ANCA-ASSOCIATED VASCULITIDES: COMPLEX DIAGNOSTIC APPROACH

BASED ON CURRENT INSIGHTS
INTO PATHOGENESIS OF THE DISEASE

(ANCA-ASOCIOVANÉ VASKULITIDY: KOMPLEXNÍ DIAGNOSTICKÝ PŘÍSTUP)

MUDr. Zdenka Hrušková (Vaňková)

Tutor:
As. MUDr. Helena Marečková, CSc.

Consultant:
Prof. MUDr. Vladimír Tesař, DrSc.

Institute of Immunology and Microbiology
Department of Nephrology

General Teaching Hospital and First Faculty of Medicine
Charles University in Prague, Czech Republic

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CONTENTS

ABBREVIATIONS AND ACRONYMS

AAV	ANCA-Associated Vasculitis (Vasculitides)
ACR	American College of Rheumatology
ANA	Anti-Nuclear Antibodies
ANCA	Anti-Neutrophil Cytoplasmic Autoantibodies
APC	Allophycocyanin
ATG	Anti-Thymocyte Globulin
AZA	Azathioprine
BFA	Brefeldin A
BPI	Bactericidal/Permeability-Increasing protein
BVAS	Birmingham Vasculitis Activity Score
c-ANCA	cytoplasmic Anti-Neutrophil Cytoplasmic Autoantibodies
CCR	C-C Chemokine Receptor
CKD	Chronic Kidney Disease
CRTH2	Chemoattractant Receptor-Homologous Molecule expressed on Th2 lymphocytes
CXCR	CXC Chemokine Receptor
CHCC	Chapel Hill Consensus Conference
cPR3	complementary Proteinase-3
CR	Complete Remission
CSS	Churg Strauss Syndrome
CTLA-4	Cytotoxic T-Lymphocyte-associated Antigen 4
CYC	Cyclophosphamide
DTH	Delayed-Type Hypersensitivity
ELISA	Enzyme-Linked Immunosorbent Assay
ENT	Ear, Nose and Throat
EUVAS	European Vasculitis Study Group
FACS	Fluorescence Activated Cell Sorter
FcγR	Receptor for the Fc fragment of immunoglobulin G
FITC	Fluorescein Isothiocyanate
FNGN	Focal Necrotizing Glomerulonephritis
FoxP3	Transcription factor forkhead box P3
FSC	Forward Scatter
GBM	Glomerular Basement Membrane
GM-CSF	Granulocyte-Macrophage Colony-Stimulating Factor
GTP	Guanosine-5'-triphosphate
HACA	Human Anti-Chimeric Antibodies

HC	Healthy Controls
IFNγ	Interferon gamma
Ig	Immunoglobulin
IIF	Indirect ImmunoFluorescence
IL	InterLeukin
IL-2R	Receptor for Interleukin-2
IRI	Immunoregulatory Index
iRPGN	idiopathic (isolated) Rapidly Progressive GlomeruloNephritis
IU	International Units
IVIg	IntraVenous Immunoglobulin
kDa	kiloDalton
LAMP-2	Lysozomal-Associated Membrane Protein 2
LPS	Lipopolysaccharide
MMF	Mycophenolate-mofetil
MPA	Microscopic Polyangiitis (Polyarteritis)
MPO	Myeloperoxidase
mRNA	messenger Ribonucleic Acid
NK	Natural Killer
n.s.	not significant
p-ANCA	perinuclear Anti-Neutrophil Cytoplasmic Autoantibodies
PE	Phycoerythrin
PerCP	Peridinin Chlorophyll Protein
PHA	Phytohaemaglutinin
PMA	Phorbol 12-Myristate 13-Acetate
PR	Partial Remission
PR3	Proteinase-3
RAG2	Recombination Activating Gene 2
RLV	Renal-Limited Vasculitis
ROS	Reactive Oxygen Species
RPMI	Roswell Park Memorial Institute medium
RTX	Rituximab
s (sCD30)	soluble (soluble CD30)
SSC	Side Scatter
T_{EMs}	Effector Memory T cells
TGFβ	Transforming Growth Factor beta
Th	T helper (cells)
TNFα	Tumor Necrosis Factor alpha
Treg(s)	regulatory T cell(s)
WG	Wegener's Granulomatosis

1. INTRODUCTION

Vasculitides are a heterogeneous group of clinico-pathologic units characterised by inflammatory infiltration and potential destruction of blood vessel walls. This leads to ischemia of the tissues supplied by the involved vessel. In some patients, systemic signs and symptoms, such as fever, fatigue or weight loss, may be present. Vasculitis can occur as the primary or sole manifestation of the disease (primary vasculitis), or in connection with another identified underlying trigger or disease, e.g. rheumatoid arthritis or systemic lupus erythematosus (secondary vasculitis). Since any type, size and location of blood vessels may be afflicted by vasculitis there is a whole spectrum of different syndromes resulting from the disease process. [1]

The classification of vasculitides has remained controversial during the past years. Various attempts have been made to develop a universally accepted classification scheme recently [2]. However, two so far still most commonly used classification systems of primary systemic vasculitides include the American College of Rheumatology (ACR) 1990 criteria [3] and the Chapel Hill Consensus Conference (CHCC) definitions proposed in 1994 [4], although the latter were not originally intended to be used as either classification or diagnostic criteria. According to the CHCC, vasculitides can be divided into large vessel, medium sized vessel and small vessel vasculitides based on the size of the involved vessel (Table 1.1).

Table 1.1: Classification of vasculitides adopted by the Chapel Hill Consensus Conference on the Nomenclature of Systemic Vasculitis (1994)

Size of involved vessels	Disease entity
Large vessel vasculitis	Giant cell (temporal) arteritis
	Takayasu's arteritis
Medium sized vessel vasculitis	Polyarteritis nodosa
	Kawasaki disease
Small vessel vasculitis	Wegener's granulomatosis*
	Churg Strauss syndrome*
	Microscopic polyangiitis* (Microscopic polyarteritis)
	Henoch Schönlein Purpura
	Essential cryoglobulinaemic vasculitis
	Cutaneous leucocytoclastic angiitis

*Strongly associated with antineutrophil cytoplasmic antibodies (ANCA).

Wegener's granulomatosis (WG), microscopic polyangiitis (MPA) and Churg Strauss syndrome (CSS) (for their CHCC definitions see Table 1.2) are a group of systemic immune-mediated diseases with a strong and highly specific association with Anti-Neutrophil Cytoplasmic Autoantibodies (ANCA). Together with idiopathic (isolated) rapidly progressive glomerulonephritis (iRPGN) they are therefore all ranked among ANCA-associated vasculitides (AAV).

Table 1.2: Definitions of ANCA-associated vasculitides according to the Chapel Hill Consensus Conference

Disease entity	**Disease definition**[+]
Wegener's granulomatosis	Granulomatous inflammation involving the respiratory tract, and necrotizing vasculitis affecting small to medium-sized vessels (e.g. capillaries, venules, arterioles, and arteries). *Necrotizing glomerulonephritis is common.*
Churg Strauss syndrome	Eosinophil-rich and granulomatous inflammation involving the respiratory tract, necrotizing vasculitis affecting small to medium-sized vessels, and associated with asthma and eosinophilia
Microscopic polyangiitis[*] **(microscopic polyarteritis)**	Necrotizing vasculitis, with few or no immune deposits, affecting small vessels (i.e. capillaries, venules, or arterioles). *Necrotizing arteritis involving small and medium sized arteries may be present. Necrotizing glomerulonephritis is very common. Pulmonary capillaritis often occurs.*

* Preferred term.

[+] Essential components are represented by normal type; italicised type represents usual, but not essential, components.

ANCA are predominantly IgG autoantibodies directed against different target antigens located in azurophilic granules of polymorphonuclear leucocytes and the peroxidase-positive lysosomes of monocytes. Nowadays, ANCA testing plays an important role in the diagnosis of vasculitides. In WG, ANCA are usually directed against proteinase 3 (PR3-ANCA) and have a cytoplasmic type of immunofluorescence

(c-ANCA). In MPA the target antigen is mostly myeloperoxidase (MPO-ANCA) and the type of immunofluorescence is perinuclear (p-ANCA). Both MPO-ANCA and, less frequently, PR3-ANCA can also be found in patients with CSS and iRPGN (sometimes referred to as renal-limited vasculitis, RLV) [5].

The annual incidence of AAV (in Europe approximately 10-20/million [6]) is not as low as it was originally believed, most probably due to higher awareness of the disease and the possibility of routine ANCA testing. AAV typically involve kidney, lungs and ENT (ear, nose and throat) organs even though, in principle, any organ may be afflicted. Diagnostics is based on clinical picture, ANCA testing and—when possible—verification by a biopsy. Untreated, generalised WG and MPA follow a progressive course with a fatal outcome due to vital organ failure. Standard combined immunosuppressive treatment consisting of cyclophosphamide and corticosteroids leads to successful remission achievement in most patients. Side effects of the therapy are, however, common; and, in the long run, many of the patients relapse.

The pathogenesis of ANCA-associated vasculitides remains to be elucidated. Nevertheless, it seems to be a complex process which likely involves impaired immune mechanisms of both innate and adaptive, and both humoral and cellular immunity. Due to close correlation of ANCA and AAV, researchers primarily focused on the potential of ANCA to induce and promote vasculitis. In recent years, there has been increasing evidence that ANCA might, indeed, play a direct pathogenic role in triggering AAV. However, as ANCA are class-switched IgG antibodies (mainly IgG1 and IgG4) [7], T cells are also involved in their production by B cells and plasma cells. During the last few years, abnormalities in T cell subpopulations in AAV have been reported repeatedly and cellular immunity in AAV has attracted substantial attention. In the future, better understanding of pathogenic mechanisms in AAV might provide clues to improve diagnostic possibilities and also develop novel, targeted, therapeutic modalities.

In this work, current insights into the pathogenesis of AAV have been summarized, with emphasis placed on the role of both ANCA and T cells in the process. The experimental part aimed to assess the cellular immunity in AAV. The expression of various surface markers and the intracellular cytokine production in peripheral lymphocytes and monocytes in patients with AAV at different stages of the disease were measured by flow cytometry.

2. CURRENT INSIGHTS INTO PATHOGENESIS OF ANCA-ASSOCIATED VASCULITIS

2.1 Anti-Neutrophil Cytoplasmic Autoantibodies (ANCA)

Anti-Neutrophil Cytoplasmic Autoantibodies (ANCA) were first described in 1982 and were originally believed to be associated with *Ross River virus* infections [8]. By 1985, however, ANCA had been linked to WG [9]. Within several more years, a relationship among ANCA, WG and MPA had been established and proteinase 3 (PR3) and myeloperoxidase (MPO) recognised as major autoantigens [10-12]. Nowadays, PR3-ANCA and MPO-ANCA testing is widely used in the diagnostics of vasculitides and, despite some controversy, also for monitoring disease activity.

2.1.1 ANCA testing

Two types of ANCA assays are currently in wide use – a more sensitive indirect immunofluorescence (IIF) assay and a more specific enzyme-linked immunosorbent assay (ELISA). To maximize diagnostic utility, combination of both methods has been recommended, with IIF used as a screening method and IIF positivity confirmed by PR3- and MPO-specific ELISAs [13-17]. Thereby, the specificity for AAV can reach up to 88-100% and sensitivity for AAV widely ranges from 34 to 92% [14, 16, 17]. Negative IIF results in patients with clinical symptoms attributable to vasculitis should also be tested by ELISA as 5% of serum samples are positive by ELISA only [13, 15]. In appropriate clinical context, ANCA testing by both IIF and ELISA can help to exclude or, conversely, support the diagnosis of pauci-immune necrotizing/crescentic glomerulonephritis (with negative and positive predictive values being 99% and 95%, respectively) [18].

2.1.1.1 Indirect immunofluorescence

When sera of the patients with AAV are incubated with ethanol fixed human neutrophils, two major immunofluorescent patterns are observed. With the classical c-ANCA pattern (Fig. 1 on the left), the granular staining is diffuse throughout the cytoplasm, with internuclear accentuation (c = cytoplasmic type of

immunofluorescence). In most cases, antibodies directed against PR3 cause this pattern, but MPO-ANCA can occasionally be responsible [13, 19].

The perinuclear or p-ANCA pattern (Fig. 1 on the right) results from a staining pattern around the nucleus (with or without nuclear extension), which represents an artefact of ethanol fixation. With ethanol fixation of the neutrophil substrate, positively-charged granule constituents rearrange themselves around the negatively-charged nuclear membrane, leading to perinuclear fluorescence [10, 19]. The antibody responsible for this pattern (detected by ELISA) is usually directed against MPO (and occasionally PR3).

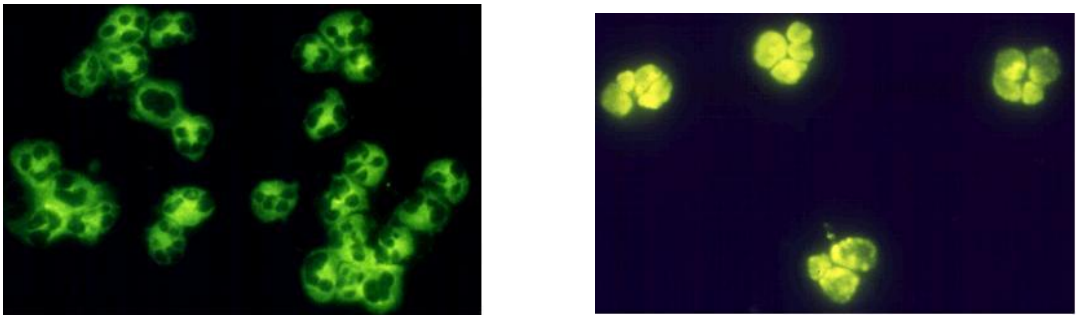

Fig. 2.1: ANCA: c - cytoplasmic type of immunofluorescence on the left; p - perinuclear type of immunofluorescence on the right [photo from the Laboratory of Immunology, Institute of Clinical Biochemistry and Laboratory Diagnostics]

However, p-ANCA (other than MPO- or PR3-specific) can also be detected in diseases different from AAV, such as in inflammatory bowel diseases and also other autoimmune disorders. Atypical cytoplasmic ANCA, with diffuse flat cytoplasmic staining without accentuation between nuclear lobules, are often linked to chronic infections and directed against bactericidal/permeability-increasing protein (BPI). Any other ANCA staining is referred to as an atypical ANCA pattern and is not considered typical for AAV. Nevertheless, in some cases it might resemble p-ANCA pattern [19].

A frequent difficulty in distinguishing the p-ANCA pattern of immunofluorescence from that caused by antinuclear antibodies (ANA) in ethanol fixation might be overcome by the use of both formalin- and ethanol-fixed neutrophil substrates, because formalin-fixed neutrophils prevent the rearrangement of charged cellular components around the nucleus [20]. However, the formalin-fixed substrates are not used in routine practice. Generally, the IIF results have to be interpreted with caution as they are highly dependent on the experience of the laboratory personnel.

2.1.1.2 Enzyme-linked immunosorbent assays

Specific ELISAs for antibodies against PR3 and MPO (and in some centres for other antigens as well) are now available, and should form an integral part of routine ANCA detection in AAV patients, as stated above. PR3-ANCA and MPO-ANCA are possibly associated with substantially higher specificities and positive predictive values than the immunofluorescence patterns to which they usually correspond (c- and p-ANCA, respectively). There are, however, significant differences in sensitivity, specificity and predictive values among commercially available direct ELISA kits as there has been no agreed international standard so far [21, 22].

It has been noted that direct ELISA results do not always correlate with IIF results. The reason might be that proteins are denatured during antigen purification or coating onto the solid phase, thereby hiding or destroying conformational epitopes on PR3. In order to avoid this, capture ELISA has been designed, where the plate is precoated with a monoclonal antibody to capture the antigen. However, the capturing antibodies may compete for epitopes recognized by some PR3-ANCA, causing occasional false-negative results. Some data suggest an advantage of capture ELISA over direct ELISA, the most important being a superior diagnostic performance and better inter-laboratory correlation in PR3-ANCA and the ability to detect immune complexes [23, 24]. In a recent report, a direct PR3-ELISA was superior to a capture PR3-ELISA as a screening test while capture ELISA was shown a potentially more useful method for monitoring serial PR3-ANCA concentrations [25].

2.1.2 Pathogenic role of ANCA in ANCA-associated vasculitis

Vasculitic lesions caused by small-vessel vasculitides associated with the presence of ANCA (i.e. WG, MPA, CSS and iRPGN) display so-called ‘pauci-immune’ pattern in indirect immunofluorescence techniques and are therefore collectively designated as pauci-immune necrotizing vasculitides. The term pauci-immune reflects the relative lack of immunoglobulin and complement depositions (particularly in the kidneys) [5] although immune complex deposits were found on electron microscopy in over a half of renal biopsies [26].

Nevertheless, the strong association of ANCA with vasculitic syndromes, their correlation with disease activity, and ability of ANCA increase to predict relapse in

some–but not all–studies [27, 28] have suggested a pathogenic role for ANCA. These findings may, however, be the cause as well as the result of the disease. In recent years, evidence for the pathogenic potential of ANCA, particularly MPO-ANCA, has emerged from both *in vitro* and *in vivo* studies, and will be summarized below. In addition to this, the first reported case of a transplacental transfer of MPO-ANCA resulting in a pulmonary-renal syndrome in the newborn has been published lately [29].

2.1.2.1 In vitro studies

2.1.2.1.1 ANCA and neutrophil and monocyte activation

In AAV, ANCA are directed against antigens located in azurophilic granules of polymorphonuclear leucocytes and the peroxidase-positive lysosomes of monocytes. The antigen is either proteinase 3 (PR3), a 29-kDa cationic protein belonging to the trypsin family of neutral serine proteases, or myeloperoxidase (MPO), a 140-kDa enzyme, which catalyses the production of hypochloric acid effective in killing phagocytized bacteria and viruses.

Although ANCA antigens are normally predominantly located in the cytoplasm of neutrophils and monocytes, preactivation (priming) of these cells, as occurs following exposure to low doses of pro-inflammatory cytokines such as TNFα (tumor necrosis factor alpha), granulocyte-macrophage colony-stimulating factor (GM-CSF), interleukin (IL)-1 or IL-8, results in the release of small amounts of ANCA antigens and their translocation to the cell surface, where they become accessible for interaction with ANCA. In vivo, these proinflammatory stimuli might, for instance, be induced by preceding or concomitant infectious agents [30, 31]. A strong association of the occurrence of relapse with chronic nasal carriage of *Staphylococcus aureus* in patients with WG has been reported [32].

The interaction of ANCA and target antigens is followed by activation of neutrophils. In vitro, IgG fractions from sera that contain PR3-ANCA or MPO-ANCA (but not IgG fractions from healthy donors) cause cytokine-primed neutrophils to undergo a respiratory burst and degranulation. The degranulation of neutrophils and release of lytic enzymes, interleukin-Iβ and reactive oxygen species (ROS) cause tissue damage [30, 33]. Recently, also IL-18 was shown to prime ANCA-stimulated neutrophils for increased superoxide production, without increasing the surface

expression of PR3 and MPO. This process was unaffected by anti-TNFα antibodies, suggesting a direct role of IL-18 in neutrophil priming [34].

Not only neutrophils but also monocytes can be activated by ANCA. Affinity-purified PR3-ANCA and MPO-ANCA are able to induce monocytes to produce oxygen radicals and to increase their synthesis of cytokines and chemokines such as monocyte chemoattractant protein-1 and IL-8 [35-37].

2.1.2.1.2 Neutrophil - endothelial cell interaction in AAV

The activation of neutrophils by ANCA only occurs when they are adherent to a surface [38]. *In vivo*, this process is assumed to occur at the endothelial surface. In *in vitro* models, binding of ANCA allows activated neutrophils and monocytes to stably adhere to the cytokine-primed endothelial layer with enhanced expression of adhesion molecules [39]. A role for adhesion molecules has been supported by the immunohistological evidence for upregulated adhesion molecules in glomerular lesions in renal biopsies of AAV patients [40]. It has been demonstrated that the interaction between neutrophils and endothelial cells depends on β2 integrins (CD11a/CD18 and CD11b/CD18) and GTP-binding chemokine receptors and that the process of neutrophil adherence might be *in vitro* inhibited by anti-CD18 antibodies [41, 42]. ANCA are able to stimulate the process of this adherence, presumably by upregulating CD11b on neutrophils as shown in an *in vitro* study [43], or by conformational change in CD11b which reveals its activation epitope as suggested by others [42]. The presence of ANCA leads to the detachment and lysis of endothelial cells [44]. In mediating the endothelial cell injury in AAV, serine proteases are supposed to play a more important role than reactive oxygen species [45]. Persistent ANCA binding to neutrophils on the endothelial surface can enhance the degree of vascular injury and also the destruction of the underlying tissues [46].

