in humans and is characterized by unmutated IgM+ IgDhi B cells (termed BND) with functional and phenotypic characteristics of anergy defined in transgenic systems (Duty 2009). The anergic B cells can undergo positive selection, if they are exposed to strongly cross-reactive antigens, excess of BAFF or sustained T-cell help, and can enter germinal centers (GCs) where they undergo affinity maturation (Basten 2010; Cambier 2007).

If the self-antigen is not accessible in the peripheral tissues, as is the case of intracellular antigens or tissue-specific antigens, some autoreactive B cells can survive as a normal non-self reactive B cells (Akkaraju 1997). In this state, named clonal ignorance, the self-reactive B cells can assume a normal follicular B cell phenotype, but they receive inhibitory signals in order to avoid the development of autoimmunity (Shlomchik 2008; Basten 2010). The inhibitory signals include sialic acid binding Ig-like lectins, like CD22 (Siglec-2) and the sialic acid acetyl esterase (SIAE). The SIAE/Siglec pathway is important to maintain a peripheral tolerance to self antigens by setting a threshold that limits activation of ignorant self-reactive B cells (Duong 2010; Pillai 2011).

3.3.2. The fate of autoreactive B cells in germinal centers

Although critical for providing the high-affinity antibody specificities required for long-term immune protection, SHM can also generate self-reactive B cells capable of differentiating into autoantibody-producing plasma cells (Shlomchik, Marshak-Rothstein, 1987). Low-affinity autoantibodies can be found in the serum of healthy donors and are frequently expressed by IgG+ memory B cells, as shown in a recent study (Tiller 2007), indicating that self-tolerance in the GC is not absolute. Nevertheless, the absence of autoantibodies from most individuals suggests that self-reactive GC B cells are normally kept in check by some mechanisms that intervene to eliminate them or to avoid their activation (Brink 2014). In particular, accessibility to the self antigen and survival signals provided by either T cell help or innate stimuli are the two main features that affect the maintenance of tolerance to self (Figure 3.3.1).

As for accessibility to the self antigen, one recent study showed that, in the case of rare or tissue-specific self antigens which have an expression that is low or physically
separate from the GC microenvironment, the autoreactive GC B cells are not eliminated, probably remaining in a state of anergy or ignorance. In addition, these B cells can undergo positive selection by a cross-reactive foreign antigen and produce plasma cells secreting high-affinity autoantibodies. By contrast, these cells undergo negative selection if the self antigen is expressed at sufficient concentration within or proximal to the GC and the B cells do not receive sufficient survival signals (Chan 2012).

In case of generation of GC B cells reactive to intracellular antigens, they can remain in an ignorant state unless they bind to intracellular antigens released after apoptosis of negatively selected centrocytes in the GC. In the particular case of release of DNA or DNA-associated proteins, the BCR engagement together with TLR stimulation by the nucleic acid can favor the survival and activation of autoreactive B cells producing anti-nuclear antibodies that are observed in patients with systemic lupus erythematosus (SLE) (Schroeder K. 2013; Detanico 2013).

The importance of costimulation for survival and activation of autoreactive B cells can be explained by the observation that in many situations where the self antigen is accessible, the lack of autoantibodies can be due to the lack of T cell help that deliver a costimulatory signal to autoreactive B cells, thus helping maintaining tolerance to self (Liu Y. 1992). Instead, when T cell help is provided, activation of autoreactive B cells can occur. In particular, costimulation can be provided by either T cells specific for the self antigen or for a foreign antigen. In the first case, self-tolerance needs to be breached at T cell level and B cells producing high-affinity pathogenic autoantibodies may be positively selected in the GC based purely on their affinity for the self antigen (Lin 1991). In the second case, autoreactive B cell activation does not need to break T cell self-tolerance, because the T cells recognize an epitope on an foreign antigen carrying a self B cell epitope that mimics a similar epitope on the self antigen (Brink 2014).

In absence of T cell help, autoreactive B cells generated in the GC can survive negative selection by activation induced by TLR-agonists. In particular, TLR9 that senses CpG-containing DNA and DNA-associated autoantigens, and TLR7 that senses single-stranded RNA and RNA-associated autoantigens can trigger B cells to produce autoantibodies (Leadbetter 2002; Lau 2005).
3. General introduction

Figure 3.3.1. Fate of autoreactive B cells generated through VDJ recombination and somatic mutations. According to the type of self antigen and its accessibility and in absence of costimulation provided by innate stimuli or T cell help, the autoreactive B cells can be either deleted, anergized or become ignorant and tolerance to self is maintained. Conversely, in presence of costimulation, autoreactive B cells can be activated and differentiate into memory B and plasma cells that produce autoantibodies.
3. General introduction

3.4. Autoantibodies and autoimmune diseases

More than 80 different autoimmune diseases have been described in the human and most of them have been associated with the production of autoantibodies. Some autoantibodies are associated with one specific disease, while other autoantibodies are detected in several autoimmune conditions. In addition, non-pathogenic autoantibodies can be found also in healthy individuals (Vinuesa 2009).

In the next paragraphs I will further analyze the origin of the autoantibodies and the mechanisms that induce their production by autoreactive B cells. Then I will give an overview of the repertoire of self-antigens recognized by autoantibodies and I will describe the effector mechanisms by which the autoantibodies contribute to the pathogenesis of the disease. Finally, I will examine in depth a particular category of autoantibodies that bind to cytokines, which comprise anti-GM-CSF autoantibodies that I studied in my experimental work.

3.4.1. Origin of autoantibodies

Although autoantibodies can be generated by V(D)J recombination during B cell development, SHM occurring during the GC reaction seems to be the preferential mechanism that generates most autoantibodies that are found in the serum of both healthy donors and patients with autoimmune diseases. This observation is sustained by a study that showed that autoantibodies are more frequently expressed by IgG+ memory B cells than mature naïve B cells or IgM+ memory B, suggesting that most self-reactive antibodies are generated by SHM during the transition between mature naïve and IgG+ memory B cells (Tiller 2007).

The first demonstration of the role of SHM in generating autoantibodies was provided by Shlomchik and colleagues that showed that autoantibodies to IgG (rheumatoid factor, RF) are not the result of a polyclonal B cell activation, but they are the product of antigen-driven somatic mutations of B cells (Shlomchik, Marshak-Rothstein, 1987; Shlomchik, Aucoin, 1987).

Due to its stochastic nature, SHM can change or broaden the fine specificity of an antibody originally reacting to a foreign antigen inducing reactivity for a self antigen. In this way non-autoreactive B cells can acquire autoreactivity de novo and can either maintain or lose the original reactivity for the foreign antigen. In the first case, the
autoantibody becomes cross-reactive, being able to recognize both the foreign and the self antigen, while in the second case, the B cell becomes completely self-reactive and produces an autoantibody that does not cross-react with the original foreign antigen (Casson 1995; Diamond 1984). It is important to consider that SHM is able not only to generate autoimmunity, but also to silence it as shown in a recent study by Sabour and colleagues (Sabour 2014).

The role of somatic mutations in the generation of autoantibodies has been further analyzed by recent studies that employed the strategy to revert the variable region sequences of highly mutated autoantibodies to the putative primary specific generated by V(D)J recombination (Brink 2014). These studies showed that anti-DNA autoantibodies from patients with SLE or anti-desmoglein 3 autoantibodies from patients with pemphigus vulgaris lost reactivity to the respective self antigens when somatic mutations were removed (Schroeder K. 2013; Mietzner 2008; Di Zenzo 2012). Despite the difficulty to find the original specificity of germlined autoantibodies, there is one study that showed that the unmutated version of an anti-thyroid-stimulating hormone receptor (TSHR) autoantibody derived from a mouse model of Grave’s disease reacted strongly with *Yersinia* antigens (Hargreaves 2013), providing support to the hypothesis that autoantibodies can be generated during the response to a foreign antigen.

### 3.4.2. Mechanisms of induction of autoantibody production

Several mechanisms have been suggested to explain the activation of autoreactive B cells to produce autoantibodies. These mechanisms take into account T-dependent or T-independent activation, dysregulation in GC constituents, location and concentration of self antigens, modifications of self antigens and epitope accessibility.

Autoreactive B cells can be stimulated to produce autoantibodies by a foreign antigen that contains self B-cell epitopes and foreign T-cell epitopes. In this case the autoreactive B cell can receive help from T cells that are specific for the foreign determinant and produce autoantibodies that cross-react with both the foreign and the self antigen, as shown by *in vivo* experiments with injection of self and xenogeneic homologous proteins (Lin 1991). In humans, an important example is given by the Guillain-Barré syndrome that arises after infection from the bacterium *Campylobacter jejuni*. In this syndrome
neuropathic autoantibodies are generated because of molecular mimicry of lipo-oligosaccharides of *C. jejuni* and gangliosides of the peripheral nerves (Ang 2004). Another example is given by pauci-immune focal necrotizing glomerulonephritis in which autoantibodies recognize the neutrophilic cytoplasmic antigen LAMP-2 (lysosomal membrane protein-2) and cross-react with the bacterial adhesin FimH which has an epitope sharing 100% homology with LAMP-2 (Kain 2008). Similarly, antinuclear autoantibodies to 60-kDa ribonucleoprotein Ro found in SLE patients cross-react with a peptide from the latent viral protein Epstein-Barr virus (EBV) nuclear antigen-1 (EBNA-1) supporting the hypothesis of molecular mimicry and an etiological role for EBV in SLE (McClain 2005). Other examples include post-streptococcal disease of the heart valves in rheumatic fever, tropical spastic paraparesis after infection with human T-lymphotrophic virus 1, reactive arthritis after *Salmonella typhimurium* infection and the cross-reactivity of autoantibodies to TSHR in Grave’s disease with *Yersinia enterocolitica* (Levin 2002; Kaplan,Suchy 1964; Kaplan,Svec 1964; Lo 2000; Hargreaves 2013).

Autoantibodies can also arise after breaching T cell self tolerance and subsequent positive selection of **autoreactive T cells** that can provide help to autoreactive B cells. One example is provided by a study showing that B cells from mice immunized with xenogeneic homologous cytochrome c allow priming of autoreactive T cells with murine cytochrome c (Lin 1991). In addition, a more recent study showed that T cells that mediate autoimmune arthritis recognized an ubiquitously expressed cellular protein, 60S ribosomal protein L23a, with which T cells and autoantibodies from RA patients reacted (Ito 2014).

In absence of T cell help, autoreactive B cells can be activated by **innate stimuli**, as shown by IgG2a-chromatin immune complexes that induce synergistic engagement of the BCR and TLR9 (Leadbetter 2002). These autoreactive B cells can also break T cell tolerance by presenting peptides of the self antigen to specific autoreactive T cells that can provide further help to augment the B cell response, thus starting a positive feedback loop that amplifies and sustains autoimmunity (Shlomchik 2009).

In case of dysregulation of germinal centers, autoantibodies can be the result of formation of ectopic GCs, aberrant persistence of GCs, alterations in removal of apoptotic cells and excess of T cell help. **Ectopic GCs** are formed at the site of tissue pathology, when the expression of the self antigen is tissue-restricted, and B cell autoreactivity is generated during SHM. Ectopic GC formation occurs in 10-35% of RA patients, in 54-63% of patients with Grave’s disease and in patients with many other autoimmune diseases (Aloisi 2006). These ectopic lymphoid structures support production of anti-
General introduction

3.1 Citrullinated autoantibodies (ACPAs) in rheumatoid synovium (Humby 2009), even though a recent study shows that synovial lymphoid neogenesis occasionally progresses to GC reactions and does not support antigen-driven autoantibody responses (Cantaert 2008).

Survival of autoreactive B cells can also result from aberrant persistence of GCs due to overexpression of Bcl2 that can interfere with apoptosis of negatively selected B cells (Hande 1998), downregulation of the inhibitory low-affinity Fc receptor for IgG (FcγRIIb) shown in memory B cells and plasma cell of patients with SLE (Mackay 2006), and mutations of certain BCR negative regulators like CD22 or SIAE, that have been found in subjects with common autoimmune disorders (Surolia 2010).

Defects in clearance of apoptotic cells in GC by tingible body macrophages can provide a larger quantity of self antigens that can determine the survival of GC autoreactive B cells, especially those producing anti-nuclear antibodies in systemic autoimmune diseases like SLE (Vinuesa 2009; Brink 2014).

Accumulation of T<sub>FH</sub> cells in GCs induced by aberrant expression of IL-17, CD95L deficiency and excessive production of IL-21 can promote aberrant positive selection of autoreactive B cells (Vinuesa 2009).

As seen before, location and concentration of self antigen play an important role in the generation of autoantibodies. In particular, B cells that produce autoantibodies that recognize rare or tissue-specific self antigens with a low expression not expressed in the GC microenvironment are not eliminated (Chan 2012). This could be the case of autoantibodies that bind systemically available self antigens. These autoantibodies are likely to arise from breaks in self-tolerance mechanism in which the self antigen itself may drive autoantibody affinity maturation (Brink 2014). For example, RF (anti-IgG) autoantibodies in hepatitis C virus-associated mixed cryoglobulinemia showed a reduced but still detectable anti-IgG activity when the somatic mutations were removed, supporting the hypothesis that the self antigen (IgG) has started the autoantibody response (Charles 2013).

Modification of self antigens by either somatic mutations or post-translational modifications can generate new antigens. In these cases, the lack of tolerance to these modified antigens can be explained by their absence in the bone marrow and in the thymus that allows B and T cells specific for these antigens to escape into the periphery and subsequently recognize them as foreign antigens, with subsequent positive selection and production of autoantibodies. In a recent study, patients with scleroderma and antibodies against the self antigen RPC1, encoded by the POLR3A gene, were shown to have an
increased risk to develop cancer. These patients had genetic alterations in the POLR3A locus, while patients without anti-RPC1 antibodies did not have any mutations. These data suggest that the "foreign" antigens in this autoimmune disease can be encoded by somatically mutated genes in the patients' incipient cancers (Joseph 2014).

**Post-translational modifications** (PTMs) are chemical modifications of proteins like phosphorylation, dephosphorylation, transglutamination, ubiquitylation, citrullination, carbamylation, acetylation, glycosylation, hydroxylation, deamidation, methylation, lipid peroxidation, oxidation and endoprotease cleavage. They can occur spontaneously during an ordered enzymatic process and are usually crucial for several cellular events, like signaling or DNA replication. Nevertheless, PTMs can also introduce modification in self-proteins that make them neo-self-antigens that trigger B and/or T cell responses. The term “autoantigenesis” has been proposed for referring to this process (Doyle 2012). Many autoimmune diseases have been shown to produce autoantibodies against post-translationally modified antigens, such as citrullinated peptides in some patients with RA, or deamidation of transglutaminase in celiac disease (Klareskog 2008; van de Wal 1998).

Antigens that have **cryptic epitopes** can become visible and trigger autoimmunity. These cryptic epitopes may not be generated at all or are generated at subthreshold levels, but if they are presented at higher concentrations they can activate self-reactive anergic or ignorant T cells that, in turn, can sustain autoantibody production by self-reactive B cells (Lanzavecchia 1995). In addition, B and T cells can diversify the response to a larger number of antigenic determinants through “**epitope spreading**”. In SLE, autoreactive B cells can be activated by a cryptic epitope of the snRNP particle of the spliceosome complex that can be internalized, processed and a broader range of epitopes can be presented to self-reactive T cells that can stimulate B cells to produce autoantibodies against different determinants of the snRNP molecule (intramolecular spreading) and also of other proteins of the same complex (intermolecular spreading) (Monneaux 2002; James 1995).
3. General introduction

3.4.3. Specificity

Autoantibodies can recognize self antigens that have either an ubiquitous or a tissue-restricted expression. Despite the large amount of human proteins in our body (estimated 20,000-60,000 different proteins), the repertoire of self-antigens that can induce autoantibody production is very limited with only 1-2% of total human proteins being target of autoimmunity. The self-antigens were shown to have specific properties that target them for an autoimmune response. Self-antigens have particular structural properties like multivalency, repetitive surface elements, high content of charged or aromatic residues, presence of a coiled-coil, bound nucleic acid. In addition, intracellular antigens can become accessible after cell death and release into extracellular space (Plotz 2003).

According to the distribution of self antigens and the pathogenicity of autoantibodies it has been possible to categorize autoantibodies into three groups. (Vinuesa 2009). The first group comprises pathogenic autoantibodies to tissue-specific antigens, while the second group includes autoantibodies that bind to tissue-specific antigens without a clear pathogenic role in the disease (Table 3.4.1 and Table 3.4.2). The third group includes autoantibodies binding to ubiquitous antigens (Watanabe 2010; Vinuesa 2009) (Table 3.4.3).

Table 3.4.1. Pathogenic autoantibodies binding to tissue-specific antigens.

<table>
<thead>
<tr>
<th>Autoantibody</th>
<th>Self antigen</th>
<th>Disease</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anti-glomerular basement membrane</td>
<td>α3(IV)NC1 collagen (in the kidney and lung)</td>
<td>Goodpasture’s disease</td>
</tr>
<tr>
<td>Thyroid stimulating antibody</td>
<td>TSH receptor (α-subunit) (in the thyroid gland)</td>
<td>Graves’ disease</td>
</tr>
<tr>
<td>Anti-acetylcholine receptors</td>
<td>Nicotinic acetylcholine receptor (α/-γ-subunits)</td>
<td>Myasthenia gravis</td>
</tr>
<tr>
<td>Anti-acetylcholine receptors</td>
<td>Ganglionic (α3 subunit) (in the autonomic ganglia)</td>
<td>Autoimmune autonomic neuropathy</td>
</tr>
<tr>
<td>Anti-platelet</td>
<td>αIβ3 integrin (in platelets)</td>
<td>Immune thrombocytopenic purpura</td>
</tr>
<tr>
<td>Anti-red blood cell</td>
<td>Rhesus antigens (in erythrocytes)</td>
<td>Autoimmune haemolytic anaemia</td>
</tr>
<tr>
<td>Anti-white blood cell</td>
<td>αMβ2 integrin (in neutrophils)</td>
<td>Autoimmune neutropenia</td>
</tr>
<tr>
<td>Intercellular cement substance antibodies</td>
<td>Desmoglein 3</td>
<td>Pemphigus vulgaris and pemphigus foliaceus</td>
</tr>
<tr>
<td>Perinuclear ANCA</td>
<td>Myeloperoxidase (in neutrophils)</td>
<td>Microscopic polyangiitis</td>
</tr>
</tbody>
</table>

Abbreviations: ANCA, anti-neutrophil cytoplasmic antibodies; TSH, thyroid stimulating hormone.
3. General introduction

Table 3.4.2. Autoantibodies binding to tissue-specific antigens with no clear pathogenic role.

<table>
<thead>
<tr>
<th>Autoantibody</th>
<th>Self antigen</th>
<th>Disease</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anti-thyroid</td>
<td>Thyroid peroxidase, Thyroglobulin</td>
<td>Autoimmune thyroid disease</td>
</tr>
<tr>
<td>Anti-islet cell</td>
<td>Glutamic acid decarboxylase 2 Insulin (in β-cells of the islets)</td>
<td>Type 1 diabetes</td>
</tr>
<tr>
<td>Anti-neuron</td>
<td>Glutamic acid decarboxylase Glutamate receptor 3</td>
<td>Stiffman syndrome, Rasmussen’s encephalitis and Batten disease</td>
</tr>
<tr>
<td>Anti-neuron</td>
<td>Aquaporin 4</td>
<td>Neuromyelitis optica</td>
</tr>
<tr>
<td>Anti-adrenal</td>
<td>Steroid 21-hydroxylase</td>
<td>Addison’s disease</td>
</tr>
<tr>
<td>Anti-skin basement membrane</td>
<td>Dystonin Hemidesmosomal 180</td>
<td>Pemphigoid</td>
</tr>
<tr>
<td>Anti-skin basement membrane</td>
<td>Tyrosinase</td>
<td>Vitiligo</td>
</tr>
<tr>
<td>Anti-liver</td>
<td>CYP1A2,CYP2A6,CYP2B6,LKM3, UDP-glucuronosyltransferase 1-1 FTCD,CYP17 (in liver microsomes)</td>
<td>Autoimmune hepatitis</td>
</tr>
<tr>
<td>Cytoplasmic ANCA</td>
<td>Protéinase 3</td>
<td>Wegener’s granulomatosis</td>
</tr>
<tr>
<td>Anti-gastric parietal cell and anti-gastric-intrinsic factor</td>
<td>Potassium-transporting ATPase-β Gastric intrinsic factor (in the stomach and intestine)</td>
<td>Pernicious anaemia</td>
</tr>
<tr>
<td>Anti-parathyroid</td>
<td>Calcium-sensing receptor</td>
<td>Hypoparathyroidism</td>
</tr>
<tr>
<td>Anti-C1q</td>
<td>C1q</td>
<td>Hypo-complementaemic urticarial vasculitis</td>
</tr>
<tr>
<td>Anti-IFNω</td>
<td>IFNω (in the thymus)</td>
<td>APECED</td>
</tr>
</tbody>
</table>

Table 3.4.3. Pathogenic autoantibodies binding to ubiquitous antigens.

