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Rituximab-induced neutropenia: clinical and pathophysiological studies

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To Heli, Hosi and Yodit

In memory of my father

Abstract

Rituximab is a monoclonal antibody directed against the CD20 antigen on normal and neoplastic B-lymphocytes. It was originally developed for treatment of lymphomas as a targeted therapy against CD20 positive non-Hodgkin lymphomas (NHL). More recently, its use has expanded into patients with rheumatic diseases. Consistent with this trend, late adverse events of rituximab are appearing, one of these is rituximab induced neutropenia also called late-onset neutropenia (LON). It is defined as an unexplained absolute blood neutrophil count (ANC) < 1.5 x 10^9/L occurring 4 weeks after termination of rituximab therapy up to one year of the follow-up time. However, incidence, mechanism, predisposing factors and clinical consequences of LON are poorly defined. The aim of this study was to address these questions in rituximab treated patients for NHL and rheumatic disease.

We studied the incidence of LON retrospectively in rituximab treated NHL patients. We found an incidence of 8% and a higher incidence was observed in autologous stem cell transplanted patients (Paper I). In this study we observed maturation arrest at the (pro)myelocyte stage of granulopoiesis in the bone marrow (BM) implying a selective depletion of granulocytes. There was no incidence report in rheumatic patients and hence we expanded our studies into this patient group (Paper II). We found similar incidence figure. However, the clinical course of LON was different and it was associated with a higher risk of infections. Moreover, flow cytometry studies on peripheral blood showed that LON patients had pronounced and longer B-lymphocyte depletion compared with non-LON matched controls. Lower IgM levels were evident in LON patients. Thus, the levels of B-lymphocyte depletion and IgM levels may identify patients at risk. Subsequently, we tried to define genetic factors for LON by analyzing polymorphisms affecting B-lymphocyte depletion and production (Paper III). Here, we studied the role of Fc gamma receptor (*FCGR*: *FCGR2A* 131 H/R, *FCGR2B* 232 I/T and *FCGR3A* 176 V/F) and B-lymphocyte activating (*BAFF*: -871C/T) gene promoter polymorphisms for the development of LON. The *FCGR3A* 176V allele was correlated with the occurrence of LON and each V allele was associated with 4-fold increase of odds-ratio for LON. Moreover, patients with this genotype had a longer time to flare of rheumatic disease. Surprisingly, patients who developed LON had also a longer time to flare demonstrating a novel correlation between LON and clinical response. In Paper IV, we tried to elucidate mechanisms of LON. We included rituximab treated NHL patients prospectively. BM and blood samples were obtained at the detection of LON. A pronounced B-lymphocyte depletion in LON patients was also evident during the LON period and this coincided with significant raise in serum BAFF levels compared to non-LON matched controls. Furthermore, BM studies revealed a selective depletion of granulopoiesis (maturation arrest at the (pro)myelocyte stage) during complete B-lymphocyte depletion.

In summary, our studies add to our understanding of LON as a distinct entity. The identification of risk factors such as levels of B-lymphocyte depletion and IgM, and possession of the high affinity *FCGR3A* 176 V allele might be helpful in future clinical practice. Moreover, this genotype as well as the presence of LON were also related to a better clinical outcome. It is, thus, tempting to suggest that LON is a good prognostic factor, but that remains to be proven in a larger prospective studies and lymphoma patients. Finally, our mechanistic studies highlight the interdependence of lymphopoiesis and granulopoiesis which might be orchestrated by BAFF.

List of Publications

This thesis is based on the following papers, which are referred to in the text by their Roman numerals.

I. DANIEL TESFA, Tobias Gelius, Birgitta Sander, Eva Kimby, Bengt Fadeel Jan Palmblad, Hans Hägglund. Late-onset neutropenia associated with rituximab therapy: evidence for a maturation arrest at the (pro)myelocyte stage of granulopoiesis. *Med Oncol*. 2008;25(4):374-9.

II. DANIEL TESFA, Sofia Ajegonova, Hans Hägglund, Birgitta Sander, Bengt Fadeel, Ingiäld Hafström, Jan Palmblad. Late-onset neutropenia following rituximab therapy in rheumatic disease: association to B-lymphocyte depletion and infections. *Arthritis Rheum*. 2011;63(8):2209-14.

III. DANIEL TESFA, Sofia Ajegonova, Hans Hägglund, Bengt Fadeel, Anna Linda Zignego, Mårten Winge, Magnus Nordensköld, Inger Vedin, Ingiäld Hafström, Jan Palmblad. Causes and consequences of late-onset neutropenia following rituximab therapy in patients with rheumatic disease: relation to FCGR and BAFF promoter polymorphisms. *Manuscript.*

IV. DANIEL TESFA,Birgitta Sander, Eva Kimby, Bengt Fadeel, Jan Palmblad, Hans Hägglund. Late-onset neutropenia following rituximab treatment in non-Hodgkin lymphoma is associated with profound B-lymphocyte depletion, elevated serum BAFF levels and perturbations of granulopoiesis. *Manuscript.*

Additional relevant publication to the thesis

DANIEL TESFA, Marianne Keisu, Jan Palmblad. Idiosyncratic drug-induced agranulocytosis: possible mechanisms and management. *Am J Hematol*. 2009; 84(7):428-34. Review.

Contents

LIST OF ABBREVIATIONS

AAV	Anti-neutrophil cytoplasmic antibody associated vasculitis
ADCC	Antibody-dependent cellular cytotoxicity
ANC	Absolute neutrophil count
APRIL	A proliferation-inducing ligand
BM	Bone marrow
B-CLL	Chronic lymphatic leukemia
BAFF	B-cell activating factor
BLYS	B-lymphocyte stimulator
CBC	Complete blood count
CDC	Complement-dependent cytotoxicity
CHOP	Cyclophosphamide, doxorubicin, vincristine and prednisolone
CNS	Central nervous system
DIAG	Drug-induced agranulocytosis
DINP	Drug-induced neutropenia
ELANE	Elastase associated neutrophil expressed protein
FDA	Food and Drug administration
GAT	Granulocyte agglutination test
GIFT	Granulocyte immunofluorescence test
G-CSF	Granulocyte colony stimulating factor
G/E	Granulopoiesis-erytropoiesis ratio
GPA	Granulomatosis with polyangiitis
FcγR	Fc gamma receptor
FCGR	Fc gamma receptor gene
HAX-1	Hematopoietic cell-specific associated protein x-1
HBV	Hepatitis-B virus
IL10	Interleukin 10
IQR	Interquartile range
LON	Late-onset neutropenia
mAb	Monoclonal antibody
MAIGA	mAb –specific immobilization of granulocyte antigen
MPA	Microscopic polyangiit
NCI-CTC	National Cancer Institute Common Toxicity Criteria
NHL	Non-Hodgkin lymphoma
NK	Natural killer
PB	Peripheral blood
PML	Progressive multifocal leukoencephalopathy
RA	Rheumatoid arthritis
SCN	Severe congenital neutropenia
SCT	Stem cell transplantation
SD	Standard deviation
SDF-1	Stromal-derived factor-1
SLE	Systemic lupus erythematosus
SNP	Single nucleotide polymorphism
T-LGL	Large granular T-lymphocytes
TNF	Tumor necrosis factor

1. INTRODUCTION
1.1 LATE-ONSET NEUTROPENIA

1.1.1 HISTORY

Drug-induced agranulocytosis (DIAG) or neutropenia (DINP) can occur as an adverse event to virtually any drug. Common for these events is that they develop during drug intake (or up to a week after cessation of the therapy), appear any time from the 1st treatment week up to (at least) one year, and typically have durations of 4 days to more than 3-4 weeks [1, 2].