In addition, primed neutrophils not only damage endothelial cells, but attract additional neutrophils to the site of damage, thereby creating an auto-amplifying loop. The release of MPO, PR3, elastase and other proteases from activated neutrophils also directly contributes to the local inflammatory process. Released enzymes can bind to the surface of unprimed neutrophils, facilitating subsequent recognition by ANCA and amplifying ANCA-potentiated neutrophil activation. Moreover, ANCA-mediated secretion of pro-inflammatory cytokines further activates other components of immune response, with monocytes and T lymphocytes involved [31, 47, 48]. Interestingly, *in*

vitro, the factors released from stimulated neutrophils have only recently been shown to also activate complement via the alternative pathway, which indicates that the so far neglected complement might play an important role in the complex pathogenesis of AAV [49].

The presence of increased numbers of circulating endothelial cells in patients with active vasculitis [50] supports the hypothesis that endothelial cells are the target of the initial injury. This results in swelling, apoptosis/necrosis and deadherence of endothelial cells. Lysed neutrophil granulocytes are found within affected vessels. In the lung, capillaries, venules and arterioles are infiltrated by polymorphonuclear leucocytes. Pulmonary microvascular necrotizing vasculitis (capillaritis) is the cause of pulmonary hemorrhage. In the kidney, rupture of the basement membrane subsequent to neutrophil degranulation gives rise to glomerular capillary thrombosis followed by a cascade of events leading to focal segmental crescentic glomerulonephritis.

2.1.2.1.3 ANCA binding to neutrophils and signal transduction pathways

Notable progress has been made lately in understanding the mechanisms of ANCA binding and, to a lesser extent, in signal transduction pathways, even though precise details still remain to be elucidated.

ANCA bind via their F(ab')2-fragments to the target antigens, i.e. surface expressed PR3 or MPO, and, at the same time, via their Fc fragments to Fc gamma receptors (FcγR) on neutrophils. Binding of ANCA to neutrophils triggers signalling events that lead to neutrophil activation. It has been discussed whether these signalling events are initiated by ANCA F(ab')2 binding to MPO or PR3 [51] or proceed via antibody binding to FcγRIIa and FcγRIIIb receptors [52-54] or both. To date, it seems highly likely that both F(ab')2 and Fc engagements are needed for effective neutrophil activation that leads to significant functional events, such as superoxide and pro-inflammatory cytokines production and degranulation. Beta-2 integrins might cooperate with the Fc gamma receptor in propagating the signal. Fc gamma receptor interactions are bound to play a role in neutrophil activation by ANCA, as blockade of FcγRIIa with monoclonal antibodies is able to prevent the process [52, 53]. Recently, it has been demonstrated that ligation of FcγRIIa and FcγRIIIb is necessary for ANCA-induced neutrophil activation. However, the signalling cascades used by ANCA are different from the signal pathways used by FcγR engagement only, suggesting that ANCA require other not yet identified membrane cofactors for neutrophil activation than FcγR

mechanisms alone [54]. In brief, the so far identified signalling pathways involved in ANCA-mediated neutrophil activation include p38 mitogen-activated protein kinase (MAPK), extracellular signal-regulated kinase and phosphatidylinositol-3 kinase control systems [54-56].

It has been reported that the intracellular signal transduction pathways differ depending on in which portion of ANCA the signal is initiated. While both ANCA IgG and ANCA IgG F(ab')2 fragment can activate inhibitory G proteins and p21ras protein activator, ANCA IgG F(ab')2 fragment is insufficient to activate tyrosin kinases pathways, thought to generate superoxide production [31, 57, 58]. However, both tyrosin kinases and inhibitory G proteins pathways proceed to the activation of p21ras, a molecule that controls a number of cellular processes in the neutrophil, including the respiratory burst. Interestingly, not all isoforms of p21ras in neutrophils are activated by ANCA. This could possibly allow a specific inhibition of ANCA-activated signalling pathways [58].

2.1.2.1.4 Apoptosis

In vitro, TNFα-primed neutrophil activation by ANCA results in accelerated and dysregulated apoptosis, dependent on reactive oxygen species generation. This subsequently leads to impaired phagocyte clearance and secondary necrosis, promoting the inflammatory process [59]. In a recent study, MPO-ANCA have been proven a stronger apoptosis activator as well as accelerator than PR3-ANCA [60]. Even though apoptotic neutrophils cannot undergo cellular activation, they also express MPO and PR3 antigens capable of binding by ANCA. Therefore, ANCA can interact with the cell surface of apoptotic neutrophils and participate in the opsonization of these cells, thereby inducing an increased uptake by macrophages and release of pro-inflammatory cytokines. *In vivo,* these processes might contribute to a failure to regulate inflammation in the normal manner [61, 62].

2.1.2.2 In vivo studies

Despite an increasing number of *in vitro* observations suggesting the pathogenic role of ANCA in vasculitis, there has been a need for *in vivo* studies to support the proposed hypotheses.

2.1.2.2.1 MPO-ANCA-associated vasculitis

In one of the first studies [63], autoimmune-prone rats were immunized with human MPO, which led to the development of anti-human-MPO antibodies that cross-reacted with endogenous rat MPO. A cellular response to MPO could also be detected in these rats. Nevertheless, no vasculitic lesions were proven until the MPO-immunized rats were injected with an extract of neutrophils, containing MPO, and hydrogen peroxide (H_20_2). After this injection, vasculitis of the lungs and the gut occurred, but still no glomerulonephritis was found. However, unilateral perfusion of the left kidney with the neutrophil extract and H_20_2 induced a severe form of necrotizing crescentic glomerulonephritis in MPO-immunized rats and no lesions in non-immunized rats [63, 64]. Interestingly, immune complex depositions were observed in the kidneys shortly after the perfusion but when the glomerulonephritis reached its maximum, immune deposits were no longer detected [63]. The degree of histological injury correlated with the amount of glomerular IgG immune deposits in a similar model [65].

On the basis of these experiments, it was concluded that when immune complexes are first deposited along the vessel wall, the presence of ANCA can induce vasculitis and pauci-immune glomerulonephritis in rats, in other words, that *in vivo*, ANCA play a role in exacerbating and augmenting inflammation initiated by a primary stimulus. The presumption that any kind of immune complex deposition along the glomerular capillary wall (not necessarily only ANCA/MPO) can enhance local immune response in glomeruli was supported by the finding that severe glomerulonephritis developed in MPO-immunized rats after low-dose injection of anti-GBM (glomerular basement membrane) antibodies, but not in the non-immunized ones [66].

More recent experiments [67] involved MPO-deficient mice that, after immunization with murine MPO, developed circulating anti-MPO antibodies. Adoptive transfer of splenocytes from these mice into recombination activating gene 2 deficient ($RAG2^{-/-}$) mice lacking both B and T lymphocytes induced circulating anti-MPO antibodies in the recipient mice. All the recipient mice developed renal failure with severe crescentic and necrotic renal lesions, but immune complex and complement depositions were also observed. Passive transfer of purified anti-MPO IgG into both $RAG2^{-/-}$ and wild type mice induced focal necrotizing crescentic glomerulonephritis with the lack of immune complexes, closely resembling the human disease. In both

adoptive and passive transfer experiments non-glomerular pathology was also noted. These data strongly support the pathogenic role of MPO-ANCA in pauci-immune glomerulonephritis and systemic vasculitis [67].

2.1.2.2.2 PR3-ANCA-associated vasculitis

Contrary to several animal models of MPO-ANCA-associated glomerulonephritis and/or vasculitis, a model for PR3-ANCA-associated vasculitis that would resemble human Wegener's granulomatosis has yet to be developed. Unlike human MPO, human PR3 does not elicit a cross-reactive antibody response against murine PR3 nor do human PR3-ANCA cross-react with murine PR3 [68]. In a model of PR3/elastase deficient mice (double-knockout mice were used since murine PR3 and elastase display a highly similar sequence), immunization with murine PR3 led to the development of anti-PR3 antibodies, which were able to bind to activated (primed) murine neutrophils. After an intradermal injection of TNFα, wild-type mice that received PR3-ANCA-containing IgG developed a significantly worse form of panniculitis than those that received control IgG. However, transfer of PR3-ANCA-containing IgG into lipopolysaccharide-primed wild type mice did not result in any signs of systemic, lung, or renal vasculitis [69]. Whether these experiments provide sufficient evidence for a pathogenic effect of PR3-ANCA at local sites of inflammatory response has been subject to discussion [68-70].

2.1.2.3 Induction of autoimmunity to antigens recognised by ANCA – mechanism and aetiology

As stated above, ANCA most probably directly participate in the pathogenesis of AAV. The truly crucial question, namely, why ANCA develop and what triggers and controls their production, remains, however, unclear. Both basic ANCA antigens, i.e. MPO and PR3, normally occur in humans. Breaking of self-tolerance is the pivotal process in autoimmunity in general. It is well known that autoreactive B and T cells exist in the blood of healthy individuals. Nevertheless, control and regulatory mechanisms in healthy individuals effectively prevent the development of an autoimmune disease [71].

In AAV patients, both B and T cell function is disturbed. On the whole, different environmental and genetic factors are suspected to contribute to the development and/or

clinical course of AAV. As for the genetic factors, a number of familial cases of AAV have been described. Suggested candidate genes include, among others, those encoding cytotoxic T-lymphocyte-associated antigen 4 (CTLA-4), interleukin-1 receptor antagonist (IL-1ra), IL-10, CD18 adhesion molecule and FcγRII/FcγRIII [72-75].

Regarding the environmental factors, exposure to certain drugs and to silica has been associated with AAV. Infection is also long thought to be one of the triggering factors in AAV. Previously postulated general mechanisms by which infectious agents might induce vasculitis include molecular mimicry, theory of autoantigen complementarity, antigen drift, abnormal presentation of self-proteins and/or abnormal stimulation of autoreactive B or T cells by e.g. superantigens. In particular the two first mentioned mechanisms have lately attracted attention and are therefore described in detail below.

2.1.2.3.1 Antibodies against lysozomal-associated membrane protein 2 (LAMP-2) and molecular mimicry

As stated above, several antigenic targets of ANCA different from MPO and PR3 have been identified, e.g. lactoferrin, elastase, BPI, lysozomal-associated membrane protein 2 (LAMP-2) and others. Clinical significance of ANCA directed against these antigens is not completely understood; until recently, they were, however, not considered typical for AAV.

In 2008, Kain et al. [76] showed that antibodies to LAMP-2 were present in most (93%) patients with biopsy-proven active pauci-immune focal necrotizing glomerulonephritis (FNGN) typical for AAV while only 83% of patients with FNGN had "classical" PR3- or MPO-ANCA. Moreover, antibodies to LAMP-2 caused pauci-immune FNGN when injected into rats, and a monoclonal antibody to human LAMP-2 caused activation of neutrophils and induced apoptosis of human endothelial cells *in vitro*. The authors also showed that anti-LAMP-2 antibodies recognized an epitope that was 100% homologous to the bacterial adhesin FimH, with which they cross-reacted. FimH is located at the tip of type 1 fimbriae that is essential for the attachment of Gram-negative pathogens such as *Escherichia coli, Klebsiella pneumoniae and Proteus mirabilis* to host epithelia. Infection with fimbriated pathogens before presentation of FNGN was proven common in the same study (confirmed in 69% individuals).

Thus, molecular mimicry may be the fundamental mechanism in the development of FNGN in AAV. In the context of prior reports, anti-LAMP-2 antibodies

might act synergistically with MPO-ANCA to cause pauci-immune glomerulonephritis. As LAMP-2 is located on the membranes of intracellular vesicles that contain PR3 and MPO, impaired function of LAMP-2 caused by anti-LAMP-2 antibodies might also lead to the synthesis of antibodies against cytoplasmic antigens, such as MPO and PR3. [77]

2.1.2.3.2 Theory of autoantigen complementarity

Theory of autoantigen complementarity has been suggested as another intriguing possible mechanism for the PR3-ANCA production [78]. Complementary PR3 (cPR3) is a protein encoded by the anti-sense DNA strand of the PR3 encoding gene. When expressed (either from endogenous DNA or from identical genetic material from an infectious pathogen), cPR3 might induce development of anti-cPR3-antibodies, which might induce anti-idiotype antibodies that would cross-react with the original antigen, i.e. PR3. Indeed, some patients with PR3-ANCA have antibodies against cPR3 translated from the middle portion of the antisense DNA strand of PR3. When mice were immunized with cPR3, they produced antibodies not only to cPR3 but also to PR3 itself (PR3-ANCA). These two types of antibodies did not cross-react and formed an idiotypic pair. In line with molecular mimicry mechanism, *Staphyloccus aureus, Entamoeba histolytica* and *Ross River virus*, pathogens known to be associated with PR3-ANCA disease, all contain peptide with an amino acid sequence that mimics the cPR3. Although not yet proven, the proposed theory of autoantigen complementarity might participate also in the pathogenesis of other autoimmune diseases [79].

2.2 Pathogenic role of T cells in ANCA-associated vasculitis

As ANCA are mainly IgG antibodies, T-cell-dependent class switch has to occur in AAV. Many other findings also support the role of T cells in the pathogenesis of AAV and will be summarized in this chapter.

2.2.1 T cells in crescentic “pauci-immune” glomerulonephritis and vasculitic lesions

Rapidly progressive, pauci-immune, crescentic necrotizing glomerulonephritis is the hallmark of ANCA-associated renal vasculitis. The term “pauci-immune” reflects the relative lack of immunoglobulin and complement depositions in affected glomeruli

as mentioned above [5]. Given that the effectors of humoral immunity are absent, the role of cellular immunity in the process of glomerular injury seems likely. In experimental models, delayed-type hypersensitivity (DTH), a manifestation of cell-mediated immunity induced by sensitized T cells, was shown important for crescent formation [80]. In patients with pauci-immune glomerulonephritis, activated T cells, macrophages, fibrin, and tissue factor, i.e. mediators of DTH, were prominent at the site of glomerular injury [81]. Furthermore, monocytes/macrophages and T cells predominate not only in renal but also in pulmonary [82] and nasal infiltrates [83] in AAV patients.

Since particularly PR3-ANCA and not MPO-ANCA associated vasculitis often displays granulomatous inflammation typically driven by T cells [84], the important role of cell-mediated immunity was supposed mainly in PR3-ANCA-associated disease. A recent study, however, also suggests that even in MPO-ANCA-associated vasculitis/glomerulonephritis effector T cells contribute to tissue injury as CD4+ T cells depletion strongly reduced glomerular crescent formation in an animal model of MPO-ANCA glomerulonephritis [85].

2.2.2 MPO- and PR3-specific T lymphocytes

T lymphocyte proliferation was observed after in vitro stimulation with PR3 and, to a lesser extent, with MPO in patients with AAV. In these patients, T cells proliferate in response to crude granular extract of neutrophils, inactivated purified PR3 or MPO, and PR3- and MPO-derived peptides. However, similarly as in other autoimmune diseases, proliferation was also observed in PR3- or MPO-stimulated T cells from healthy controls [86-89]. Whilst an increased frequency of MPO-specific T lymphocytes was reported in patients with active MPO-ANCA associated vasculitis [88], other authors found no correlation between ANCA titres/activity of the disease, and PR3- or MPO-stimulated T cell proliferation [87, 89]. Even though some PR3-derived peptide sequences were suggested as potential targets of T cell responses in AAV, this field clearly requires further studies and the exact role of PR3- and MPO-specific T cells in the pathogenesis of AAV remains unclear [89-91].

2.2.3 T cell activation in AAV

Several studies found increased serum markers of T cell activation in AAV, including soluble IL-2 receptor or soluble CD30. In patients with WG, the levels of soluble IL-2 receptor (sIL-2R) correlate well with disease activity [92, 93] and might even indicate imminent relapse according to some authors [92] even though this was not confirmed by others [94]. Soluble CD30 (sCD30) was also shown to correlate with disease activity [94, 95]. Although immunosuppressive treatment leads to a decrease in levels of both sIL-2R and CD30, their levels remain increased in comparison with healthy controls. Interestingly, increased levels of these soluble markers were associated with persistent or renewed ANCA positivity in a recent study, but did not predict a risk for relapse [94]. On the basis of these observations, the authors concluded that ANCA positivity might relate to a complex, potentially T-cell-driven, immune activation in AAV patients.

Moreover, in contrast to soluble T cell markers and to activation markers on B lymphocytes, activation markers on T cells (e.g. CD25 and HLA-DR) seem not to correlate with disease activity; they are up-regulated even in remission and despite treatment [96, 97]. In a recent study, this phenomenon called persistent T cell activation (defined as either increase in CD25+ naïve CD4+ lymphocytes and/or decrease in the total number of CD4+CD45RO− naïve cells) was shown to be associated with disease severity [98].

2.2.4 T cell subpopulations in AAV

A number of different T cell subpopulations with presumed regulatory, effector and/or helper functions have been described during the last decade. Based on the cytokines and co-stimulatory signals involved, a naïve T cell can follow several differentiation pathways (some of them portrayed in Fig. 2.2). It is, however, necessary to stress that this area has been undergoing rapid development and completely novel pathways might be described in the near future.

Dysregulation of immune pathways has been hypothesized to play role in many autoimmune diseases, including AAV. The most often discussed subpopulations of T cells in AAV, i.e. regulatory T cells, T helper cells type 1 and 2 (Th1/Th2), recently described Th17 cells, and effector memory T cells, will be mentioned in detail below.

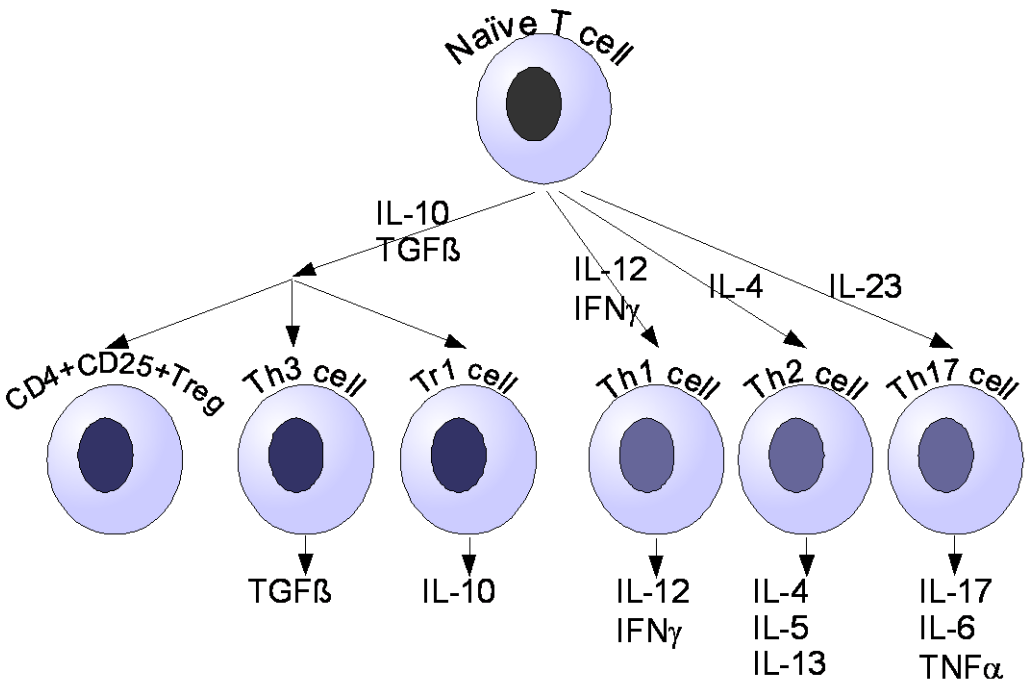

Fig. 2.2: Different ways of naïve T cell differentiation. Th = T helper cells, Treg = regulatory T cell, Tr1 = a subset of regulatory T cells, TGF-β = transforming growth factor-beta. For other abbreviations see text.

2.2.4.1 Regulatory T cells in AAV

In general, several mechanisms might contribute to T cell activation in AAV, e.g. aberrant co-stimulation or expression of co-stimulatory molecules, or homeostatic expansion [99]. Nevertheless, special emphasis was placed on the role of regulatory T cells (Tregs) in AAV in the last few years. Tregs are a subset of CD4+ T cells, characterised by a high-level surface expression of CD25 and intracellular expression of the transcription factor forkhead box P3 (FoxP3) [100]. Decreased frequency and/or impaired function of Tregs have been described in several autoimmune diseases, e.g. multiple sclerosis [101], rheumatoid arthritis [102] and systemic lupus erythematosus [103]. Until recently, no difference in the numbers or function of Tregs had been described in AAV [97, 99]. Contrary to previous findings, Abdulahad et al. [104] reported on expanded proportion of Tregs that poorly suppressed CD4+CD25– effector T cell proliferation in patients with WG in remission. Such a functional defect of Tregs might support the development of inflammation and autoimmunity in WG but data from animal and further *in vitro* studies are still needed [105].