<table>
<thead>
<tr>
<th>Autoantibody</th>
<th>Self antigen</th>
<th>Disease</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anti-chromatin</td>
<td>dsDNA, histones, chromatin, nucleosomes</td>
<td>SLE</td>
</tr>
<tr>
<td>Anti-ENA (SS-A, SS-A, SS-B, Sm, nRNP, ScI70)</td>
<td>Ro52, Ro60, LA, snRNP, Ribonuclear proteins, Topoisomerase 1</td>
<td>SLE, Sjogren’s syndrome, scleroderma polymyositis, rheumatoid arthritis and scleroderma</td>
</tr>
<tr>
<td>Jo-1</td>
<td>Histidyl-tRNA synthases</td>
<td>Polymyositis (also known as Jo-1 syndrome)</td>
</tr>
<tr>
<td>Phospholipids antibodies</td>
<td>Cardiolipin and β, glycoprotein 1</td>
<td>Phospholipid antibody syndrome</td>
</tr>
<tr>
<td>Rheumatoid factor and anti-CCP antibodies</td>
<td>IgG CCPS</td>
<td>Rheumatoid arthritis</td>
</tr>
<tr>
<td>Anti-mitochondrial (M2)</td>
<td>Pyruvate dehydrogenase complexE2 Dihydrolipoamide acetyltransferase</td>
<td>Primary biliary cirrhosis</td>
</tr>
<tr>
<td>Endomysial antibodies</td>
<td>Transglutaminase 2</td>
<td>Coeliac disease</td>
</tr>
<tr>
<td>Anti-GM-CSF antibodies</td>
<td>GM-CSF</td>
<td>Pulmonary alveolar proteinosis</td>
</tr>
<tr>
<td>Anti-EPO antibodies</td>
<td>EPO</td>
<td>Pure red-cell aplasia</td>
</tr>
<tr>
<td>Anti-IL-8 antibodies</td>
<td>IL-8</td>
<td>ARDS</td>
</tr>
</tbody>
</table>

Abbreviations: APECED, autoimmune polyendocrinopathy-candidiasis-ectodermal dystrophy; ARDS, acute respiratory distress syndrome; C1q, complement component 1q; CCPs, cyclic citrullinated peptides; CYP, cytochrome P450; ds, double-stranded; ENA, extractable nuclear antigen; FTCD, formiminotransferase cyclodeaminase; EPO, erythropoietin; GM-CSF, granulocyte/macrophage colony-stimulating factor; IFNω, interferon-ω; SLE, systemic lupus erythematosus; snRNP, small nuclear ribonucleoprotein; SSB, Sjogren’s syndrome antigen B; UDP, uridine diphosphate.
3.4.4. Pathogenicity

Autoantibodies can induce pathology by several mechanisms including agglutination, complement-dependent citotoxicity, antibody-dependent citotoxicity, deposition of immune complexes in the tissues, neutralization or enhancement of the biological activity of the target molecule and agonistic activity on receptors (Figure 3.4.1). Below I will show some examples.

In autoimmune haemolytic anaemia, autoantibodies to anti-red blood cell cause their lysis leading to insufficient plasma concentration. The hemoagglutination is induced by complement activation by IgM, IgG1, IgG3, and IgA or by antibody-dependent citotoxicity induced by IgG1 and IgG3 (Gibson 1988).

In Goodpasture’s disease, anti-glomerular basement membrane antibodies bind to type IV collagen inducing a linear deposition of IgG along the glomerular and alveolar capillary basement membranes that cause a rapidly progressive renal failure due to necrotizing crescentic glomerulonephritis and potentially fatal pulmonary hemorrhage due to alveolitis. The autoantibodies cause the disease by activating the classical pathway of the complement system, which starts a neutrophil-dependent inflammation and also induces cell death of tagged cells (Hellmark 2014).

As described in more detail in the next paragraph, in many autoimmune diseases mediated by cytokine autoantibodies, the disease is caused by neutralization of the cytokine activity by the autoantibodies, as is the case of anti-GM-CSF autoantibodies causing pulmonary alveolar proteinosis (Trapnell 2003), or by enhancement of the cytokine activity, like anti-IL-8 autoantibodies in acute respiratory distress syndrome (Fudala 2008).

Finally, in Graves’ disease, the thyroid stimulating antibody binds to TSH receptor in the thyroid gland, mimicking the TSH, thus causing hyperthyroidism and all dependent effects (Menconi 2014).
Figure 3.4.1. Pathogenic mechanisms of autoantibodies binding to self antigens. i) Agglutination is the clumping of cells resulting from the interaction of antibodies with multiple antigens on the membrane of different cells forming a large complex. ii) Complement-dependent cytotoxicity (CDC) and iii) antibody-dependent cytotoxicity (ADCC) result from the interaction of the Fc portion of antibodies bound to the cell surface with either the C1q protein of the complement or with the FcR of natural killer (NK) cells, respectively, thus inducing the activation of their cytotoxic activity that leads to cell lysis. iv) Polyclonal antibodies can bind to different antigenic sites of an antigen making different immune complexes that can deposit in tissues. v) Neutralization occurs when the antibody binds to an epitope that is critical for the interaction of the molecule with the receptor, thus preventing intracellular signaling. vi) Enhancement occurs when the antibody favors the interaction of a molecule with the receptor and subsequent signaling by binding to an epitope not involved in such interaction. vi) Agonism occurs when the antibody binds to a receptor and, mimicking its ligand, induces intracellular signaling.
3.4.5. Cytokine autoantibodies

Among autoantibodies that recognize ubiquitously expressed self antigens we can find autoantibodies against cytokines. Several studies report their presence in different autoimmune and infectious diseases. In most cases only an association between autoantibodies against a specific cytokine and one or more specific pathologies has been demonstrated, but their pathogenic significance remains unclear. Interestingly, autoantibodies targeting proinflammatory cytokines like IL-1α, tumor necrosis factor-α (TNF-α), IL-6 and granulocyte-macrophage colony-stimulating factor (GM-CSF), have been reported more frequently compared to autoantibodies against anti-inflammatory cytokines like IL-10 and transforming growth factor-β (TGF-β). A list of cytokines targeted by pathogenic autoantibodies has been reviewed in (Watanabe 2010), (Cappellano 2012) and (de Lemos Rieper 2009) (Table 3.4.4).

<table>
<thead>
<tr>
<th>Cytokine</th>
<th>Disease</th>
<th>Cytokine</th>
<th>Disease</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-1α</td>
<td>RA, SjS, MCTD, PM, SLE, SSC, ILD, self-limiting synovitis</td>
<td>IFN-α</td>
<td>APS-1, RA, SLE, MCTD, PM, SCLC, thymoma ± MG, infection</td>
</tr>
<tr>
<td>IL-1β</td>
<td>RA</td>
<td>IFN-β</td>
<td>RA, SLE, SCLC, thymoma ± MG</td>
</tr>
<tr>
<td>IL-2</td>
<td>HIV (cross reactivity w/ gp41)</td>
<td>IFN-γ</td>
<td>MS, AM, SLE, NTM, infection</td>
</tr>
<tr>
<td>IL-3</td>
<td>FS</td>
<td>IFN-ω</td>
<td>APS-1, RA, SLE, thymoma ± MG</td>
</tr>
<tr>
<td>IL-4</td>
<td>MS, AM, stroke, lung infection</td>
<td>TNF-α</td>
<td>RA, SLE, HIV, GNB, MS, AM</td>
</tr>
<tr>
<td>IL-6</td>
<td>SSC, RA, PM, DM, SLE, MCTD, ALC, recurrent bacterial infection</td>
<td>NGF</td>
<td>SLE, autoimmune thyroiditis, RA, spondylarthropathy, mental disorder</td>
</tr>
<tr>
<td>IL-8</td>
<td>ARDS, ovarian cancer, periodontitis, arthritis</td>
<td>GM-CSF</td>
<td>PAP, thymoma ± MG, PM, DM, FS, MS</td>
</tr>
<tr>
<td>IL-10</td>
<td>MS, AM, stroke</td>
<td>G-CSF</td>
<td>FS, SLE</td>
</tr>
<tr>
<td>IL-12</td>
<td>Thymoma ± MG, SLE, RA, SCLC</td>
<td>EPO</td>
<td>PRCA, SLE</td>
</tr>
<tr>
<td>IL-17/IL-22</td>
<td>APS-1</td>
<td>OPN</td>
<td>T1DM, RA</td>
</tr>
</tbody>
</table>

Abbreviations: ALC, alcoholic liver cirrhosis; AM, aseptic meningitis; APS-1, autoimmune polyendocrine syndrome-1; ARDS, acute respiratory distress syndrome; CNS, central nervous system; DM, dermatomyositis; EPO, erythropoietin; FS, Felt’s syndrome; GNB, gram negative bacteria; HIV, human immunodeficiency virus; MCTD, mixed connective tissue disease; MG, myasthenia gravis; MS, multiple sclerosis; OPN, osteopontin; NGF, nerve growth factor; NTM, nontuberculous mycobacteria; PAP, pulmonary alveolar proteinosis; PF, pleural fluid; PM, polymyositis; PRCA, pure red-cell aplasia; RA, rheumatoid arthritis; SCLC, small cell lung cancer; SjS, Sjögren syndrome; SLE, systemic lupus erythematosus; SSC: systemic sclerosis; T1DM, type 1 diabetes mellitus.
In few cases cytokine autoantibodies have been demonstrated to have a pathogenic role in the disease by either neutralizing or enhancing the cytokine activity. Examples of neutralizing autoantibodies include autoantibodies against GM-CSF in autoimmune PAP and against EPO in pure red-cell aplasia (PRCA) and SLE with anemia (Trapnell 2009; Casadevall 2002; Tzioufas 1997). In PAP neutralization of GM-CSF impairs the terminal differentiation of alveolar macrophages that play a central role in surfactant catabolism with increased risk of uncontrolled infections. Neutralization of EPO induces arrest in red blood cell production.

Interferon-γ (IFN-γ) is important for antimycobacterial activity of macrophages and autoantibodies against IFN-γ have been detected in patients with both mycobacterial and nontuberculous mycobacterial infections (Madariaga 1998; Patel 2005). Autoantibodies to IL-6 that stimulates the synthesis of acute phase reactants that contribute to opsonin and complement activation, have been associated with increased risk of bacterial infections and mortality in patients with alcoholic cirrhosis (Homann 1996). Autoantibodies to IL-1α have been associated to attenuation of disease severity in RA, Sjögren syndrome (SjS) and self-limiting synovitis: levels of IL-1α autoantibodies were higher in patients with non-destructive arthritis than in those with destructive arthritis, indicating a better prognosis (Jouvenne 1997; Graudal 2002). More recently, autoantibodies against osteopontin (OPN), IL-17 and IL-22 have been identified. OPN is pleiotropic cytokine present at the site of inflammation and in the extracellular matrix of mineralized tissues (Stepien 2011). High levels of OPN and anti-OPN autoantibodies have been detected in patients with RA (Sennels 2008; Sakata 2001). OPN could have a role in supporting inflammation and bone erosion, while OPN autoantibodies could counteract OPN action and ameliorate the disease (Chabas 2001; Cappellano 2012). Autoantibodies to IL-17A, IL17F and IL-22 have been detected in patients with autoimmune polyendocrine syndrome-1 (APS-1) with concomitant mucocutaneous candidiasis. IL-17A, IL17F and IL-22 are cytokines produced by T_{H}17 T cells that can counteract Candida infection and it has been hypothesized that autoantibodies binding to these cytokine may favor the infection by causing a partial immune deficiency (Puel 2010).

Unlike autoantibodies that neutralize the activity of a cytokine, autoantibodies against IL-8 have been found to **enhance the activity** of IL-8 in acute respiratory distress syndrome (ARDS), an inflammatory disease of the lung characterized by acute hypoxemic respiratory failure. IL-8 immune complexes have been detected in the lung of ARDS patients and in individuals with higher risk to contract ARDS. IL-8 immune complexes
were shown to be potent chemoattractant of neutrophils *in vitro* and to reduce apoptosis of neutrophils by binding to IL-8 receptor and FcγRIIa and transmitting the IL-8 signal that inhibits caspase-3 and caspase-9 (Fudala 2008).

Some cytokine autoantibodies have been apparently **induced by viral infections or tumors**. In patients infected with the human immunodeficiency virus (HIV) and hepatitis virus higher levels of autoantibodies against IFN-α, IL-12 and TNF-α have been detected compared to healthy individuals, suggesting that increased levels of proinflammatory cytokines may trigger autoantibody production (Capini 2001; Ikeda 1991). Interestingly, antibodies against a peptide of the HIV envelope cross-reacting with an epitope of IL-2 molecule have been found in HIV patients, indicating that HIV infection can induce a potentially suppressive autoimmune response (Bost 1988). Tumor involvement in autoantibody production has been shown in patients with myasthenia gravis (MG), an autoimmune channelopathy due to autoantibodies that bind to acetylcholine receptor and block the stimulating effect of the neurotransmitter acetylcholine causing muscle weakness. Up to 75% of MG patients have abnormal thymus and about 25% have a thymoma, and thymic cells have been reported to produce both IFN-α and IL-12 autoantibodies without mitogen stimulation (Shiono 2003). In addition, another study showed that patients with thymic neoplasia and concurrent opportunistic infections developed autoantibodies neutralizing IFN-α, IFN-β, IL-α, IL-12p35, IL-12p40 and IL-17A *in vitro* (Burbelo 2010).

Cytokine autoantibodies have been detected both in sera and in intravenous immunoglobulin preparations (IVIG) of **healthy individuals**. Serum autoantibodies against IL-1α, IL-2, IL-6, IL-8, granulocyte colony-stimulating factor (G-CSF) and GM-CSF, and IVIG autoantibodies against IL-1α, IL-6, GM-CSF, IL-10, IFN-α and IL-5 have been reported. Currently there is not any consensus about their frequency in the human population that has been reported in different studies with very variable values (from 0.3 to 100%) (Watanabe 2010). It has been suggested that cytokines and their related autoantibodies are stoichiometrically balanced in healthy individuals and that cytokines are present in the serum in the form of immune complexes that could interfere with any measurement of their actual serum concentration made with conventional assays. Immune complexes formed by autoantibodies and the cytokines IL-2, IL-4, IL-8, IL-10, TNF-α, G-CSF and GM-CSF have been reported to be present in the serum of healthy individuals (Watanabe 2007; Uchida 2009). Given the common frequency of cytokine autoantibodies in healthy individuals, Watanabe and colleagues have proposed that cytokine
autoantibodies can have different physiological roles in vivo (Watanabe 2010). First, cytokine autoantibodies can regulate cytokine activity and maintain homeostasis by acting as scavengers of the excess of cytokines that are produced at the site of inflammation. In this way cytokines can achieve signal transduction at active inflammatory sites, while unnecessary tissue damage can be prevented at distal sites. Second, cytokine autoantibodies can increase the half-life of a cytokine, thus creating a reservoir of bioavailable cytokine. The cytokine should be always in equilibrium between its free form and the immune complex form. Third, cytokine autoantibodies can transmit a physiological cytokine signal through interaction with FcRs as shown by IL-2 in immune complexes with an anti-IL-2 monoclonal antibody that induces proliferation of CD8 T cells only if the Fc part of the antibody is maintained, whereas the IL-2 F(ab’)2 does not mediate the proliferation signal (Boyman 2006).

An effective alteration (neutralization, enhancement or modulation) of the bioactivity of a cytokine by autoantibodies, both in healthy and pathologic conditions, is the result of a combination of different factors including binding properties and relative concentrations of both cytokine and autoantibodies, epitope specificity and type of B cell immune response. The avidity of autoantibodies binding to their specific cytokine is quite high (4-400 pM for GM-CSF, IL-1α, IL-5, IL-6, IL-10 and IFN-α) and comparable to the avidity of cytokines binding to their natural receptors. As a consequence, cytokine autoantibodies can compete and interfere with the binding of a cytokine to its receptor affecting the depending signaling (Watanabe 2010). The neutralizing activity can occur when the molar ratio of autoantibodies compared to cytokines is high. In addition, when present at a lower molar ratio, cytokine autoantibodies can enhance the bioactivity and increase the half-life of a cytokine. As an example, IL-2 autoantibodies were found in healthy donors and neutralized IL-2 induced peripheral blood lymphocytes proliferation in vitro, but they also increased IL-2 half-life and bioactivity in vivo by forming immune complexes (Monti 1993; Boyman 2006). IL-3 and IL-4 autoantibodies were also found to increase the bioactivity of IL-3 and IL-4, respectively, which stimulate proliferation of murine mucosal mast cells (Finkelman 1993).

Cytokine autoantibodies can be generated to recognize different epitopes of the cytokine. Autoantibodies that recognize an epitope of the cytokine bound by the corresponding cytokine receptor are likely to be neutralizing, while autoantibodies binding to the cytokine portion not interacting with the receptor can enhance the cytokine activity by favoring the interaction of the free epitope with the receptor (Watanabe 2010).
A distinction between **monoclonal and polyclonal B cell responses to cytokines** should be taken into account while describing the possible pathogenic mechanisms. This issue has been addressed by few pharmacokinetics studies that have analyzed the efficacy of neutralization of antibodies against toxins like botulinum and anthrax toxins, and cytokines like IL-3, IL-4, IL-6 and IFN-α. These studies suggest that monoclonal antibodies can be non-neutralizing *in vivo*, but they can increase the half-life and bioactivity of their target. Conversely, combinations of oligoclonal antibodies targeting different epitopes on the same molecule can efficiently neutralize the biological toxicity of the toxin or the biological activity of the cytokine, supporting the important role of polyclonal responses in different conditions and also in the choice of antibody-based treatments (Lu 1992; Finkelman 1993; Kontsek 1991; Saurat 1991; Nowakowski 2002; Chow 2013; Pohl 2013; Montero-Julian 1994). In addition, polyclonal autoantibodies can form immune complexes with their targets that can be cleared *in vivo*, suggesting that FcRs may play a role for the efficient neutralization of either a toxin or a cytokine (Montero-Julian 1994; Pohl 2013; Abboud 2010). The main FcRs that can mediate endocytosis and clearance of immune complexes are the low-affinity receptors human FcγRIIA (CD32A) and FcγRIIB2 (CD32B2) both expressed on myeloid cells (Bruhns 2012; Zhang 2011).

The **origin** of cytokine autoantibodies can be explained by the different mechanisms shown in the previous paragraphs. In particular, the IgG isotype and the high affinity of most cytokine autoantibodies suggest a germinal center origin of these antibodies. Interestingly, two studies reported the presence of IgM autoantibodies to G-CSF and GM-CSF in cord blood, indicating that cytokine autoantibodies can be produced in the fetus (Revoltella 2001; Revoltella 2000). Even though these autoantibodies were not neutralizing, these data support the hypothesis that some autoreactive B cells can escape the B-cell central tolerance and enter the germinal center reaction after cytokine stimulation. Repeated and long-term cytokine exposure seem to be associated to autoantibody production, since high frequency of autoantibodies can be found in patients where the specific cytokine level is high, as is the case for G-CSF in FS and SLE, or EPO in PRCA and SLE (Hellmich 2002; Hara 2008). Furthermore, production of cytokine autoantibodies can be accelerated by other conditions like infections, neoplasms and genetic abnormalities (Watanabe 2010).
3. General introduction

3.5. GM-CSF autoantibodies

Among different cytokine autoantibodies we can find autoantibodies that neutralize GM-CSF, causing a severe autoimmune disease named pulmonary alveolar proteinosis (PAP). In my thesis I will focus my analysis on GM-CSF autoantibodies to study their origin and their pathogenic mechanism in PAP.