Rituximab induced neutropenia also called late-onset neutropenia (LON) is a unique type of delayed drug-induced reaction, not known before the era of rituximab. LON occurs months after the cessation of rituximab therapy. LON was first reported in two pivotal prospective clinical trials evaluating efficacy of rituximab in relapsed low-grade non-Hodgkin lymphoma (NHL) [3, 4]. These authors demonstrated an unexplained isolated late-onset neutropenia occurring from 4 up to 10 months after the end of rituximab therapy. Surprisingly, most of the cases resolved with in a month without treatment with granulocyte colony stimulating factor (G-CSF). Another study, assessing the efficacy and safety of re-treatment with rituximab in relapsed low-grade or follicular lymphoma, reported similar isolated neutropenia 1 month after the termination of rituximab treatment [5]. Interestingly, one patient developed a similar pattern of LON upon re-exposure to rituximab. However, no defined criteria for LON were established in the aforementioned prospective trials and it was difficult to discern the clinical characteristics of LON.

Since then, several case reports and few retrospective studies with specific aims to study LON have tried to define this clinically significant event in lymphoma patients [6-13]. The validation of LON as a distinct entity is also established by reevaluating data from randomized trials on NHL patients where no LON cases were reported in the historical group, consisting of patients who had been treated with non-rituximab-containing regimens [7, 10, 11]. In addition, recent studies have reported the occurrence of LON in rituximab-treated patients with autoimmune and rheumatic disease [14-19]. However, prospective studies of LON are lacking. The aim of the studies comprised in this thesis has been to understand the incidence, mechanism, predisposing factors and clinical consequences of LON.

1.1.2 DEFINITION

LON is defined here as an unexplained absolute blood neutrophil count (ANC) < 1.5 x 10^9/L (corresponding to neutropenia of grade 2-4 according to National Cancer Institute Common Toxicity Criteria (NCI-CTC) [20] starting from 4 weeks after termination of rituximab therapy, until the end of follow-up. The follow-up period for detection of LON is up to 12 months. Thus, alternative causes of neutropenia should be ruled out by a medical history, physical examination and a thorough clinical investigation. In addition, during these 4 weeks after rituximab treatment the patient must have recovered to normal ANC after previous chemotherapy. Moreover, during these 4 weeks the patient must not receive any other chemotherapy or neutropenia causing drugs. For stem cell transplanted (SCT) patients the same definition was used, but to qualify as LON the neutropenic episode could occur at earliest 4 weeks after transplantation. Inclusion criteria and definition of LON for each study are given in detail in the Papers included in this thesis.

1.2 NEUTROPHILS AND IMMUNITY

1.2.1 DEVELOPMENT

Granulopoiesis starts with the differentiation of promyeloblast to promyelocyte (Figure 1). Promyelocytes are large cells with purple staining non-specific or primary azurophilic granules. Promyelocytes develop into myelocytes that are characterized by the presence of smaller specific or secondary granules. The cell loses its basophilic cytoplasma and granule production ceases at the end of the myelocyte stage. At the metamyelocyte stage, the cell size decreases and the nucleus becomes flattened and the chromatin condenses. This stage is followed by a horseshoe-shaped nucleus, called band cell. Then, the nucleus gets segmented into lobes and a mature neutrophil is formed.

Developmental defects at specific stages of granulopoiesis are reported in patients with severe congenital neutropenia (SCN) and LON after rituximab treatment. Thus, maturation arrest at the (pro)myelocyte stage of granulopoiesis is a characteristic of SCN and it is related to mutations in *HAX-1* and *ELANE* genes [21, 22]. This has been attributed to excessive apoptosis of neutrophil progenitors in patients with *HAX-1* mutations [23]. Likewise, mutations in the *ELANE* gene results in the production of a mutant protein, neutrophil

elastase, with abnormal packaging in the neutrophils primary granules. This leads to apoptosis and unfolded protein response, resulting in the diminishing of neutrophil progenitors in the bone marrow (BM) [24].

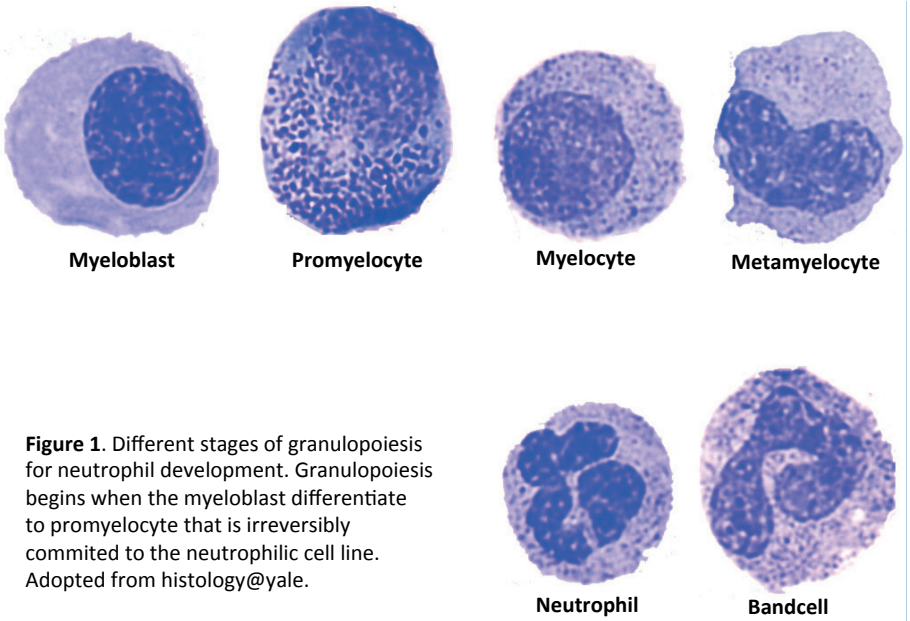


Figure 1. Different stages of granulopoiesis for neutrophil development. Granulopoiesis begins when the myeloblast differentiate to promyelocyte that is irreversibly commited to the neutrophilic cell line. Adopted from histology@yale.