2.2.4.2 Th1/Th2 paradigm in AAV

Based on their cytokine profile and related ability to generate different types of immune effector responses, CD4+ T helper lymphocytes used to be classified into two distinct subsets: type 1 (Th1) and type 2 (Th2) (Figure 2.3). In general, Th1 cells characteristically produce interferon gamma (IFNγ) and are involved in cell-mediated inflammatory reactions. They promote macrophage activation and delayed-type hypersensitivity (DTH). Th2 cells, producing interleukin (IL)-4, IL-5, IL-10 and IL-13, are associated with phagocyte-independent host responses, encourage non-complement-fixing IgG and IgE production and enhance eosinophil proliferation and function. [106, 107]

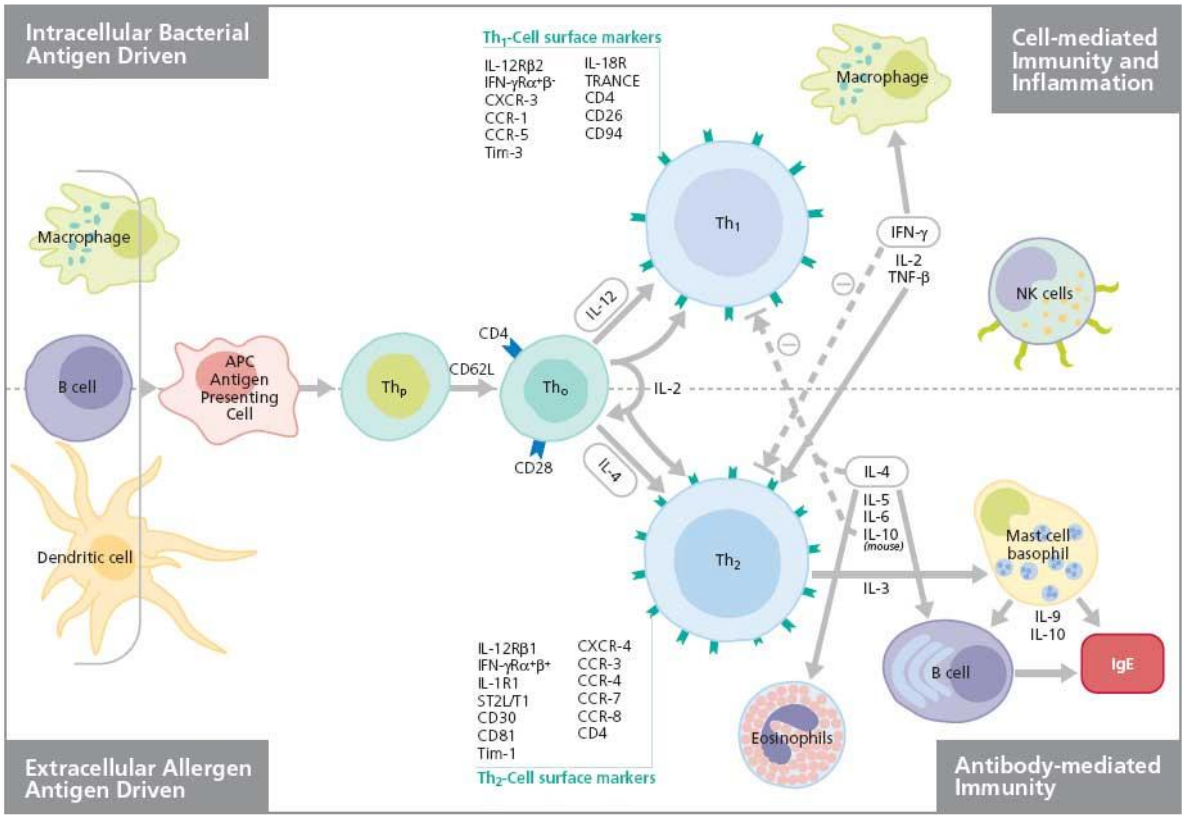

Fig. 2.3: Th1/Th2 differentiation, associated cytokines and surface molecules [adapted from 108]. For abbreviations see text.

Both Th1 and Th2 cells differentiate from naïve precursors (Th0) following specific antigen stimulation via their α/β T cell receptor (TCR). Antigen dose and antigen affinity can influence Th subset development. The key factor in directing Th cell polarization is the cytokine milieu but co-stimulatory signals are also important.

The main inducers of the Th1 profile are IFNγ and IL-12. In recent years, IL-18 and IL-27 have been recognized as promoters of Th1 polarization. IFNγ but not IL-12 may participate in inhibiting Th2 responses [109]. Of the Th2-related cytokines, IL-4 promotes differentiation of naive CD4+ cells into Th2 cells, whereas IL-10 inhibits Th1 cytokine synthesis. As for the co-stimulatory molecules, CD80 and CD86 signal via CD28, constitutively expressed on T cells. Inhibition of CD28 blocks Th2 responses without blocking Th1 responses [110].

Differences in surface membrane markers on Th1 and Th2 cells subsequently result in different intracellular signalling events. In addition, Th1 and Th2 cells seem to express different patterns of chemokine receptors. CXCR3, the receptor for IFNγ inducible chemokines (e.g. IP-10, Mig and I-TAC), and CCR5 are predominantly expressed at high levels on Th1 cells. On the other hand, CCR3 and CCR4 are associated with Th2 cells [111].

The Th1/Th2 paradigm in AAV, in particular in WG, has been discussed recently in many studies [95, 112-121, 123]. In localized WG, i.e. early WG restricted to the respiratory tract, T cells in nasal inflammatory infiltrates were shown to abundantly express CD26, an optional Th1 marker. In the same study, higher numbers of IFNγ-positive cells in localized WG than in generalized disease were found both in nasal infiltrates and in peripheral blood. On the contrary, IL-4 mRNA was detected in higher amounts in nasal biopsies in generalized WG [112]. Th2 environment in nasal granuloma in generalized WG was also detected in another study [113], in which increased IL-4 expression but no IFNγ were observed in nasal biopsies in generalized active WG.

Both localized and generalized WG displayed up-regulated expression of both Th1-associated CCR5 and Th2-associated CCR3 chemokine receptors in a flow cytometric analysis of circulating T cells. Nevertheless, predominant CCR5 expression on T cells as well as in granulomatous lesions was noted in localized WG, which may favour stronger recruitment of Th1-cytokine-secreting cells into inflammatory lesions [114, 115].

As mentioned above, plasma levels of sCD30, a member of the tumour necrosis factor receptor family and a Th2 marker, have been shown to be significantly increased and to correlate with disease activity in generalized WG. These findings further support the hypothesis that generalized WG may be associated with Th2 type of immune response [95].

In summary, there seems to be an aberrant Th1 type response that might play role during initiation of WG in patients with localized WG, when PR3 is often not yet detected. On the contrary, there seems to be significant appearance of Th2 cells and less prominent Th1 phenotype and Th1 cytokine production in granulomatous lesions of the upper respiratory tract (and eventually also in peripheral circulation) in patients with generalized vasculitis. This increasing complexity and "shift" of immune response have been hypothesized to be a consequence of B cell expansion and T cell dependent PR3-ANCA production during disease progression [116], potentially triggered by interaction between neutrophils and autoreactive T and B cells within granulomatous lesions. Persistent antigenic stimulation, e.g. by *Staphylococccus aureus* (a known risk factor for relapse in WG [32]), might contribute to the shift in the immune response [117].

Nevertheless, data regarding the type of Th response in WG are not entirely consistent. Predominant IFNγ production by T cells from peripheral blood, bronchoalveolar lavage, and granulomatous nasal lesions was reported in patients with generalized WG [118]. Furthermore, in renal lesion of the patients with generalized AAV, the polarization towards Th1 type response was found but the lesions contained Th2 cells as well [113, 119, 120].

Increased IFNγ production in WG patients was noted in another study, possibly induced by increased amounts of IL-12, produced by monocytes in both active and inactive patients with WG. Moreover, the authors demonstrated that *in vitro* IFNγ production might be inhibited by exogenous IL-10 [121]. In addition to its presumed association with Th2 type of immune response, IL-10 is a cytokine with immunosuppressive and anti-inflammatory potential. Levels of IL-10 were high at diagnosis in patients with WG and decreased subsequently. Intriguingly, low levels of IL-10 were associated with increased relapse rate in a recent study [94]. Polymorphisms in genes for IL-10 associated with WG have already been described [122].

Even though less thoroughly studied, Th2 immune response was reported in CSS, where T cells producing IL-4 and IL-13 may drive the eosinophilic inflammation [123]. Only little information is available on the Th1/Th2 polarization in MPA.

2.2.4.3 Th17 lineage in Wegener's granulomatosis

Nowadays, it is obvious that apart from Th1 and Th2 cells other subsets of T helper cells can be defined. The recently described IL-23-dependent-IL-17-producing

CD4+ T helper cell subset has been termed Th17 lineage. The Th17 immune pathway has been hypothesized to play an evolutionary significant role in rapid recruitment of neutrophils to the sites of acute infection [124]. This rapid response likely serves to "wall-off" the damaged tissue and presumably "buys time" for the induction of antimicrobial Th1-IFNγ response which takes several days to develop. If dysregulated, the Th17 pathway might lead to the development of a chronic inflammatory response and thus participate in the pathogenesis of various autoimmune diseases [124].

In patients with WG, a skewed Th17 response that suggests the role of IL-17 in the disease pathogenesis has recently been proven by Abdulahad et al. [125] but further studies are needed to clarify the significance of the Th17 lineage in AAV.

2.2.4.4 The role of effector memory T cells in AAV

Numerous studies have given attention to the altered phenotype of T cells in AAV, especially in WG. The expansion of a subset of circulating T cells lacking co-stimulatory molecule CD28 has been reported repeatedly [126-129]. However, surprisingly very little is still known about this immunological phenomenon.

The expansion of CD28– T cells starts early in the disease process and correlates with organ involvement and disease progression from localized to generalized WG [127, 128]. Most studies show that circulating peripheral blood CD4+CD28– T cells as well as those within granulomatous lesions are a major source of Th1 cytokine secretion [128]. Moreover, expanded CD28– T cells express the differentiation marker CD57, the activation marker and adhesion molecule CD18, Th1 type chemokine receptor CCR5, and upregulate HLA-DR and CD152 (CTLA-4), which indicates that these cells belong to so-called late differentiated or effector memory T cells (T_{EMs}) [126-132]. Shortened telomers and oligoclonality indicate cytokine- and/or antigen-driven expansion and replicative senescence of T_{EMs} [133, 134].

Intriguingly, patients in remission display higher levels of circulating T_{EMs} than patients with active disease. It is speculated that these T_{EMs} migrate from peripheral blood to the sites of inflammation causing a decrease in circulating memory T cells during active disease [135].

As proliferation of T cells toward T_{EMs} requires a strong and persistent immune trigger, presence of such an antigenic stimulus in WG patients seems likely. Contrary to central memory T cells, T_{EMs} do not express CCR7, the lymph node homing receptor,

and thus fail to migrate to lymphoid organs (Figure 2.4). However, they are capable of migrating to the sites of inflammation and producing pro-inflammatory cytokines [131].

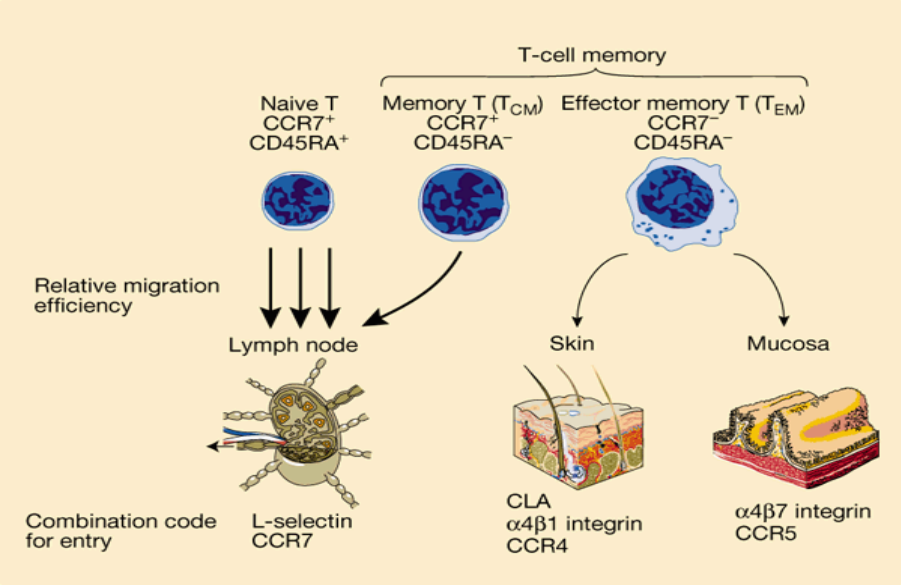

Fig. 2.4: Two types of memory T cells with different migration preferences [adapted from 136]

The apoptosis process was proven impaired in CD28– T_{EMs} due to increased expression of anti-apoptotic protein Bcl-2 [137]. Although not yet proven, CTLA-4 could contribute to this Bcl-2 increased expression. In addition, the aforementioned defective function of Tregs in WG may also account for T_{EMs} expansion [104]. Last but not least, two recently identified T-cell-function-associated genetic factors predisposing to granulomatous inflammation (HLA-DPB1*0401) and PR3-ANCA positivity (PTPN22*620W) might be related to both persistent T cell activation and T_{EMs} expansion in WG [138, 139].

Taken together, Th1 type T_{EMs} might contribute to the chronic granulomatous inflammation and development of autoimmunity in WG. In accordance with previous findings regarding the Th1/Th2 concept, exaggerated Th1 response in the respiratory tract with clonal expansion of T_{EMs} could sustain the chronic granulomatous inflammation and promote the formation of ectopic "lymphoid-like" tissue within granulomatous lesions. Subsequently, this might induce PR3-ANCA formation which results in ANCA-induced vasculitis [132]. Therefore, suppression of T_{EMs} cells might become an important goal in therapeutic strategies in AAV in the future.

2.3 Therapeutic implications

Without treatment, the prognosis of systemic AAV is poor. In 1958, the average patient survival after diagnosis of WG was 5 months [140]. The unfavourable outcome of patients with AAV was dramatically improved in the early 1970s when Fauci and Wolff introduced an empirical therapeutic scheme consisting of daily oral cyclophosphamide (CYC) and corticosteroids administered for at least one year [141]. Thus, remission was induced in approximately 80% of cases [142]. However, the toxicity of the regimen caused considerable morbidity and mortality [142]. Moreover, a long-term follow-up showed that at least 50% of the patients relapsed, even under continuing immunosuppression, or when the therapy was reduced [143].

Therefore, there has been an urgent need to find new effective treatment strategies and modalities with lower toxicity. Such an approach has been lately supported by major advances in understanding the disease pathogenesis. At present, remission induction treatment of AAV may involve pulsed cyclophosphamide, methotrexate or plasma exchange, according to disease severity. Azathioprine was proven successful for remission maintenance therapy. Newer promising therapeutic possibilities include mycophenolate-mofetil, deoxyspergualin or leflunomide.

Development of biological therapy has revealed a completely new area of therapeutic options in AAV. At present, biologics are still used more as a rescue therapy after conventional therapeutic regimens have failed. Nevertheless, in the oncoming years they are likely to play a more important role in the therapy of AAV and are therefore mentioned in detail below.

2.3.1 Biological therapy in ANCA-associated vasculitis

Biologics currently used in AAV include intravenous immunoglobulins, anti-thymocyte globulin, alemtuzumab, and mainly rituximab and anti-TNFα therapy.

A number of studies have reported the beneficial effects of intravenous immunoglobulin (IVIg) in patients with chronic grumbling vasculitis not reacting to conventional treatment or in patients with acute disease. Jayne et al. [144] conducted a small controlled trial of IVIg given as a single course of a total dose of 2 g/kg in patients with chronic active disease. This treatment resulted in a significant clinical improvement, but the effect was short-lived and did not last beyond 3 months.

As for anti-T-cell-directed biologics, fifteen patients with active refractory WG were treated with anti-thymocyte globulin (ATG) in a trial designed by the European Vasculitis Study Group (EUVAS). Thirteen of 15 patients showed a favourable response to ATG. While the authors conclude that anti-T-cell-directed treatment with ATG may be a therapeutic option for severe refractory Wegener's granulomatosis, they at the same time warn that the therapy is, however, associated with a high risk of infection and toxicity, and the risk-to-benefit ratio always needs to be considered [145].

Rituximab (RTX) is a genetically engineered chimeric monoclonal antibody that contains a human IgG1 constant region plus murine heavy and light chain variable regions directed against CD20. Rituximab causes a selective transient depletion of the CD20+ B cell subpopulation (i.e. the whole B cell population with the exception of plasma cells and pre-B cells), which typically lasts for at least 6 months with subsequent gradual reconstitution of B cell numbers. Elimination of B cells by RTX (4 weekly doses of 375 mg/m2 or, occasionally, 2 doses of 1g 2 weeks apart) successfully induced complete but temporary remissions in patients with AAV refractory to conventional therapy in several smaller studies [146, 147]. However, some patients, especially those with predominantly granulomatous manifestations, seem not to respond to RTX [148]. Rituximab therapy was usually accompanied by prednisolone, and the previous immunosuppressive therapy with cytotoxic agents was also often continued. Safety did not appear to be a major problem but continued vigilance is warranted. Repeated rituximab administration was suggested and proven successful after B cell reconstitution but human anti-chimeric antibodies (HACA) and prolonged hypogammaglobulinaemia might occur [149]. Randomised controlled studies are needed to clarify the effects of RTX and its indication in AAV.

The expansion of circulating TNFα-producing Th1 type CD28– effector memory T cells and their presence as Th1-type-cytokine-profile-driving cell population within granulomatous lesions, together with further *in vitro* data supporting the role of TNFα in the pathogenesis of AAV, provide the rationale for using TNFα-blocking agents in refractory AAV. Both the chimeric monoclonal anti-TNFα antibody infliximab and the fusion protein consisting of a part of the human TNFα receptor linked to the Fc portion of human IgG1 etanercept have been successfully applied as additional therapy for refractory Wegener’s granulomatosis in smaller studies [150, 151]. Nevertheless, relapses were quite common and some severe infectious complications were noted.

The randomised WGET trial (Wegener’s Granulomatosis Etanercept Trial), in

which patients were randomised to receive either etanercept or placebo in addition to standard immunosuppressive treatment, did not, however, confirm the efficacy of etanercept [152]. The time to remission, duration of remission or relapse rate did not differ between the groups. Intriguingly, 6 patients in the etanercept group developed solid organ cancers [153]. On the contrary, no cancer was observed in the placebo group even though the patients were also treated with CYC.

Despite the lack of efficacy of etanercept in the therapy of AAV, it is still possible that TNFα blockade with infliximab might be beneficial in the induction therapeutic regimens in AAV, which has to be clarified in future controlled trials. While etanercept binds to circulating TNFα only, infliximab also binds to TNFα complexed with TNFα receptors and might therefore have a different therapeutic mechanism. As in inflammatory bowel disease [154], infliximab but not etanercept may therefore be proven useful in the therapy of AAV.

Lymphocyte depletion using humanized monoclonal anti-CD52 antibodies (alemtuzumab) has been reported in a series of patients with relapsing/refactory disease. Although most patients achieved remission, relapses and adverse events were common in the long run and further studies are needed [155].

2.3.2 Therapeutic trends for the future

Many monoclonal antibodies to molecules involved in the disease pathogenesis have already been developed. For instance, interaction of CD28 on T cells with CD80 and CD86 on antigen-presenting cells is required for T cell activation. As CTLA-4 (cytotoxic T-lymphocyte-associated antigen 4, receptor for both CD80 and CD86) is upregulated on effector memory T cells in WG, blocking of CTLA-4-mediated co-stimulation and upregulation of anti-apoptotic Bcl-2 expression by CTLA-4-immunoglobulin (Abatacept) might help to modulate pathologic immune response in AAV [104].

Another therapeutic approach might, for instance, target Kv1.3 K+ channels, which are specific functional markers of $T_{EMs.}$ In rat models of rheumatoid arthritis, specific Kv1.3 blockers have already been successfully used [156]. In the future, monoclonal antibodies inhibiting e.g. endothelium-neutrophil interactions, ANCA or directly suppressing signalling cascades triggered by ANCA could be developed and once even terminate the need for cytotoxic therapeutic regimens in AAV.

3. MATERIAL AND METHODS

3.1 Patient characteristics

3.1.1 Basic cohort of patients

A total of 69 patients with AAV, followed-up at the Department of Nephrology, General Teaching Hospital and First Faculty of Medicine, Charles University in Prague, were originally included into this flow cytometric study during a 16-month period (September 2004 - December 2005). The basic characteristics of the patients are summarized in Table 3.1.