3.5.1. GM-CSF and GM-CSF receptor

Hematopoiesis is an important process that generates about $3 \times 10^9$ erythroid cells/kg and $10^9$ myeloid cells/kg every day in the adult. Colony stimulating factors (CSFs) are glycoproteins that stimulate proliferation, maturation and function of both hematopoietic cells and mature myeloid populations. CSFs include GM-CSF (or CSF-2), macrophage-CSF (M-CSF or CSF-1) and granulocyte-CSF (G-CSF or CSF-3). M-CSF is constitutively and ubiquitously expressed in steady state, while GM-CSF and G-CSF are usually expressed during the host response to infection or injury. M-CSF regulates numbers and maturation of several macrophage lineage populations important for tissue homeostasis and it also controls the differentiation of specific DC subsets like Langherans cells and kidney, lung and gut CD11b+ DCs. Furthermore, M-CSF suppresses the development of monocytes/macrophages into cells with the DC-like phenotype initiated by GM-CSF. G-CSF controls neutrophils numbers by inducing proliferation and maturation of myeloid progenitors and by promoting neutrophils release from the bone marrow (Gasson 1991; Hamilton 2013; Bogunovic 2009; Eyles 2008).

GM-CSF can be synthesized by different cells in response to specific activating signals. Physiological sources of GM-CSF include: T lymphocytes, B lymphocytes, macrophages, mast cells, fibroblasts, endothelial cells, mesothelial cells and osteoblasts. GM-CSF production can be induced by TCR and co-receptor stimulation, TLR agonists, TNF, IL-1 and prostaglandin E2. GM-CSF is present in the serum at very low levels that are often undetectable, indicating that it behaves in a paracrine way in which GM-CSF is produced and acts locally. GM-CSF can be found also in pathologic samples from patients suffering from acute and chronic myeloid lymphomas, some solid tumors and rheumatoid arthritis, thus making this cytokine a potential therapeutic target (Gasson 1991; Zupo 1992; Williamson 1988; van Nieuwenhuijze 2013). Besides the original function of growth factor for the production and differentiation of monocytes and neutrophils from immature bone marrow-derived precursors, GM-CSF has many different functions on
mature hematopoietic cells. GM-CSF enhances antigen presentation (Cook 2004), induces complement- and antibody-mediated phagocytosis (Fleetwood 2005), promotes leukocyte chemotaxis and adhesion (Gomez-Cambronero 2003), polarises macrophages into “M1-like” inflammatory macrophages (Fleetwood 2007), promotes maturation of alveolar macrophages (Stanley 1994), regulates differentiation of invariant natural killer cells (Bezbradica 2006), activates macrophage-like microglia in central nervous system (Parajuli 2012), promotes survival and activation of eosinophils and basophils (Esnault 2002; Hirai 1997), upregulates integrin CD11b in mature neutrophils increasing adhesion to vascular endothelium and tissue entry (Hansen 2008), primes neutrophils increasing antimicrobial functions like phagocytosis and oxidative burst (Condliffe 1998), increases uptake capacity and cross-presentation of mature DCs (Zhan 2011), induces proliferation of CD103+ intestinal DC in vivo (Schulz 2009), promotes induction and survival of T\textsubscript{H}17 cells via IL-6 and IL-23 and is required for the pathogenicity of T\textsubscript{H}17 cells in experimental allergic encephalopathy (Codarri 2011).

Even though GM-CSF is a pleiotropic cytokine, many of its functions are redundant because they can be easily performed by the other CSFs. The only function for which GM-CSF is not dispensable is the induction of the terminal differentiation of alveolar macrophages that seem to develop from fetal monocytes, rather than from adult circulating monocytes, as it is the case for other tissue-resident macrophages (Stanley 1994; Guilliams 2013).

According to its chemical properties, human GM-CSF is a secreted glycoprotein of 127 amino acids with a molecular weight of 14.65 kDa. Its final molecular mass can vary from 18 to 25 kDa depending on the glycosylation of the molecule. Glycosylation of GM-CSF has implications only in prolonging the half-life of the molecule, but it does not reduce its biological activity as confirmed by studies where oligosaccharides where removed from the cytokine (Chiarini 2004; Moonen 1987). The structure of GM-CSF has been solved by x-ray crystallography (Protein Data Bank code: 2GMF) and shows an open bundle of four α-helices combined with two anti-parallel β-sheets. The tertiary structure is stabilized by two disulfide bridges (Diederichs 1991; Rozwarski 1996) (Figure 3.5.1). Human GM-CSF shares 54% identity at amino acid level with the murine counterpart, and there is no cross-species reactivity between mouse and human GM-CSF (Gasson 1991).

GM-CSF interacts with GM-CSF receptor (GM-CSFr or CSFR2) expressed at very low levels (100-1000 molecules per cell) on the surface of sensitive target cells that are myeloid cells and also non-hematopoietic cells like endothelial cells. GM-CSFr is formed
by two different transmembrane glycosylated polypeptide chains named α-chain (CSFR2α) and β-chain (CSFR2β). The structure of GM-CSF receptor ternary complex (CSFR2α + CSFR2β + GM-CSF) has been solved by Hansen and colleagues (Hansen 2008). They observed a higher-order dodecamer composed of two hexamers, each with a stoichiometry of 2 GM-CSF: 2 CSFR2α: 2 CSFR2β. The α-chain binds specifically to GM-CSF with low affinity ($K_D=0.2-100$ nM), while the β-chain, that is shared also with IL-3 and IL-5 receptors and is known as common β-chain (βc), is required for signal transduction (Gearing 1989; Hayashida 1990). High-affinity binding to GM-CSF ($K_D=100$ pM) occurs only after dimerization of the GM-CSF-α-chain complex with free β-chains forming the high-affinity hexamer complex (Figure 3.5.1). Lateral aggregation of hexamer complexes forms the dodecamer (or higher-order) complex that transmits signal mainly through Janus kinase/signal transducer and activator of transcription (JAK/STAT) pathway. Low GM-CSF concentrations (0.01-10 pM) induce phosphorylation of serine$^{585}$ of the intracytoplasmic β-chain motif that transmits signal to the adapter protein 14-3-3 that in turn signals to phosphatidylinositol-3-kinase (PI3K) and Akt resulting in cell survival without proliferation. Instead, high GM-CSF concentrations (10-10,000 pM) induce phosphorylation of tyrosine$^{577}$ of the intracytoplasmic β-chain motif that, together with JAK2 that is activated after dimerization of α- and β-chain of GM-CSFr, transmits signal either to STAT5 or to She that couples with the adapter protein 14-3-3 that in turn signals to PI3K and Akt stimulating cell survival, cell activation and cell proliferation (Figure 3.5.2). Both pathways eventually stimulate expression of PU.1, an important transcription factor that regulates the expression of many genes involved in different immune and non-immune functions consistent with the terminal differentiation of alveolar macrophages in the lung. Among PU.1 targets we can find BCL2A1 for cell survival, CD11b for cell adhesion, mannose receptors for pathogen recognition, FeRs for phagocytosis, TLR2, TLR4 and TNF-α for proinflammatory cytokine signaling, IL-12 and IL-18 for IFN-γ production that mediates both innate and adaptive responses, GM-CSF and M-CSF for macrophage activation, reactive oxygen species (ROS) for microbial killing and unknown genes involved in surfactant catabolism (Hansen 2008; Jenkins 1998; Trapnell 2009).

In addition to the membrane form of GM-CSFr, a soluble form of the receptor has been described as a result of alternative splicing and/or ectodomain shedding of cell surface α-chain (Brown 1995; Prevost 2002).
Different domains of GM-CSF are involved in binding to GM-CSFr. The first studies identified up to 8 different epitopes on GM-CSF that can be targeted by different monoclonal antibodies (mAbs), and 3 of them are functional domains since the related mAbs could neutralize GM-CSF in a colony assay for normal marrow progenitor cells (Kanakura 1991). In particular, the distal two-thirds of the third α-helix and immediate downstream residues (amino acids 78-94) and the extreme carboxy-terminus of GM-CSF are mainly involved in GM-CSFr binding (Brown 1994; Hercus 1994; Chiarini 2004).

**Figure 3.5.1.** Structure of GM-CSFr ternary complex on the membrane surface. GM-CSF is highlighted in blue and GM-CSFr α-chain in yellow. One monomer of GM-CSFr β-chain is shown in magenta (chain a) and the other in green (chain b). Labels correspond to domain names. Reproduced from (Hansen 2008), with permission from Elsevier.

**Figure 3.5.2.** Model of transduction of GM-CSF signal. The low-affinity complex consists of GM-CSF (GM) bound to GM-CSFr α-chain (GMRα). Interaction with free β-chains (βc) forms the high-affinity hexamer complex. Lateral aggregation of hexamer complexes form the dodecamer complex that is fully competent to transduce signal intracellularly through JAK2 associated with βc (shown as red spheres). Reproduced from (Hansen 2008), with permission from Elsevier.
3.5.2. Autoimmune pulmonary alveolar proteinosis

Autoimmune pulmonary alveolar proteinosis (PAP) is a severe disease resulting from the accumulation of surfactant within the alveoli causing respiratory insufficiency and increased risk of uncontrolled infections. The disease, that was first described by Rosen and colleagues in 1958 (Rosen 1958), is very rare with a prevalence of 0.37 per 10,000 people. The median age at diagnosis is 39 years, most patients are men and 72% have a history of smoking. The disease exists in three forms either autoimmune, congenital or secondary. Autoimmune PAP occurs in 90% of cases and is caused by the production of autoantibodies that neutralize GM-CSF activity. The disease should be distinguished from congenital PAP which is caused by mutations in the genes encoding surfactant protein B or C or the β-chain of GM-CSF, and from similar clinical forms that can be secondary to conditions that cause functional impairment or reduced numbers of alveolar macrophages; such conditions include some hematologic cancers, pharmacologic immunosuppression, inhalation of inorganic dust (e.g., silica) or toxic fumes, and certain infections. PAP patients have high risk of infections from a variety of pathogens, including both common respiratory pathogens and opportunistic pathogens, especially Nocardia spp., Streptococcus spp., Pseudomonas aeruginosa, Listeria monocytogenes, Pneumocystis carinii, Plasmodium chabaudi, Mycobacterium tuberculosis and adenovirus (Rosen 1958; Trapnell 2003; Trapnell 2009; Suzuki 2010). These infections frequently occur at sites outside the lung, suggesting systemic defects in host defense (Seymour 2002).

Clinically, the patients present dyspnea, cough and fatigue, although 30% of cases can be asymptomatic. Less common symptoms include fever, chest pain, or hemoptysis, especially if secondary infection is present. Typical chest radiographs of PAP patients show widespread, patchy and asymmetric bilateral air-space disease. High-resolution computed tomographic scan of the chest usually shows patchy areas of ground-glass opacification and interlobular septal thickening. Clinical and radiographic findings help to diagnose PAP, but in most cases analysis of bronchoalveolar-lavage specimens can confirm the diagnosis. The lavage fluid has an opaque and milky appearance and contains large and foamy alveolar macrophages or monocytes-like alveolar macrophages and increased numbers of lymphocytes together with large, acellular eosinophilic bodies and high levels of surfactant proteins (Rosen 1958; Trapnell 2003). Surfactant is composed by lipids (90%), especially phospholipid, proteins (10%), named surfactant proteins A, B, C and D, and carbohydrates (less than 1%). The role of surfactant is to reduce surface tension.
at the air-liquid interface of the alveolar wall to prevent alveolar collapse and transudation of capillary fluid into the alveolar lumen. The accumulation of surfactant in the lungs of PAP patients is due to insufficient clearance by alveolar macrophages, rather than to overproduction by alveolar type II epithelial cells (Pattle 1955; Trapnell 2003) (Figure 3.5.3).

Figure 3.5.3. Surfactant homeostasis and impaired surfactant catabolism in PAP. (A) GM-CSF has a critical role in surfactant homeostasis in the normal lung. (B) Interruption of GM-CSF signaling in the lung by neutralizing anti-GM-CSF autoantibodies results in the accumulation of surfactant aggregates and cellular debris that fill the alveoli and reduce the size of the available gas-exchange surface eventually leading to the clinical syndrome. Reproduced with permission from (Trapnell 2003), Copyright Massachusetts Medical Society.
3. General introduction

Autoimmune PAP is treated by whole-lung lavage since 1960s and still remains the standard of care today as it improves clinical, physiological and radiographic findings in PAP patients. An alternative treatment is based on inhalation of GM-CSF that has been shown to be safe and effective in autoimmune PAP (Ramirez 1963; Luisetti 2010; Tazawa 2010).

The first evidence that **GM-CSF has a crucial role in alveolar macrophages function** was shown in 1994 with the discovery that GM-CSF deficient mice accumulated surfactant in the lungs and developed a lung phenotype similar to that of patients with autoimmune PAP. In addition, these mice had an increased mortality from pulmonary and systemic infections. Interestingly, GM-CSF deficient mice did not have defect in myeloid cell development suggesting that GM-CSF is not an essential growth factor for basal hematopoiesis, but it has a critical role in pulmonary homeostasis (Stanley 1994; Dranoff 1994). Mice lacking CSFR2β also developed a phenotype resembling PAP with normal development of hematopoietic cells (Nishinakamura 1996). GM-CSF replacement by either pulmonary but not systemic administration of GM-CSF or expression of the GM-CSF gene in the lung resulted in resolution of PAP (Reed 1999; Huffman 1996; Zsengeller 1998). Knocking-in human GM-CSF in the mouse GM-CSF locus and engrafting mice with human CD34+ hematopoietic cells improved myeloid cell reconstitution supporting, in particular, the development of human alveolar macrophages that partially rescued the PAP syndrome in GM-CSF-deficient mice (Willinger 2011).

The **evidence of causality for GM-CSF autoantibodies in the onset of PAP** was demonstrated by Sakagami and colleagues who were able to reproduce the disease in nonhuman primates. They administered highly purified GM-CSF autoantibodies derived from a PAP patient to healthy nonhuman primates (*Macaca fascicularis*) observing a phenotype resembling that of PAP patients (Sakagami 2009).
3.5.3. Pathogenic and non-pathogenic GM-CSF autoantibodies

After discovering PAP in GM-CSF deficient mice, GM-CSF autoantibodies were found in both serum and bronchoalveolar lavage fluid (BALF) of patients with common clinical autoimmune PAP. The autoantibodies are polyclonal IgG, primarily IgG1 and IgG2, that are highly specific for human GM-CSF and bind to the cytokine with high affinity (20±7 pM). The autoantibodies can target different epitopes that vary among patients with the amino acid 78 to 84, that belong to the main domain of GM-CSF interacting with GM-CSFr, being consistently recognized (Uchida 2004; Trapnell 2009). IgM antibodies to GM-CSF can be detected in 81% of PAP patients, with a binding avidity to GM-CSF 100-fold lower than that of IgGs and with weak or no neutralizing activity (Nei 2012). Also IgA antibodies to GM-CSF were detected in 63% of PAP patients (Nei 2012). High levels of GM-CSF autoantibodies were found only in patients with autoimmune PAP and not in patients with either congenital or secondary PAP, or individuals with other lung diseases or healthy people. The serum levels of GM-CSF autoantibodies in PAP patients were high enough to neutralize a large excess of GM-CSF compared to that present at physiological levels suggesting that these autoantibodies eliminate GM-CSF bioactivity in vivo (Trapnell 2003; Uchida 2004).

**GM-CSF neutralization** by autoantibodies can be the result of steric inhibition of binding of the cytokine to its receptors, as proposed by a recent study (Wang 2013). GM-CSF neutralization results in functional defects in human alveolar macrophages that show increased cell diameter, decreased cell adhesion, decreased expression of PU.1, mannose receptors, TLR4, TLR2, FcγRs and M-CSF, decreased phagocytosis of latex microspheres and decreased ability of killing microorganisms like *E. coli*, *S. aureus* and *Candida* (Trapnell 2009). Interestingly, the finding that PU.1 is consistently expressed only in the nuclei of normal alveolar macrophages, but not other normal tissue macrophages or blood monocytes, supports the notion that GM-CSF is specifically acting on alveolar macrophages (Nakata 2006). GM-CSF is not crucial for differentiation of neutrophils as neither PU.1 levels nor expression of differentiation markers on neutrophils are affected in GM-CSF deficient mice and PAP patients. Nevertheless, GM-CSF neutralization negatively impairs different neutrophil functions like adhesion to the vascular endothelium, phagocytosis of latex beads, production of ROS, bacterial killing, STAT5 phosphorylation and CD11b stimulation index (Uchida 2007).
More recently GM-CSF autoantibodies were also detected in non-immunocompromised patients with metastatic colorectal carcinoma treated with recombinant human GM-CSF to stimulate immune responses to tumor cells. These patients developed GM-CSF IgG autoantibodies after the third therapeutic cycle causing decreased half-life of exogenously administered GM-CSF and reduced GM-CSF-dependent enhancement of granulocytes. The levels of GM-CSF antibodies gradually disappeared after discontinuation of GM-CSF therapy, probably as a result of no further antigenic stimulation (Ragnhammar 1994). In another study, high titers of autoantibodies to GM-CSF were found in patients with acute myeloid leukemia, chronic myeloid leukemia or myelodysplastic syndrome, without developing PAP. GM-CSF autoantibodies of either IgG, or IgM or IgA isotype were induced by GM-CSF used as adjuvant to solid tumor vaccination and their titers were higher in patients with active disease compared to those in complete remission (Sergeeva 2008). Taken together, these reports show that there are situations in which GM-CSF autoantibodies are not associated with development of PAP.

GM-CSF autoantibodies are often found in IVIG preparations and were also reported to be present in healthy individuals at very low serum level compared to PAP patients. These autoantibodies are mostly bound to GM-CSF, forming immune complexes that make difficult their detection in serum, and it has been reported that they neutralize GM-CSF activity (Uchida 2009). Also IgM and IgA antibodies to GM-CSF have been reported in 22% and 57% of healthy donors, respectively (Nei 2012). It has been hypothesized that GM-CSF autoantibodies are ubiquitously present in all individuals, but they become pathogenic and cause PAP only when they exceed a critical threshold that can block GM-CSF bioactivity completely. Under the critical threshold (estimated between 8.5 and 19 μg/ml), GM-CSF autoantibodies can act as scavenger of free GM-CSF and may negatively regulate myeloid cell reactivity and functions depending on GM-CSF signaling (Uchida 2009).
3. General introduction

3.6. Autoantibodies to citrullinated protein antigens

As mentioned above, post-translational modifications can generate new self antigens against which an immune response can arise because immunologic tolerance to these self antigens was not induced during B cell and T cell development. In rheumatoid arthritis (RA) patients autoantibodies to proteins modified by post-translational modifications, like citrullination, carbamylation and oxidation, have been detected (Burska 2014). In particular, antibodies to citrullinated protein antigens (ACPAs) have been found in ~60% of patients with RA and it has been proposed a role for ACPAs in the pathogenesis of the disease (Schellekens 2000; Klareskog 2008). In my thesis I will extend to ACPAs the study on the origin of autoantibodies.