1.2.2 FUNCTION

Neutrophils are the essential part of the innate immune system, also called the first line of defense. They are immediately recruited to the site of inflammation. They have an average half-life of 5 days and survive for 1-2 days after they have migrated into tissues. They directly attack microbes through phagocytosis and release of soluble anti-microbial substances (granule proteins, e.g. neutrophil elastase). Recently, Brinkmann and colleagues reported a new mechanism for killing of microbes by neutrophils. Activated neutrophils release web-like structure of DNA, called neutrophil extracellular traps consisting of web of fibers made of

chromatin and serine proteases that trap and kill microbes extracellular [25]. Moreover, neutrophils produce chemotactic signals that recruit monocytes and dendritic cells important for antigen presenting of adaptive immunity.

Recently, neutrophils have been shown to play a major role in adaptive and cellular immunity. Briefly, activated neutrophils are major sources of B-cell activating factor (BAFF), also called B-lymphocyte stimulator (BLYS) [26]. BAFF is needed for proliferation and maturation of B-lymphocytes [27]. In addition, neutrophils produce interferon-γ which helps to drive differentiation of T-cells and activation of macrophages [28]. Moreover, a recent report demonstrated that neutrophils stimulate immunoglobulin diversification and production [29]. Hence, neutrophils help not only in initiating of the immunological memory but also, assist in the process of the specific immunity.

1.3 RITUXIMAB

1.3.1 MECHANISM

Rituximab is an IgG1 chimeric human/mouse monoclonal antibody (mAb) which targets the CD20 antigen, a transmembrane phosphorylated protein located on normal and neoplastic B-lymphocytes. Rituximab consists of glycosylated human kappa and gamma-1 constant regions (Fc domain) and IgG1 kappa immunoglobulin with murine light- and heavy-chain variable regions (Fab domain) that recognizes the CD20 antigen.

CD20 expression begins at the pre-B cell stage (before IgM expression) and is lost prior to differentiation into immunoglobulin-secreting plasma cells [30]. Thus, CD20 is not expressed on hematopoietic progenitor cells or on mature, antibody secreting plasma cells [31]. Although the exact physiological role remains unclear, it is believed play a role in Ca^{2+} influx across plasma membranes, maintaining intracellular Ca^{2+} concentrations and allowing B-lymphocytes activation [32]. Mice lacking CD20 display no distinguishing phenotype [33]. The CD20 antigen has characteristics that render it a suitable target for treatment: CD20 does not circulate freely in the plasma, CD20 does not shed from the surface of B-cells after binding of anti- CD20 antibodies, and CD20 does not internalize or modulate upon antibody binding [34, 35].

As a result, rituximab clusters densely and persistently close to the cell surface facilitating mechanisms for antibody and complement binding. After binding, its Fc part binds to Fc gamma receptors on natural killer, granulocytes or macrophages (the effector cells). This ligation induces cell activation, leads to the release of cytotoxic substances, proteases and reactive oxygen species, conferring cell death of targeted B-lymphocytes. This causes a rapid depletion of normal and malignant B-lymphocytes, making it attractive for treating diseases characterized by having overactive production or dysfunctional B-lymphocytes. However, mechanisms actions of rituximab are not fully understood [36].

Mechanisms of actions of rituximab may occur by antibody-dependent cellular cytotoxicity (ADCC), complement-dependent cytotoxicity (CDC) and direct signaling (apoptosis). All appear to play a role in rituximab efficacy and most agree on ADCC to be the most important one. However, studies of rituximab mechanisms of action have been challenging and they are derived mainly from studies in vivo and in vitro lymphocytes, and animal lymphoma models. Rituximab has long half-time life and is present at therapeutic levels in the circulation of patients for up to a month. Moreover, lymphocytes have short life in vivo. Therefore, most studies of rituximab mechanisms of action often utilize tumor cell lines that have been selected based on their ability to grow rapidly in vitro, and sometimes their relative sensitivity to therapy. Most in vitro assays involve incubation times of minutes (analysis of direct signaling effects of rituximab) to hours (cytotoxicity assays), but never weeks—the time frame of clinical response to rituximab. Thus, in vitro assays usually focus on one mechanism. Indeed, animal models reflect the clinical situation although they significantly differ from clinical lymphoma with respect to growth kinetics, phenotype, infiltrating benign cells, and heterogeneity. Hence, clinical trials and correlative studies have been valuable to understand mechanisms of actions of rituximab.

Antibody-dependent cellular cytotoxicity antibody-coated lymphocytes may be killed by effector cells, including natural killer (NK) cells, granulocytes, and macrophages expressing Fc gamma receptors (FcγRs) through ADCC mechanism [36, 37]. These processes require that the Fc of the antibody bound to the target cell bind to FcγRs on the effector cells triggering immune cell activation and death of the target cell [38]. In vitro, animal model and correlative clinical studies suggest that interaction of antibody Fc with *FCGR3A* contributes

to the clinical anti-tumor activity of single-agent rituximab. Clynes and colleagues, demonstrated rituximab was effective in wild-type mice but not in mice lacking the common FcRγ chain [39]. Furthermore, the anti-tumor effect of mAb therapy was enhanced in mice lacking FcγRIIb, which is an inhibitory receptor, providing additional evidence that the interactions between the antibody and FcR is central to determining the efficacy of therapy [40]. There is also a strong evidence that ADCC is mechanistically involved in clinical response to rituximab therapy from correlative studies demonstrating an association between *polymorphisms* on *FCGR3A* and clinical response to rituximab in both NHL [41, 42] and autoimmune diseases [43]. *FCGR3A* homozygous for valine at 176 V/V (also called 158 V/V) has a higher affinity for IgG1 leading to a better binding of rituximab and more profound B-lymphocyte depletion than does *FCGR3A* with phenylalanine at that position (V/F or F/F) [44-49]. These data highlight the importance of Fc–FcγR interactions in the anti-tumor effects of rituximab and suggest that ADCC is a major mechanism of action.

Complement-Dependent Cytotoxicity (CDC) binding of rituximab to CD20 may activate the complement cascade through C1q, leading to cell death or deposition of complement proteins on the cell membrane, a phenomenon known as CDC. Several in vitro studies have demonstrated that rituximab is highly efficient at mediating CMC of various B-cell lines, as well as fresh malignant B-cell samples and in serum samples. The expression of complement inhibitory molecules (CD55 and CD59) on malignant B cells correlates with the extent of in vitro lysis [50-55]. A number of in vivo tumor models suggested that CDC plays a role in the anti-tumor effect of rituximab, e.g. depletion of complement through use of cobra venom factor abolished the therapeutic response [56, 57]. Recently, clinical studies have demonstrated lymphocytes that remain after rituximab treatment have a higher surface expression of the complement inhibitor CD59 when compared to pre-therapy expression of this marker [58, 59]. However, no correlation has been found between expression of complement inhibitors and clinical response to rituximab treatment [55].