Table 3.1: Patient characteristics at the time of inclusion into the study (No. of patients = 69)

Characteristics		**Data**[1]
Age (years)		56 (22-76)
Male gender		40 (58%)
Type of vasculitis		
	WG	39 (56.5%)
	MPA	19 (27.5%)
	RLV	9 (13%)
	CSS	2 (3%)
ANCA specificity		
	PR3-ANCA	42 (60.9%)
	MPO-ANCA	27 (39.1%)
Duration of vasculitis before inclusion (months)		15 (0-168)
Organ involvement (anytime in patients' history)		
	Kidney	68 (98.5%)
	Lungs	36 (52.2%)
	ENT	25 (36.2%)
Serum creatinine (μmol/L)		173 (46-602)
No. of patients on haemodialysis		14 (20.3%)

[1]Data are presented as No.(%), or median (range).

Patients with WG, MPA and CSS matched the definitions for those diseases as formulated by the Chapel Hill Consensus Conference [4]. Patients with WG and CSS

also met the American College of Rheumatology criteria [3]. The diagnosis of renal-limited vasculitis (RLV) was based on renal biopsy showing focal or diffuse segmental necrotizing crescentic glomerulonephritis and absence or paucity of immune deposits by immunofluorescence, together with the lack of other systemic manifestations of vasculitis.

Disease activity was documented with the use of Birmingham Vasculitis Activity Score (BVAS) in its BVAS2003 modification [157], based on clinical observations and laboratory results performed routinely at patients' regular visits to the clinic.

The patients' initial status was classified as:

- remission – in 36 patients; defined as the absence of clinical signs and symptoms of active vasculitis, irrespective of ANCA levels, BVAS=0; or
- active disease – this group of patients consisted of:
  - *newly diagnosed* patients - 20 patients; diagnosed ≤ 6 weeks before inclusion into the study,
  - patients with *chronic grumbling disease* - 9 patients; BVAS=1-5, with no signs of new or worse disease, and
  - patients with *relapse* - 4 patients; defined as clinical signs of new or worse disease activity in combination with laboratory results reflecting disease activity and/or new/worse finding on imaging methods compatible with disease activity, BVAS>1.

All patients were subsequently monitored up until May 2008 at their regular visits to the clinic. Clinical status, BVAS, ANCA levels, renal parameters, relapses and deaths were registered.

Blood samples (a total of 12 millilitres of venous blood in several different collection tubes) were collected from each patient at entry. Ongoing infection in the patients was ruled out. Occasionally, some of the patients were examined repeatedly when either their disease relapsed or, on the contrary, they achieved remission. Thus, a total of 94 analyses of peripheral blood samples of 69 patients with AAV were performed. The repeated samples were included only in the subgroup analysis of AAV patients but not in the basic comparison (i.e. within each subgroup all patients are unique). A total of 43 patients were examined in the active phase of the disease (20 patients with newly diagnosed disease, 9 patients with chronic grumbling disease and 14 patients with relapse). Fifty-one patients were examined in remission. Table 3.2

shows clinical characteristics of patients in each subgroup. Patients with chronic grumbling disease were later excluded from the subgroup analysis due to their low number.

Table 3.2: Clinical characteristics of patients in subgroups according to disease activity (at the time of blood sample examination)

	Newly active	**Chronic grumbling**	**Relapse**	**Remission**
No. of patients	20	9	14	51
Male gender[1]	14 (70%)	7 (77.8%)	8 (57.1%)	26 (51%)
Age (years)[2]	58 (24-73)	49 (30-65)	52 (22-68)	57 (27-76)
PR3-ANCA/MPO-ANCA[3]	10/10	9/0	13/1	29/22
S-creatinine (μmol/L)[2]	255.5 (46-602)	147 (81-401)	153 (54-512)	148 (54-298)
No. of patients on haemodialysis[1]	4 (20%)	2 (22.2%)	3 (21.4%)	7 (13.7%)
ANCA (ELISA–IU/mL[4])[2]	74 (9->100)	35 (7->100)	38 (11->100)	0 (0-82)
BVAS[2]	25.5 (10-44)	2 (1-5)	16 (6-23)	0

Data presented as [1] No. (%) or as [2] median (range). [3] Anytime in patients' history. [4] Normal range 0-5.
PR3 = proteinase 3, MPO = myeloperoxidase, BVAS = Birmingham Vasculitis Activity Score.

Clinical characteristics of the subgroups of patients with PR3-ANCA and MPO-ANCA-associated disease are summarized in Table 3.3.

Table 3.3: Clinical characteristics of patients with PR3-ANCA- and MPO-ANCA-associated disease

	PR3-ANCA	**MPO-ANCA**
No. of patients	42	27
Male gender[1]	25 (59.5%)	15 (55.6%)
Age (years)[2]	54 (22-72)	62 (24-76)
S-creatinine (μmol/L)[2]	148 (46-538)	209 (54- 602)
No. of patients on haemodialysis[1]	9 (21.4%)	5 (18.5%)
No. of samples examined	60	34
Active disease	32	11
Remission	28	23

Data presented as [1] No. (%) or as [2] median (range). PR3 = proteinase 3, MPO = myeloperoxidase.

Induction immunosuppressive treatment consisted of corticosteroids (usually started with 3 pulses of intravenous methylprednisolone, then continued with oral prednisolone) and cyclophosphamide (either oral or intravenous pulse) in all newly active patients. In patients with severe disease, adjunctive therapy with plasma exchange was prescribed. After 3 months of stable remission, the patients were switched to azathioprine or mycophenolate-mofetil for at least 18 months. Relapses were treated with regard to their severity and patients' history (e.g. new series of pulsed cyclophosphamide; addition or change of an immunosuppressive drug; increase in dose of respective immunosuppressive agent and/or corticosteroids). Patients treated with biological therapy (i.e. rituximab) were analysed separately (see below).

In this study, a total of 10 active patients (8 newly diagnosed patients and 2 patients with relapse) were examined before any immunosuppressive therapy was administered. Sixteen remission samples were collected from patients in whom the immunosuppressive therapy had been stopped before.

The control group consisted of 30 healthy volunteers or blood donors (16 men, 14 women; median age 53 years, range 23-81 years). While surface molecules were examined in all healthy controls, results on the intracellular cytokine production were available in 24 out of 30 healthy individuals only (13 men, 11 women; median age 54.5 years, range 23-81 years).

Another control group of 20 patients with chronic kidney disease caused by "non-immunological" conditions such as hypertensive or diabetic nephropathy (13 men, 7 women; median age 59 years, range 24-83 years; median serum creatinine 181 μmol/L; 4 patients on haemodialysis, 1 patient treated with peritoneal dialysis) was also examined in this study.

Biochemical and haematological tests, and ANCA testing were performed in routine laboratories of the General Teaching Hospital in Prague. ANCA were determined by both IIF and ELISA (Orgentec, Mainz, Germany). The design of the work conformed to currently applied ethical standards.

3.1.1.1 Long-term follow-up of the basic cohort of AAV patients - clinical characteristics

During the follow-up (median time 43 months, range 1-44), 28 out of 69 patients relapsed (40.6%). The median time to relapse was 19 months (range 2-38 months) after

the inclusion into the study. Out of 51 patients who were examined in remission, relapse was observed in 19 patients. Clinical characteristics of patients with and without relapse are summarized in Table 3.4.

Table 3.4: Clinical characteristics of patients in remission without and with relapse during the follow-up (at the time of blood sample examination)

	Without relapse	**With relapse**
No. of patients	32	19
Male gender[1]	18 (56.3%)	10 (52.6%)
Age (years)[2]	57.5 (27-76)	57 (33-71)
PR3-ANCA/MPO-ANCA[3]	18/14	11/8
S-creatinine (μmol/L)[2]	163 (54-260)	147 (79-298)
No. of patients on hemodialysis	5 (15.6%)	2 (10.5%)
ANCA (ELISA–IU/mL[4])[2]	0 (0-48)	0 (0-82)

Data presented as [1]No. (%) or as [2]median (range). [3]Anytime in patients' history.
[4]Normal range 0-5. PR3 = proteinase 3, MPO = myeloperoxidase.

Eight out of 69 patients died (11.6%) during the follow-up. The cause of death was attributable to active disease in 4 patients. In 2 patients the death was related to infectious complications of immunosuppressive therapy. One patient died of lung cancer; the influence of previous long-term immunosuppressive treatment cannot be excluded. In the last patient, the death was caused by heart failure, and related neither to vasculitis nor its treatment.

3.1.2 Patients treated with rituximab

One of the aims of this work was to monitor the influence of administered biological therapy on the lymphocyte subpopulations and intracellular cytokine production. From August 2005 to June 2007, rituximab (RTX) was administered to 6 patients with AAV (for their basic characteristics see Table 3.5). In 4 patients with refractory disease 2x1 g of RTX two weeks apart was administered. Two newly diagnosed patients were treated with 4x 375 mg/m2 of RTX weekly. In all cases, RTX administration was accompanied by 2 pulses of cyclophosphamide and corticosteroids. Blood samples (5 mL of venous blood) were examined regularly at 0-1.5-3-6-9-12-18-24 months after RTX administration. In March 2009, the median time of follow-up of the patients was 36 months (range 21-42 months).

Table 3.5: Characteristics of the patients with AAV treated with rituximab (No. of patients = 6)

Characteristics		Data[1]
Age (years)		42.5 (36-74)
Male gender		5 (83.3%)
Type of vasculitis		
	WG	4 (66.7%)
	MPA	1 (16.7%)
	RLV	1 (16.7%)
ANCA specificity		
	PR3-ANCA	4 (66.7%)
	MPO-ANCA	2 (33.3%)
Duration of vasculitis before inclusion (months)		43 (0-150)
Organ involvement (anytime in patients' history)		
	Kidney	4 (66.7%)
	Lungs	3 (50%)
	ENT	1 (16.7%)
Serum creatinine (μmol/L)		195 (76-350)
No. of patients on hemodialysis		1 (16.7%)

[1]Data presented as No.(%), or median (range). ENT = ear, nose and throat.

3.2 Media, reagents and antibodies

For flow cytometric analyses of cell surface markers and intracellular cytokines the following material was used:

Tubes:

- BD Vacutainer 5mL blood collection tubes with Natrium Heparin (BD Biosciences, San Jose, CA, USA)
- Polypropylene tubes for blood cell cultivation (Nunc, Thermo Fisher Scientific, Rochester, NY, USA)
- BD Falcon tubes 12x75 mm for cell staining (BD Biosciences)

Reagents for cell cultivation and stimulation:

- RPMI „Roswell Park Memorial Institute“ medium 1640 (Gibco, Invitrogen, Carlsbad, CA, USA)
- Brefeldin A (BFA) – transport inhibitor protein; reconstitued and diluted, used at a final concentration of 10 μg/mL (Sigma Aldrich, St.Luis, MO, USA)
- Phorbol 12-myristate 13-acetate (PMA) – mitogen; reconstitued and diluted, used at a final concentration of 20 ng/mL (Sigma Aldrich)
- Ionomycin – ionophore; reconstitued and diluted, used at a final concentration of 1 μg/mL (Sigma Aldrich)
- Lipopolysaccharide (LPS) – mitogen; reconstitued and diluted, used at a final concentration of 1 μg/mL (Sigma Aldrich)
- Phytohaemaglutinin (PHA) – reconstitued and diluted, used at a final concentration of 1 μg/mL (Wellcome, Dartford, England)

Monoclonal antibodies – directly conjugated with a fluorochrome:

BD Biosciences:

- CD4-PerCP (peridinin chlorophyll protein)
- CD19-PerCP
- CD80-PE (phycoerythrin)
- CD86-PE
- CD3-FITC (fluorescein isothiocyanate)
- CD3-PerCP
- HLA-DR-PE
- CD45RA-FITC
- CD28-PE
- CD30-PE
- CCR5 (chemokine receptor 5) -PE
- CD14-FITC
- TNFα-FITC
- IL-2-PE
- IFNγ-FITC
- IL-4-PE
- IL-10-PE
- IL-12-PE
- CD20-APC (allophycocyanin)

Immunotech – Beckman Coulter (Fullerton, CA, USA)

- CRTH2 (chemoattractant receptor-homologous molecule expressed on Th2 lymphocytes)-PE

R&D (Minneapolis, MN, USA)

- CXCR3 (CXC chemokine receptor 3)-FITC

Other material:

- FACS Permeabilizing Solution (BD Biosciences)
- FACS Lysing Solution (BD Biosciences)
- Washing solution: CellWASH (BD Biosciences)
- Fixative solution: CellFIX (BD Biosciences)

Analysis equipment:

- FACSCalibur (Fluorescence Activated Cell Sorter) with G3 Macintosh computer (BD Biosciences) + CellQuest software (BD Biosciences)
- FACSCanto (BD Biosciences) + BD FACSDiva 5.03 (BD Biosciences) – since 2007

3.3 Flow cytometry

3.3.1 Intracellular cytokine production

3.3.1.1 Cell activation

In this study, a previously described and validated modification [158] of the commonly used procedure for cell activation was applied. In routine laboratory practice, this modification enables to examine blood samples delivered to laboratory during the whole day (and not only in the early morning hours as required for the common procedure).

3.3.1.1.1 Lymphocyte cultivation

1. 500 μL of RPMI 1640 + 10 μL of PHA with 500 μL of whole peripheral blood pipetted into the cultivation tube.
2. The tube inserted into the incubator (at temperature 37°C under 5% CO_2) for 20-22 hours.

3. Afterwards, 10 μL of BFA + 10 μL of PMA + 10 μL of ionomycin added into the tube.
4. The tube inserted into the incubator (at temperature 37°C under 5% CO_2) for 4.5 hours.

3.3.1.1.2 Monocyte cultivation

1. 10 μL of LPS with 500 μL of whole peripheral blood pipetted into the cultivation tube.
2. The tube inserted into the incubator (at temperature 37°C under 5% CO_2) for 20-22 hours.
3. Afterwards, 10 μL of BFA added into the tube.
4. The tube inserted into the incubator (at temperature 37°C under 5% CO_2) for 4.5 hours.

3.3.1.2 Procedure for intracellular cytokine determination

1. 100 μL of cultivated blood (see above) + 20 μl of monoclonal antibody directed against surface antigens incubated for 20 minutes in the dark at room temperature.
2. Erythrocytes lysing and lymphocyte fixing: Lysing solution (2 mL) added, incubated for 10 minutes in the dark at room temperature. Centrifugation (1,000 rpm for 5 minutes) followed, supernatant poured off afterwards.
3. 500μl of permeabilizing solution added, incubated for 10 minutes in the dark at room temperature. Washing solution added, centrifugation followed, and supernatant poured off afterwards.
4. 20μl of respective monoclonal antibody against intracellular antigen added, incubated for 25 minutes in the dark at room temperature. Afterwards, the samples washed twice.
5. Intracellular cytokines analysis by flow cytometer. T lymphocytes gated using FSC (forward scatter) and CD3; monocytes gated using SSC (side scatter) and CD14.

3.3.2 Cell surface marker staining

Whole peripheral blood samples were incubated in the presence of respective monoclonal antibodies in the dark at room temperature (20-25 °C) for 15 minutes. After the lysing and washing phases, three-colour flow cytometric analysis was performed by FACSCalibur (six-colour analysis enabled by FACSCanto since 2007).

Basic subpopulations of lymphocytes (CD3, CD4, CD8, CD19, CD16 and CD56) were determined by a routine flow cytometric laboratory procedure, using the BD Biosciences monoclonal antibodies and equipment.

3.4 Statistical analysis

Statistical analysis was performed applying SPSS and STATGRAPHICS Plus software. Continuous variables are presented as mean ± standard deviation or as median with range. Differences in continuous variables were compared by means of Student's *t*-test in the case of normal distribution. Otherwise, the Wilcoxon signed rank test was used for comparison of paired data, and the Mann-Whitney test for comparison of unpaired data. For multiple comparisons, the non-parametric Kruskal-Wallis test, or ANOVA with further analysis using Tukey's method were used. Proportions between two groups were compared with Fisher's exact test or, for larger samples, with chi-square test. The correlation analysis was performed using the non-parametric Spearman's test. A two-sided $P < 0.05$ was considered statistically significant in all cases.

The statistical analysis was performed by and the results consulted with statisticians from the National Institute of Public Health and from the Department of Statistics and Probability, Faculty of Informatics and Statistics, University of Economics.

4. RESULTS

4.1 Basic cohort of patients with AAV

4.1.1 Surface molecules

The absolute number of lymphocytes was significantly reduced in patients with AAV (both in patients with active disease and in patients in remission) when compared to healthy controls (HC, p<0.001, Table 4.1). In contrast to the absolute numbers of lymphocytes, the percentage of CD3+ cells (T lymphocytes) did not significantly differ between AAV and HC.

Table 4.1: Lymphocyte levels in patients with AAV and healthy controls

	Total No. of lymph. (cells x 10^9 /l)	**P value**	**CD3+ cells (% of lymph.)**	**P value**
Healthy controls (N=30)	2.2 ± 0.4		70.9 ± 5.7	
AAV – all (N=69)	1.3 ± 0.7	<0.001	68.7 ± 12.9	n.s.
AAV – active (N=43)	1.1 ± 0.6	<0.001	67.3 ± 12.1	n.s.
AAV – remission (N=51)	1.5 ± 0.7	<0.001	69.8 ± 13.3	n.s.

Data presented as mean ± standard deviation. P value represents the difference between patients with AAV and healthy controls. No. = number, lymph. = lymphocytes, n.s. = not significant.

Table 4.2: Basic lymphocyte subpopulations in patients with AAV and healthy controls

	CD4+ (% of ly.)	**P value**	**CD8+ (% of ly.)**	**P value**	**CD19+ (% of ly.)**	**P value**
Healthy controls (N=30)	48.2 ± 7.5		22.0 ± 6.0		10.4 ± 3.2	
AAV – all (N=69)	40.4 ± 13.5	<0.001	29.0 ± 13.7	<0.05	9.6 ± 8.0	n.s.
AAV – active (N=43)	42.1 ± 10.4	< 0.01	25.7 ± 12.1	n.s.	13.1 ± 8.4	n.s.
AAV – remission (N=51)	39.1 ± 15.3	<0.001	31.6 ± 14.1	<0.001	6.9 ± 6.4	<0.01

Data presented as mean ± standard deviation. P value represents the difference between patients with AAV and healthy controls. Ly. = lymphocytes, n.s. = not significant.

However, the so-called immunoregulatory index (IRI, defined as CD3+CD4+/CD3+CD8+) was decreased in our AAV patients, i.e. we found lower percentage of CD4+ cells together with higher percentage of CD8+ in the AAV patients

(both active and, even more so, in remission, Table 4.2). In active AAV patients, the percentage of CD19+ cells (B lymphocytes) was higher than in HC, but this difference did not reach statistical significance. Significantly lower levels of B cells were observed in remission ($p<0.01$, Table 4.2); as shown below, even in patients in that immunosuppressive treatment had already been stopped.

The number of natural killer (NK) cells (CD3–CD16,56+) in AAV did not differ from HC (Table 4.3). On the contrary, a significant decrease in naïve (CD4+CD45RA+) T cells was noted in all AAV patients when compared to HC, in particular in AAV patients in remission ($p<0.001$, Table 4.3). Patients with AAV in remission (and not active patients) had also higher numbers of activated HLA-DR+ CD3+ T cells than HC ($p<0.01$, Table 4.3).

Table 4.3: NK cells, naïve T cells and activated T cells in patients with AAV and healthy controls

	CD3– CD16,56+ (% of ly.)	**P value**	**CD4+ CD45RA+ (% of ly.)**	**P value**	**CD3+ HLA-DR+ (% of ly.)**	**P value**
Healthy controls (N=30)	15.3 ± 9.1		30.4 ± 11.7		4.6 ± 2.6	
AAV – all (N=69)	13.5 ± 9.7	n.s.	19.6 ± 8.9	<0.001	6.9 ± 6.8	<0.01
AAV – active (N=43)	11.6 ± 8.0	n.s.	22.8 ± 9.4	<0.01	5.3 ± 3.4	n.s.
AAV – remission (N=51)	15.0 ± 10.6	n.s.	17.2 ± 7.7	<0.001	8.1 ± 5.3	<0.01

Data presented as mean ± standard deviation. P value represents the difference between patients with AAV and healthy controls. Ly. = lymphocytes, n.s. = not significant.

Furthermore, chemokine receptors expressed on CD4+ cells were examined. Even HC displayed low-level cell surface expression of inducible chemokine receptors, which may occur in response to any antigenic challenge. However, the expression of Th1-associated receptor CCR5 on CD4+ T lymphocytes was significantly increased in our patients when compared to HC ($p<0.01$, Table 4.4). In contrast to CCR5, neither active patients with AAV nor patients with remission differed from HC in the expression of another Th1-associated chemokine receptor, CXCR3 (Table 4.4).