3.6.1. Rheumatoid arthritis

RA is a chronic disease characterized by inflammation of the synovial joints. As the disease progresses, the inflamed synovium invades and damages the cartilage, followed by erosion of the bone. Patients with RA suffer chronic pain, loss of function and disability. RA was at first considered an autoimmune disease driven mainly by B cells producing anti-IgG autoantibodies (RF) (Waaler 2007). However, the identification of RF in healthy individuals and its absence in some patients with RA, suggested that more complex mechanisms are involved in this pathology. In particular, self antigens such as type II collagen, proteoglycans, aggrecan and fibrinogen have been proposed as potential antigens because of their ability to induce arthritis in mice. However, their pathogenic role in humans is not clear. Recently, new clues have been provided by the discovery of ACPAs that, unlike RF, are highly specific for RA as they exist in around 2% of normal population and are also quite rare in other systemic inflammatory conditions, such as SLE, Sjongren’s disease or myositis. RA patients develop ACPAs several years before the onset of the disease (Schellekens 2000; Avouac 2006). Several studies suggest that immune response to citrullinated proteins contributes to the development of RA. In rodents, monoclonal antibodies to citrullinated fibrinogen enhance arthritis development and immunization with citrullinated type II collagen causes a more severe arthritis compared to immunization with the non-citrullinated protein (Kuhn 2006; Lundberg 2005). In humans, autoantibodies to citrullinated vimentin have been shown to induce
osteoclastogenesis and subsequent bone resorption activity that leads to bone loss in RA patients (Harre 2012).

It has been suggested that both genetic and environmental determinants predispose to RA. The occurrence of antibodies to citrullinated proteins is closely linked to particular MHC class II alleles, characterized by the HLA-DRB1 shared epitope (SE), and to the 1858T polymorphism in the gene encoding the PTPN22 tyrosine phosphatase. The combination of both genes increases the risk of ACPA-positive RA 20-fold. Smoking is long known to be the major environmental factor to increase risk for RA and its association with HLA-DRB1 increases the relative risk to develop ACPA-positive RA 20-fold compared to non-smokers with no HLA-DRB1 alleles (Klareskog 2008; van der Helm-van Mil 2007; Wesoly 2005; Law 2012).

The presence of ACPAs has been included in the 2010 RA classification criteria. The current diagnostic test uses cyclic citrullinated peptide (CCP) as an artificial antigen to detect ACPAs in RA patients (Aletaha 2010; Neogi 2010; van Venrooij 2011). However, the specificity of the autoantibodies has not been completely defined, although it has been reported reactivity of serum IgG of RA patients against citrullinated synovial proteins like fibrinogen, vimentin, type II collagen and alpha-enolase (Masson-Bessiere 2001; Vossenaar, Despres, 2004; Young 1979; Klareskog 2008). More recently, new citrullinated proteins, like apoliprotein E, myeloid nuclear differentiation antigen and β-actin, have been found in the synovial fluid and have been proposed as additional targets of ACPAs (van Beers 2013).

### 3.6.2. PAD enzymes and citrullination

Citrullination is a posttranslational process of deimination of charged peptidyl-arginine to the neutral peptidyl-citrulline mediated by a series of enzymes denoted peptidyl arginine deiminases (PADs). The activity of these enzymes is dependent on high concentrations of calcium and deimination can occur intracellularly in conjunction with apoptosis (Figure 3.6.1). Citrullination seems to be a physiological event: it is likely that changes in the charge and subsequent intra and intermolecular interactions could lead to alter protein folding making protein more prone to degradation by proteolytic enzymes (Gyorgy 2006). Increased citrullination was observed in many inflamed tissues, including arthritic joints in several forms of arthritis, lungs, extraarticular inflammatory sites in RA,
human brain, inflamed muscles and inflamed lymphoid organs (Masson-Bessiere 2001; Vossenaar, Despres, 2004; Young 1979; Klareskog 2008). When citrullination spans the entire range of molecular weights, the process is named “cellular hypercitrullination” and it has been shown to be induced by two immune-mediated membranolytic pathways mediated by perforin and the membrane attack complex of the complement, which are active in RA joints and play an important role in RA pathogenesis (Romero 2013).

Five PAD enzymes (PAD1-4 and PAD6) have been identified in humans, PAD2 and PAD4 being the most likely candidates for the citrullination of synovial proteins in RA since they are expressed in myeloid cells and are detected in RA synovial fluid closely associated with neutrophils infiltrates (Vossenaar, Radstake, 2004). In addition to human PAD enzymes, it has been discovered that the bacterium Porphyromonas gingivalis, responsible for periodontal infections, expresses a unique PAD enzyme (PPAD) that converts arginine residues to citrulline, thus providing new sources of citrullinated antigens in RA. Unlike human PADs, PPAD is not calcium dependent and can convert free L-arginine and only carboxy-terminal arginines (Maresz 2013; Routsias 2011).

**Figure 3.6.1.** Deimination of arginine to citrulline by PAD. Adapted from (Klareskog 2008).
3.6.3. Etiopathogenic model for rheumatoid arthritis

The most recent model for the etiopathogenesis of ACPA-positive RA is based on three consecutive stages (Klareskog 2008) (Figure 3.6.2). In stage 1 (immune response) environmental risk factors, such as smoking or infections, may induce inflammation by stimulating influx of immune cells into the lung. Toxic components of smoke activate the cells and make them more prone to apoptosis. PAD becomes activated due to a rise in intracellular calcium concentration during the apoptotic process and deiminates intracellular proteins. The apoptotic cells are usually removed by neighboring phagocytic cells, but when the number of dying cells is too high or there is a defect in the clearance of apoptotic cell remnants, the cells become necrotic and release their contents into the extracellular space. Citrullinated proteins are released together with PAD enzymes that can also citrullinate extracellular proteins present in the lung. Since proteins undergoing processing in APCs can be citrullinated before presentation to autoreactive T cells, as shown in a recent study (Ireland 2006), presentation of citrullinated peptides in genetically predisposed individuals may activate autoreactive T cells, which in turn induce B cell help and stimulate the production of ACPAs.

In stage 2 (pathologic inflammatory response) arthritis develops by an unspecific stimulus that induces citrullination of proteins in the joints. ACPA from the circulation are recruited and bind to citrullinated proteins present in the inflamed joint forming immune complexes. Such immune complexes activate APCs that present more citrullinated antigens, more B cells that increase ACPA and RF production and more T cells that produce cytokines, which in turn stimulate monocytes/macrophages, endothelial cells, fibroblasts to produce more proinflammatory cytokines (TNF, IL-1, IL-6) or chemokines (CXCL8, CCL2) and metalloproteinases which are responsible for tissue destruction. In addition, neutrophils can cause tissue damage and promote autoimmunity by enhanced formation of neutrophils extracellular traps (NETs) that are formed by a chromatin meshwork decorated with antimicrobial peptides typically present in neutrophil granules. NETs can be released by a particular form of cell death called NETosis and can externalize proinflammatory molecules and several autoantigens, especially histone and vimentin citrullinated by PAD enzymes (Khandpur 2013; Li 2010).
In stage 3 (chronic RA) more immune cells are recruited into the joint perpetuating the joint inflammation into chronic RA. In conclusion, according to the model proposed by Klareskog and colleagues, ACPAs may play an important role in the pathogenesis of RA. (Klareskog 2008; van Venrooij 2011).

**Figure 3.6.2.** Three-stage etiological model for the development of ACPA-positive RA. Adapted from (Klareskog 2008).
4. Research plan

4.1. Aims of the research

As seen in the introduction, autoantibodies can be generated by V(D)J recombination during B cell development or by somatic mutations acquired during the GC reaction. The autoreactive B cells resulting from these processes can pose a risk for developing autoimmune diseases if they receive stimuli by TLR agonists or by helper T cells that induce their activation and sustain their survival and differentiation into memory B cells and plasma cells that produce autoantibodies.

In this process, the origin of autoantibodies that are involved in the pathogenesis of autoimmune diseases is still object of debate. Therefore, the first aim of my thesis is to test whether the autoantibodies are originated from autoreactive naïve B cells or by fortuitous mutations acquired during the response to a foreign antigen. In particular I have addressed this question in two autoantibody-mediated autoimmune diseases: pulmonary alveolar proteinosis (PAP) and rheumatoid arthritis (RA), where autoantibodies to the granulocyte-macrophage colony-stimulating factor (GM-CSF) and to citrullinated proteins are produced.

For many disorders, the mechanism of action of autoantibody is known, but for PAP it is still a matter of debate. Indeed, autoantibodies to GM-CSF used in therapy do not cause PAP raising the question of the particular mechanism by which GM-CSF autoantibodies cause a complete deficiency of the cytokine in vivo. Therefore the second aim of my thesis is to understand how they participate to the pathogenesis of the disease once they bind to the self-antigen. I want to understand whether the pathogenicity is dependent on the amount, on the specificity or on the combination of particular autoantibodies. In particular, also in view of the reports indicating that anti-GM-CSF autoantibodies can be found in healthy individuals, I think it is important to establish an assay to detect such autoantibodies.
4.2. Experimental approach

The first experimental approach is to reconstruct the development of the clone that gives rise to the autoantibody. This can be done by isolating autoantibodies using a protocol developed in our laboratory (Traggiai 2004). The antibody genes will be sequenced and the antibodies will be produced in a recombinant form. Somatic mutations will be removed to generate antibodies that represent the so called “unmutated common ancestor” (UCA), i.e. the antibody produced by the naïve B cell that gave rise to the clone producing the autoantibody. The specificity and the affinity of binding to the self antigen will be analyzed by ELISA or surface plasmon resonance (SPR). In particular I will use SPR to determine the kinetics parameters, as I also did for the characterization of influenza antibodies in a recent study in which I am coauthor (Pappas 2014).

The second experimental approach is to determine the fine specificity and affinity of the autoantibodies, their cytokine neutralizing activity in vitro and in vivo. First, I will define the epitopes recognized by the autoantibodies and I will analyze the possible formation of immune complexes using high-performance liquid chromatography (HPLC). GM-CSF neutralization will be studied in vitro using a bioassay based on GM-CSF-dependent TF-1 cells. Pharmacokinetics will be studied in vivo to assess the clearance of GM-CSF induced by single or multiple autoantibodies and the role of Fc receptors in this process will be analyzed using autoantibodies that carry mutations in the constant region of the heavy chain which make them lose the binding to Fc receptors.
5. Results and discussion

5.1. Neutralization and clearance of GM-CSF by autoantibodies in pulmonary alveolar proteinosis

A manuscript by:

Luca Piccoli,1,2 Ilaria Campo,3 Chiara Silacci Fregni,1 Blanca Maria Fernandez Rodriguez,1 Andrea Minola,1 Federica Sallustio,1 Maurizio Luisetti,3 Davide Corti,1,4 and Antonio Lanzavecchia1,2

1 Institute for Research in Biomedicine (IRB), Bellinzona, Switzerland
2 Institute of Microbiology, Eidgenössische Technische Hochschule (ETH), Zürich, Switzerland
3 Respiratory Disease Unit, IRCCS San Matteo Hospital Foundation, Pavia, Italy
4 Humabs Biomed SA, Bellinzona, Switzerland
5.1.1. Abstract

Pulmonary alveolar proteinosis (PAP) is a severe autoimmune disease caused by the production of autoantibodies that neutralize GM-CSF and cause a deficiency of alveolar macrophages and the consequent accumulation of lipoproteinaceous material in the alveoli. We isolated and characterized twenty-one monoclonal autoantibodies from PAP patients and found that three antibodies can bind to non-overlapping sites on the same GM-CSF molecule. When tested in a cell proliferation bioassay, we found that single antibodies can only partially neutralize GM-CSF depending on the concentration of the cytokine and the number of cell receptors, while three antibodies binding to non-overlapping sites can sequester GM-CSF and fully neutralize its activity even under the most unfavorable conditions. Furthermore, when tested in vivo, administration of single antibodies led to the accumulation of a large pool of bioavailable GM-CSF, while administration of three antibodies promoted a rapid degradation of GM-CSF in a Fc-dependent fashion. We also found that somatic mutations contributed to the specificity of the autoantibodies and that low levels of anti-GM-CSF antibodies can be detected also in healthy donors using a new complementation bioassay. Taken together, these findings provide a plausible explanation for the severe phenotype of PAP patients and the safety of treatments based on single anti-GM-CSF monoclonal antibodies.
5. Results and discussion

5.1.2. Introduction

Autoantibodies against cytokines have been frequently reported both in healthy individuals and in patients with autoimmune or infectious diseases (Watanabe 2010). In several instances, a pathogenic role for cytokine autoantibodies has not been formally demonstrated, as it is the case for autoantibodies to IL-17 in patients with mucocutaneous candidiasis or autoantibodies to IFN-γ in patients with mycobacterial infections (Watanabe 2010; Puel 2010; Madariaga 1998; Patel 2005). In other instances, autoantibodies have been shown to cause severe pathology by neutralizing the biological activity of the target cytokine, as it is the case for autoantibodies to the granulocyte-macrophage colony stimulating factor (GM-CSF) in autoimmune pulmonary alveolar proteinosis (PAP) and autoantibodies to erythropoietin (EPO) in pure red-cell aplasia (Trapnell 2009; Casadevall 2002). While in some cases autoantibody production has been linked to the administration of recombinant cytokines, such as EPO, GM-CSF or IFN-β (Casadevall 2002; Sergeeva 2008; Sethu 2013), in most cases the stimuli that elicit the production of cytokine autoantibodies remain unknown.

The reason why cytokine autoantibodies may or may not cause pathology is not entirely clear. The prevailing view is that, when of enough affinity and present above a certain threshold of concentration, an autoantibody can neutralize the biological activity of the cytokine by simply binding and preventing its interaction with the cognate cellular receptor, a mechanism that can be recapitulated in vitro using cell proliferation bioassays with cytokine-dependent cell lines. Interestingly, however, several studies with toxins (Pohl 2013; Chen 2012; Nowakowski 2002) and cytokines (Montero-Julian 1994) demonstrated a synergy between different antibodies binding to the same molecule, suggesting that in some cases neutralization may be dependent on the production of antibodies targeting multiple antigenic sites, thus leading to the formation of immune complexes with the cytokine that can be efficiently cleared in vivo. In addition, it has been reported that monoclonal antibodies to IL-2, that neutralize the cytokine activity in vitro, can paradoxically enhance and extend IL-2 activity when injected in vivo (Boyman 2006).

Autoimmune PAP is a rare and severe disease caused by the production of autoantibodies that neutralize the biological activity of GM-CSF. GM-CSF was shown to be essential to promote the terminal differentiation of lung-resident fetal monocytes into alveolar macrophages that are then responsible for the catabolism of surfactant lipids and proteins in the lungs (Guilliams 2013). The lack of alveolar macrophages as a
5. Results and discussion

consequence of GM-CSF neutralization leads to the accumulation of lipoproteinaceous material within the alveoli causing respiratory insufficiency and increased risk of infections (Trapnell 2009). In addition, GM-CSF deficiency results in impaired antimicrobial activity of neutrophils (Uchida 2007). The pathogenic role of GM-CSF autoantibodies has been demonstrated by adoptive transfer of GM-CSF autoantibodies purified from PAP patients into non-human primates (Sakagami 2009). The sera of PAP patients contain levels of autoantibodies that exceed the pathogenic threshold of 10 μg/ml, and that bind and neutralize GM-CSF at high titers (Uchida 2004). A recent study reported the isolation of several monoclonal autoantibodies from PAP patients that showed different binding affinity and suggested that single human monoclonal antibodies neutralize the cytokine activity by blocking binding to the GM-CSF receptor (Wang 2013).

In this study we isolated a panel of monoclonal autoantibodies from PAP patients and show that single antibodies can only partially neutralize GM-CSF activity in vitro, depending on the experimental conditions of the bioassay, whereas three non-cross-competing antibodies can completely neutralize GM-CSF activity. In addition, we show that, in vivo, single antibodies enhance the levels of bioavailable GM-CSF, while three non-cross-competing antibodies induce a rapid Fc-dependent clearance of the cytokine. Finally we show that in most cases somatic mutations are critical for binding to GM-CSF, suggesting that clones that give rise to autoantibodies derive from memory rather than naïve B cells.
5. Results and discussion

5.1.3. Methods

**Isolation and production of monoclonal antibodies from PAP patients.** Peripheral blood samples were obtained from five PAP patients. Memory B cells were isolated from cryopreserved or fresh PBMCs using anti-FITC microbeads (Miltenyi Biotec) following staining of PBMCs with CD22-FITC (BD Pharmingen), and were immortalized with Epstein-Barr virus and CpG in multiple wells as described previously (Traggiai 2004). Culture supernatants were tested for binding to human GM-CSF by ELISA. cDNA was synthesized from positive cultures and both heavy chain and light chain variable regions were sequenced. All monoclonal antibodies were produced recombinantly as IgG1 by transient transfection of HEK 293 Freestyle Cells (Invitrogen) using polyethylenimine (PEI), and tested for binding to GM-CSF by ELISA. The study was conducted in accordance with the Declaration of Helsinki guidelines and was approved by the Ethics Committee of IRCCS San Matteo Hospital Foundation of Pavia, Italy. All patients gave informed consent.

**Sequence analysis of antibodies and reversion to germline.** The usage of VH and VL genes and the amount of somatic mutations were determined by analysing the homology of VH and VL sequences of mAbs to known human V, D and J genes by the IMGT (international ImMunoGeneTics information system) database (Lefranc 2009). Sequences of unmutated common ancestors (UCA) were determined by reverting mutations to the germline sequence while retaining the original CDR3 junctions and terminal deoxy-nucleotidyl transferase (TdT) N nucleotides. UCA sequences in which putative mutations of the HCDR3 were not removed were also determined. VH UCA, VH UCA (HCDR3 WT) and VL UCA nucleotide sequences were synthesized by Genscript, and their accuracies were confirmed by sequencing. Four different versions of 7 selected mAbs were produced recombinantly: UCA, UCA-HCDR3 WT, and shuffled mAbs either with VH UCA + wild-type VL or with wild-type VH + VL UCA.

**Antibody purification and ELISA assays.** Human mAbs and total IgG from PAP sera were purified by protein A or protein G chromatography (GE Healthcare). Total IgG from healthy donors were purified by HiTrap Protein A HP columns (GE Healthcare) and concentrated by Amicon Ultra filter units (100K, Millipore). Total GM-CSF antibodies were affinity-purified from PAP sera using magnetic beads (Invitrogen) conjugated with human GM-CSF. Total IgGs were quantified by ELISA plates coated with anti-human IgG (SouthernBiotech) using Certified Reference Material 470 (ERMs-DA470, Sigma-
Aldrich) as standard. Binding to GM-CSF was tested by ELISA using 384-well SpectraPlates (PerkinElmer) for primary screenings or 96-well MaxiSorp plates (Nunc) for any following test. Briefly, ELISA plates were coated with 1 μg/ml of recombinant human GM-CSF (Gentaur), blocked with 1% BSA and incubated with titrated antibodies, followed by AP-conjugated anti-human IgG secondary antibodies (SouthernBiotech). Plates were then washed, substrate (p-NPP, Sigma) was added and plates were read at 405 nm. EC50 (ng/ml) was calculated for every sample by nonlinear regression analysis using GraphPad Prism 5 software.

**Surface plasmon resonance (SPR) assays.** Protein A (450 nM) was stabilized in 10 mM acetate buffer, pH 4.5, and immobilized onto a EDC/NHS pre-activated ProteOn sensor chip (Biorad) through amine coupling; unreacted groups were blocked by injection of ethanolamine HCl (1 M). HEPES buffered saline (HBS) (10 mM HEPES, pH 7.4, 150 mM NaCl, 3 mM EDTA, 0.005% surfactant Tween-20) was used as running buffer. All injections were made at flow rate of 100 μl/min. Monoclonal antibodies were diluted in HBS (200 nM) and injected onto the protein A coated chip for capturing, followed by injection of different concentrations of human GM-CSF (400 nM, 200 nM, 100 nM, 50 nM, 25 nM); one channel of the chip was injected with HBS and used as reference for the analysis. Injection time and dissociation time were 120 s and 600 s, respectively. Each binding interaction of mAbs with GM-CSF was assessed using a ProteOn XPR36 instrument (Biorad) and data processed with ProteOn Manager Software. Ka, Kd and KD were calculated applying the Langmuir fit model. To determine the epitope specificity, GM-CSF autoantibodies (150 mM) were directly immobilized on a sensor chip, followed by injection of GM-CSF (100 nM) and autoantibodies (200 nM). To assess simultaneous binding to GM-CSF, different mAbs (200 nM each) were serially injected after GM-CSF capture (50 nM). Injection time and dissociation time were 60 s and 20 s, respectively.