Signaling-Induced Cell Death antibody binding to CD20 may have direct anti-proliferative effects or may actively induce cell death (apoptosis). In the absence of immune effector mechanisms, rituximab can induce death of malignant B cell lines in vitro. Thus, changes that have been identified in response to rituximab in vitro include inhibition of p38 mitogen-activated protein kinase, nuclear factor-κB, extracellular signal-regulated kinase 1/2, and AKT anti-apoptotic survival pathways [60-63]. Interestingly, rituximab injected directly into the cerebrospinal fluid in patients with central nervous system (CNS) lymphoma has been

reported to have local anti-lymphoma effects [64]. Likewise, synergy between rituximab and cytotoxic chemotherapy has been demonstrated in vitro study [65]. Moreover, clinical data in a variety of B-cell malignancies provide strong evidence that rituximab and chemotherapy can work well together and are more effective.

The discussion above addresses the major mechanisms of action of rituximab independently. However, different mechanisms may interact to each other synergistic (*interacting mechanisms*). Several studies point to more than one mechanism play a role for response of the therapy including a decrease in complement inhibitory molecules, enhanced expression of anti-apoptotic molecules and enhanced antibody binding by ADCC mechanism [66-67].

1.3.2 PHARMACOKINETICS AND PHARMACODYNAMICS

The pharmacokinetics of rituximab is similar to that seen with human IgG [3]. Rituximab distributes slowly in both the intravascular and extravascular compartments, and it is present within the involved lymph nodes environment that include not only malignant B cells but also stromal cells, benign lymphocytes, extracellular matrix, vasculature, proteins in the extravascular fluid, and a complex mixture of cytokines and chemokines. As a single agent, rituximab is usually administered parenteral weekly for 4 weeks in lymphoma patients or once every two weeks or weekly in patients with autoimmune diseases. When used in combination with chemotherapy, it is often administered every 3 to 4 weeks. Oral administration is precluded by the molecular size, hydrophilicity and gastric degradation of rituximab. Distribution into tissue is slow because of the molecular size of rituximab, and volumes of distribution are generally low. Serum half-life time is 20 days [68, 69]. Then, it is metabolized to peptides and amino acids in several tissues, by circulating phagocytic cells or by their target antigen-containing cells.

Population pharmacokinetic analyses have been applied in assessing covariates in the disposition of rituximab. Possible factors influencing elimination of rituximab include the amount of the target antigen, immune reactions to the antibody and patient demographics. Bodyweight and/or body surface area are generally related to clearance of rituximab, but clinical relevance is often low. However, dose-finding studies are limited and the actual choice of dosage/schedule is not based on optimal anti-tumor activity. Thus, the optimal dose

regimen is not established. Whether given weekly or monthly in all investigated dose regimen (375 mg/m^2, 500 mg/m^2, 1000 mg every two weeks and 500 mg or 100 mg weekly), rituximab is present at therapeutic levels in the circulation of patients for weeks. Although, the pharmacokinetic pattern is not well studied, dose dependent increases in serum concentration are reported [3]. The mean serum half-life of rituximab often increases when repeated cycles are given. This suggests that the reduction in CD20 positive tumor mass demands a lower dose rituximab [68].

Metabolic drug-drug interactions are rare. This was demonstrated by the achievement of a similar dose intensity and toxicity of the CHOP component in the CHOP arm and the R-CHOP arm suggesting that rituximab exerts no influence on the pharmacokinetics of the CHOP drugs. Furthermore, rituximab does not affect any of the cytochrome P450 enzymes responsible for metabolizing cyclophosphamide, doxorubicin, or vincristine or their excretion pathways [69]. Whether the same holds true for rheumatic patients with concomitant immunosuppressive treatment remains to be determined.

The immediate toxic side effects of rituximab are characterized by acute allergic and cytokine associated reactions. They are common and manageable. Complement activation was recently found to play a role in antibody-induced infusion toxicity in both animal models and patients. Use of antibodies modified to have a reduced ability to fix complement induced fewer infusion reactions. This reduction in infusion reaction had little effect on anti-tumor activity [70]. However, the immediate toxicity of rituximab still compares favourably with most cytostatic agents. There is a strong evidence for its efficacy in NHL patients. In an autoimmune setting, repeated doses are needed. Although few autoimmune patients achieve long-term remission, it has been favored compared to long-term corticosteroid and immunosuppressive treatments.

1.3.3 RITUXIMAB USAGE

Rituximab was originally developed for treatment of lymphomas as a targeted therapy against CD20 positive non-Hodgkin lymphomas (NHL) [3, 4]. Subsequently, because of the rapid depletion of autoantibody producing B-lymphocytes and following a resolution of joint inflammation in a patient with lymphoma treated with rituximab, its use was proposed in rheumatoid arthritis (RA) [71]. Since then rituximab has been successfully used in a variety of

autoimmune diseases [72], e.g. systemic lupus erythematosus (SLE) and anti-neutrophil cytoplasmic antibody associated vasculitis (AAV).

Rituximab use in NHL Rituximab was the first antibody approved by the US Food and Drug Administration (FDA) for use in the treatment of lymphoma. In 1997, it was approved by FDA for the treatment of relapsed or refractory, low-grade or follicular lymphomas. The recognition that rituximab could have a substantial therapeutic effect in cases of relapsed, indolent NHL opened a new era of monoclonal antibody therapy for cancer [3, 4]. Since then, it has become a backbone in the therapy of NHL including diffuse large B-cell lymphoma, follicular lymphoma, mantel cell lymphoma, chronic lymphatic leukemia (B-CLL), marginal zone lymphoma and Mb Waldenström. It has been investigated as either a single agent or in combination with standard chemotherapy regimens for lymphoma, such as CHOP (immunochemotherapy). It is also used in vivo purging before SCT and for maintenance therapy after remission [4, 69, 73-78].

Rituximab use in rheumatic diseases Rituximab is FDA approved for the treatment of moderate to severe RA not responsive to TNF antagonists [79, 80]. Most recently, it is also approved for two forms of AAV, granulomatosis with polyangiitis (GPA) (formerly known as Wegners granulomatosis), and microscopc polyangiit (MPA) [16]. In patients with refractory SLE, randomized placebo-controlled trials [81-83] failed to show a significance response rate for the rituximab arm. However, open-label studies have shown benefit in the use of rituximab [84-86]. Rituximab is regarded as the rescue treatment for severe or refractory manifestations, but it is used mostly on empirical basis in the rheumatic diseases. And hence, the number of patients receiving rituximab is increasing largely. Rituximab is often used repeatedly in part due to the early negligible effects of rituximab on the hematological parameters. However, the indirect consequences of repeated B-lymphocyte depletion on the remaining humoral and cellular immunity are not well investigated. Moreover, reports of unexpected and unusual late complications are appearing, highlighting consequences of the disturbance of this balance [87-91].