Table 4.4: Th1-associated chemokine receptors on CD4+ T cells in AAV and healthy controls

	CCR5+ (% of CD4+ ly.)	P value	CXCR3+ (% of CD4+ ly.)	P value
Healthy controls (N=30)	16.4 ± 8.4		37.1 ± 10.0	
AAV – all (N=69)	23.0 ± 11.6	<0.01	39.3 ± 16.6	n.s.
AAV – active (N=43)	22.2 ± 14.4	<0.01	35.8 ± 10.4	n.s.
AAV – remission (N=51)	23.7 ± 8.5	<0.01	42.3 ± 15.0	n.s.

Data presented as mean ± standard deviation. P value represents the difference between patients with AAV and healthy controls. Ly. = lymphocytes, n.s. = not significant.

On the other hand, the expression of Th2 associated chemoattractant receptor-homologous molecule CRTH2 on CD4+ T lymphocytes and the expression of Th2 associated surface marker CD30 was increased in our group of patients with AAV taken as a whole and in active patients in comparison to HC ($p<0.05$). In patients in remission, the expression of these molecules did not differ from HC (Table 4.5).

Table 4.5: Expression of Th2-associated surface markers on lymphocytes in AAV and healthy controls

	CRTH2+ (% of CD4 + ly.)	P value	CD30+ (% of ly.)	P value
Healthy controls (N=30)	6.5 ± 2.2		4.6 ± 2.8	
AAV – all (N=69)	10.6 ± 7.6	<0.05	7.4 ± 4.6	<0.05
AAV – active (N=43)	12.1 ± 8.6	<0.05	8.0 ± 5.4	<0.05
AAV – remission (N=51)	7.8 ± 2.8	n.s.	6.3 ± 2.1	n.s.

Data presented as mean ± standard deviation. P value represents the difference between patients with AAV and healthy controls. Ly. = lymphocytes, n.s. = not significant.

Finally, the expression of CD28 and its ligands CD80 and CD86 on lymphocytes were assessed in this study. A significant increase in CD28–CD8+ subpopulation of lymphocytes (compared to HC) was observed in patients with AAV in remission ($p<0.001$, Table 4.6). This was accompanied by the increased expression of both ligands of CD28, i.e. CD80 and CD86, on CD19– lymphocytes (i.e. most probably T cells, $p<0.01$, Table 4.6).

Table 4.6: Expression of CD28 and its ligands CD80 and CD86 on lymphocytes in AAV and healthy controls

	CD28– CD8+ (% of ly.)	**P value**	**CD80+ CD19– (% of ly.)**	**P value**	**CD86+ CD19– (% of ly.)**	**P value**
Healthy controls (N=30)	13.3 ± 5.3		4.9 ± 3.1		6.1 ± 3.4	
AAV – all (N=69)	21.4 ± 15.3	<0.01	6.7 ± 4.0	<0.05	9.0 ± 4.2	<0.05
AAV – active (N=43)	14.6 ± 11.1	n.s.	6.5 ± 4.6	n.s.	8.0 ± 3.7	n.s.
AAV – remission (N=51)	26.5 ± 15.9	<0.001	6.9 ± 3.6	<0.01	10.9 ± 4.0	<0.01

Data presented as mean ± standard deviation. P value represents the difference between patients with AAV and healthy controls. Ly. = lymphocytes, n.s. = not significant.

4.1.2 Intracellular cytokines

In this study, the intracellular production of IFNγ, the hallmark cytokine of Th1 cells, did not differ between active patients with AAV and HC (Table 4.7). On the contrary, patients in remission displayed significantly higher production of IFNγ than HC ($p<0.01$, Table 4.7).

The intracellular production of IL-4 (associated with Th2 cells and Th2 type of immune response) was low in both patients with AAV and HC in this study. No significant difference in the IL-4 production was observed (Table 4.7).

Table 4.7: Intracellular cytokine (IFNγ and IL-4) production in CD3+ T lymphocytes in patients with AAV and healthy controls

	IFNγ[1]	**P value**	**IL-4**[1]	**P value**
Healthy controls (N=24)	29.8 ± 9.8		3.6 ± 3.4	
AAV – all (N=69)	34.8 ± 16.5	n.s.	4.5 ± 4.0	n.s.
AAV – active (N=43)	28.3 ± 13.0	n.s.	4.6 ± 3.4	n.s.
AAV – remission (N=51)	41.8 ± 18.9	<0.01	4.5 ± 4.9	n.s.

Data presented as mean ± standard deviation. P value represents the difference between patients with AAV and healthy controls. [1]In % of CD3+ cells; n.s. = not significant.

The TNFα production in AAV patients was also similar to HC (Table 4.8) in this study. Furthermore, we did not find any significant difference in the IL-2 production between patients with AAV at any stage of the disease and HC (Table 4.8).

Table 4.8: Intracellular cytokine (TNFα and IL-2) production in CD3+ T lymphocytes in patients with AAV and healthy controls

	TNFα[1]	P value	IL-2[1]	P value
Healthy controls (N=24)	45.4 ± 19.4		31.1 ± 11.8	
AAV – all (N=69)	49.0 ± 24.0	n.s.	34.5 ± 18.9	n.s.
AAV – active (N=43)	45.2 ± 20.0	n.s.	35.9 ± 17.0	n.s.
AAV – remission (N=51)	52.0 ± 20.4	n.s.	31.9 ± 17.2	n.s.

Data presented as mean ± standard deviation. P value represents the difference between patients with AAV and healthy controls. [1]In % of CD3+ cells; n.s. = not significant.

Regarding the intracellular cytokine production in monocytes, no significant difference in the IL-10 production between AAV patients and HC was noted. As for the IL-12, significantly higher IL-12 production was observed in patients with AAV than in HC, both in active patients and in patients in remission ($p<0.01$ and 0.05, respectively, Table 4.9).

Table 4.9: Intracellular cytokine (IL-10 and IL-12) production in monocytes in patients with AAV and healthy controls

	IL-10[1]	P value	IL-12[1]	P value
Healthy controls (N=24)	14.3 ± 9.8		10.2 ± 9.8	
AAV – all (N=69)	14.4 ± 10.3	n.s.	17.9 ± 13.2	<0.05
AAV – active (N=43)	17.3 ± 14.2	n.s.	20.8 ± 14.2	<0.01
AAV – remission (N=51)	12.2 ± 6.4	n.s.	16.5 ± 13.0	<0.05

Data presented as mean ± standard deviation. P value represents the difference between patients with AAV and healthy controls. [1]In % of monocytes; n.s. = not significant.

4.1.3 Results of correlation analysis

In this study, no significant difference in the measured parameters between genders was found. In the correlation analysis, we prospected for a possible correlation between the acquired laboratory data and age, glomerular filtration rate (GFR), ANCA levels and BVAS. However, no correlation between age and laboratory parameters was observed, nor did GFR significantly correlate with any of the surface markers or intracellular cytokines (Table 4.10).

Results of the correlations between ANCA levels or BVAS and measured variables are summarized in Table 4.10. ANCA levels significantly positively correlated with the percentage of IL-10 and IL-12 producing cells, and with the percentage of CD19 cells. On the other hand, a significant negative correlation between ANCA levels and CD28–CD8+ cells was observed. Furthermore, BVAS but not ANCA levels significantly positively correlated with the numbers of CRTH2+, and CD30+ cells. A positive correlation of the number of IL-10 producing cells and BVAS was also found.

Table 4.10: Correlation matrix

	Age	**GFR**[5]	**ANCA**	**BVAS**
CD4+[1]	0	0	0.1, n.s.	0.1, n.s.
CD8+[1]	0	0	-0.1, n.s.	-0.1, n.s.
CD19+[1]	0	0	**0.4****	0.2, n.s.
CD3+HLA-DR+[1]	0.3, n.s.	-0.1, n.s.	0	0.2, n.s.
CD4+45RA+[1]	-0.2, n.s.	0.1, n.s.	0.1, n.s.	0.1, n.s.
CD28–CD8+[1]	0.3, n.s.	0.1, n.s.	**-0.3***	-0.2, n.s.
CD30+[1]	0.2, n.s.	0.2, n.s.	0.3, n.s.	**0.8****
CCR5+[2]	0.2, n.s.	0	-0.2, n.s.	- 0.1, , n.s.
CXCR3+[2]	0.2, n.s.	0	-0.2, n.s.	- 0.1, n.s.
CRTH2+[2]	0.1, n.s.	0.1, n.s.	0.2, n.s.	**0.6***
IFNγ[3]	0.3, n.s.	0.2, n.s.	-0.1, n.s.	-0.1, n.s.
IL-4[3]	0	0	0.2, n.s.	0.1, n.s.
TNFα[3]	0.3, n.s.	0.2, n.s.	-0.1, n.s.	0
IL-2[3]	0.1, n.s.	0.1, n.s.	0.1, n.s.	0
IL-10[4]	0.1, n.s.	0	**0.4*****	**0.3***
IL-12[4]	0.1, n.s.	0	**0.3****	0.2, n.s.

Expressed as the correlation coefficient ρ; 0 = ρ <0.1;
* = p < 0.05, ** = p < 0.01, *** = p < 0.001, n.s. = not significant
[1]In % of lymphocytes, [2] in % of CD4+ lymphocytes, [3] in % of CD3+ cells, [4]in % of monocytes
[5] GFR = glomerular filtration rate, calculated with the help of the MDRD formula [159].

4.1.4 Comparison of active patients and patients in remission

The numeric results of surface molecule and intracellular cytokine examination in both active patients with AAV and patients with AAV in remission have been shown above (Tables 4.1-4.9). In this chapter, results of the comparison of active patients and patients in remission have been summarized.

Several significant differences in the basic lymphocytes subpopulations and the expression of surface molecules between active patients and patients in remission were noted (Fig. 4.1 and 4.2). Patients with AAV in remission had significantly higher percentage of CD8+ cells when compared to active AAV ($p<0.05$, Fig. 4.1). On the contrary, CD19+ cell count in remission was significantly lower than in active patients ($p<0.001$, Fig. 4.1). A decrease in CD4+45RA+ naïve T cells was also observed in remission (Fig. 4.1).

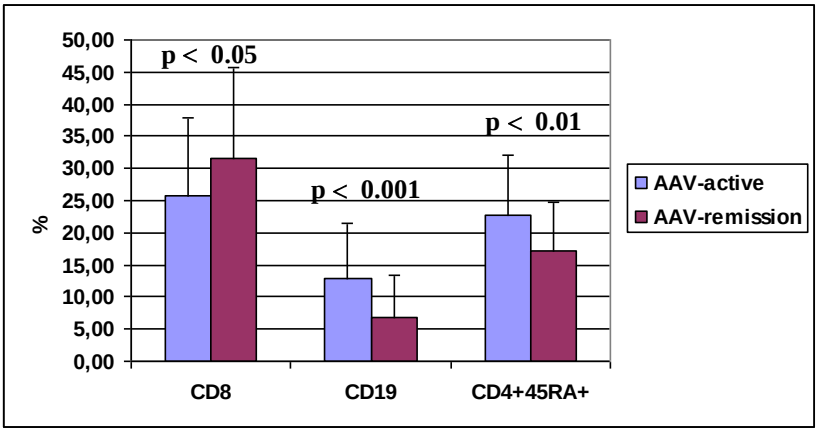

Fig. 4.1: Significant differences in lymphocyte subpopulations between active patients with AAV and patients in remission. Data presented as mean, error bars represent standard deviation.

On the other hand, the number of activated (HLA-DR+) T cells and also of CXCR3+ CD4+ cells was significantly higher in remission than in active disease (Fig. 4.2). Finally, patients in remission had a significant expansion of CD28– subpopulation of CD8+ cells (Fig. 4.2). No other significant difference in the surface molecule expression between active patients and patients in remission was found in this study.

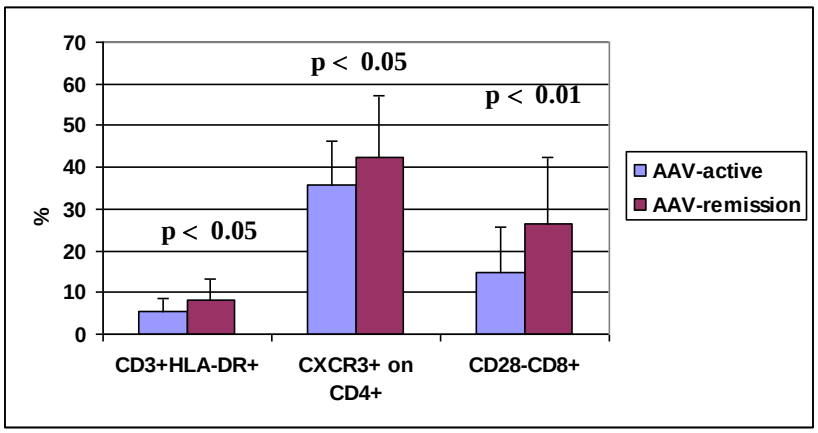

Fig. 4.2: Significant differences in surface molecule expression between active patients with AAV and patients in remission. Data presented as mean, error bars represent standard deviation.

As for the intracellular cytokine production, active patients with AAV had significantly lower production of IFNγ than patients in remission ($p<0.01$, Fig. 4.3). On the contrary, the production of IL-10 was significantly higher in active patients than in remission ($p<0.05$, Fig. 4.3). The difference between active patients and patients in remission in the intracellular production of other determined cytokines, i.e. IL-2, IL-4, TNFα and IL-12, was not statistically significant.

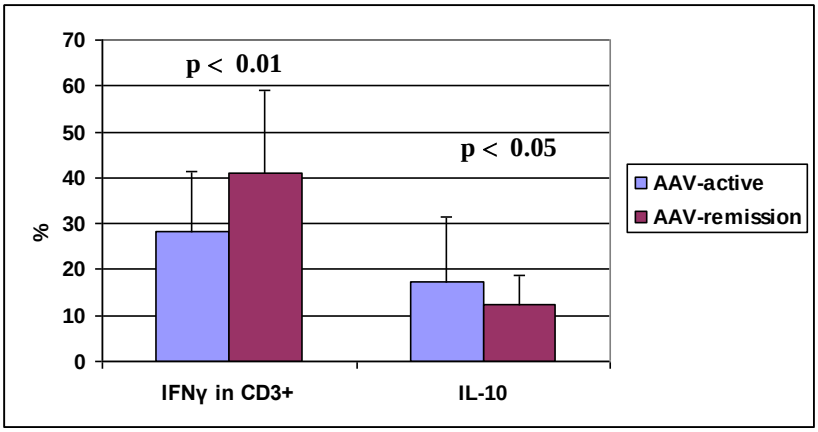

Fig. 4.3: Significant differences in the intracellular cytokine production between active patients with AAV and patients in remission. Data presented as mean, error bars represent standard deviation.

In the subsequent subgroup analysis, results of newly active patients were compared to patients with relapse. In the surface molecules expression, the only significant difference between these two subgroups of active patients was a lower

number of CD19+ cells (B lymphocytes) in patients with relapse; the other results did not significantly differ.

Nevertheless, more intriguing results were obtained in the comparison of the intracellular cytokine production. The production of IL-10 was significantly higher in newly diagnosed patients than in patients with relapse but did not significantly differ from healthy controls (Fig. 4.4.). Furthermore, newly diagnosed patients had increased IL-12 production when compared to patients with relapse, and, in this case, also to healthy controls (Fig. 4.4). No other significant difference in the intracellular cytokine production between newly active patients and patients with relapse was found.

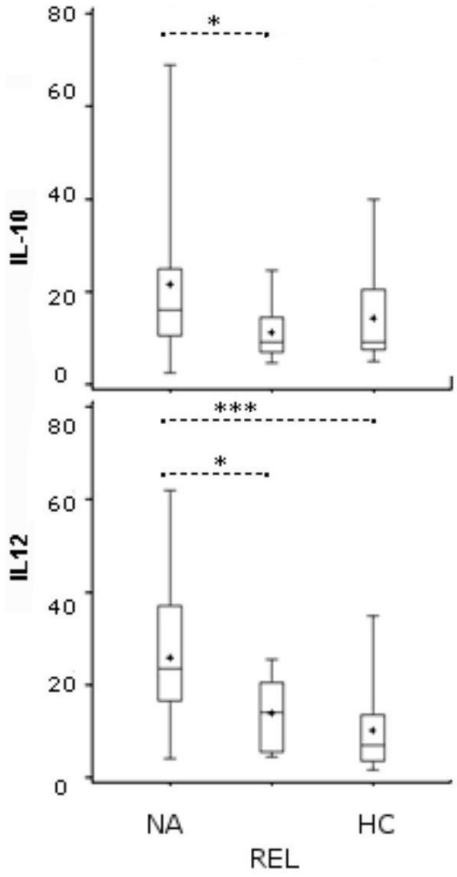

Fig. 4.4: Intracellular cytokine production of IL-10 and IL-12 in monocytes. Box plots indicate 5-95% range (error bars), 25-75% range (boxes), median value (horizontal lines) and mean (+). * $p<0.05$, *** $p<0.001$. NA = newly active patients with ANCA-associated vasculitis (N=20); REL = active patients with ANCA-associated vasculitis with relapse (N=14); HC = healthy controls (N=24).

4.1.5 Comparison of PR3-ANCA and MPO-ANCA-associated disease

Table 4.11: Comparison of surface molecule expression and intracellular cytokine production between PR3-ANCA-associated and MPO-ANCA-associated disease

	PR3-ANCA-active (N=32)	MPO-ANCA-active (N=11)	P value	PR3-ANCA-remission (N=28)	MPO-ANCA-remission (N=23)	P value
CD4+[1]	42.3 ± 9.7	41.6 ± 12.3	n.s.	41.4 ± 16.2	36.2 ± 13.6	n.s.
CD8+[1]	27.7 ± 12.9	19.5 ± 6.3	n.s.	31.3 ± 12.5	31.9 ± 15.9	n.s.
CD19+[1]	12.4 ± 8.8	15.0 ± 6.6	n.s.	7.3 ± 7.4	6.3 ± 4.7	n.s.
CD3+HLADR+[1]	5.1 ± 3.6	5.8 ± 2.5	n.s.	6.7 ± 5.0	9.8 ± 4.6	n.s.
CD4+45RA+[1]	22.0 ± 9.3	25.3 ± 9.1	n.s.	18.3 ± 8.0	15.8 ± 7.1	n.s.
CD3–CD16,56+[1]	9.9 ± 5.7	16.7 ± 11.1	**<0.05**	13.6 ± 8.0	16.7 ± 12.8	n.s.
CD28–CD8+[1]	14.0 ± 11.3	16.6 ± 10.1	n.s.	22.7 ± 15.5	31.5 ± 15.0	n.s.
CD30+[1]	7.1 ± 5.6	9.8 ± 4.4	n.s.	6.6 ± 2.3	5.4 ± 1.4	n.s.
CCR5+[2]	20.6 ± 8.4	25.7 ± 22.0	n.s.	22.6 ± 8.4	24.9 ± 8.5	n.s.
CXCR3+[2]	35.3 ± 11.2	37.0 ± 7.8	n.s.	38.3 ± 7.7	47.1 ± 19.6	n.s.
CRTH2+[2]	15.0 ± 9.8	9.8 ± 3.6	n.s.	8.3 ± 2.0	7.3 ± 3.3	n.s.
IFNγ[3]	27.0 ± 11.9	31.5 ± 15.4	n.s.	39.2 ± 18.6	43.2 ± 16.8	n.s.
IL-4[3]	4.3 ± 3.2	4.7 ± 3.4	n.s.	5.6 ± 5.7	3.1 ± 2.2	n.s.
TNFα[3]	42.5 ± 17.1	49.1 ± 23.5	n.s.	51.9 ± 19.0	54.1 ± 21.9	n.s.
IL-2[3]	35.4 ± 16.4	36.4 ± 16.8	n.s.	34.1 ± 17.5	31.5 ± 15.7	n.s.
IL-10[4]	15.1 ± 9.4	20.8 ± 12.9	n.s.	13.5 ± 6.9	11.6 ± 6.1	n.s.
IL-12[4]	18.8 ± 15.1	26.8 ± 12.6	n.s.	15.3 ± 8.7	18.0 ± 15.5	n.s.

Data presented as mean ± standard deviation. P value represents the difference between patients with PR3-ANCA-associated disease and MPO-ANCA associated disease (for active patients and patients in remission, respectively). n.s. = not significant. [1]In % of lymphocytes, [2] in % of CD4+ lymphocytes, [3] in % of CD3+ cells, [4]in % of monocytes

Basic clinical and demographic characteristics of patients with PR3-ANCA- and MPO-ANCA-associated disease have been shown above (Chapter 3). Patients with MPO-ANCA-associated disease were significantly older than patients with PR3-ANCA-associated disease ($p<0.05$). No other significant difference in the basic parameters was observed.