**Size-exclusion HPLC.** Three non-cross competing mAbs were diluted in phosphate buffered saline (PBS) singularly or as a three-antibody-combination (10 μg of total antibody amount), and mixed with GM-CSF (1:1 or 10:1 molar ratios) for 1 hour, RT. Samples were analyzed by Agilent 1100 HPLC machine using TSK-GEL G3000SW columns (Tosoh, bed volume: 13 ml, void volume: 4.6 ml) with PBS as mobile phase (flow rate: 1 ml/min). A universal solvent 2 μm filter (Agilent) was put between injector and column. Detection was performed by a Variable Wavelength Detector (VWD, Agilent) with ultraviolet absorption at 220 nm.
**TF-1 proliferation bioassays.** TF-1 cells (CLS, Cell Lines Service) were maintained in RPMI 1640 medium supplemented with 10% Fetal Bovine Serum (HyClone), 1% GlutaMAX, 1% Penicillin/Streptavidin, 1% non-essential amino acids, 1% sodium pyruvate, 1% 2-mercaptoethanol (all from GIBCO), 5 ng/ml human GM-CSF (Gentaur), 10 ng/ml human IL-3 (ImmunoTools). Cells were grown at 37°C in a humidified incubator with 5% CO₂. A GM-CSF neutralization assay was performed by serially diluting mAbs (or combination of mAbs, total IgG, or affinity-purified antibodies) in growth medium with neither GM-CSF nor IL-3, adding GM-CSF at a concentration of 100 pg/ml, and preincubating in 96-well flat-bottom cell culture plates (Costar) at 37°C for 1 hour. TF-1 cells were washed 5 times, diluted in growth medium with neither GM-CSF nor IL-3, and 10,000 cells per well were seeded (final GM-CSF concentration equal to 50 pg/ml). In other tests, GM-CSF was used at final concentration of 500 and 5,000 pg/ml, and 1,000 cells per wells were seeded. Cells with or without GM-CSF in absence of antibodies were used as control to determine maximum and minimum levels of cell proliferation. Plates were incubated at 37°C in a humidified incubator with 5% CO₂ for 72 hours, and cell proliferation was measured after 6-hour incubation with 0.2 μCi/well of [3H]-thymidine (PerkinElmer). GM-CSF neutralization was calculated as percentage of inhibition of TF-1 growth with the following formula: \[ \frac{1 - (\text{CCPM of a single well} - \text{average CCPM of control cells grown without GM-CSF})}{\text{average CCPM of control cells grown with GM-CSF} - \text{average CCPM of control cells grown without GM-CSF})} \times 100 \] (CCPM = corrected counts per minute). IC90 (μg/ml) was calculated for every sample by a nonlinear regression analysis using GraphPad Prism 5 software. In some experiments mouse sera were titrated in TF-1 growth medium and preincubated at 37°C for 30 min. TF-1 cells were washed and seeded (1,000 cells per well). A titration of GM-CSF (60,000 to 0.3 ng/ml) was added as growth control. CCPM of each single well were plotted against the serum titration.

**In vivo clearance of GM-CSF immune complexes.** Groups of 6-8 week-old female BALB/c mice were injected intravenously with 100 μg of purified mAbs or 2 mg of total IgG purified from PA96 patient. After 16 hours, 2 μg of human GM-CSF were injected. Sera samples were collected on day 1 and day 5. GM-CSF was quantified by a sandwich ELISA. Briefly, 10 μg/ml of an antibody that bound to site II of GM-CSF was used to coat 96-well Maxisorp plates (Nunc), which were then blocked with PBS + 10% FBS (Gibco). All sera and GM-CSF, which was used as standard (range 3.4-600,000 pg/ml), were titrated and tested in parallel under different conditions: in one plate all samples were
supplemented with 25% (vol/vol) of an alkaline dissociation buffer (2.5% Triton X100, 2 M ethanolamine, 0.15 M NaCl, pH 11.6), in the other plate all samples were supplemented with 25% (vol/vol) of PBS + 10% FBS. Plates were left overnight at RT. Detection of captured GM-CSF was made with 1 µg/ml of a biotinylated antibody that bound to site I of GM-CSF for 1 h, RT, followed by binding of 0.5 µg/ml streptavidin-AP (Jackson ImmunoResearch) for 1 h, RT. Plates were then washed, substrate (p-NPP, Sigma) was added and plates were read at 405 nm.

**Binding of GM-CSF immune complexes to FcγRs.** Four TZM-bl cell lines (NIH AIDS Research & Reference Reagent Program) each transfected with a specific Fcγ receptor (FcγRI, FcγRIIa, FcγRIIb or FcγRIIIa) were maintained in DMEM medium supplemented with 10% Fetal Bovine Serum (Hyclone), 0.025 M Heps, 10 µg/ml Gentamicin and 20 µg/ml Blasticidin. Untransfected TZM-bl cells were used as negative control and were maintained in DMEM medium supplemented with 10% Fetal Bovine Serum (Hyclone) and 2% Penicillin/Streptavidin. Cells were grown at 37°C in a humidified incubator with 5% CO₂. Expression of specific FcγRs was assessed by staining TZM-bl cells with FITC-conjugated anti-CD64 (anti-FcγRI), anti-CD32 (anti-FcγRIIa and anti-FcγRIIb) and anti-CD16 (anti-FcγRIIIa) antibodies (all from BD Pharmingen). Untrasfected and transfected TZM-bl cells were washed with staining buffer (PBS with 10% Fetal Bovine Serum and 2 mM EDTA) and seeded in 96-well-plates at a density of 50,000 cells per well. A single anti-GM-CSF mAb (GCA21) or a combination of three non-cross-competing mAbs (GCA21, GCA7 and GCB59) at final concentration of 2.5 µg/ml were mixed with 0.05 µg/ml GM-CSF, or staining buffer (PBS with 10% Fetal Bovine Serum and 2 mM EDTA). The LALA versions of all antibodies and a mAb with a different specificity were included as controls. Samples were incubated at 37°C for 30 min to allow the formation of immune complexes and then cooled down to 4°C before adding them to TZM-bl cells for 30 min. Cells were washed twice and stained with anti-human IgG Fcγ fragment specific F(ab’)2 fragment (Jackson ImmunoResearch). Samples were analyzed on BD FACSCanto (BD Biosciences) and median intensity fluorescence was analyzed and compared between samples.
5. Results and discussion

5.1.4. Results

Isolation and characterization of GM-CSF-specific autoantibodies from PAP patients. Peripheral blood mononuclear cells (PBMCs) and sera were collected from five chronic PAP patients of age ranging from 32 to 69 years. All patients had high serum titers of GM-CSF autoantibodies, ranging from 46 to 805 µg/ml (Table 5.1.1). Memory B cells were isolated and immortalized with Epstein-Barr virus and CpG, as described (Traggiai 2004). The supernatants were screened for the presence of GM-CSF-specific IgG by ELISA and 21 clones that produced GM-CSF autoantibodies (mAbs) were identified. The antibody V genes were sequenced and analyzed using the IMGT database (Lefranc 2009). Most antibodies were IgG1 (20/21) and used κ light chain (19/21), with no clear preference for V gene usage. The load of somatic mutations was comparable to that characteristic of T-cell-dependent responses against non-self antigens, ranging from 3.8% to 17.4% in the VH gene segment and from 0% to 14.5% in the VL gene segment. All antibodies were produced recombinantly as IgG1 and tested for binding to GM-CSF (Table 5.1.2 and Table 5.1.3). The EC50 values determined by ELISA ranged from 16 to 836 ng/ml and the KD values, as determined by surface plasmon resonance (SPR), ranged from 0.2 to 5.1 nM, consistent with high-affinity binding. Of notice, the kinetics values were heterogeneous. For instance, antibodies GCA7 and GCE402, which had comparable EC50 and KD values, showed different kinetics, being GCA7 characterized by a low on-/low off-rate and GCE402 by a high on-/high off-rate. The properties of the monoclonal antibodies isolated are consistent with a polyclonal response of somatically mutated B cells.

Binding to GM-CSF is dependent on somatic mutations. The role of somatic mutations in the development of GM-CSF autoantibodies was investigated by testing versions of the autoantibodies in which the somatic mutations of the whole V(D)J gene were removed. We refer to these antibody variants as the unmutated common ancestors (UCA). We also produced shuffled variants in which only the VH or the VL were reverted to the UCA sequence. When analyzed by SPR and ELISA, 5 out of the 7 UCA antibodies tested did not bind to GM-CSF, while the remaining 2 showed a reduced, but still detectable binding (Figure 5.1.1 and Table 5.1.4). Shuffling experiments showed that somatic mutations in the heavy chain played a major role in high-affinity binding. Since a
precise identification of somatic mutations in the HCDR3 is not possible due to the difficulty of unequivocally assign the D segment and determine the junctional rearrangement, we also tested antibody variants in which only the V gene segment was reverted to the germline sequence, while the HCDR3 was left in the wild-type form. Interestingly, in 4 out of 7 cases removal of mutations in the V segment was sufficient to abolish binding (Table 5.1.4), providing formal evidence for the role of somatic mutations in determining autoantibody specificity.

**PAP autoantibodies target multiple sites of GM-CSF and can form high-molecular-weight immune complexes.** Using SPR cross-competition experiments we identified multiple antigenic sites on GM-CSF and a complete antigenic map based on a matrix of cross-competition experiments was generated (Figure 5.1.2A). Sites I, II, III and IV are defined by four non-cross-competing autoantibodies GCA21, GCA7, GCB59 and GCC9. Interestingly, using SPR we could show that three non-cross-competing autoantibodies can bind simultaneously to a single molecule of GM-CSF (Figure 5.1.2B). Furthermore, when GM-CSF was incubated with an excess of three antibodies, formation of high-molecular-weight immune complexes could be detected by size-exclusion chromatography (SEC-HPLC) (Figure 5.1.2C).

**Potent in vitro neutralization of GM-CSF by combinations of three autoantibodies.** The neutralizing activity of the autoantibodies was assessed by measuring their ability to inhibit the proliferation of TF-1 cells in response to recombinant GM-CSF. Polyclonal IgG and autoantibodies purified from the sera of PAP patients showed potent and complete neutralizing activity, with IC90 values ranging from 0.53 to 36 µg/ml and from 0.018 to 0.181 µg/ml, respectively (Figure 5.1.3A). From these values it was estimated that GM-CSF autoantibodies account for 0.1 up to 5.6% of total IgG in the serum of PAP patients (i.e. 7.6 to 1300 µg/ml). These findings are consistent with previous reports (Uchida 2004; Uchida 2009) and indicate that PAP patients have very high levels of GM-CSF autoantibodies capable of neutralizing the biologic activity of the cytokine.

Surprisingly, in the same bioassay, most monoclonal autoantibodies failed to neutralize GM-CSF, even when tested at the concentration of 1 mg/ml (Figure 5.1.3B and data not shown). The only exception was GCE536 which neutralized GM-CSF activity with an IC90 value of 2.43 µg/ml, while the therapeutic antibodies Namilumab and MOR103 (Krinner 2007; Steidl 2008), showed IC90 values of 0.80 and 0.16, respectively.
Interestingly, when combined together, two non-cross-competing antibodies showed enhanced neutralizing activity both in terms of dose-response and percent inhibition, the combination of GCA21 (site I) and GCB59 (site IV) being the most effective (Figure 5.1.3B). Furthermore, a combination of three non-cross-competing antibodies (GCA21, GCA7 and GCB59, specific for sites I, II and IV) led to a complete inhibition of proliferation with an IC90 value of 0.08 µg/ml (expressed as the total concentration of the three mAbs), which was lower than that of the therapeutic antibodies MOR103 and Namilumab. A strong synergy between three non-cross-competing antibodies was observed for most combinations tested, with IC90 values comparable to those of affinity-purified antibodies from PAP sera (Figure 5.1.4).

Considering the law of mass action, we hypothesized that in the presence of a single antibody a small fraction of GM-CSF may continuously dissociate from the antibody and become available to trigger the high-affinity GM-CSF receptor. In contrast, in the presence of three antibodies, GM-CSF may be sequestered irreversibly in stable immune complexes. As expected from the law of mass action, we found that by varying the cell number and the GM-CSF concentration the sensitivity of the assay was dramatically affected. In particular, lowering the number of TF-1 cells and the concentration of GM-CSF led to a more sensitive test that showed increased neutralization by single and multiple antibodies (Figure 5.1.3C). In contrast, when high number of TF-1 cells and high doses of GM-CSF were used, even the most potent neutralizing antibodies MOR103 and Namilumab, failed to neutralize GM-CSF, even when present in a 400-fold molar excess. Strikingly, in all conditions, a combination of three non-cross-competing antibodies was capable of completely neutralizing GM-CSF.

**Fc-dependent clearance of GM-CSF immune complexes in vivo.** Having established that GM-CSF can form complexes with three antibodies resulting in efficient in vitro neutralization of the cytokine biological activity, we were interested in understanding the effect of single versus multiple autoantibodies in vivo. Mice were injected with a total of 100 µg of single or multiple monoclonal antibodies or with 2 mg of the IgG fraction isolated from the serum of a PAP patient, followed by injection of 2 µg of human GM-CSF. At different time points serum was collected and the amount of GM-CSF present was measured by a sandwich ELISA using an antibody specific for site II for capture and site I for detection. The assay was performed on serum either untreated or after alkaline treatment to dissociate the immune complexes (Figure 5.1.5). In the absence of
antibodies, the injected GM-CSF disappeared rapidly from the serum and was undetectable after 24 hours (Figure 5.1.6A). In contrast, when single antibodies (GCA21 or MOR103) were used, high levels of GM-CSF were recovered from serum on day 1 and were still present on day 5. Of note, GM-CSF detection required alkaline dissociation in the case of MOR103 but not for GCA21, consistent with the different dissociation rates of the two antibodies (Table 5.1.1). In striking contrast, when mice received three non-cross-competing antibodies (GCA21, GCA7 and GCB59) or PAP IgG, GM-CSF was rapidly cleared since only low or undetectable amounts of the cytokine could be detected in the day-1 and day-5 sera, respectively, after alkaline dissociation.

To address the possible role of Fc receptors in the clearance of GM-CSF, we tested the same antibodies in a variant form, called LALA, that does not bind to C1q nor to Fcγ receptors. Similarly to the wild-type antibodies, single LALA antibodies led to an increase in GM-CSF levels in serum. However, in contrast to what observed for three wild-type antibodies, three LALA antibodies failed to clear GM-CSF, which was quantitatively recovered in the sera following alkaline dissociation even on day 5 (Figure 5.1.6A).

To ask whether the antibody-bound GM-CSF would be bioavailable, we tested the sera of mice for their ability to support TF-1 proliferation (Figure 5.1.6B). Sera of mice receiving GCA21 or MOR103 led to a robust proliferation of TF-1 cells, consistent with a GM-CSF dissociation rate sufficient to engage the cytokine receptor. In contrast, sera of mice receiving three wild-type antibodies or PAP IgG were not able to stimulate proliferation, consistent with clearance of the immune complexes in vivo. In addition, although containing high level of GM-CSF, sera of mice receiving three LALA antibodies were not stimulatory, a finding consistent with irreversible sequestration of GM-CSF in stable immune complexes.

To further address the role of Fcγ receptors, we tested immune complexes formed between GM-CSF and wild-type or LALA antibodies for their capacity to bind to TZM-bl cells expressing different Fcγ receptors. Strong binding was observed only on FcγRIIa- and FcγRIIb-expressing cells and when immune complexes were formed by three wild-type, but not LALA, antibodies (Figure 5.1.6C and Figure 5.1.7). Taken together, the above results indicate that single antibodies, even when potently neutralizing in vitro, increase the half-life of GM-CSF and build up a circulating pool of bioavailable cytokine. In contrast, three or more antibodies lead to the formation of immune complexes that are efficiently cleared through an Fc-dependent mechanism.
Low levels of anti-GM-CSF antibodies devoid of neutralizing activity can be detected in sera of healthy donors. To address whether low levels of autoantibodies to GM-CSF may be found in the serum of healthy donors, we took advantage of the synergy between autoantibodies in GM-CSF neutralization. When tested for their capacity to neutralize GM-CSF bioactivity on TF-1 cells, the IgG fraction isolated from serum of healthy donors did not show detectable neutralizing activity. However, when serum IgG was supplemented with a single non-neutralizing monoclonal antibody, a clear neutralizing activity was measured, which varied depending on the serum donor and on the site-specificity of the antibodies used. In one case we could observe complete neutralization using the serum IgG from donor 6 (D6) combined with GCB9 and GCA21 antibodies (Figure 5.1.8). These results suggest that low levels of anti-GM-CSF antibodies devoid of neutralizing activity are present in the sera of healthy donors and can be detected using a sensitive complementation assay.
5. Results and discussion

5.1.5. Figures and tables

**Figure 5.1.1.** Somatic mutations critically contribute to the specificity of GM-CSF autoantibodies. Binding of WT (red), UCA (blue) and shuffled variants VK UCA (green) and VH UCA (violet) to GM-CSF as measured by SPR. The table shows equilibrium dissociation constants (KD).

<table>
<thead>
<tr>
<th>mAb</th>
<th>KD (M)</th>
</tr>
</thead>
<tbody>
<tr>
<td>GCA21</td>
<td>1.7E-10, 1.1E-08, nc</td>
</tr>
<tr>
<td>GCB8</td>
<td>1.0E-09, 1.9E-08, nc</td>
</tr>
<tr>
<td>GCB9</td>
<td>1.8E-09, 8.4E-09, nc</td>
</tr>
<tr>
<td>GCB9</td>
<td>2.5E-09, 8.2E-09, 7.5E-08, 8.2E-09, 7.5E-08, nc</td>
</tr>
<tr>
<td>GCA7</td>
<td>2.3E-10, 3.5E-10, nc</td>
</tr>
<tr>
<td>GCA43</td>
<td>6.2E-10, 6.6E-10, nc</td>
</tr>
<tr>
<td>GCE536</td>
<td>2.5E-10, 1.3E-09, 1.1E-09, 9.0E-08</td>
</tr>
</tbody>
</table>
Figure 5.1.2. Three antibodies can bind simultaneously to a single molecule of GM-CSF forming high-molecular-weight immune complexes. (A) Map of the antigenic sites targeted by GM-CSF-specific autoantibodies as defined by SPR cross-competition. Four reference antibodies are in bold. (B) A multichannel chip coated with antibodies to site I (GCA21, red line), site II (GCA7, blue line) or site IV (GCB59, green line) was saturated with GM-CSF and serially exposed to an excess of the same antibodies. (C) SEC-HPLC profile of samples containing the three non-cross-competing antibodies, alone or with GM-CSF added in equimolar concentrations (1:1) or in 10-fold antibody excess (10:1).
**Figure 5.1.3.** Potent *in vitro* neutralization of GM-CSF by a combination of three non-cross-competiting antibodies. A fixed amount of GM-CSF (final concentration 50 pg/ml) was incubated with serial dilutions of one or more antibodies, added to TF1 cells (10,000/well) and cell proliferation was measured on day 3 by thymidine incorporation. (A) IC90 values of polyclonal IgG and affinity-purified polyclonal antibodies isolated from the serum of 5 PAP patients. The numbers indicate the percentage of anti-GM-CSF antibodies relative to total IgG. (B) Serial dilutions of single monoclonal antibodies, or mixtures of two and three non-cross-competiting antibodies were tested for their capacity to neutralize GM-CSF. (C) The sensitivity of the test was changed by varying the number of cells and the concentration of GM-CSF as indicated. Shown is for each experimental condition the inhibition obtained using single antibodies or a combination of three non-cross-competiting antibodies.
5. Results and discussion

Figure 5.1.4. Comparison of \textit{in vitro} neutralization of GM-CSF by single, or multiple autoantibodies or affinity-purified antibodies from PAP sera. (A) Rank of neutralizing activity (IC90 values) of 140 different combinations of GM-CSF mAbs. (B) A selection of the most potent neutralizing combinations is shown and compared to therapeutic anti-GM-CSF antibodies (MOR103 and Namilumab) and to autoantibodies purified from PAP sera.
Figure 5.1.5. A sandwich ELISA to detect GM-CSF in the presence of specific antibodies. A fixed amount of GM-CSF was added to mouse serum together with three monoclonal antibodies (GCA21, GCA7, GCB59) added separately or in combination. The quantification of GM-CSF was performed by a sandwich ELISA using an antibody specific for site II for capture and site I for detection. Serial dilutions of serum in neutral (left) or alkaline buffer (right) were added and GM-CSF concentration was determined with reference to a GM-CSF standard. The dotted line represents the concentration of GM-CSF measured in the absence of antibodies.
Figure 5.1.6. 