1.4 **OTHER LATE-ADVERSE EFFECTS OF RITUXIMAB**

1.4.1 THE EFFECT OF RITUXIMAB ON IMMUNITY

Rituximab has direct effects on CD20+ B-lymphocytes and depletes B-lymphocytes from the peripheral blood (PB), bone marrow and lymph nodes, killing tumor cells and presumably disrupting pathological production of autoantibodies. After depletion, B-lymphocytes return to the peripheral blood at a mean of 8 months [81]. The repopulation of the B-lymphocyte starts by the appearance of immature (CD38++, CD10+, CD24+), followed by naïve (CD27) B-lymphocytes, while CD27+ memory B-lymphocytes may remain reduced for up to 2 years [92-93]. CD20 expression begins in the late pre-B cell phase and rituximab interrupts the generation of plasmablasts from memory B cells [94] which may interfere with the survival of long-lived, CD20+ plasma cells in secondary lymphoid tissue [95]. Moreover, rituximab response in patients with multiple sclerosis (previously considered T-cell mediated autoimmune disease) and clinical response in immune thrombocytopenic purpura patients with out anti-platelet antibodies, suggest that B-lymphocytes may not be the only target of rituximab therapy.

Several studies have also suggested that B-lymphocytes have indirect effects on cellular immunity by activating *T-lymphocytes* through antigen presentation. An animal model exploring the immunological consequences of B-lymphocyte depletion showed impairment in CD4 T-lymphocyte and clonal expansion in response to protein antigens or pathogens, but no direct effect on T-lymphocyte subsets or activation status, and CD8 T-lymphocyte activation. In this model the combination of B-lymphocytes and dendritic cells were required for optimal antigen-specific CD4 T cell priming [96]. Likewise, there are reports on T-lymphocyte changes, including increase in regulatory and activated T-lymphocytes, and large granular T-lymphocytes (T-LGL) [97-98]. Moreover, removal of the CD20 B-lymphocyte pool in autoimmune diseases indirectly causes the normalization of T-helper cell type 1 and 2 ratio, and increase in regulatory T-lymphocytes [99]. In another animal study, one could demonstrate a regulatory role of B-lymphocytes by producing interleukin 10 and B-lymphocyte depletion could prevent autoimmune relapse through the expansion of regulatory T and B-lymphocytes [100].

Hypogammaglobulinaemia has been reported in several studies, mainly in patients who are treated with intensive chemotherapy, SCT or with increasing courses of rituximab needed in relapsing autoimmune patients, probably reflecting bone marrow depression and the depth of B-lymphocyte depletion [101-105]. In support of this notion, very few cases of hypogammaglobulinaemia have been reported in patients treated with one course of rituximab [106]. Most hypogammaglobulinaemia is contributed by a significant fall of IgM levels, in contrast to slight decrease in IgG and IgA [107]. Consequences of these alterations such as infections remain to be determined.

Recent studies show an increase incidence of *infections* in rituximab treated patients. On the contrary, a systematic review of rituximab in cancer patients did not show an increased risk of infection [108]. However, an evaluation in a randomized trial in follicular lymphoma patients on rituximab maintenance showed a significant increase in rate of infections [109]. Likewise, in a randomized trial of patients with RA, more serious infections occurred in the rituximab group compared with controls [110-111]. Similarly, in patients with HIV-lymphoma, rituximab may be associated with an increased risk of bacterial and opportunistic infections [112]. Recently, progressive multifocal leukoencephalopathy (PML) a rare but potential life-threatening side-effect of rituximab with 90% fatality rate has been reported. It is caused by a reactivation of a latent JC virus, present in 80% of adults which may disseminate during compromised cellular immunity [113]. However, casual relationship between rituximab and PL is not established. Hepatitis-B virus (HBV) reactivation is rather a well-known complication of rituximab therapy due to the disturbance of immune surveillance by B-lymphocyte depletion [114]. However, the limiting susceptibility is not known yet. A recent report suggested that hypo-Ig-emia prior to rituximab treatment was a risk factor for the development of severe infections in RA patients [115]. Finally, infectious complications will require long-term follow up data to be determined. Nevertheless, vigilance is warranted.

1.4.2 THE EFFECT OF RITUXIMAB ON HEMATOPOIESIS

The early effects of rituximab on hematological parameters are often negligible. Anemia and thrombocytopenia are uncommon. Few cases with severe thrombocytopenia have been

reported in patients who developed infusion related toxicity, suggesting cytokine-associated mechanisms [116-117]. However, neutropenia following rituximab has been more extensively reported. It is frequently of late onset character (> 4 weeks after treatment). Although, LON can occur when rituximab is used alone, it is more common when rituximab is used in a combination with chemotherapy or SCT [7]. The phenomenon of maturation arrest at the (pro)myelocyte stage of granulopoiesis in the BM of LON patients [12] have highlighted that B-lymphocytes may not be the only target of rituximab therapy but also indirectly affect granulocytes.

A recent case report of LON patient treated with rituximab, showed extraordinary high-levels of serum BAFF during neutropenia period [118]. BAFF is, a member of TNF superfamily, produced and secreted mainly by myeloid cells (macrophages, monocytes and neutrophils) [27]. The biological role of BAFF is mediated by three specific receptors, two high affinity receptors, namely BAFF receptor (BAFF-R) and transmembrane activator-calcium interacting ligand, a low affinity receptor, B-cell maturation antigen. Binding to one the receptors gives BAFF different functions and they are found on B-lymphocytes, effector T-cells, plasma cells and plasmablasts [100].BAFF-R is expressed by all peripheral B-lymphocytes and it is a potent regulator of B-lymphocyte survival [119, 120]. BAFF-deficient mice exhibit defects in peripheral B-lymphocyte maturation and decreased levels of immunoglobulins [119]. Another animal model demonstrated that overexpression of BAFF leads to hyperplasia, lymphoproliferation, hyper-gammaglobulinemia and symptoms of autoimmunity [122]. Increased levels of BAFF have also been correlated with different types of autoimmune diseases [123]. On the other hand, lymphocytes produce numerous cytokines needed for granulocytes proliferation and differentiation suggesting the interdependence of each other. The intricate balance of lymphopoiesis and granulopoiesis governed by a complex cytokine balance in the BM environment may be hampered by rituximab [124]. Thus, causes and consequences of this disturbance such as late-onset neutropenia is the aim of our study and it will be discussed in detail in this thesis.