Few differences were also found in the measured laboratory parameters. Patients with active PR3-ANCA-associated disease had significantly lower numbers of CD3–CD16,56+ cells (NK cells) than patients with active MPO-ANCA-associated disease ($p<0.05$, Table 4.11). The difference in all other parameters did not reach statistical significance (Table 4.11). Similarly, no significant difference between patients with PR3-ANCA and MPO-ANCA-associated disease in remission was found even though a tendency to differ was indicated in some parameters (e.g. in CXCR3+ or CD28–CD8+ cells).

4.1.6 Comparison of patients with different therapies

To elucidate the possible influence of immunosuppressive therapy on the results, a total of 10 yet untreated patients with active disease were examined in this study. Results of the comparison of untreated active patients with AAV and active patients with AAV on immunosuppressive therapy are shown in Table 4.12.

In comparison with active patients on immunosuppressive therapy, the active untreated patients had significantly lower numbers of CD8+ cells ($p<0.05$). They also had significantly lower percentage of CD80+ cells within the CD19+ subpopulation of lymphocytes ($p<0.01$, Table 4.12). No other significant differences between treated and untreated active patients were found.

Table 4.12: Comparison of surface molecule expression and intracellular cytokine production between active untreated patients with AAV and active patients with AAV on therapy

	Active untreated (N=10)	**Active on therapy** (N=33)	**P value**
CD4+[1]	40.2 ± 11.1	42.7 ± 10.1	n.s.
CD8+[1]	19.8 ± 7.6	27.3 ± 12.6	**<0.05**
CD19+[1]	15.6 ± 5.3	12.3 ± 8.9	n.s.
CD3+HLA-DR+[1]	5.3 ± 4.3	5.3 ± 3.1	n.s.
CD4+45RA+[1]	21.6 ± 8.7	23.0 ± 9.5	n.s.
CD3–CD16,56+[1]	15.2 ± 8.5	10.6 ± 7.5	n.s.
CD28–CD8+[1]	14.6 ± 11.7	14.6 ± 10.9	n.s.
CCR5+[2]	18.1 ± 6.1	23.4 ± 15.8	n.s.
CXCR3+[2]	38.9 ± 8.5	34.8 ± 10.7	n.s.
CD80+[3]	18.6 ± 9.2	34.1 ± 20.3	**<0.01**
CD86+[3]	21.1 ± 10.9	34.1 ± 17.9	n.s.
IFNγ[4]	28.8 ± 14.1	28.1 ± 12.8	n.s.
IL-4[4]	5.9 ± 4.0	3.8 ± 2.7	n.s.
TNFα[4]	41.7 ± 22.2	46.5 ± 19.5	n.s.
IL-2[4]	29.9 ± 16.8	37.5 ± 16.0	n.s.
IL-10[5]	18.3 ± 14.5	16.8 ± 14.4	n.s.
IL-12[5]	20.4 ± 15.6	20.9 ± 14.0	n.s.

Data presented as mean ± standard deviation. P value represents the difference between active untreated patients with AAV and active patients with AAV on therapy. n.s. = not significant.
[1]In % of lymphocytes, [2] in % of CD4+ lymphocytes, [3] in % of CD19+ lymphocytes, [4] in % of CD3+ cells, [5]in % of monocytes.

Table 4.13: Comparison of surface molecule expression and intracellular cytokine production between patients with AAV in remission on therapy and patients in remission without therapy

	Remission on therapy (N=35)	**Remission untreated** (N=16)	**P value**
CD4+[1]	40.3 ± 17.0	36.4 ± 10.3	n.s.
CD8+[1]	32.7 ± 13.6	29.2 ± 14.8	n.s.
CD19+[1]	6.6 ± 6.7	7.4 ± 5.5	n.s.
CD3+HLA-DR+[1]	8.7 ± 9.2	6.8 ± 5.4	n.s.
CD4+45RA+[1]	16.5 ± 7.7	18.5 ± 7.6	n.s.
CD3–CD16,56+[1]	12.2 ± 9.5	21.3 ± 10.2	**<0.01**
CD28–CD8+[1]	25.5 ± 15.6	27.1 ± 15.3	n.s.
CD80+CD19-[1]	7.5 ± 3.4	5.2 ± 2.5	**<0.05**
CCR5+[2]	24.6 ± 8.2	21.0 ± 8.8	n.s.
CXCR3+[2]	43.6 ± 15.2	38.5 ± 13.8	n.s.
CD80+[3]	38.5 ± 17.6	17.1 ± 11.0	**<0.001**
IFNγ[4]	40.8 ± 17.8	42.1 ± 18.1	n.s.
IL-4[4]	5.0 ± 4.9	3.0 ± 2.1	n.s.
TNFα[4]	49.2 ± 20.0	55.6 ± 19.1	n.s.
IL-2[4]	27.6 ± 16.2	40.4 ± 15.8	**=0.05**
IL-10[5]	11.7 ± 7.1	12.7 ± 5.4	n.s.
IL-12[5]	18.0 ± 14.0	13.2 ± 6.4	n.s.

Data presented as mean ± standard deviation. P value represents the difference between patients with AAV in remission on therapy and patients in remission without therapy. n.s. = not significant.
[1] In % of lymphocytes, [2] in % of CD4+ lymphocytes, [3] in % of CD19+ lymphocytes, [4] in % of CD3+ cells, [5] in % of monocytes.

When patients in remission on maintenance immunosuppressive therapy were compared to patients in remission in whom the immunosuppressive therapy had been stopped before the examination, the patients on therapy had significantly lower number of NK cells ($p<0.01$, Table 4.13). Moreover, higher expression of CD80 both on CD19–

lymphocytes ($p<0.05$) and also within the CD19+ subpopulation ($p<0.001$) was found in patients on immunosuppressive therapy. The IL-2 production was lower in patients in remission on therapy than in those without therapy, even though this difference reached borderline significance only ($p=0.05$). The remaining results of the surface molecule expression and the intracellular cytokine production did not significantly differ between these two subgroups of patients with AAV.

We also separately analysed and compared patients in remission treated by cyclophosphamide (CYC) with patients in remission treated by azathioprine (AZA) or mycophenolate-mofetil (MMF). The subgroups of patients were overall small and thus difficult to assess statistically. However, an increased number of CD3+HLA-DR+ cells was noted in the CYC subgroup of remission patients compared to the AZA/MMF subgroup. No other significant difference between patients on CYC and patients on AZA/MMF was found (Table 4.14).

Table 4.14: Comparison of surface molecule expression and intracellular cytokine production between patients in remission treated with CYC and patients in remission treated with AZA or MMF

	Remission on CYC (N=7)	**Remission on AZA/MMF** (N=18)	**P value**
CD4+[1]	44.8 ± 13.2	40.1 ± 17.8	n.s.
CD8+[1]	32.4 ± 11.7	32.8 ± 11.7	n.s.
CD19+[1]	5.0 ± 3.2	6.8 ± 7.7	n.s.
CD3+HLA-DR+[1]	15.4 ± 16.0	5.6 ± 4.1	**<0.05**
IFNγ[2]	33.4 ± 11.5	43.1 ± 20.1	n.s.
IL-4[2]	5.8 ± 3.6	5.0 ± 6.3	n.s.
TNFα[2]	53.6 ± 16.9	48.2 ± 23.5	n.s.
IL-2[2]	34.3 ± 15.5	26.5 ± 17.4	n.s.
IL-10[3]	9.8 ± 5.5	12.8 ± 7.0	n.s.
IL-12[3]	12.9 ± 9.6	18.9 ± 9.1	n.s.

Data presented as mean ± standard deviation. P value represents the difference between patients with AAV in remission on CYC and patients in remission on AZA/MMF. CYC=cyclophosphamide, AZA=azathioprine, MMF=mycophenolate-mofetil, n.s. = not significant.
[1] In % of lymphocytes, [2] in % of CD3+ cells, [3]in % of monocytes.

4.2 Long-term follow-up

Special emphasis in this study was placed on discerning any lymphocyte subpopulations or cytokine patterns potentially associated with the prognosis. As mentioned above, 8 patients died during the follow-up. Nevertheless, no relationship between the measured parameters and subsequent death was found.

On the contrary, intriguing results were obtained when patients in remission who subsequently relapsed during the follow-up (N=19) were compared to those without relapse (N=32). Clinical characteristics of these two subgroups of patients were shown above (see Chapter 3). There was no significant difference in age, gender, ANCA specificity or serum creatinine levels between these two subgroups. Patients with and without relapse also did not significantly differ in the proportion of the basic lymphocyte subpopulations and surface molecule expression.

In contrast to surface molecules, significant differences were noted in the intracellular cytokine production, namely in the IL-10 production. The IL-10 production in remission was significantly lower in the patients who subsequently relapsed than in those who did not ($p<0.01$, Fig. 4.5); it was even significantly lower than in HC ($p<0.05$, Figure 4.5). However, no correlation between the length of remission and IL-10 production was observed. There was also no correlation between the IL-10 production and the time to relapse.

Patients in remission without relapse during the follow-up tended to display higher IL-12 levels than those with relapse but the difference between these two subgroups was not statistically significant (Figure 4.5). Nevertheless, as shown in Fig. 4.5, the IL-12 production was higher in patients in remission without subsequent relapse than in HC.

The production of IFNγ, TNFα, IL-2 and IL-4 did not significantly differ between patients in remission with subsequent relapse and those without relapse.

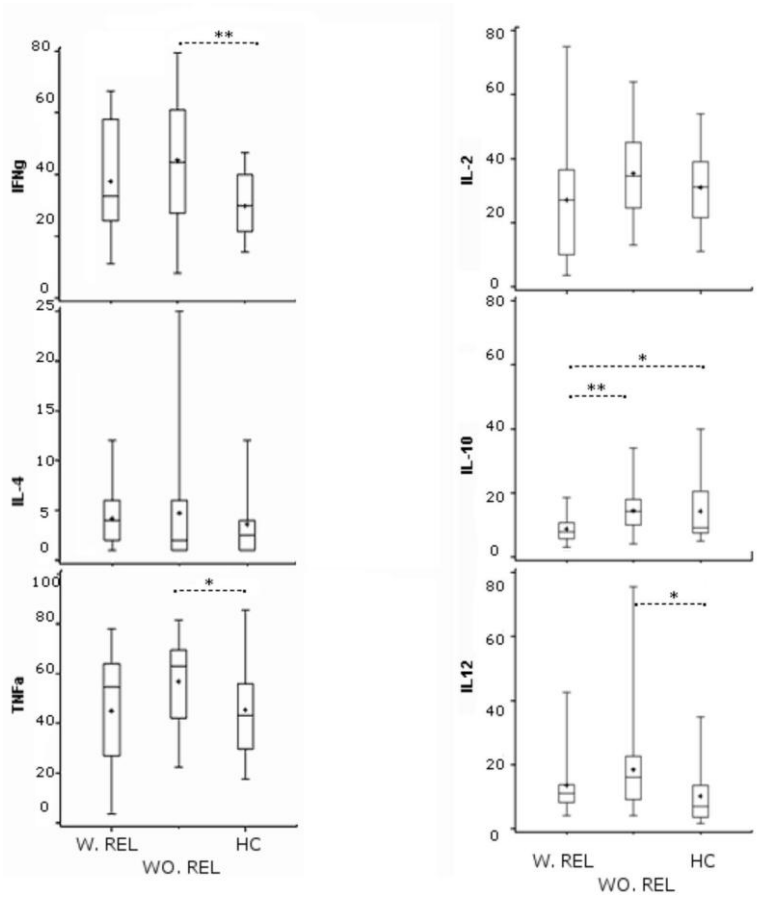

Fig. 4.5: Intracellular cytokine production in patients with AAV and healthy controls. Box plots indicate 5-95% range (error bars), 25-75% range (boxes), median value (horizontal lines) and mean (+). * $p<0.05$, ** $p<0.01$. W.REL = patients with ANCA-associated vasculitis who relapsed during follow-up (N=19); WO. REL = patients with ANCA-associated vasculitis who did not develop relapse during follow-up (N=32); HC = healthy controls (N=24).

4.3 Patients treated with rituximab – preliminary results

Long-term B cell depletion lasting 9-30 months (median 13.5 months) after rituximab (RTX) administration was observed in all treated patients with AAV (see Fig. 4.6 for an example of flow cytometric results in a RTX treated patient). Whilst in 2 newly diagnosed patients with MPA, and RLV respectively, complete remission (CR) was achieved, in 4 patients with refractory AAV (WG in all cases) RTX administration led to partial remission/stabilization (PR) of the disease in 2 patients and did not have any favourable effect in 2 patients with predominant pulmonary granulomatous involvement.

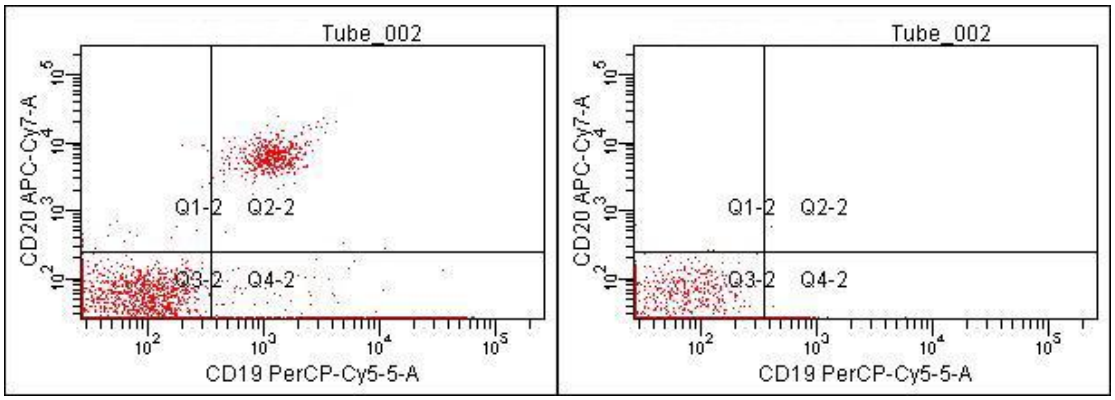

Fig. 4.6: Example of flow cytometric results (patient with normal B cell subpopulation on the left, patient treated with RTX with complete B cell depletion on the right)

The intracellular cytokine production varied deeply among the RTX treated patients. However, interestingly, an increase in IL-10 production 3 months after RTX administration was noted in 4 patients who achieved CR or PR ($p<0.05$) but not in the other two patients without favourable effect of RTX (Fig. 4.7).

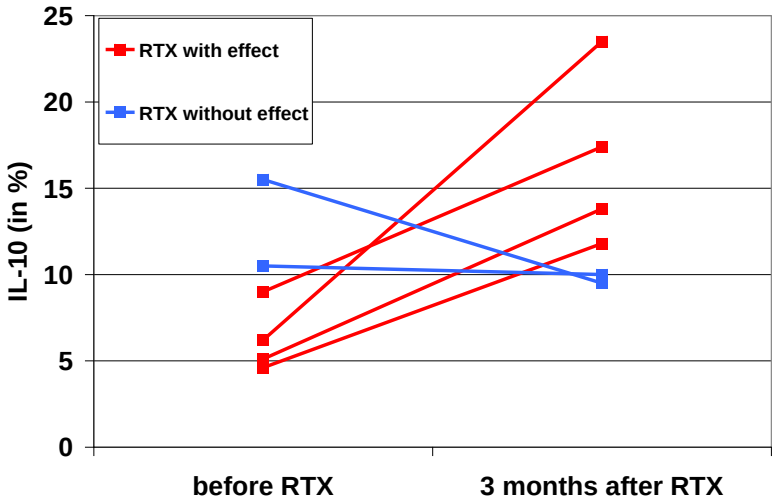

Fig. 4.7: Intracellular production of IL-10 in AAV patients before and 3 months after RTX administration.

Furthermore, complete B cell depletion induced by RTX was accompanied by significant changes in the lymphocyte subpopulations and altered also T cell phenotype in all patients. One of the prominent findings was a significant increase in CD3+HLA-DR+ cells observed early after RTX administration in all patients. The development of the number of CD19+ cells, number of CD3+HLA-DR+ cells, and also ANCA and IgG levels after RTX administration is illustrated in Fig. 4.8 in the example of a patient with RLV who responded well to the therapy and achieved CR.

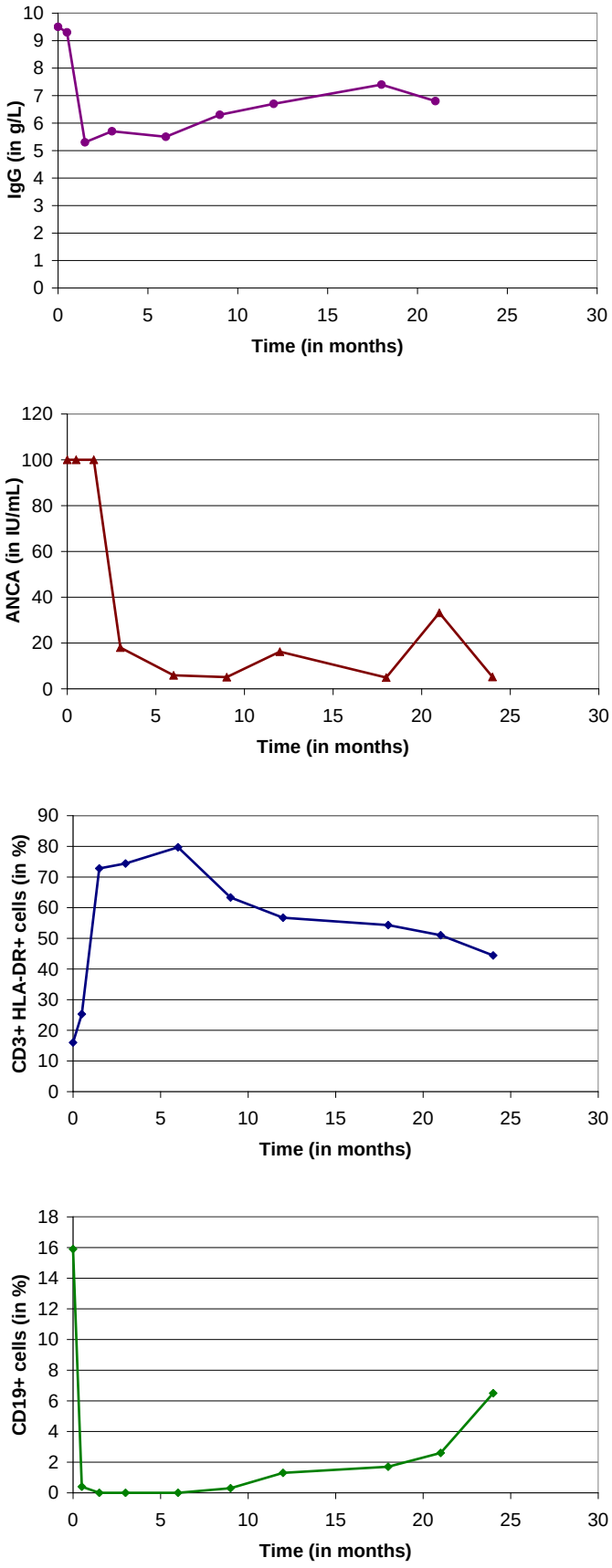

Fig. 4.8: Development of IgG and ANCA levels, and the percentage of CD3+HLA-DR+ cells and CD19+ cells (in the top-down order) after rituximab administration in a patient with AAV.

5. DISCUSSION

ANCA-associated vasculitis is an autoimmune inflammatory disease in which tissue injury most probably results from the interaction of ANCA, neutrophils, and endothelial cells, with presumed role of impaired T cell functions throughout the pathogenic process. While most authors focused on the role of ANCA in the pathogenesis of AAV, the cellular immunity in AAV has only recently attracted attention. This study therefore aimed to assess various cellular immunity parameters (i.e. lymphocyte subpopulations, surface molecule expression, and intracellular cytokine production) in patients with AAV at different stages of the disease, with different treatment modalities, and with respect to the long-term prognosis of the patients.

Since the patients were recruited from a Nephrology Department, renal involvement was present in all but one of our patients. Given the low incidence and prevalence of the disease, 69 patients with AAV included into our study represent a relatively large and homogenous cohort of patients with generalized vasculitis and major organ (renal) involvement. We were also able to examine a total of 26 patients (including 10 active patients) without any immunosuppressive treatment, which has been a logistic problem in many previous studies [94, 97, 115]. Moreover, the demographic and clinical data of our patients as presented in Chapter 3 bear striking resemblance to the data obtained from a long-term follow-up of all consecutive patients with AAV performed at our centre previously [160]. For instance, in agreement with the long-term follow-up (and also other studies), patients with MPO-ANCA-associated disease in this study were significantly older than patients with PR3-ANCA-associated disease. Male gender also slightly predominated in both studies. Thus, the cohort of our patients likely forms a representative sample of AAV patients with renal involvement.