Fc-dependent clearance of GM-CSF immune complexes in vivo. (A) Female Balb/c mice (5/group) were injected with 100 μg of monoclonal antibody, either GCA21 (1 mAb) or GCA21+GCA7+GCB59 (3 mAbs) in the IgG or IgG-LALA format, or with 2 mg total IgG from a PAP patient, followed by 2 μg GM-CSF after 16 hours. Sera were collected after 1 or 5 days and GM-CSF concentrations were measured by ELISA in untreated serum and in serum treated at pH 11.6 to dissociate immune complexes. Shown is the GM-CSF concentration on day 1 and on day 5 in untreated serum (left) or alkaline-treated serum (right). (B) Proliferation of TF-1 cells in response to different dilutions of serum of mice injected 24 hours before with GM-CSF and the indicated antibodies. (C) Binding of GM-CSF immune complexes formed by one or three antibodies (in the IgG1 or IgG1-LALA format) to TZM-bl cells expressing FcγRIIa or FcγRIIb, as measured by flow cytometry using an anti-IgG Fc specific antibody.
Figure 5.1.7. Expression of FcγRs on transfected TZM-bl cells and binding of immune complexes. (A) Expression of FcγRs on TZM-bl cells transfected with FcγRI, FcγRIIa/b and FcγRIIIa, as detected by antibodies to CD64, CD32 and CD16, respectively. Untransfected TZM-bl cells were used as negative control. (B) Shown is also the binding of GM-CSF immune complexes containing one or three antibodies to TZM-bl cells expressing FcγRI or FcγRIIIa or to untransfected TZM-bl cells.
5. Results and discussion

**Figure 5.1.8.** Low levels of anti-GM-CSF antibodies in the blood of healthy donors can be detected by measuring their synergy with single monoclonal antibodies in GM-CSF neutralization. (A, B) IgG purified from the serum of two healthy donors (D10 and D6) were tested in the presence or absence of single monoclonal antibodies for their capacity to neutralize GM-CSF using the TF-1 bioassay (50 pg/ml GM-CSF and 10,000 cells/well).
5. Results and discussion

Table 5.1.1. Features of 5 PAP patients. WLL = whole lung lavage.

<table>
<thead>
<tr>
<th>ID</th>
<th>Code</th>
<th>Gender</th>
<th>Birth year</th>
<th>Diagnosis year</th>
<th>Therapy</th>
<th>Follow up (years)</th>
<th>Serum GM-CSF antibodies (μg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PA93</td>
<td>GCA</td>
<td>M</td>
<td>1943</td>
<td>2010</td>
<td>-</td>
<td>2</td>
<td>804.5</td>
</tr>
<tr>
<td>PA26</td>
<td>GCB</td>
<td>F</td>
<td>1966</td>
<td>2005</td>
<td>-</td>
<td>7</td>
<td>454.9</td>
</tr>
<tr>
<td>PA40</td>
<td>GCC</td>
<td>F</td>
<td>1954</td>
<td>2005</td>
<td>WLL</td>
<td>7</td>
<td>100.6</td>
</tr>
<tr>
<td>PA96</td>
<td>GCD</td>
<td>M</td>
<td>1980</td>
<td>2010</td>
<td>-</td>
<td>2</td>
<td>741.2</td>
</tr>
<tr>
<td>PA65</td>
<td>GCE</td>
<td>F</td>
<td>1959</td>
<td>2005</td>
<td>WLL</td>
<td>7</td>
<td>46</td>
</tr>
</tbody>
</table>

Table 5.1.2. Binding properties of GM-CSF autoantibodies (EC50, as determined by ELISA, and Ka, Kd, and KD, as determined by SPR) are reported. Binding properties of four control mAbs (mouse clones 3092 and 1089 and therapeutic anti-GM-CSF antibodies MOR103 and Namilumab are reported at the bottom of the table).

<table>
<thead>
<tr>
<th>mAb</th>
<th>Patient</th>
<th>Isotype</th>
<th>EC50 (ng/ml)</th>
<th>Ka (1/Ms)</th>
<th>Kd (1/s)</th>
<th>KD (M)</th>
</tr>
</thead>
<tbody>
<tr>
<td>GCA7</td>
<td>PA93</td>
<td>IgG1</td>
<td>186.8</td>
<td>2.4E+05</td>
<td>6.0E-05</td>
<td>3.8E-10</td>
</tr>
<tr>
<td>GCA14</td>
<td>PA93</td>
<td>IgG1</td>
<td>213.0</td>
<td>3.1E+05</td>
<td>9.6E-05</td>
<td>3.1E-10</td>
</tr>
<tr>
<td>GCA21</td>
<td>PA93</td>
<td>IgG1</td>
<td>59.4</td>
<td>9.5E+05</td>
<td>6.5E-04</td>
<td>6.9E-10</td>
</tr>
<tr>
<td>GCA43</td>
<td>PA93</td>
<td>IgG1</td>
<td>835.6</td>
<td>1.7E+05</td>
<td>1.6E-04</td>
<td>9.8E-10</td>
</tr>
<tr>
<td>GCA101</td>
<td>PA93</td>
<td>IgG1</td>
<td>291.5</td>
<td>3.8E+05</td>
<td>2.8E-04</td>
<td>7.8E-10</td>
</tr>
<tr>
<td>GCA102</td>
<td>PA93</td>
<td>IgG1</td>
<td>208.1</td>
<td>8.5E+05</td>
<td>7.8E-04</td>
<td>5.6E-10</td>
</tr>
<tr>
<td>GCB6</td>
<td>PA26</td>
<td>IgG1</td>
<td>92.4</td>
<td>4.9E+05</td>
<td>2.8E-04</td>
<td>7.8E-10</td>
</tr>
<tr>
<td>GCB9</td>
<td>PA26</td>
<td>IgG1</td>
<td>228.3</td>
<td>2.1E+05</td>
<td>7.6E-04</td>
<td>3.6E-09</td>
</tr>
<tr>
<td>GCB14</td>
<td>PA26</td>
<td>IgG1</td>
<td>32.9</td>
<td>4.3E+05</td>
<td>2.0E-03</td>
<td>4.5E-09</td>
</tr>
<tr>
<td>GCB41</td>
<td>PA26</td>
<td>IgG1</td>
<td>605.3</td>
<td>8.6E+05</td>
<td>4.9E-04</td>
<td>6.2E-10</td>
</tr>
<tr>
<td>GCB53</td>
<td>PA26</td>
<td>IgG1</td>
<td>222.6</td>
<td>1.9E+06</td>
<td>4.0E-04</td>
<td>2.5E-10</td>
</tr>
<tr>
<td>GCB59</td>
<td>PA26</td>
<td>IgG1</td>
<td>307.6</td>
<td>1.7E+06</td>
<td>1.2E-03</td>
<td>6.8E-10</td>
</tr>
<tr>
<td>GCC9</td>
<td>PA40</td>
<td>IgG1</td>
<td>43.2</td>
<td>1.2E+06</td>
<td>9.6E-04</td>
<td>9.4E-10</td>
</tr>
<tr>
<td>GCC11</td>
<td>PA40</td>
<td>IgG1</td>
<td>55.4</td>
<td>6.3E+05</td>
<td>1.8E-03</td>
<td>2.7E-09</td>
</tr>
<tr>
<td>GCC13</td>
<td>PA40</td>
<td>IgG1</td>
<td>16.1</td>
<td>1.0E+06</td>
<td>7.5E-04</td>
<td>9.8E-10</td>
</tr>
<tr>
<td>GCC21</td>
<td>PA40</td>
<td>IgG1</td>
<td>68.1</td>
<td>4.5E+05</td>
<td>2.1E-04</td>
<td>1.1E-09</td>
</tr>
<tr>
<td>GCD10</td>
<td>PA96</td>
<td>IgG3</td>
<td>241.7</td>
<td>2.0E+06</td>
<td>3.3E-03</td>
<td>1.9E-09</td>
</tr>
<tr>
<td>GCD22</td>
<td>PA96</td>
<td>IgG1</td>
<td>205.4</td>
<td>4.1E+05</td>
<td>1.9E-03</td>
<td>5.1E-09</td>
</tr>
<tr>
<td>GCD27</td>
<td>PA96</td>
<td>IgG1</td>
<td>166.4</td>
<td>9.9E+05</td>
<td>1.5E-04</td>
<td>1.5E-10</td>
</tr>
<tr>
<td>GCE402</td>
<td>PA65</td>
<td>IgG1</td>
<td>107.8</td>
<td>1.2E+06</td>
<td>4.5E-04</td>
<td>4.0E-10</td>
</tr>
<tr>
<td>GCE536</td>
<td>PA65</td>
<td>IgG1</td>
<td>61.4</td>
<td>6.6E+05</td>
<td>1.1E-04</td>
<td>1.8E-10</td>
</tr>
<tr>
<td>Clone 3092</td>
<td>mIgG1</td>
<td>κ</td>
<td>61.0</td>
<td>5.9E+05</td>
<td>3.4E-04</td>
<td>5.7E-10</td>
</tr>
<tr>
<td>Clone 1089</td>
<td>mIgG1</td>
<td>κ</td>
<td>1080.0</td>
<td>1.8E+05</td>
<td>7.9E-05</td>
<td>4.4E-10</td>
</tr>
<tr>
<td>MOR103</td>
<td>IgG1</td>
<td>λ</td>
<td>90.0</td>
<td>2.7E+05</td>
<td>1.5E-05</td>
<td>1.9E-10</td>
</tr>
<tr>
<td>Namilumab</td>
<td>IgG1</td>
<td>κ</td>
<td>75.3</td>
<td>3.1E+05</td>
<td>7.7E-05</td>
<td>2.4E-10</td>
</tr>
</tbody>
</table>
### Table 5.1.3. V(D)J gene usage of GM-CSF autoantibodies isolated from PAP patients.

<table>
<thead>
<tr>
<th>mAb</th>
<th>Heavy chain VDJ genes (% identity to GL)</th>
<th>Light chain VJ genes (% identity to GL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>GCA7</td>
<td>VH3-66 (91.2) D3-10 JH4 (93.8) VK4-1 (96) JK3 (100)</td>
<td></td>
</tr>
<tr>
<td>GCA14</td>
<td>VH1-58 (92.4) D3-9 JH6 (84.9) VK4-1 (97.6) JK2 (86.8)</td>
<td></td>
</tr>
<tr>
<td>GCA21</td>
<td>VH3-30-3 (83.3) D2-15 JH2 (84.9) VK1-5 (92.8) JK4 (97.2)</td>
<td></td>
</tr>
<tr>
<td>GCA43</td>
<td>VH1-69 (91.7) D5-24 JH3 (88) VK4-1 (95.3) JK2 (100)</td>
<td></td>
</tr>
<tr>
<td>GCA101</td>
<td>VH4-59 (90.5) D1-26 JH2 (94.3) VK4-1 (91.6) JK2 (92.1)</td>
<td></td>
</tr>
<tr>
<td>GCA102</td>
<td>VH3-23 (90.7) D6-13 JH6 (90.3) VK1-16 (94.3) JK4 (97.4)</td>
<td></td>
</tr>
<tr>
<td>GCB6</td>
<td>VH3-23 (87.5) D1-7 JH6 (80.7) VK1-16 (95.7) JK4 (88.9)</td>
<td></td>
</tr>
<tr>
<td>GCB9</td>
<td>VH1-18 (82.6) D4-23 JH4 (85.4) VK3-20 (91.5) JK1 (97.2)</td>
<td></td>
</tr>
<tr>
<td>GCB14</td>
<td>VH4-59 (90.9) D2-2 JH5 (88.2) VK3-15 (98.2) JK2 (94.7)</td>
<td></td>
</tr>
<tr>
<td>GCB41</td>
<td>VH1-18 (96.1) D6-13 JH4 (85.4) VK1-16 (100) JK3 (92.1)</td>
<td></td>
</tr>
<tr>
<td>GCB53</td>
<td>VH3-73 (95.9) D3-3 JH6 (83.9) VK1D-39 (89.5) JK1 (94.7)</td>
<td></td>
</tr>
<tr>
<td>GCB59</td>
<td>VH3-21 (86.8) D2-15 JH6 (77.4) VL3-21 (92.1) JL2 (91.9)</td>
<td></td>
</tr>
<tr>
<td>GCC9</td>
<td>VH4-30-2 (87.6) D3-10 JH5 (92.2) VK1-NL1 (97.5) JK4 (97.4)</td>
<td></td>
</tr>
<tr>
<td>GCC11</td>
<td>VH4-39 (95) D6-13 JH1 (78.3) VK3-20 (97.5) JK1 (94.7)</td>
<td></td>
</tr>
<tr>
<td>GCC13</td>
<td>VH3-21 (96.2) D5-24 JH2 (88.7) VK4-1 (97.6) JK1 (100)</td>
<td></td>
</tr>
<tr>
<td>GCC21</td>
<td>VH3-23 (95.4) D5-24 JH4 (89.6) VK3-11 (97.1) JK4 (97.3)</td>
<td></td>
</tr>
<tr>
<td>GCD10</td>
<td>VH3-30 (85.1) D6-25 JH3 (82) VK4-1 (89.9) JK4 (86.8)</td>
<td></td>
</tr>
<tr>
<td>GCD22</td>
<td>VH3-11 (87.2) D2-2 JH5 (86.3) VK3-11 (93.6) JK5 (100)</td>
<td></td>
</tr>
<tr>
<td>GCD27</td>
<td>VH7-4-1 (89.2) D3-10 JH6 (79.4) VK1-27 (89.6) JK3 (94.7)</td>
<td></td>
</tr>
<tr>
<td>GCE402</td>
<td>VH4-4 (84.9) D6-13 JH6 (85.5) VK1D-39 (85.5) JK3 (92.1)</td>
<td></td>
</tr>
<tr>
<td>GCE536</td>
<td>VH1-46 (87.9) D2-2 JH6 (85.5) VK3-20 (91.5) JK2 (92.1)</td>
<td></td>
</tr>
</tbody>
</table>
Table 5.1.4. Binding of WT, UCA, UCA-HCDR3 WT and shuffled variants VK/VL UCA and VH UCA to GM-CSF as measured by ELISA and SPR. nc = not calculable.

<table>
<thead>
<tr>
<th>mAb</th>
<th>Version</th>
<th>EC50 (ng/ml)</th>
<th>K on (1/Ms)</th>
<th>K off (1/s)</th>
<th>KD (M)</th>
</tr>
</thead>
<tbody>
<tr>
<td>GCA21</td>
<td>WT</td>
<td>33.3</td>
<td>1.3E+06</td>
<td>5.5E-04</td>
<td>5.7E-10</td>
</tr>
<tr>
<td></td>
<td>VK UCA</td>
<td>77.3</td>
<td>3.1E+06</td>
<td>2.6E-02</td>
<td>1.1E-08</td>
</tr>
<tr>
<td></td>
<td>VH UCA</td>
<td>nc</td>
<td>nc</td>
<td>nc</td>
<td>nc</td>
</tr>
<tr>
<td></td>
<td>UCA</td>
<td>nc</td>
<td>nc</td>
<td>nc</td>
<td>nc</td>
</tr>
<tr>
<td></td>
<td>UCA - HCDR3 WT</td>
<td>nc</td>
<td>nc</td>
<td>nc</td>
<td>nc</td>
</tr>
<tr>
<td>GCA7</td>
<td>WT</td>
<td>52.4</td>
<td>1.8E+05</td>
<td>3.1E-05</td>
<td>2.3E-10</td>
</tr>
<tr>
<td></td>
<td>VK UCA</td>
<td>64.8</td>
<td>4.9E+04</td>
<td>1.7E-05</td>
<td>3.5E-10</td>
</tr>
<tr>
<td></td>
<td>VH UCA</td>
<td>nc</td>
<td>nc</td>
<td>nc</td>
<td>nc</td>
</tr>
<tr>
<td></td>
<td>UCA</td>
<td>7724</td>
<td>nc</td>
<td>2.1E-03</td>
<td>nc</td>
</tr>
<tr>
<td></td>
<td>UCA - HCDR3 WT</td>
<td>354.2</td>
<td>3.4E+04</td>
<td>2.1E-03</td>
<td>6.3E-08</td>
</tr>
<tr>
<td>GCB59</td>
<td>WT</td>
<td>428.7</td>
<td>1.0E+06</td>
<td>1.7E-03</td>
<td>1.8E-09</td>
</tr>
<tr>
<td></td>
<td>VL UCA</td>
<td>24039</td>
<td>1.3E+06</td>
<td>1.0E-02</td>
<td>8.4E-09</td>
</tr>
<tr>
<td></td>
<td>VH UCA</td>
<td>nc</td>
<td>nc</td>
<td>nc</td>
<td>nc</td>
</tr>
<tr>
<td></td>
<td>UCA</td>
<td>nc</td>
<td>nc</td>
<td>nc</td>
<td>nc</td>
</tr>
<tr>
<td></td>
<td>UCA - HCDR3 WT</td>
<td>nc</td>
<td>nc</td>
<td>nc</td>
<td>nc</td>
</tr>
<tr>
<td>GCB9</td>
<td>WT</td>
<td>322</td>
<td>3.1E+05</td>
<td>7.5E-04</td>
<td>2.5E-09</td>
</tr>
<tr>
<td></td>
<td>VK UCA</td>
<td>258.3</td>
<td>4.8E+05</td>
<td>2.8E-03</td>
<td>6.2E-09</td>
</tr>
<tr>
<td></td>
<td>VH UCA</td>
<td>956</td>
<td>4.2E+05</td>
<td>3.9E-03</td>
<td>1.5E-08</td>
</tr>
<tr>
<td></td>
<td>UCA</td>
<td>6765</td>
<td>1.7E+05</td>
<td>1.2E-02</td>
<td>8.2E-08</td>
</tr>
<tr>
<td></td>
<td>UCA - HCDR3 WT</td>
<td>3664</td>
<td>5.9E+04</td>
<td>8.5E-03</td>
<td>1.5E-07</td>
</tr>
<tr>
<td>GCA43</td>
<td>WT</td>
<td>258</td>
<td>2.5E+05</td>
<td>1.5E-04</td>
<td>6.2E-10</td>
</tr>
<tr>
<td></td>
<td>VK UCA</td>
<td>286.7</td>
<td>3.0E+05</td>
<td>1.7E-04</td>
<td>6.5E-10</td>
</tr>
<tr>
<td></td>
<td>VH UCA</td>
<td>nc</td>
<td>nc</td>
<td>nc</td>
<td>nc</td>
</tr>
<tr>
<td></td>
<td>UCA</td>
<td>nc</td>
<td>nc</td>
<td>nc</td>
<td>nc</td>
</tr>
<tr>
<td></td>
<td>UCA - HCDR3 WT</td>
<td>nc</td>
<td>nc</td>
<td>nc</td>
<td>nc</td>
</tr>
<tr>
<td>GCB6</td>
<td>WT</td>
<td>31.1</td>
<td>5.2E+05</td>
<td>4.4E-04</td>
<td>1.0E-09</td>
</tr>
<tr>
<td></td>
<td>VK UCA</td>
<td>35.1</td>
<td>6.5E+05</td>
<td>1.0E-02</td>
<td>1.9E-08</td>
</tr>
<tr>
<td></td>
<td>VH UCA</td>
<td>nc</td>
<td>nc</td>
<td>nc</td>
<td>nc</td>
</tr>
<tr>
<td></td>
<td>UCA</td>
<td>nc</td>
<td>nc</td>
<td>nc</td>
<td>nc</td>
</tr>
<tr>
<td></td>
<td>UCA - HCDR3 WT</td>
<td>nc</td>
<td>nc</td>
<td>nc</td>
<td>nc</td>
</tr>
<tr>
<td>GCE536</td>
<td>WT</td>
<td>54.3</td>
<td>5.8E+05</td>
<td>1.4E-04</td>
<td>2.5E-10</td>
</tr>
<tr>
<td></td>
<td>VK UCA</td>
<td>37.5</td>
<td>7.3E+05</td>
<td>9.5E-04</td>
<td>1.3E-09</td>
</tr>
<tr>
<td></td>
<td>VH UCA</td>
<td>56.3</td>
<td>4.7E+04</td>
<td>5.4E-05</td>
<td>1.1E-09</td>
</tr>
<tr>
<td></td>
<td>UCA</td>
<td>19773</td>
<td>2.4E+03</td>
<td>8.7E-05</td>
<td>9.0E-08</td>
</tr>
<tr>
<td></td>
<td>UCA - HCDR3 WT</td>
<td>13.7</td>
<td>5.5E+05</td>
<td>6.0E-03</td>
<td>1.0E-08</td>
</tr>
</tbody>
</table>
5. Results and discussion

5.1.6. Discussion

Our study identifies two mechanisms by which polyclonal anti-GM-CSF antibodies produced by PAP patients inhibit the biological activity of GM-CSF. The first is the irreversible sequestration of GM-CSF in high-molecular-weight immune complexes that can be readily detected in vitro using cell proliferation bioassays. The second is the in vivo Fc-dependent degradation of immune complexes formed between GM-CSF and multiple autoantibodies.