2. AIMS OF THE STUDY

2.1 GENERAL AIMS

To study incidence, mechanism, predisposing factors and clinical consequences of LON in lymphoma and rheumatic patients

2.2 SPECIFIC AIMS

-To study incidence, risk factors and clinical features of LON in lymphoma patients (Paper I)

-To study incidence, risk factors and clinical features of LON in rheumatic patients (Paper II)

-To elucidate the role of FCGR and BAFF promoter polymorphisms for the development of LON and clinical outcome in relation to genotypes and LON (Paper III)

-To understand mechanisms of LON in relation to BAFF production in lymphopoiesis and granulopoiesis (Paper IV)

3. PATIENTS AND METOHDS

In this section materials and methods will be briefly summarized. Detailed descriptions are found in the "Materials and Methods" section of each paper. All studies were approved by the Ethics committee at Karolinska Institutet Stockholm, Sweden, and were performed in accordance with the Helsinki declaration.

3.1 PATIENTS

3.1.1 RETROSPECTIVE COHORT OF NHL PATIENTS (Paper I)

We reviewed the medical records of all 113 consecutive lymphoma patients treated with rituximab alone or combined with chemotherapy or SCT during the period from July 2002 to June 2004 to identify periods of LON. All patients were treated at the Hematology Center at the Karolinska University Hospital, Huddinge. All patients with LON were detected at routine follow-up or emergency visit due to neutropenia. This cohort is used in Paper I. Details over NHL diagnoses and study design is given in this Paper.

3.1.2 RETROSPECTIVE COHORT OF RHEUMATIC PATIENTS (Paper II and Paper III)

The medical records of all 214 consecutive adult patients treated with rituximab for rheumatic diseases from June 2003 through March 2009 at the Department of Rheumatology at Karolinska University Hospital, Huddinge were reviewed to identify LON patients. Medical records were also reviewed for 2 years before start of rituximab treatment, in order to determine if the included patients had a previous history or other identifiable cause of neutropenia. Thus, patients with propensity for developing neutropenia as a consequence of autoimmune disease or prior therapy were excluded. This cohort is used in Paper II and III. Flow chart of the study population is given each Paper. For the purpose of this study, a control group was established after the detection of LON patients from the same rituximab cohort in order to control for confounding factors of neutropenia. Two control patients were chosen for each case of late-onset neutropenia (Paper II). Where as, in Paper III we expanded our control group to 50 patients i.e. 5 controls to 1 LON case (genotype and clinical outcome study).

3.1.3 PROSPECTIVE COHORT OF NHL PATIENTS (Paper IV)

We included prospectively 174 consecutive NHL adult patients, treated with rituximab during the period of April 2009 until March 2011 at the Hematology Center, Karolinska University Hospital, Huddinge. Patients were treated and followed-up according to standard care protocol at the discretion of the treating physician. BM and blood samples were collected at the detection of LON. For the purpose of this mechanistic study, we have included LON patients with ANC $\leq 0.5 \times 10^9$/L, only. Flow chart of patients included and excluded from the study, and their diagnosis is given in Figure 1, Paper IV. A control group including 2 controls for each LON case was established from the same rituximab cohort at the time of detection of LON patients.

3.2 METHODS

3.2.1 MORPHOLOGICAL EVALAUATIONS

Morphological evaluations were performed on BM and PB. Morphological evaluations included all hematopoietic series: erythrocytes, megakaryocytes, lymphocytes, plasma cells, monocytes and all stages of granulopoiesis (myeloblasts, promyelocytes, myelocytes/ metamyelocytes, band cells and segmented granulocytes). Differential counts were performed on BM smears. And then, granulopoiesis and erythropoiesis (GE) ratio was calculated; the normal value is 2:1-5:1 (Paper I and IV).

The *maturation index* (MI) of the granulopoiesis was also calculated according to the formula (myeloblasts + promyelocytes + myelocytes/ metamyelocytes + band cells and segmented granulocytes); the normal value is 1:3-1:5 (Paper I and IV).

3.2.2 *HAX-1* MUTATIONS

Sequencing of HAX-1 gene was performed on DNA extracted from PB cells, according to the procedures reported by Klein and colleagues (Paper I) [125].

3.2.3 FLOW CYTOMETRY

Flow cytometry were performed by using three-color fluorescence (Paper II) and by four or eight-color fluorescence (Paper IV) immunophenotyping. This was done according to standard procedures at the Departments of Pathology and of Laboratory Medicine, Division of Clinical Immunology and Transfusion Medicine, Karolinska University Hospital. Monoclonal

antibodies used for analysis of B-, T-, NK- and myeloid cells are given in Paper II and IV. Additional details are given in each Paper. All samples were analyzed by setting appropriate side and forward scatter gates. For the cell subpopulation analysis, a minimum of 10 000 events were collected in the gate. Results were reported as the percentage of cells positive for each marker. For PB flow cytometry analysis, the numbers of cells were calculated from the percent of these cells in the gate, the percent of specified cells and the complete blood counts. B-lymphocyte depletion is divided as follows: *complete B-lymphocyte depletion* is defined as B-lymphocyte count $<0.01 \times 10^9$/L. B-lymphocyte counts between 0.01 and 0.09 x 10^9/L were classified as "under depletion" and $>0.09 \times 10^9$/L as "recovered", respectively.

3.2.4 ANTI-NEUTROPHIL ANTIBODIES

Tests for anti-neutrophil antibodies were performed at the Department of Laboratory Medicine, Division of Clinical Immunology and Transfusion Medicine, Karolinska University Hospital, Huddinge. The initial assay included granulocyte agglutination test (GAT) and granulocyte immunofluorescence test (GIFT). Complementary analysis by monoclonal antibody-specific immobilization of granulocyte antigen (MAIGA) was used in positive cases to characterize the specificity and to rule out false positivity.

3.2.5 GENOTYPING

Genomic DNA was isolated from peripheral blood mononuclear cells using QIAamp® DNA mini kit, according to the recommendations of the manufacturer (Qiagen, Hilden, Germany). *FCGR* genotyping of the single nucleotide polymorphisms (SNPs) in the *FCGR3A* 176 V/F and *FCGR2A* 131 H/R gene was performed with allelic discrimination using two Taqman assays. *FCGR2B* 232 I/T genotyping was performed using oligonucleotide probing based on fluorescence resonance energy transfer technology. *BAFF* promoter genotyping was performed by restriction fragment length polymorphism analysis. Details are given in Paper III. All genotyping results were in consistent with Hardy-Weinberg equilibrium and genotyping efficiency were validated (Haploview v.4.1 software). All samples were run in du-triplicates and non-working samples were also rerun in du-triplicates.

3.2.6 ENZYME-LINKED IMMUNOSORBENT ASSAY (ELISA)

Serum levels of human BAFF, APRIL (a proliferation-inducing ligand) and G-CSF were determined by ELISA using Quantikine BAFF, APRIL and G-CSF immunoassay (R&D Systems Europe). Analysis were done according to the recommendations of the manufacturer. Plasma levels of human SDF-1 (stromal-derived factor-1) were determined by using Quantikine SDF-1 immunoassay (R&D Systems Europe) according to the recommendations of the manufacturer. All assays specificity and reproducibility were ascertained by the manufacturer. All samples were analyzed in duplicates.