As discussed in detail below, we found distinct abnormalities in the numbers and phenotype of peripheral blood lymphocytes as well as in the intracellular cytokine production in patients with generalized AAV. Despite somewhat different methodology, our findings strengthen and further extend results of a number of earlier studies [94-98, 112-115, 118-119, 121, 127-128, 161-164]. Nevertheless, it is difficult to make direct comparisons with other studies, in particular in the case of the cytokine production assessment. While most authors measured cytokine levels with enzyme immunoassays either directly in blood samples [94, 118, 161, 162] or after stimulation in culture

supernatants [86, 121], and others measured cytokine mRNA with reverse transcriptase-polymerase chain reaction [112, 113, 118], the flow cytometric analysis of intracellular cytokine production similar to our study was less frequent [97, 119] and not related to the long-term prognosis. The advantage of this method is that the intracellular cytokine production after stimulation reflects the ability of blood cells to produce the respective cytokine after activation, and not only cytokine levels themselves. *In vivo*, this activation might be mediated by various infectious stimuli (in WG e.g. by nasal carriage of *Staphylococcus aureus* as suggested earlier [117]). Another advantage of flow cytometric analysis is also the possibility to precisely define the cytokine-producing population. On the other hand, we are aware that the need of artificial stimulation is a possible limitation of flow cytometric analyses as the spectrum of activated cytokine production might differ from activation induced *in vivo* by antigen-presenting cells.

Compatibly with findings of others [97, 163, 164], patients with AAV in this study had significantly lower total number of lymphocytes, diminished proportion of CD4+ cells and enlarged proportion of CD8+ cells when compared to healthy individuals. Notably, lower numbers of lymphocytes and CD4+ cells were observed in all subgroups of AAV patients, including the untreated active ones. Therefore, immunosuppressive therapeutic regimens themselves cannot be blamed for these findings. On the contrary, patients in remission on cyclophosphamide had relatively highest CD4+ cell counts among AAV patients. We presume that the decrease in the total number of circulating lymphocytes might reflect the initial pathogenic vasculitic process with lymphocyte pooling in peripheral tissues. The inflammatory lesions and granulomas in AAV are known to contain significant numbers of CD4+ T cells along with macrophages and neutrophils [81, 128, 131], which might at least partly explain low numbers of CD4+ cells in peripheral blood in AAV patients.

In line with this, it is necessary to have a closer look at the population of CD8+ cells. The significant expansion of CD8+ cells in this study was observed in remission phase of the disease but not in active patients, in particular not in the untreated ones. The influence of administered immunosuppressive therapy (i.e. mostly corticosteroids and pulsed intravenous cyclophosphamide) on the numbers of CD8+ cells seems therefore likely. Indeed, the increase in the percentage of CD8+ cells and the decline of the IRI after cyclophosphamide administration have been previously reported in patients with multiple sclerosis [165] or systemic lupus erythematodes [166].

However, we show that increased CD8 numbers prevail long after cyclophosphamide (and any other immunosuppressive) treatment have been stopped and no difference in CD8+ cell counts was found between patients in remission on cyclophosphamide and those on maintenance immunosuppressive therapy (i.e. azathioprine or mycophenolate-mofetil). Therefore, other factors that account for the expansion of CD8+ cell population in AAV are likely to be identified.

Interestingly, Giscombe et al. [126] described upregulated expression of co-stimulatory molecules of tumour necrosis factor receptor (TNFR) superfamily on expanded CD8+ T cells but not on CD4+ cells in WG. Thus, TNFR expression on CD8+ cells might augment clonal expansion and differentiation, and sustain longevity of cytotoxic T cell response [132].

Furthermore, Iking-Konert et al. [164] reported on the presence of a small population of CD8+CD28+ cells expressing activation marker CD11b during active vasculitis. This population is considered a transient "intermediate" phenotype in the process of CD8+ activation, giving rise to a more persistent phenotype CD8+CD28–CD11b+ found in remission and under immunosuppressive therapy. Activated CD8+ cells may therefore play an important role in the pathogenesis of AAV, either by inducing differentiation of polymorphonuclear neutrophils [164] or by direct contribution to the damage of vascular endothelium, as suggested by others [163].

In accordance with these findings, a significant increase in CD28–CD8+ cells was noted in patients in remission in our study as well as in previous studies [126, 127]. Similarly as others [164], we also observed that low IRI (CD4:CD8 ratio) correlated with a high percentage of CD28–CD8+ cells (Fig. 5.1). Iking-Konert et al. [164] reported that a low IRI was generally associated with a long lasting, severe disease and renal involvement. As renal involvement was present in nearly all our patients, we were only able to prospect for a possible relationship between disease duration and low IRI. No such relationship was, however, found in this study as well as some previous studies [127].

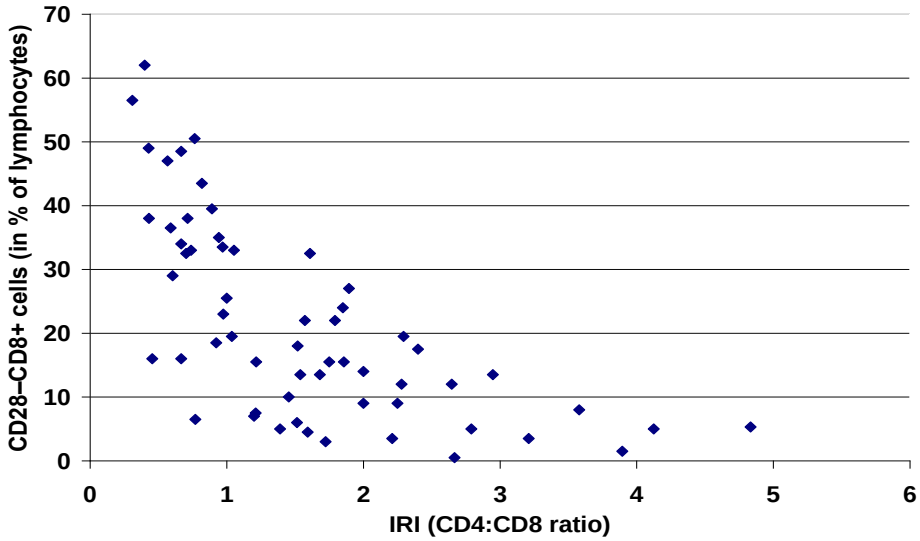

Fig. 5.1: Low immunoregulatory index (IRI) correlates with a high percentage of CD28–CD8+ cells.

In contrast to observations of others [127], we did find a significant difference in the numbers of CD28–CD8+ cells between active patients and patients in remission in this study. In our experience, CD28–CD8+ cells were lower in active disease than in remission; they even negatively correlated with ANCA levels. Notably, no difference between newly diagnosed patients and patients with relapse was found (14.8±11.0, and 14.4±10.9 respectively). Taken the findings of Iking-Konert et al. [164] into account, the explanation might be that in active disease a population of CD8+CD28+ cells prevails and the loss of CD28 expression occurs later in the period of active disease and persists in remission. In that case the timing of blood sample collection during active disease would be important; in our study, most of the active patients were examined soon after the disease activity appeared.

Molecules CD80 and CD86 are CD28 ligands. Both are normally expressed on antigen-presenting cells and their interaction with CD28 on T cells provides a co-stimulatory signal required for T cell activation. In this study, the expression of neither CD80 nor CD86 on CD19+ (B) cells in AAV differed from healthy controls (data not shown). Nevertheless, the expression of CD80 within the CD19+ population was significantly lower in untreated active patients than in active patients on immunosuppression, and decreased after the withdrawal of immunosuppressive therapy in remission. Thus, while B cell numbers generally decrease on therapy, the expression of CD80 per B cell increases. At this point, it is important to highlight that this finding

would not be noted if only the total numbers of CD80 expressing B cells were considered. Instead of this, the proportion of CD19+ cells expressing CD80 was calculated in this study (as the percentage of CD80+CD19+ cells divided by the percentage of CD19+ cells, multiplied by 100). In our opinion, such a calculation helps to prevent misinterpretation of the results, e.g. in the case of low CD19+ cell numbers under immunosuppression, and was therefore used repeatedly.

Intriguingly, increased expression of both CD80 and CD86 on CD19– cells was observed in AAV patients in remission when compared to healthy controls. At the time of the blood sample examination, FACS with three-colour imaging only was available at our centre. However, as the up-regulation of CD80 and CD86 on T cells in patients with AAV had been reported before [127], we supposed that the CD19 negative population represented T cells. Later on, with six colours available, we were, indeed, able to verify the unusual presence of CD80 and CD86 on both CD4+ and CD8+ cells in AAV, as portrayed in Fig. 5.2

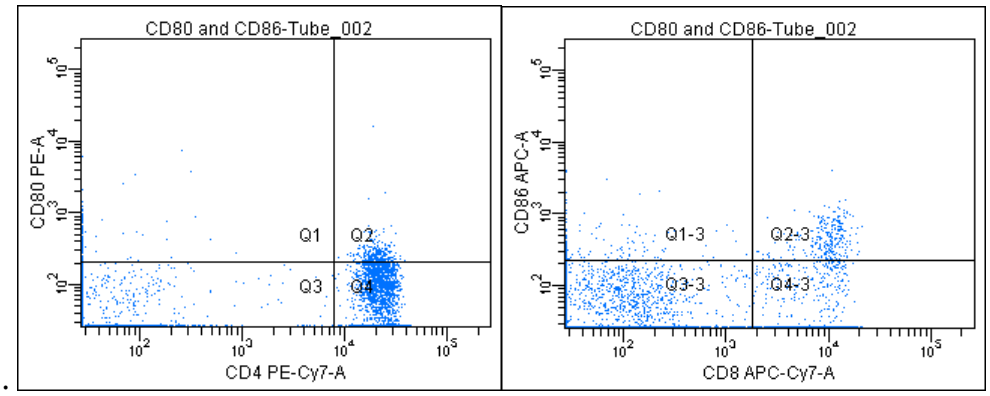

Fig. 5.2: Example of unusual expression of CD80 on CD4+ cells (on the left) and expression of CD86 on CD8+ cells (on the right).

Both the expansion of CD28– cells and the up-regulation of CD28 ligands on T cells seem to go hand in hand with the ongoing inflammation in AAV patients. The loss of CD28 expression on T cells is considered a consistent biological indicator of aging in the human immune system. It has been noted that in immunopathological processes, including AAV, CD28– T cells likely represent prematurely senescent lymphocytes due to persistent immune activation [167]. Regarding the increased expression of CD80 and CD86 on T cells, a self-co-stimulation of these cells is, theoretically, conceivable [127]. Thus, the increased expression of co-stimulatory molecules on T cells may lead to peripheral blood T cell stimulation and contribute to the persistent T cell activation in AAV.

As mentioned in Chapter 2, most authors agree that in contrast to soluble T cell markers and to activation markers on B lymphocytes, activation markers on T cells (e.g. CD25 and HLA-DR) in AAV seem not to correlate with disease activity; they are up-regulated even in remission and despite treatment [96, 98]. The hypothesis of persistent immune system activation in remission of AAV has been supported by several results of our study. On the whole, these findings indicate the possible importance of ongoing immunosuppressive treatment in patients with AAV in remission.

Marinaki et al. [98] defined persistent T cell activation (PTA) in AAV as either of two activation phenotypes, i.e. a low percentage of CD4+CD45RO– (naïve) T cells or a high percentage of CD25 in the naïve CD4+ population. In agreement with the findings of Marinaki et al., a significant decrease in CD4+CD45RA+ naïve T cells was observed in all subgroups of patients with AAV in our study, including the subgroup of yet untreated active patients and also patients with sustained remission. Notably, the decrease in the CD4+CD45RA+ cell population was even more evident in patients in remission.

Correspondingly, the numbers of activated HLA-DR positive CD3+ (T) cells were significantly higher in patients in remission than in healthy controls and active patients in our study. However, in contrast to previous studies [96, 121], significantly increased numbers of circulating HLA-DR+ T cells were not found in our active patients with AAV. Accordingly, higher IFNγ production than in healthy individuals was observed in our patients with AAV in remission but not in active disease. The production of IFNγ in remission of AAV might be sustained by expanded CD28– population of CD4+ T effector memory cells as reported in previous studies [128].

Taken together, our data give enough evidence for the presence of activated T cells in remission of AAV. The explanation for relatively low markers of T cells activation found in active disease in this study is not entirely clear. Marinaki et al. [98] have previously reported on the strong variation of HLA-DR+ cell numbers during longitudinal follow-up. CD25+ cells of non-regulatory phenotype, which were not assessed in our study, were therefore considered a more appropriate marker of persistent T cell activation than HLA-DR by those authors [98]. On the other hand, HLA-DR and CD25 appear on T cells at different stages of activation. HLA-DR appears later than CD25 and persists for a longer period [126]. Thus, the above-mentioned timing of blood sample examination may have contributed to lower numbers of HLA-DR+ cells in active disease in this study.

In theory, more intense immunosuppressive therapy administered in active patients might have also influenced the results; the results of untreated patients did, however, not differ from patients on immunosuppression in the parameters of T cell activation. Moreover, patients in remission treated with cyclophosphamide had higher CD3+HLA-DR+ numbers than patients on maintenance immunosuppressive therapy (azathioprine or mycophenolate-mofetil). In addition, based on our preliminary results, rituximab also leads to an increase in the numbers of CD3+HLA-DR+ cells in AAV patients. Thus, T cell activation in AAV seems to persist despite complete peripheral B cell depletion. Nonetheless, the stimulus that triggers persistent T cell activation in remission of AAV remains to be identified [98]. It is also questionable whether this finding is a cause, a bystander phenomenon or rather a consequence of the inflammatory process in AAV. In agreement with findings of others [94], no relationship between markers of T cell activation and subsequent relapse was revealed in this study, but an association with renewed or persistent ANCA positivity had been described earlier [94].

A detailed analysis of results obtained from active patients might help to shed more light on the immune processes participating in AAV. These results closely relate to the topic of Th1/Th2 polarization in AAV that has been studied thoroughly [reviewed in 116, 117] both in granulomatous lesions of the upper respiratory tract [112, 113, 118] and in renal biopsies [113, 119], as well as in peripheral blood [94, 97, 112, 114, 119, 121, 161, 162], and described in detail in Chapter 2. In brief, most authors agree that there seems to be an aberrant Th1 type response that might play role during initiation of WG in patients with localized WG. On the contrary, there seems to be a significant appearance of Th2 cells in patients with generalized vasculitis.

In this study, we were not able to detect outright Th1 or Th2 polarization in patients with generalized AAV. Levels of both Th1- (CCR5, IL-12, IFNγ) and Th2-associated markers (CD30, CRTH2) were increased in patients with AAV when compared to healthy individuals. Nevertheless, while both CD30 and CRTH2 together with IL-10 production prevailed in active disease and correlated with disease activity measured with BVAS, the Th1-associated markers were either similarly increased in both active disease and remission (CCR5, IL-12) or significantly higher in remission than in active disease (CXCR3, IFNγ). Thus, we speculate that Th2 type of immune response may play an important role in the pathogenesis of active generalized vasculitis, whereas Th1 type of immune response seems to predominate later in the pathogenic

process and in remission.

Soluble CD30 (sCD30) has been previously shown to correlate with disease activity in AAV [94, 95] but the authors did not agree on whether its levels remained increased in remission compared to healthy controls [94, 95]. In this study, we demonstrate that also the total expression of CD30 on lymphocytes as well as CD30 expression on CD4+ lymphocytes (data not shown) correlate well with disease activity measured with BVAS. In agreement with Wang et al. [95] and unlike Sanders et al. [94] we were not able to detect significantly increased CD30 expression in remission of AAV. On the other hand, the significant correlation with ANCA levels described by Wang et al. [95] was not proven in this study. CRTH2 (chemoattractant receptor-homologous molecule expressed on Th2 lymphocytes) expression is a less frequently used marker of Th2 type of immune response. In this study, the expression of CRTH2 on CD4+ T cells correlated with disease activity, and also with the CD30 expression. Neither CRTH2 expression in remission differed from healthy individuals, suggesting that Th2 cell numbers in peripheral blood tend to normalize in remission of AAV.

In agreement with previous reports [115], the increased expression of Th1-associated chemokine receptor CCR5 on CD4+ T cells persisted in patients in remission in this study. The increased expression of CCR5 in AAV has been ascribed to the expanded population of effector memory T cells (T_{EMs}) [115]. Most of T_{EMs} in WG lack CD28 expression, and display upregulated CCR5, HLA-DR, CD18 (β_2 integrin/adhesion molecule), CD152 (CTLA-4), and CD57 expression (differentiation marker) [132]. The majority of authors agree that CD28– T_{EMs} secrete Th1 cytokines [132]. Even though not all of these markers were measured in this study, we presume that our findings in patients in remission (i.e. low numbers of CD4+CD45RA+ cells, increased numbers of HLA-DR+ cells, increased expression of CCR5, and increased IFNγ production) conform to the presence of expanded population of T_{EMs} in AAV. Increased numbers of T_{EMs} in remission of WG when compared to active disease have been reported before by Abdulahad et al. [135] and explained by the migration of T_{EMs} to the sites of inflammation during active disease.

Regarding the intracellular cytokine production, one of the prominent findings in our study was the increased IL-12 production in patients with active generalized vasculitis (both PR3-ANCA- and MPO-ANCA-associated) that persisted even in remission, even though a positive correlation with ANCA levels was observed. This is in agreement with previous studies [97, 121] and might imply a skewed regulation

towards Th1 cell differentiation as IL-12 is a major inducer of IFNγ and as such is necessary for differentiation and proliferation of Th1 cells. However, in our patients, no correlation between IL-12 and IFNγ production was observed. As mentioned before, the IFNγ production in our active patients did not differ from controls despite increased IL-12 production. On the contrary, in remission, IL-12 production remained higher than in healthy individuals but tended to decrease compared to active patients, whereas a significant increase in IFNγ production was noted.

It has been proven that cytokine-primed neutrophils and monocytes (e.g. after stimulation with an infectious agent) are capable of translocating small amounts of ANCA antigens to the cell surface where they become accessible for interaction with ANCA, which subsequently leads to neutrophil activation [31]. Given that the IL-23-IL-17 immune axis is probably closely associated with the rapid influx of neutrophils during acute infections and is thought to "buy time" for the induction of Th1 response [124], it is conceivable that dysregulated Th17 response found in AAV [125] participates in the early stages of disease pathogenesis. We speculate that activated neutrophils not only contribute to tissue damage and attract mononuclear cells to the sites of inflammation, but might also be a potential source of IL-12 [168], thus promoting a Th1 cytokine response in the infiltrating mononuclear cells. This process seems to occur especially in the initial phases of the disease. The IL-12 production in monocytes in our study was particularly high in newly diagnosed patients whereas in patients with relapse the levels were relatively low. We presume that after a long-term cultivation increased IFNγ production in active patients might also be observed.

Theoretically, IL-12-induced IFNγ production might be to some extent inhibited by the counterbalancing IL-10 in active patients. IL-10 has pleiotropic effects in immunoregulation and inflammation. It is an important cytokine with anti-inflammatory and immunosuppressive potential, suppressing especially the Th1 immune response. In our active patients, the IL-10 production was significantly higher than in patients in remission, similarly to findings of others [94] even though in our study the production did not significantly differ from healthy individuals.

Importantly, IL-10 was the only marker in this study related to the long-term prognosis of patients in the prospective follow-up. As mentioned earlier, many of the patients with AAV who successfully achieve remission undergo a relapse. Identifying patients at risk for relapse is one of the lasting challenges in the field of vasculitides.

We demonstrate that low IL-10 production at anytime in remission of the disease is associated with a higher probability of subsequent relapse, which in our study occurred in the range of 2 to 38 months after the cytokine measurement. Similar findings were reported in previous studies. However, in the study by Ohlsson et al. [161] low levels of IL-10 were observed 3 months before relapse and in the study by Sanders et al. [94] low levels of IL-10 were found 3 months after diagnosis and relapse was observed within following 60 months. We prospected for a possible correlation between the time to relapse and IL-10 production in our patients. Nevertheless, no such correlation was proven.

In accordance with the above mentioned significance of activation (e.g. infectious) stimuli in the disease pathogenesis, it seems that patients with low IL-10 are particularly prone to relapse after an intercurrent infection due to the lack of the anti-inflammatory potential of IL-10. Based on the available data, IL-10 might serve as a marker of imminent relapse in patients with AAV, including those who seem to be in stable remission. This could have important therapeutic consequences, yet longitudinal studies monitoring intra-individual variability of IL-10 production are needed.