The autoantibodies isolated show affinities in the nanomolar range and distinct kinetics of binding, differing in particular in their off-rates. Overall they were comparable to those reported in a recent study (Wang 2013). Interestingly, while this study claimed that all the antibodies were capable of neutralizing GM-CSF in the TF-1 bioassay, we show that neutralization by single antibodies is strictly dependent on the concentration of the cytokine and the number of receptors in the system, a finding that can be explained by the reversibility of antibody-antigen interaction, according to the law of mass action, and the affinity of GM-CSF receptor for its ligand, which is higher than that of antibodies (Niu 2000; Hansen 2008). It was therefore interesting to discover that, when added together, three antibodies that bind to non-overlapping sites, can completely neutralize GM-CSF bioactivity, irrespective of the concentration of cytokine or receptor. When tested in stringent conditions, a cocktail of three antibodies was, on a weight basis, more potent than two anti-GM-CSF antibodies that are currently developed for therapy of autoimmune and inflammatory diseases (Behrens 2014; Krinner 2007; Steidl 2008). We envisage that, when complexed with three antibodies, GM-CSF becomes completely sequestered and no longer available for interaction with the receptor.

A key observation in this study was that, when injected in mice, single antibodies led to the accumulation of a large pool of long-lived GM-CSF that was still able to dissociate and trigger the receptor, as shown by the capacity of the sera to stimulate proliferation of TF-1 cells. The capacity of a single antibody to build up a large cytokine reservoir, as we observed for GM-CSF, could be the basis of the enhancing activity of monoclonal antibodies to common gamma-chain cytokines in vivo (Boyman 2006). In striking contrast, we showed that three antibodies formed immune complexes that were rapidly degraded in vivo in a Fc-dependent fashion. The degradation of GM-CSF induced by polyclonal antibodies is reminiscent of a previous study where three antibodies were shown to cause degradation of IL-6 in vivo (Montero-Julian 1994). Our result extend this
concept to autoantibodies and suggest that degradation is the main mechanism by which polyclonal autoantibodies, such as those found in the serum of PAP patients, can lead to complete clearance of the cytokine, thus explaining the severe phenotype characteristic of these patients. In summary our result suggest a two-step mechanism by which polyclonal antibodies lead to a severe GM-CSF deficiency: first, they irreversibly sequester GM-CSF thus completely preventing its interaction with the receptor, second, by inducing its degradation in a Fc-dependent fashion. Our findings also help explain why pulmonary alveolar proteinosis has not been reported following administration of an anti-GM-CSF monoclonal antibody in humans (Behrens 2014). We suggest that the main effect of the therapeutic anti-GM-CSF antibody would be to redistribute the cytokine into the circulation, thus decreasing its concentration in inflammatory sites, while leaving enough free GM-CSF for the development of alveolar macrophages.

An interesting observation of this study is related to the role of somatic mutations and the presence of low levels of GM-CSF antibodies in healthy individuals. The finding that the UCA of the autoantibodies did not bind, or showed minimal binding to GM-CSF, is consistent with previous reports of autoantibodies found in patients with pemphigus and lupus (Di Zenzo 2012; Mietzner 2008; Schroeder K. 2013). In contrast, we observed that the UCA versions of antiviral antibodies showed only a slightly reduced binding to the specific antigen ((Corti 2011; Corti 2013 and data not shown). These findings support the notion that autoantibodies are preferentially generated from activated memory B cells that have been triggered and have undergone somatic mutation in response to foreign antigens. It is tempting to speculate that the preferential origin of autoantibodies from memory B cells is due to the fact these cells have a lower activation threshold or may be more difficult to anergize as compared to naïve B cells. In addition, it has been suggested that memory B cells may be able to efficiently prime naïve T cells (Liu Y. 1992).

GM-CSF autoantibodies have been described not only in PAP patients, but also in the serum of healthy donors or in patients with inflammatory bowel disease with normal pulmonary function (Uchida 2009; Dabritz 2013). Building our findings, we developed a sensitive complementation assay that detects in the serum of healthy donors non-neutralizing GM-CSF autoantibodies based on their capacity to synergize with non-neutralizing monoclonal autoantibodies. These findings suggest that healthy donors may have anti-GM-CSF antibodies in serum at low concentrations and/or in combinations that cannot promote GM-CSF sequestration and degradation. Conversely, PAP patients
developed high levels of GM-CSF autoantibodies to multiple sites that form immune complexes that mediate sequestration and degradation of the cytokine.
5. Results and discussion

5.2. Somatic mutations play a crucial role in the generation of autoantibodies binding to GM-CSF and citrullinated vimentin

5.2.1. Abstract

In a previous study we provided evidence that removal of somatic mutations from autoantibodies binding to the granulocyte-macrophage colony-stimulating factor (GM-CSF) isolated from patients suffering from pulmonary alveolar proteinosis (PAP) resulted in loss of binding to GM-CSF in most cases. Here we have isolated from a patient with rheumatoid arthritis (RA) a high-affinity autoantibody specific for citrullinated vimentin and found that, similarly to GM-CSF autoantibodies, removal of somatic mutations abolished the binding of the autoantibody to citrullinated vimentin. Furthermore, we provided anti-influenza H1 hemagglutinin (H1-HA) antibodies as essential controls and found that removal of somatic mutations resulted in antibodies binding to HA with similar or only slightly reduced affinity compared to the mutated antibodies. Taken together, these findings are consistent with a model where self-reactivity of B cells is acquired through somatic mutations during the response to a foreign antigen.

5.2.2. Introduction

An important process occurring in the germinal center (GC) is somatic hypermutation (SHM) that introduces point mutations in the variable region of both the heavy chain and light chain of an immunoglobulin and is responsible for affinity maturation and changes in fine specificity of the antibodies (Neuberger 2008). Because of its stochastic nature, SHM can be also responsible for the generation of self-reactive B cell clones that produce autoantibodies (Brink 2014). In a previous study (shown is section 5.1) we sustained this model by removing somatic mutations from seven anti-GM-CSF antibodies isolated from PAP patients to produce the “unmutated common ancestor” (UCA), i.e. the antibody produced by the naïve B cell that gave origin to the autoantibody. We found that in 5 out 7 cases, the UCA of anti-GM-CSF antibodies were not able to bind to GM-CSF, while in the remaining 2 cases there was a very low but still detectable binding.

Rheumatoid arthritis (RA) is a chronic disease characterized by inflammation of the synovial joints that damages the cartilage with subsequent erosion of the bone. Sixty
percent of RA patients develop autoantibodies to citrullinated proteins (ACPAs), in particular against citrullinated vimentin, fibrinogen and α-enolase (Klareskog 2008). Citrullination is a physiological posttranslational process that deiminates arginine to citrulline and is mediated by peptidyl arginine deiminases (PADs) (Gyorgy 2006). As many other posttranslational modifications, protein citrullination can lead to the generation of neo-self antigens that can become available for an immune response (Doyle 2012). In particular, increased citrullination has been observed in inflamed synovium in RA patients and autoantibodies to citrullinated vimentin have been shown to induce osteoclastogenesis and subsequent bone resorption activity that leads to bone loss in RA patient, suggesting a critical role for citrullinated peptides in the etiopathogenesis of RA (Klareskog 2008; Harre 2012). The origin of ACPAs remains unclear, but a likely hypothesis is that they can be generated through SHM during the response to unrelated antigens.

To further study the origin of autoantibodies and extend the results obtained with GM-CSF autoantibodies, we isolated and characterized one monoclonal antibody binding with very high affinity and specificity to citrullinated vimentin, and found that reversion to the UCA completely abolished the binding to citrullinated vimentin. In addition, we provided anti-influenza H1 hemagglutinin (H1-HA) antibodies as essential controls and found that the UCA antibodies bound to H1-HA with similar or only slightly reduced affinity compared to the mutated antibodies. These findings support the important role of somatic mutations that, beyond increasing the affinity of antibodies reacting to foreign antigens, can also broaden the original specificity of the antibodies making them able to bind to self antigens.
5. Results and discussion

5.2.3. Methods

**Isolation and production of monoclonal antibodies to citrullinated peptides.** Isolation and production of monoclonal antibodies to citrullinated vimentin and their reversion to germline were performed as described for GM-CSF autoantibodies in section 5.1.3. Isolation and characterization of GM-CSF autoantibodies and influenza antibodies were described in section 5.2 and in (Pappas 2014), respectively.

**ELISA assays.** Citrullinated vimentin peptide (amino acids 60-75), fibrinogen peptide (amino acids 36-52) and α-enolase peptide 1 were kindly provided by Karoline Lundberg from Karolinska Institute, Stockholm, Sweden. Control non-citrullinated peptides were synthesized by Schafer-N, Copenhagen, Denmark. Vimentin and fibrinogen peptides were biotinylated at the C-terminus, while α-enolase was not biotinylated. ELISA plates were coated with avidin (10 μg/ml), blocked with PBS 1% BSA and biotinylated vimentin and biotinylated fibrinogen peptides were added (10 μg/ml) for capturing by avidin. A-enolase (10 μg/ml) was directly coated on the ELISA plates. The plates were incubated with titrated antibodies, followed by AP-conjugated anti-human IgG secondary antibodies (SouthernBiotech). Plates were then washed, substrate (p-NPP, Sigma) was added and plates were read at 405 nm. EC50 (ng/ml) was calculated for every sample by nonlinear regression analysis using GraphPad Prism 5 software.

**SPR assays.** SPR analysis of GM-CSF autoantibodies was described in section 5.1.3. SPR. The same protocol was applied to study the interaction between influenza antibodies and H1-HA with some differences. Briefly, monoclonal antibodies (10 nM) were injected for 30 s onto the protein-A-coated chip for capturing, followed by injection of different concentrations (30 nM, 10nM, 3.3 nM, 1.1 nM) of A/H1/California/07/09 hemagglutinin (Protein Sciences). Injection time was 240 s and dissociation time was 900 s. The antibodies that did not show clear binding were re-tested using more sensitive conditions: monoclonal antibodies (100 nM) were injected for 180 s to saturate the protein A chip, followed by injection of higher concentrations of A/H1/California/07/09 (300nM, 100 nM, 33.3nM, 11.1 nM, 3.7nM). Injection time was 240 s and dissociation time was 600 s.
5.2.4. Results

Isolation and characterization of an antibody that binds to citrullinated vimentin with high affinity and specificity. PBMCs and serum were collected from one ACPA+ RA patient. Presence of high titers of antibodies to citrullinated vimentin and citrullinated fibrinogen peptides was assessed by ELISA (Figure 5.2.1). Memory B cells were isolated and immortalized as described (Traggiai 2004). The supernatants were screened for the production of specific IgGs to citrullinated vimentin, citrullinated fibrinogen and citrullinated α-enolase peptides by ELISA and one clone (BVCA1) producing antibodies to citrullinated vimentin was identified. The monoclonal antibody BVCA1 was sequenced and analyzed using IMGT database (Lefranc 2009). BVCA1 was an IgG1 and used a κ light chain. The antibody was highly mutated in both heavy chain and light chain variable regions (Table 5.2.1) with a load of mutations of 19% and 14.3% in VH and VL gene segments, respectively. BVCA1 was produced recombinantly as IgG1 and tested for binding to citrullinated vimentin by ELISA where it showed a very high-affinity binding with an EC50 of 7.2 ng/ml. In particular, BVCA1 showed a much higher affinity than that of other ACPAs reported in previous studies (Amara 2013). The binding of BVCA1 to citrullinated vimentin peptide was highly specific as it did not cross-react with either the unmutated vimentin peptide or other citrullinated or unmutated peptides like fibrinogen and α-enolase (Figure 5.2.2). BVCA1 is likely to recognize citrullinated vimentin produced by macrophages in the inflamed synovium of RA patient as it positively stained RA synovial tissues with a distribution similar to that of macrophages (Figure 5.2.3).

Binding to citrullinated vimentin is dependent on somatic mutations. Similarly to GM-CSF autoantibodies, we analyzed the role of somatic mutations in the generation of autoantibodies to citrullinated vimentin by reverting the sequence of BVCA1 to the germline. We produced both the unique common ancestor (UCA) and the UCA variant in which the HCDR3 was left in the wild-type form (UCA-HCDR3 WT). In addition, we produced also the shuffled variants in which only the VH or the VL were reverted to the UCA sequence. When tested by ELISA, both the UCA and the UCA-HCDR3 WT versions of BVCA1 completely lost their ability to bind to citrullinated vimentin peptide (Figure 5.2.4). In addition, the shuffled versions showed a detectable low-affinity binding only when the VH was reverted to the UCA sequence, while the binding was completely lost.
when the VL was reverted to the UCA sequence (Figure 5.2.4). These results suggest that somatic mutations in both heavy and light chains are crucial for developing the specificity and the high affinity of autoantibodies binding to citrullinated vimentin.

**Removal of somatic mutations from anti-influenza H1-HA UCA antibodies results in similar or slightly reduced binding to H1-HA.** To support the hypothesis that autoantibodies are generated through somatic mutations, we provided anti-influenza H1-HA antibodies as controls and we compared the binding of both mutated and UCA versions of the antibodies to H1-HA by SPR. Unlike UCAs of antibodies binding to GM-CSF, the UCA versions of anti-influenza H1-HA antibodies showed a similar or only slightly reduced binding to H1-HA when tested with low-sensitivity conditions (low amount of both antibodies and HA) due to a little increase in the off-rate (Figure 5.2.5). In addition, some influenza antibodies showed a great reduction in the binding to H1-HA when reverted to the UCA form as measured by SPR using low-sensitivity conditions (Figure 5.2.6). Nevertheless, when the same antibodies were tested with high-sensitivity conditions (high amount of both antibodies and HA), they showed an improved binding to H1-HA that was only slightly reduced in comparison to muted antibodies (Figure 5.2.7).

To test whether high-sensitivity conditions in SPR could show binding of UCA autoantibodies to the self antigen, we tested the 7 UCA anti-GM-CSF autoantibodies described in section 5.1 using high amount of both antibodies and GM-CSF with up to 4-fold GM-CSF excess. Unlike the UCA of anti-influenza antibodies, we did not detect any improvement in the binding of UCA of anti-GM-CSF antibodies to GM-CSF using high-sensitivity conditions. Therefore we confirmed the previous findings that in 5 out 7 cases both the UCA and the UCA-HCDR3 WT autoantibodies did not show any detectable and specific binding to GM-CSF. The remaining two antibodies showed a drastic reduction in binding to GM-CSF in which either the on-rate of GCE536 UCA or the off-rates of GCB9 UCA, GCB9 UCA-HCDR3 WT and GCE536 UCA-HCDR3 WT were mainly affected (Figure 5.2.8).

Altogether these results indicate an additional role for somatic mutations that, beyond increasing the affinity of antibodies reacting to foreign antigens, can also broaden the original specificity of the antibodies making them able to bind to self antigens.
5. Results and discussion

5.2.5. Figures and tables

**Figure 5.2.1.** The serum of one ACPA⁺ RA patient contains high levels of autoantibodies to citrullinated vimentin and citrullinated fibrinogen. Shown is the serial dilution of the serum from one ACPA⁺ RA patient (left) and from one healthy donor (right) tested for binding to citrullinated vimentin, citrullinated fibrinogen and citrullinated α-enolase peptides by ELISA.

![Figure 5.2.1](image)

**Figure 5.2.2.** BVCA1 specifically binds to citrullinated vimentin peptide with high affinity as determined by ELISA. Binding of BVCA1 to both citrullinated (citr-) and unmutated (arg-) vimentin, fibrinogen and α-enolase peptides is shown.

![Figure 5.2.2](image)
Figure 5.2.3. BVCA1 binds citrullinated vimentin peptide expressed in the RA synovium. Staining of synovial tissue with BVCA1 (upper-left), anti-CD3 (upper-right), anti-CD68 (lower-left) and isotype control (lower-right) made at the University Hospitals of Geneva by Prof. Cem Gabay’s research assistants.

Figure 5.2.4. Somatic mutations are crucial for the specificity of autoantibodies to citrullinated vimentin. Binding of WT (red), UCA (blue) and UCA-HCDR3 WT (black), shuffled variants VK UCA (green) and VH UCA (violet) of BVCA1 to citrullinated vimentin peptide as measured by ELISA.
5. Results and discussion

Figure 5.2.5. UCA versions of influenza neutralizing antibodies show only a slightly reduced binding to H1-HA. Binding of UCA (left) and WT (right) antibodies (10 nM) to H1-HA at different concentrations (red, 30 nM; blue, 10 nM; green, 3.3 nM; purple, 1.1 nM) from three representative clones as measured by SPR. These results have been published in (Pappas 2014).
Figure 5.2.6. UCA versions of influenza neutralizing antibodies show only a slightly reduced binding to H1-HA. Binding of 15 WT (red) and UCA (blue) antibodies to H1-HA by SPR using stringent conditions (10 nM antibody, 10 nM H1-HA). These results have been published in (Pappas 2014).
Figure 5.2.7. UCA versions of influenza neutralizing antibodies show only a slightly reduced binding to H1-HA. Binding of the same 15 UCA antibodies shown in Figure 5.2.6 to H1-HA by SPR using more sensitive conditions (100 nM antibody, 100 nM H1-HA). These results have been published in (Pappas 2014).
Figure 5.2.8. GM-CSF autoantibodies acquire specificity for GM-CSF through somatic mutations. Binding of WT, UCA and UCA-HCDR3 WT autoantibodies (200 nM) to GM-CSF at different concentrations (red, 400 nM; green, 200 nM; violet, 100 nM; blue, 50 nM; light blue, 25 nM), as measured by SPR using sensitive conditions. RU, resonance units.
Table 5.2.1. V(D)J gene usage of BVCA1 autoantibody.