3.2.7 STATSTICAL ANALYSIS

Values are given as mean ± SD (standard deviation) or median (and interquartile range, IQR) depending on value distributions. The chi-squared, 2-sided Fisher exact t-tests, Mann-Whitney U-test and Spearmans correlation analysis were used for comparisons as suitable. Wilcoxon's matched pairs/signed rank test was performed to test within-group changes at different time-points. Associations between gene polymorphisms, LON and clinical outcomes were analyzed by logistic binary or ordinal regression or Spearman's correlation analysis. The Kaplan-Meier method were used to display the cumulative probability of remaining flare-free time at 12 months with log-rank test to assess between-group differences.

All tests were 2-sided and P values less than 0.05 were considered significant.

4. RESULTS AND DISCUSSIONS

4.1 INCIDENCE (Papers I, II and IV)

The incidence of LON is studied retrospectively in Paper I and Paper II. In Paper I, we reviewed the medical records of all 113 consecutive NHL patients treated with rituximab alone or combined with chemotherapy or SCT, described in details in methods and patients. LON was diagnosed in 8 (7%) of the patients. LON was defined here in as an unexplained ANC < 1.5 x 10^9/L corresponding to neutropenia of grade 2-4 according to NCICT criteria starting from 4 weeks after end of rituximab therapy. The follow-up period for detection of LON is 9 months. Thus, alternative causes of neutropenia were ruled out by a thorough review of medical history including concurrent drug intake. The characteristics and diagnoses of these patients are given in Table 1-Paper I. All LON patients presented at routine follow-up or at emergency visit. Although, this study was not powered enough to identify incidence differences between diagnoses and treatments, we observed a higher incidence of LON (20%) among patients who underwent SCT compared to those treated with standard chemotherapy alone.

The occurrence of LON in rituximab treated autoimmune patients was suggested only in a few case reports when we performed the study of LON rheumatic patients (Paper II). In this paper, we evaluated the medical records of all 214 consecutive patients treated with rituximab for rheumatic diseases; details are given in Methods and Patients. Here, we have used the same definition of LON as in Paper I but we extended the follow-up period to 12 months since the reported mean B-lymphocyte recovery times was reported to range up to 12 months [126]. Indeed, we have observed a case of LON with a neutropenia episode at 295 days after rituximab treatment in Paper I-Table 1. Alternative causes of neutropenia were ruled out by a thorough medical review. For instance, tests were performed also of for the presence of anti-neutrophil antibodies. Moreover, medical records were reviewed for 2 years before the start of rituximab in order to determine if the included patients had a previous history of neutropenia as a consequence of the autoimmune disorder or prior concomitant immune suppressive treatment. Thus, 5 patients were excluded from the analysis due to possible alternative causes of neutropenia (Figure 1-Paper II). In the end, 209 patients remained for LON analysis. LON was diagnosed in 11 (5%) of the patients depending on diagnosis (3-23%). A higher incidence of LON was observed in SLE and GPA patients (23% and 20%, respectively) than RA patients (3%).

For the purpose of this study (Paper II), the cohort was divided in to 2 consecutive groups (group A and B), according to frequency of blood sample collection and flow cytometry follow-up. In group A (N=99), complete blood cell counts (CBCs) were performed once a month and flow cytometry of PB was done every 3 months (June 2003-December 2007). In group B (N=110), only CBCs were performed (at most) every 3 months (January 2008-March 2009). A higher incidence of LON was evident in group A (n=9) (9%) than group B (n=2) (2%). However, higher numbers of SLE and GPA patients were included in group A than in group B. The observation that GPA and SLE patients appeared to have a higher incidence of LON was intriguing. However, the numbers of SLE and GPA patients were too low to draw a conclusion as to a specific disease-drug relationship. Nevertheless, the more often the CBCs were obtained, the higher was the LON incidence.

In Paper IV, a prospective mechanistic study of 174 consecutive NHL adult patients (described in detail in Methods and Patients), we included only patients with ANC < 0.5 x 10^9/L as it was not aimed to study the incidence of LON. All patients were included at start of rituximab treatment and followed-up at least 12 months. Nevertheless, we found 14 (8%) of LON with agranulocytosis after we ruled out alternative causes of neutropenia by a medical history, physical examination and a thorough clinical investigation. A higher incidence of LON was also observed in patients after SCT (57%) compared to those treated with standard chemotherapy, confirming our previous result of a higher LON incidence in SCT group. Finally, our incidence reports are probably an underestimation, as CBCs were not collected on regular basis and patients with a shorter period of neutropenia might be missed.

4.2 TIME TO ONSET AND DURATION (Papers I, II and IV)

The median duration of LON was 54 days (range, 10-120 days) in our retrospective lymphoma study (Paper I, Table 1). On the contrary, we noted a shorter duration of LON in our prospective NHL analyses (Paper IV, Table 2), i.e. 15 days (range, 7-33 days). However, we included only patients with severe neutropenia for this mechanistic study and majority of patients needed G-CSF treatment. Likewise, the median duration of neutropenia in Paper II was 9 days (range, 4-20) (Paper II, Table 1). Similarly, the majority of LON cases in this rituximab treated rheumatic patients were also treated with G-CSF. Thus, sustained neutropenia was observed in patients who were not receiving G-CSF. It is not known how

much G-CSF treatment shortens the period of agranulocytosis in this set of patients, who otherwise, due to more profound neutropenia, is expected to have a longer duration of neutropenia than those with mild neutropenia.

The median time to onset of LON in our studies after the termination of rituximab treatment was 88 days (Paper I), 102 days (Paper II) and 96 days (Paper IV). Some LON cases had a recurrence of LON during the follow-up period or with retreatment of rituximab. However, they had similar clinical features as in the first LON episode. The cumulative incidence of late-onset neutropenia in relation to follow-up time is given in Figure 2A, Paper II. Interestingly, this coincides with the period of B-lymphocyte depletion, implying that LON occurs before B-lymphocyte recovery after rituximab therapy. The relationship between B-lymphocyte depletion and neutropenia is given in Figure 2D, Paper II.

4.3 RISK FACTORS AND CAUSES OF LON

4.3.1 TREATMENT RELATED FACTORS (Paper I, II and IV)

We observed a higher incidence of LON in lymphoma patients after SCT (Paper I and IV) implying that high cumulative doses of myelotoxic agents, such as those used in SCT, might be a risk factor for LON development. Likewise, we found a higher incidence of LON in GPA and SLE patients compared to RA patients (Paper III) and those patients had received more myelotoxic and immunosuppressive treatment compared to RA patients (Supplementary Table, Paper II). This is suggestive of that higher doses of myelotoxic agents could be a risk factor. But, we did not find a correlation to a single agent cumulative dose, e.g. the cumulative doses of methotrexate and rituximab (Papers II and IV).