Of note, an increase in IL-10 production following rituximab (RTX) administration seemed to be associated with favourable treatment outcome in a small group of RTX-treated patients in this study. Relatively low levels of IL-10 before RTX administration in patients with refractory active WG correspond to our finding that patients with relapse had significantly lower IL-10 production than newly active patients. The increase in IL-10 production after RTX in patients with successful treatment course further highlights the importance of the anti-inflammatory and regulatory potential of IL-10 in AAV.

Talking about cellular immunity in AAV, we have to bear in mind that most researchers exclusively attended to patients with WG, or PR3-ANCA-associated disease, respectively, in their studies. Patients with MPA, or MPO-ANCA-associated disease, were included in few studies only [97, 98, 161, 162, 164], and detailed data are missing. Given the known difference in the amount of evidence for the pathogenic potential of PR3-ANCA and MPO-ANCA in *in vivo* models, with the unique presence of granulomatous inflammation in WG, one would also expect a considerable difference in cellular immunity parameters. Surprisingly, hardly any significant difference was found between patients with PR3-ANCA and MPO-ANCA-associated disease in this study, as well as in other studies [161, 164].

Schönermarck et al. [162] reported on the difference in sCD30 and sCD26 levels between generalized WG and MPA. Increased sCD30 levels and decreased sCD26 levels, suggesting a shift towards Th2 type of immune response, were found in patients with active generalized WG but not in patients with MPA. In contrast to these observations, the increase in CD30 expression was observed in both PR3-ANCA- and MPO-ANCA-associated disease in our study. It was even slightly—not significantly—more prominent in active MPO-ANCA-associated disease than in active PR3-ANCA-associated disease, in which CRTH2 expression predominated.

Furthermore, we noted lower numbers of NK cells in active PR3-ANCA-associated disease than in active MPO-ANCA-associated disease; the biological significance of this is, however, uncertain. In theory, the decrease of NK cells in peripheral blood in WG might reflect their local role in the first wave of immune response to a (yet unknown) stimulus triggering granuloma formation.

Collectively, the examination of cellular immunity parameters in peripheral blood does not provide enough clues to distinguish PR3-ANCA-associated disease from MPO-ANCA-associated disease. It is indisputable that granulomas are not found in all patients with PR3-ANCA-associated disease nor do patients with PR3-ANCA uniformly respond to immunosuppressive therapy or follow the same course of the disease. Further studies might help to discern more homogenous groups within patients with AAV, and thus contribute to the development of "patient-tailored" therapeutic strategies.

Last but not least, we investigated the possible influence of impaired renal function on the results of this study. Nevertheless, no correlation between glomerular filtration rate and measured parameters was found. Moreover, we separately analysed patients with serum creatinine levels below and above 300 μmol/L. However, even in this case no significant difference was observed (data not shown).

Although a decrease in renal function is present in many patients with generalized vasculitis, most authors did not deal with direct comparison of patients with AAV and patients with chronic kidney disease. Ohlsson et al. [161] measured cystatin C as a marker of glomerular filtration and concluded that IL-6 but not IL-8 or IL-10 seemed to be affected by decreased renal function. To elucidate this topic, we decided to examine a control group of patients with chronic kidney disease (CKD) caused by "non-immunological" conditions. Compatibly with findings of others [169], patients with CKD displayed signs of immune system activation and depletion of naïve T

lymphocytes (increased HLA-DR, CCR5 and CXCR3 expression, decreased numbers of CD4+CD45RA+, higher IFNγ and TNFα production when compared to healthy individuals). Selected parameters are compared between patients with CKD and patients with AAV in Fig. 5.3-5.5.

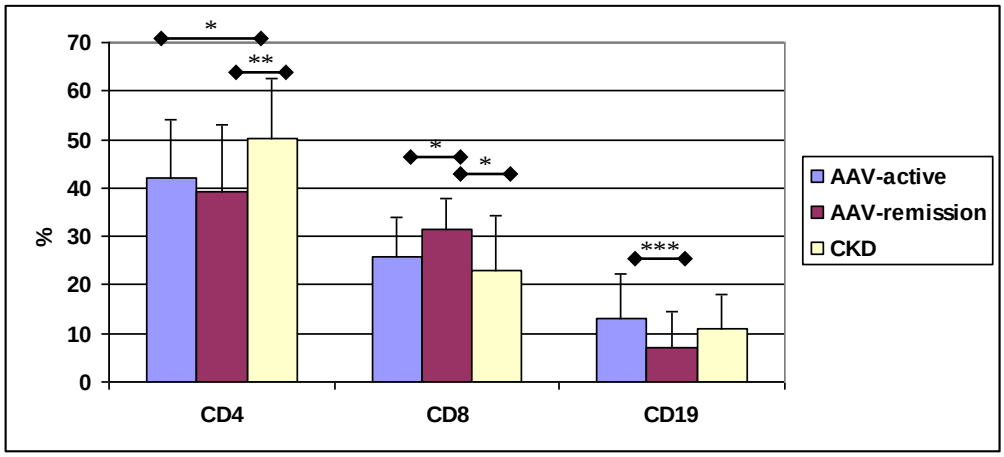

Fig. 5.3: Comparison of basic subpopulations in patients with chronic kidney disease (CKD) and patients with AAV. Data presented as mean, error bars represent standard deviation. *p<0.05, ** p<0.01, *** p<0.001

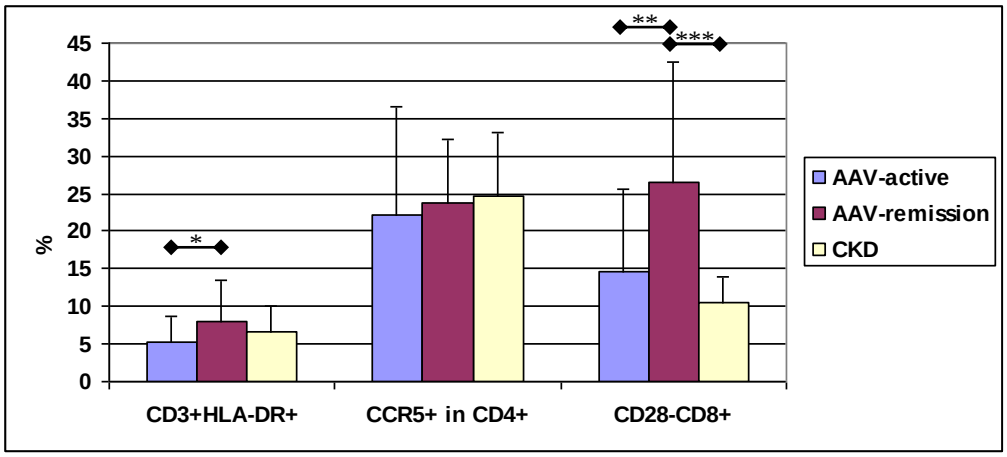

Fig. 5.4: Comparison of the expression of selected surface molecules in patients with chronic kidney disease (CKD) and patients with AAV. Data presented as mean, error bars represent standard deviation. * p<0.05, ** p<0.01, *** p<0.001

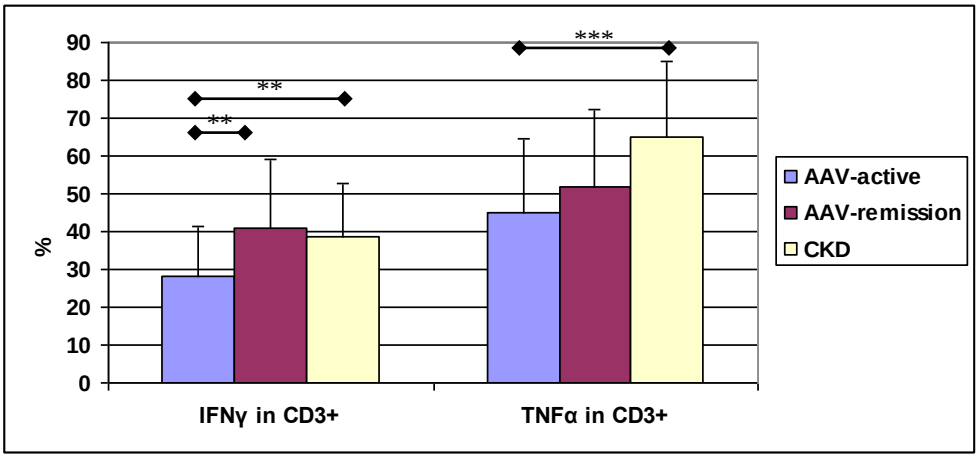

Fig. 5.5: Comparison of intracellular cytokine production in patients with chronic kidney disease (CKD) and patients with AAV. Data presented as mean, error bars represent standard deviation. * $p<0.05$, ** $p<0.01$, *** $p<0.001$

It is obvious that some markers of immune system activation (HLA-DR and CCR5 expression) were equally elevated in CKD patients and AAV patients, or were even higher in CKD than in AAV, in particular than in active AAV (IFNγ and TNFα production). However, no shift in CD4:CD8 ratio was found in CKD. A profound difference between CKD patients and AAV was also observed in CD28 expression. Importantly, in CKD neither CD80 nor CD86 expression (increased on CD19− cells in remission of AAV) differed from healthy individuals. Contrary to active AAV patients, patients with CKD did not display increased CD30 and CRTH2 expression.

Taken together, activation of the immune system is present in CKD but differs from that in AAV in several aspects. We conclude that whilst renal insufficiency may to some extent contribute to persistent T cell activation in AAV, it does not seem to be the crucial factor for two following reasons. Firstly, we observed higher parameters of T cell activation in patients in remission than in active disease even though active patients had generally higher levels of serum creatinine than patients in remission. Secondly, the signs of persistent immune system activation in AAV were also described in previous studies in that renal involvement was missing in the majority of patients [126].

In conclusion, our own findings and previous published reports of others lead us to a current working hypothesis on the pathogenic mechanisms in AAV (Fig. 5.6) that, indeed, involve both humoral and cellular immune mechanisms. Even though much is already known, the proposed model of the disease pathogenesis provides important clues for future research that might help to identify possible therapeutic targets and improve diagnostic possibilities to predict and early detect AAV relapse.

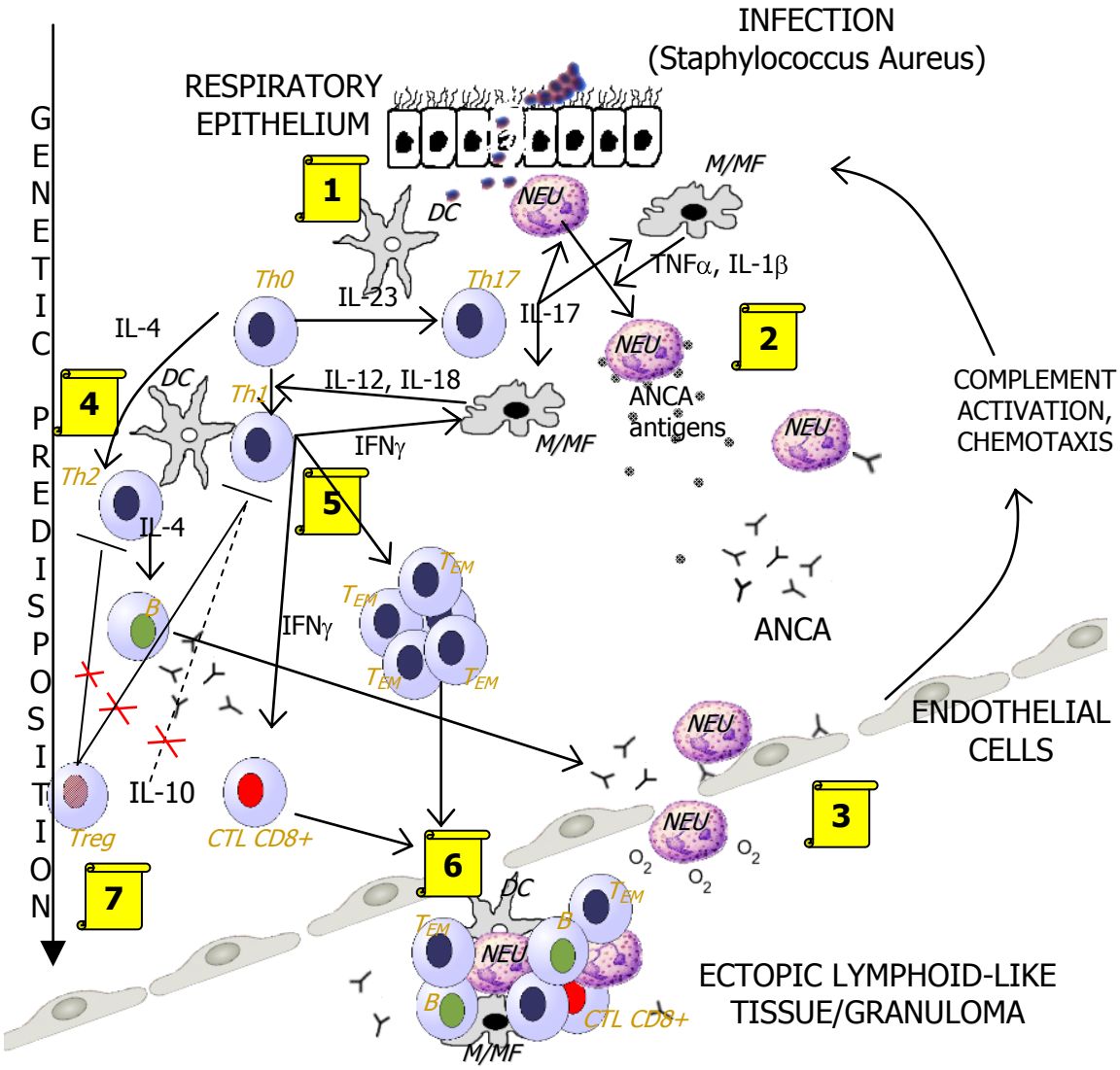

Fig. 5.6: Current working hypothesis on the pathogenic mechanisms in AAV [freely adapted from 47, 132, 170].

NEU = neutrophil, DC = dendritic cell, M/MF = monocyte/macrophage, CTL = cytotoxic T lymphocytes. For other abbreviations see text.

(1) The presence of infectious agents stimulates antigen-presenting cells (e.g. DC) to produce IL-23, which induces differentiation and proliferation of Th17 cells. Th17 cells play role in the rapid recruitment of neutrophils and also stimulate monocytes/macrophages to produce pro-inflammatory cytokines.

(2) Chemokines and pro-inflammatory cytokines attract neutrophils to the infected tissue and cause neutrophil priming with subsequent release and increased membrane expression of ANCA antigens, which become accessible for interaction with ANCA.

(3) The inflammatory cytokines also lead to upregulation of adhesion molecules both on neutrophils and endothelial cells. ANCA activate neutrophils adherent to endothelial cells which results in respiratory burst and neutrophil degranulation. Reactive oxygen species and proteolytic enzymes cause tissue damage. Released antigens also bind to endothelial cells and in the presence of ANCA form transient immune complexes which leads to complement activation and amplification of the immune response.

(4, 5) Activated DCs induce both Th2 and Th1 type of immune response. Th2 cells further participate in B cell activation with subsequent ANCA production (4). Exaggerated Th1 type of immune response results in macrophage activation and plays role in the activation of cytotoxic CD8+ T lymphocytes. Cytokine- and/or antigen-driven expansion of CD28– T effector memory cells (T_{EM}) occurs (5).

(6) Both T_{EM} and cytotoxic T lymphocytes directly contribute to the endothelial and tissue damage. Moreover, T_{EM} sustain chronic granulomatous inflammation with ectopic lymphoid-like tissue formation, which further promotes ANCA formation.

(7) Genetic predisposition helps to sustain the inflammatory process. Regulatory immune mechanisms with impaired function fail to resolve inflammation in a normal manner.

6. CONCLUSIONS

The extent and complexity of our study have enabled us comparison with numerous partial studies of others. Therein, the patient cohort selection and proper description have been proven very important. In our experience, the studies that do not consider the disease activity status or the influence of immunosuppressive treatment may often produce distorted results.

Main results of our study may be summarized into several following points:

- Patients with AAV had decreased total number of lymphocytes and CD4+ cells in peripheral blood. These findings were observed even in untreated patients, and cannot be therefore explained by immunosuppressive therapy only. We suppose that decreased numbers of the cells in peripheral blood may reflect their increased numbers found in the inflammatory lesions.
- An expansion of CD28−CD8+ subpopulation that correlated with low CD4:CD8 ratio and negatively correlated with ANCA levels was noted in remission. Further studies are needed to unambiguously elucidate the significance of this cell subpopulation in AAV.
- Increased expression of CD80 and CD86 on CD19− cells found in this study may be ascribed to their increased expression on T cells and likely contributes to the persistent T cell activation in AAV.
- The presence of T cell activation in remission of AAV was supported by the decrease in CD4+CD45RA+ and the increase in CD3+HLA-DR+ cells. These results indicate the importance of ongoing immunosuppressive treatment in remission.
- We did not acquire enough evidence to detect outright Th1/Th2 polarization in generalized AAV. However, CRTH2 expression on CD4+ cells correlated with disease activity but CCR5 expression on CD4+ cells persisted in remission. Increased IL-12 production also persisted in remission but did not correlate with IFNγ production.
- Low IL-10 production in remission was associated with increased risk of relapse.

- Mechanisms of immune system activation clearly differ between non-AAV renal insufficiency and AAV.

Taken together, the results reveal intriguing possibilities of further research on the cellular immunity in AAV. Nevertheless, they particularly demonstrate the persistent immune system activation in remission of AAV and warn against early withdrawal of immunosuppressive therapy in remission. This prompts us to future studies focused on the possible benefit from the long-term follow-up of cellular immunity parameters for guiding the maintenance immunosuppressive therapy.

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APPENDIX

I. Publications that formed the basis of the Thesis

II. Further publications related to the topic of the Thesis

I. Publications that formed the basis of the Thesis[1]

- **Vankova-Hruskova Z,** Rihova Z, Mareckova H, Tesar V. New Insights in the Pathogenic Role of ANCA (Anti-Neutrophil Cytoplasmic Antibodies). In: Autoantibodies Research Progress, 1st Edition, New York: Nova Science Publishers, Inc., 2008, pp. 193-213.
- **Hrusková Z**, Marecková H, Ríhová Z, Rysavá R, Jancová E, Merta M, Tesar V. T cells in the pathogenesis of ANCA-associated vasculitis: current knowledge. Folia Biol. 2008; 54:81-7. **IF** = 1.14
- **Hruskova Z**, Rihova Z, Tesar V. Current treatment of ANCA-associated renal vasculitis. Port J Nephrol Hypert. 2008; 22: 149-55.
- **Hruskova Z**, Rihova Z, Mareckova H, Jancova E, Rysava R, Zavada J, Merta M, Löster T, Tesar V. Intracellular cytokine production in ANCA-associated vasculitis: low levels of interleukin-10 in remission are associated with a higher relapse rate in the long-term follow-up. Arch Med Res. 2009; 40:276-84. **IF** = 1.703 (2008)

[1] Full text of the publications inserted.

II. Further publications related to the topic of the Thesis[2]

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- Závada J, Kideryová L, Pytlík R, **Hrusková Z**, Tesar V. Reduced number of endothelial progenitor cells is predictive of early relapse in anti-neutrophil cytoplasmic antibody-associated vasculitis. Rheumatology. 2009 (In Press). **IF** = 4.136 (2008)
- Frausova D, Brejnikova M, **Hruskova Z**, Rihova Z, Tesar V. Outcome of thirty patients with ANCA-associated renal vasculitis admitted to the intensive care unit. Ren Fail. 2008; 30:890-5. **IF** = 0.657
- **Hrušková Z**, Tesař V. Léčba ANCA-asociované vaskulitidy. Pokroky v oboru Nefrologie. 2008; 2 (1): 43-52.
- Závada J, Kideryová L, Pytlík R, **Vaňková Z**, Tesař V. Circulating Endothelial Progenitor Cells in Patients with ANCA-Associated Vasculitis. Kidney Blood Press Res. 2008; 31:247-54. **IF** = 1.268
- **Vaňková Z**, Říhová Z, Jančová E, Ryšavá R, Merta M, Tesař V: Optimizing the therapeutic strategies in ANCA-associated vasculitis – single centre experience with randomized international trials. Prague Med Rep. 2006; 107:189-198.
- Rihova Z, Honsova E, Zavada J, **Vankova Z**, Jancova E, Reiterova J, Tesar V.: Two familial cases of antineutrophil cytoplasmic antibody (ANCA)-associated vasculitis. Rheumatology. 2006; 45:356-7. **IF** = 4.052
- Rihova Z, Maixnerova D, Jancova E, Pelclova D, Bartunkova J, Fenclova Z, **Vankova Z**, Reiterova J, Merta M, Rysava R, Tesar V: Silica and asbestos exposure in ANCA-associated vasculitis with pulmonary involvement. Ren Fail. 2005; 27: 605-8. **IF** = 0.54

[2] Title page of the publications inserted only.