<table>
<thead>
<tr>
<th>Heavy chain VDJ genes (% identity to GL)</th>
<th>Light chain VJ genes (% identity to GL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>VH3-49 (81.0)</td>
<td>VK1-6 (85.7)</td>
</tr>
<tr>
<td>D3-10</td>
<td>JK2 (84.2)</td>
</tr>
<tr>
<td>JH4 (81.3)</td>
<td></td>
</tr>
</tbody>
</table>

5.2.6. Discussion

Reversion to germline to analyze the specificity of the UCAs is a potent approach to reconstruct the development of high-affinity antibodies. We have recently characterized 197 antibodies binding to the stem region of influenza H1-HA isolated from a single donor, and found key somatic mutations that are required for the initial development of high-affinity anti-H1-HA antibodies (Pappas 2014). In particular, the SPR experiments that I performed in the course of this study, showed that, when compared to the wild-type antibodies, all the UCA antibodies tested bound to soluble H1-HA with only a slight reduction in the affinity, as compared to the mature mutated antibodies. The increased affinity showed by mutated antibodies was mainly due to decreased dissociation rates, while association rates remained relatively constant. These results are consistent with previous reports from our laboratory that showed that FI6, a monoclonal antibody to influenza, and MPE8, a monoclonal antibody to human respiratory syncytial virus (RSV) were still able to bind and neutralize the viruses when reverted to the UCA form (Corti 2011; Corti 2013). Interestingly, in the influenza study we observed that additional favorable mutations can increase the breadth of reactivity against different HAs, making the initial mutations functionally redundant (Pappas 2014). Similarly, wild-type MPE8 was able to cross-bind and cross-neutralize human metapneumovirus, bovine RSV and pneumonia virus of mice. Taken together, these data indicate that the UCAs of antiviral antibodies bind to the virus with moderate to high affinity and mutations can broaden the original specificity.

Consistent with this observation, SHM may also introduce mutations that change or broaden specificity making the antibody able to bind to a self antigen. In this situation the generation of autoantibodies can be considered a possible by-product of germinal center reaction (Vinuesa 2009). Our findings that the UCAs of antibodies against GM-CSF and citrullinated vimentin do not generally bind the self antigen may be consistent with the
hypothesis that autoantibodies could be generated during the response to a foreign antigen where non-autoreactive GC B cells acquired autoreactivity \textit{de novo} during the process of SHM. These results are in agreement with previous reports that showed that germline versions of autoantibodies binding to citrullinated peptides in RA, desmoglein 3 in pemphigus and DNA in lupus, lost their ability to bind the respective self antigens (Amara 2013; Di Zenzo 2012; Schroeder K. 2013; Mietzner 2008). In particular, our anti-citrullinated vimentin autoantibody showed a much higher affinity than that of other ACPAs reported in previous studies (Amara 2013), and the complete loss of binding of the UCA version of this antibody highly supports the model that self-reactivity of B cells can be generated through somatic mutations during the response to a foreign antigen.

Nevertheless, we observed two cases where GM-CSF UCA and UCA-HCDR3 WT autoantibodies showed a reduced but still detectable binding to GM-CSF. This results can be explained by the difficulty to precisely identify the somatic mutation in the HCDR3 because of the difficulty of unequivocally assign the D segment and determine the junctional rearrangement. In these situations the real germline HCDR3 could have contributed to show a complete loss in the residual binding observed in some recombinant UCA or UCA-HCDR3 WT autoantibodies.

The importance of somatic mutations in generating autoantibodies supports also the possibility that memory B cells contribute more to autoimmunity than naïve B cells (Figure 5.2.9). This hypothesis is consistent with recent studies that showed that autoantibodies were found to be more frequently expressed by IgG$^+$ memory B cells than naïve and IgM$^+$ memory B cells in healthy donors (Tiller 2007). These data suggest that autoantibodies are created \textit{de novo} by SHM during the transition between mature naïve and IgG$^+$ memory B cells, rather than by the variable region gene rearrangement occurring during the development of B cells in the bone marrow (Tiller 2007; Brink 2014). In addition, the diversity of naïve repertoire is related to the large number of naïve clones and is based on VDJ recombination and combinatorial paring of heavy and light chains. In contrast, the diversity in the memory repertoire is dependent on a relatively small number of clones which, however, have been extensively diversified through SHM. The BCR repertoire of memory B cells may also contain particular sequences that need fewer rounds of SHM to gain self-reactivity compared to that of naïve B cells. In addition, memory B cells that produce high-affinity antibodies against a foreign antigen have higher chances to survive GC negative selection compared to naïve B cells producing low-affinity antibodies. In this situation, if acquisition of self-reactivity does not change the affinity for
the foreign antigen, memory B cells survive and produce autoantibodies. In addition, memory B cells have a lower activation threshold compared to naïve B cells, thus making them more susceptible to BCR triggering by the self antigen and less prone to become anergic. As depicted in Figure 3.3.1, another important aspect that favors memory B cells to become self-reactive is their ability to circulate from secondary lymphoid organs to peripheral tissues, thus having more chances to encounter tissue-specific antigen and become activated in comparison to naïve B cells that traffic only in the secondary lymphoid organs. Once back in the lymph nodes, memory B cells can undergo new GC reactions that further increase or even abolish self reactivity (Sabouri 2014). Finally, it has been shown that activated/memory B cells can prime autoreactive naïve T cells that, in the presence of the self antigen, may sustain the autoantibody response (Lin 1991) (Figure 5.2.9).

Figure 5.2.9. The relative contribution of naïve/activated memory B cells to autoimmunity.
6. Concluding remarks and future perspectives

The aim of this thesis was to study the origin of autoantibodies and the pathogenic mechanism by which they cause the associated autoimmune disease. In particular, I focused my attention on two diseases, pulmonary alveolar proteinosis and rheumatoid arthritis, where autoantibodies to GM-CSF and to citrullinated proteins are produced.

My data have shown that these autoantibodies are more likely to be generated during the T cell dependent response to an unrelated antigen where the B cells acquire somatic mutations that broaden the original specificity making them self-reactive. I came to this conclusion by observing that in most cases the UCA versions of GM-CSF and citrullinated vimentin autoantibodies lost the ability to bind the self antigen. Importantly, in control experiments I could show that when somatic mutations were removed from antiviral antibodies, the UCAs always showed a clear binding to the viral antigen. These results show that somatic hypermutation is a very important process to increase affinity of an antibody to a foreign antigen, but it can also introduce new mutations that generate antibodies recognizing self antigens.

These findings open new questions on the role of somatic mutations in the generation of autoantibodies. First, is the origin of autoantibody from somatic mutations a common rule in autoimmune diseases? This aspect can be studied by isolating new ACPAs from RA patients and new autoantibodies from patients suffering from other autoantibody-mediated autoimmune diseases and analyzing the binding of UCA autoantibodies to the respective self antigens. Second, which are the funder mutations sufficient to introduce self-reactivity in an antibody and which are the mutations responsible for the high affinity of the autoantibodies? For this question it would be nice to find an autoimmune disease mediated by monoclonal or oligoclonal antibodies in order to follow the affinity maturation and reconstruct the genealogic tree of related self-reactive clones by deep-sequencing analyses. A possible candidate could be the Sjögren’s syndrome that is strongly associated with malignant B-cell lymphomas producing antibodies with RF activity. Third, what is the original specificity of the autoantibodies? For some diseases, like Guillain-Barré syndrome or Grave’s disease, cross-reactivity of autoantibodies to self antigens and pathogens has been demonstrated, but the isolation of a panel of cross-reactive monoclonal antibodies that specifically bind to the pathogen, but not the self antigen, in the UCA form could help to sustain the model of the origin autoantibodies through somatic mutations from responses to foreign antigens.
Forth, are autoreactive B cells triggered to produce autoantibodies by innate stimuli or by T cell help? In the latter case, is it necessary to break T cell self-tolerance? Autoantibodies often show a high level of somatic mutations suggesting that they are generated during a T cell dependent response and, in the case of PAP, there is no evidence of T cell help to develop GM-CSF autoantibodies. Unfortunately we did not find T cell specific for GM-CSF (data not shown), but we could try to find those specific for citrullinated peptides.

A second important aim of my thesis was to understand the pathogenic mechanism of GM-CSF autoantibodies in PAP. The main finding was that three non-cross-competing autoantibodies can sequester GM-CSF in stable immune complexes and can completely neutralize the cytokine activity in vitro, while in vivo the same immune complexes are rapidly cleared in a Fc-dependent fashion. In contrast, single autoantibodies could only partially neutralize GM-CSF activity in vitro and enhanced the levels of bioavailable GM-CSF in vivo. Furthermore, I could develop a new complementation bioassay by which I could detect low levels of anti-GM-CSF autoantibodies in healthy donors. Taken together, these findings provide a plausible explanation for the severe phenotype of PAP patients that developed high levels of GM-CSF autoantibodies to multiple epitopes of the molecule forming immune complexes that mediate GM-CSF sequestration and degradation. Conversely, healthy donors may have anti-GM-CSF antibodies in serum at low concentrations and/or in combinations that cannot promote sequestration and degradation of the cytokine.

Future studies could try to elucidate some details of the pathogenic mechanism in PAP. Where are the immune complexes with GM-CSF and autoantibodies formed and where are they cleared from the body? Which are the FcR-expressing cells responsible for the clearance of immune complexes? Unfortunately, the lack of cross-reactivity of GM-CSF autoantibodies with mouse GM-CSF makes it difficult to generate a model where to assess the proposed pathogenic mechanism and to study additional details. Anyway, it could be interesting to assess whether neutralization and clearance are common steps in the pathogenesis of other cytokine-mediated autoimmune diseases or in situations where administration of biological treatments, like IFN-β for multiple sclerosis, induced production of anti-drug antibodies that reduce the efficacy of the therapeutic cytokine.
7. References


cells, which is essential for the effector phase of autoimmune neuroinflammation." Nat Immunol 12(6): 560-567.


generated through somatic mutations target the desmoglein-3 cis-interface." J Clin Invest 122(10): 3781-3790.


Autoimmunity **46**(2): 121-127.

"Intestinal CD103+, but not CX3CR1+, antigen sampling cells migrate in lymph and serve classical dendritic cell functions."  


Leukemia **22**(4): 783-790.


8. Appendix

8.1. List of abbreviations

A  adenine/adenosine
Ab antibody
ACPA antibody to citrullinated protein antigens
Ag antigen
AID activation-induced deaminase
AIRE autoimmune regulator
APC antigen-presenting cell
APE apurinic/apyrimidic endonuclease
APS-1 autoimmune polyendocrine syndrome-1
ARDS acute respiratory distress syndrome
BAFF B-cell activating factor
BALF bronchoalveolar lavage fluid
βc β-chain
BCL B-cell lymphoma
BCR B-cell receptor
BER base excision repair
BIM BCL-2-interacting mediator of cell death
C cytosine/cytidine or constant
CCL CC-chemokine ligand
CCP cyclic citrullinated peptide
CCPM corrected counts per minute
CCR CC-chemokine receptor
CD cluster of differentiation/classification determinant
CD(nr)L CD(nr) ligand
CDR term complement-determining region
CDR C_H constant region of the heavy chain
CR complement receptor
CSF colony stimulating factor
CSFR2α GM-CSF receptor α-chain
CSFR2β GM-CSF receptor β-chain
CSR class-switch recombination
CTLA cytotoxic T-lymphocyte antigen
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>CXCL</td>
<td>CXC-chemokine ligand</td>
</tr>
<tr>
<td>CXCR</td>
<td>CXC-chemokine receptor</td>
</tr>
<tr>
<td>D</td>
<td>diversity</td>
</tr>
<tr>
<td>DC</td>
<td>dendritic cell</td>
</tr>
<tr>
<td>dC</td>
<td>deoxycytidine</td>
</tr>
<tr>
<td>DMARD</td>
<td>disease-modifying antirheumatic drug</td>
</tr>
<tr>
<td>EBNA-1</td>
<td>EBV nuclear antigen-1</td>
</tr>
<tr>
<td>EBV</td>
<td>Epstein-Barr virus</td>
</tr>
<tr>
<td>ELISA</td>
<td>enzyme-linked immunosorbent assay</td>
</tr>
<tr>
<td>EPO</td>
<td>erythropoietin</td>
</tr>
<tr>
<td>ERK</td>
<td>extracellular-signal-regulated kinases</td>
</tr>
<tr>
<td>FAB</td>
<td>fragment antigen binding</td>
</tr>
<tr>
<td>FcR</td>
<td>Fc receptor</td>
</tr>
<tr>
<td>fDC</td>
<td>follicular dendritic cell</td>
</tr>
<tr>
<td>FR</td>
<td>framework region</td>
</tr>
<tr>
<td>FS</td>
<td>Felty's syndrome</td>
</tr>
<tr>
<td>G</td>
<td>guanine/guanosine</td>
</tr>
<tr>
<td>GC</td>
<td>germinal center</td>
</tr>
<tr>
<td>G-CSF</td>
<td>granulocyte colony-stimulating factor</td>
</tr>
<tr>
<td>GM-CSF</td>
<td>granulocyte-macrophage colony-stimulating factor</td>
</tr>
<tr>
<td>GM-CSFr</td>
<td>GM-CSF receptor</td>
</tr>
<tr>
<td>GMRα</td>
<td>GM-CSF receptor α-chain</td>
</tr>
<tr>
<td>H</td>
<td>heavy</td>
</tr>
<tr>
<td>H1-HA</td>
<td>hemagglutinin H1</td>
</tr>
<tr>
<td>HBS</td>
<td>HEPES buffered saline</td>
</tr>
<tr>
<td>HIV</td>
<td>human immunodeficiency virus</td>
</tr>
<tr>
<td>HPLC</td>
<td>high-performance liquid chromatography</td>
</tr>
<tr>
<td>ICOS</td>
<td>inducible T-cell costimulator</td>
</tr>
<tr>
<td>IF</td>
<td>intermediary filament</td>
</tr>
<tr>
<td>IFN</td>
<td>interferon</td>
</tr>
<tr>
<td>Ig</td>
<td>immunoglobulin</td>
</tr>
<tr>
<td>IL</td>
<td>interleukin</td>
</tr>
<tr>
<td>IMGT</td>
<td>international ImMunoGeneTics information system</td>
</tr>
<tr>
<td>ITIM</td>
<td>immunoreceptor tyrosine-based inhibition motif</td>
</tr>
<tr>
<td>IVIG</td>
<td>intravenous immunoglobulin preparation</td>
</tr>
<tr>
<td>J</td>
<td>joining</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Definition</td>
</tr>
<tr>
<td>--------------</td>
<td>------------</td>
</tr>
<tr>
<td>JAK</td>
<td>Janus kinase</td>
</tr>
<tr>
<td>Ka</td>
<td>association constant</td>
</tr>
<tr>
<td>Kd</td>
<td>dissociation constant</td>
</tr>
<tr>
<td>KD</td>
<td>equilibrium dissociation constant</td>
</tr>
<tr>
<td>L</td>
<td>light</td>
</tr>
<tr>
<td>LAMP-2</td>
<td>lysosomal membrane protein-2</td>
</tr>
<tr>
<td>LPS</td>
<td>lipopolysaccharide</td>
</tr>
<tr>
<td>mAb</td>
<td>monoclonal antibody</td>
</tr>
<tr>
<td>M-CSF</td>
<td>macrophage colony-stimulating factor</td>
</tr>
<tr>
<td>MCV</td>
<td>mutated citrullinated vimentin</td>
</tr>
<tr>
<td>MG</td>
<td>myasthenia gravis</td>
</tr>
<tr>
<td>MMR</td>
<td>mismatch-repair</td>
</tr>
<tr>
<td>NET</td>
<td>neutrophils extracellular trap</td>
</tr>
<tr>
<td>NF-κB</td>
<td>nuclear factor kappa-light-chain-enhancer of activated B cells</td>
</tr>
<tr>
<td>NHEJ</td>
<td>nonhomologous end-joining</td>
</tr>
<tr>
<td>NK</td>
<td>natural killer</td>
</tr>
<tr>
<td>OPN</td>
<td>osteopontin</td>
</tr>
<tr>
<td>PAD</td>
<td>peptidyl arginine deiminase</td>
</tr>
<tr>
<td>PALS</td>
<td>peri-arteriolar lymphoid sheath</td>
</tr>
<tr>
<td>PAP</td>
<td>pulmonary alveolar proteinosis</td>
</tr>
<tr>
<td>PBMC</td>
<td>peripheral blood mononuclear cell</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate buffered saline</td>
</tr>
<tr>
<td>PCNA</td>
<td>proliferating cell nuclear antigen</td>
</tr>
<tr>
<td>PEI</td>
<td>polyethylenimine</td>
</tr>
<tr>
<td>PI3K</td>
<td>phosphatidylinositol-3-kinase</td>
</tr>
<tr>
<td>PPAD</td>
<td>peptidyl arginine deiminase from <em>P. gingivalis</em></td>
</tr>
<tr>
<td>PRCA</td>
<td>pure red-cell aplasia</td>
</tr>
<tr>
<td>PTM</td>
<td>post-translational modification</td>
</tr>
<tr>
<td>RAG</td>
<td>recombination-activating gene</td>
</tr>
<tr>
<td>RF</td>
<td>rheumatoid factor</td>
</tr>
<tr>
<td>ROS</td>
<td>reactive oxygen species</td>
</tr>
<tr>
<td>RSS</td>
<td>recombination signal sequences</td>
</tr>
<tr>
<td>RSV</td>
<td>respiratory syncytial virus</td>
</tr>
<tr>
<td>S</td>
<td>switch region</td>
</tr>
<tr>
<td>SAP</td>
<td>SLAM-associated protein</td>
</tr>
<tr>
<td>SE</td>
<td>shared epitope</td>
</tr>
</tbody>
</table>
Appendix

SEC  size-exclusion chromatography
SH2  src-homology domain 2
SHIP SH2-domain-containing inostil-5-phosphatase
SHM somatic hypermutation
SHP1 SH2-domain-containing protein tyrosine phosphatase 1
SIAE sialic acid acetyl esterase
SjS Sjögren syndrome
SLAM signalling lymphocytic activation molecule
SLC surrogate light chain
SLE systemic lupus erythematosus
SPR surface plasmon resonance
STAT signal transducer and activator of transcription
T thymidine/thymine
TCR T-cell receptor
TD T-dependent
TdT terminal deoxy-nucleotidyl transferase
T_FH follicular helper T cells
TGF transforming growth factor
TI T-independent
TI-1 T-independent type 1
TI-2 T-independent type 2
TLR Toll-like receptor
TNF tumor necrosis factor
TSHR thyroid-stimulating hormone receptor
U uracil/uridine
UCA unmutated common ancestor
UNG uracil DNA N-glycosylase
V variable
VH heavy chain variable region
VL light chain variable region
8.2. Curriculum vitae

Personal details

Name Luca Piccoli
Nationality Italian
Address Via Monte Gaggio 15a, 6500 Bellinzona, Switzerland
Date of birth July 12, 1984
Place of birth Breno (BS), Italy
E-mail luca.piccoli@irb.usi.ch

Education and research activities

Nov 2011 to date Doctoral studies at ETH, D-BIOL, Zurich, Switzerland.

PhD thesis dissertation at the Institute for Research in Biomedicine, Bellinzona, Switzerland, under the supervision of Prof. A. Lanzavecchia.

Thesis subject: “Origin and pathogenicity of autoantibodies in cytokine-mediated and tissue-specific autoimmune diseases”.

Jul 2009 – Oct 2011 Recipient of a grant from IRCCS San Matteo Hospital Foundation, Department of Infectious and Tropical Diseases, Pavia, Italy. Supervisor: Dr. Enrico Brunetti.

Projects: serum cytokine profiling in patients with cystic echinococcosis, genotyping of echinococcal cysts.

Oct 2006 – Dec 2008 Master’s degree in Medical and Pharmaceutical Biotechnologies, 110/110 with distinction, at the University of Pavia, Italy.

Thesis: “Analysis of dysplastic histogenesis of cartilage in a mouse model of diastrophic dysplasia”.

Oct 2003 – Sep 2006 Bachelor’s degree in Biotechnologies, 110/110 with distinction, at the University of Pavia, Italy.

Thesis: “Development and evaluation of a nested PCR test on amniotic fluid in prenatal diagnosis of congenital toxoplasmosis”.

Jul 2003 Diploma di Liceo Scientifico (certificate of secondary school focusing on sciences), 100/100, Breno, Italy.
8. Appendix

Language skills

Italian  mother tongue
English  proficient user (European level: C1)
Spanish independent user (European level: B1)
French independent user (European level: B1)
German basic user (European level: A2)

8.3. List of relevant publications