4.3.2 B-LYMPHOCYTE DEPLETION (Paper II and IV)

LON occurred during the period of B-lymphocyte depletion and therefore we studied the course of B-lymphocyte depletion and recovery in detail in Paper II. This was a cohort and case-controlled retrospective study in rituximab-treated rheumatic patients. For this patient group under concomitant immune modulating treatment a matched control group with similar treatment pattern is used in order to control for confounding factors of neutropenia. Flow cytometry follow-up were performed on PB at baseline and 3, 6 and 12 months after rituximab treatment. We compared the levels of B-lymphocyte depletion between LON patients and non-LON matched controls. There was no significant difference in B-lymphocyte counts between LON and control non-LON patients at base line. All patients showed

complete depletion at 3 months after treatment (except for 2 non-LON matched controls). At 6 months, complete B-lymphocyte depletion persisted in all LON patients where as only 2 control patients exhibited complete depletion (p=0.002) (Figure 2B, Paper II). Interestingly, all LON cases with available complete flow cytometry results presented with neutropenia during the period of B-lymphocyte depletion. The relationship between LON and B-lymphocyte depletion over time is depicted in Figure 2D. Thus, the levels of B-lymphocyte depletion may help in identifying patients at risk for LON development.

In Paper IV, flow cytometry tests were performed in the BM. BM aspirates were collected during LON for LON patients and at the corresponding time of LON for non-LON matched controls. Ninety two% of LON patients developed LON during complete depletion. However, only 30% of controls displayed a complete depletion. Thus, there was a significant difference in the numbers of CD19+CD20+ B-lymphocytes between LON and non-LON controls (p=0.002). Similar to results in Paper II, we did not find significant differences in the numbers of subpopulations of T-cells. A direct relationship between B-lymphocyte depletion and LON could not be described in Paper II, since blood samples were not available during LON period. Here, in a cohort of NHL patients, we confirmed the observation holds true even during the LON period (Paper IV).

4.3.3 T-CELL SUBPOPULATIONS (Paper II and IV)

The numbers of T-cells ($CD3^+$) and NK cells ($CD56^+$, $CD3^-$) did not differ significantly between the LON or control groups (Paper II and IV). There were no significant differences in the numbers of subpopulations of T-cells (i.e. $CD4^+$ T-cells, $CD8^+$ T-cells) or for $CD4^+$ and $CD8^+$ ratios. Moreover, we did not observe significant differences in the numbers of T-LGL cells ($CD3^+$, $CD56^+$) between LON and control groups (Paper II and IV). In addition, none of the cases, LON or non-LON controls, exhibited a proliferation of T-LGL, as assessed morphologically (Paper IV).

4.3.4 GENOTYPES (Paper III)

We analyzed *FCGR* single nucleotide polymorphisms (*FCGR2A* 131H/R, *FCGR2B* 232I/T and *FCGR3A* 176V/F) and *BAFF* gene promoter polymorphism -871C/T, in Paper III, since the binding of rituximab to Fc gamma receptors (FcγR) bearing macrophages and natural killer cells is postulated to be an important step in the ADCC mechanism of a rapid depletion of $CD20^+$ B-lymphocytes. Certain single nucleotide polymorphisms (SNP) in the *FCGR* gene, particularly the *FCGR3A* 176 V/V genotype, enhance the ligation of rituximab to this receptor [127-128]. Moreover, Serum BAFF level raises before the return of B-lymphocytes to the PB after rituximab therapy and an enhanced generation of BAFF has been associated with the presence of a certain SNP of *BAFF* gene promoter (the -871 T/C genotype) [129-130].

Genotyping was performed on 11 LON patients and 50 non-LON controls, matched regarding diagnosis, and had similar age, gender and treatments. The *FCGR3A* 176V allele was significantly more often associated with LON compared to the *FCGR3A* 176 F allele (comparison in V/V vs V/F or F/F groups) (p=0.03) (Figure 2a, Paper III). The number of V alleles was significantly correlated to LON (r=0.42; p=0.01). We, then, determined how much each V allele affected the risk for LON development by logistic regression, and found that each additional V allele was associated with 4-fold increase in the odds of ratio for LON (p=0.017).

There were no significant associations between LON and the *FCGR2A* 131 H/R, *FCGR2B* 232 I/T and *BAFF* 871 I/T genotypes. However, there was a reciprocal relationship for LON versus non-LON groups in the distribution of the *FCGR2B* and the *FCGR3A* genotypes (Figure 2a and 2c, Paper III). Forty-five per cent of LON patients exhibited the *BAFF* 871 T/T genotype compared to 24% in the non-LON group (P=0.1) (Figure 2d, Paper III).

4.3.5 SERUM IMMUNOGLOBULIN LEVELS (Paper II and III)

Immunoglobulin levels were measured after rituximab treatment and compared between LON and non-LON group in Paper II and III. There were no significant differences in serum IgG, IgA and IgM levels at baseline. None of the patients had hypogammaglobulinemia at baseline. All patients showed a decrease in IgM levels after rituximab treatment. However, a significant difference in IgM levels between the LON and non-LON controls was detected at 6 months (P=0.027) (Paper II), probably, reflecting differences in the depth of B-lymphocyte depletion. Likewise, we found that LON patients had a more pronounced decrease in IgM levels during

the first follow-up year by using an extended non-LON control material in Paper III. However, there were no significant differences in the decreases of IgG and IgA levels between LON and controls. Thus, the levels of IgM may helpful in identifying patients at risk.

We related serum IgM changes to *FCGR* and *BAFF* genotypes since an enhanced binding of rituximab to FcγRs might confer more pronounced and/or prolonged IgM reduction and recovery of immunoglobulin production is partly governed by BAFF [27]. There was no significant difference for IgM levels in the *FCGR3A* V/V and V/F or F/F groups at baseline. However, patients with V/V genotype, compared to V/F or F/F, had a more pronounced decrease in IgM levels the first year, measured between the baseline and the lowest value. Thus, patients homozygous for high-affinity V-allele of *FCGR3A* had lower IgM levels over time than those with the low-affinity F-allele, independently of gender and previous treatment ($p=0.016$). There was no significant association between serum IgM and other tested *FCGR* and *BAFF* promoter polymorphisms. Moreover, there was no association between serum IgG levels at baseline or over time and LON occurrence or the gene polymorphisms studied here. Thus, high-affinity V-allele of *FCGR3A,* leading to an enhanced binding of rituximab to FcγRs, might confer more pronounced or prolonged IgM reductions as a result of deeper and sustained B-lymphocyte depletion.

4.3.6 ANTI-NEUTROPHIL ANTIBODIES (Paper II and IV)

We studied the role of anti-neutrophil antibodies for the development of LON in Paper II and IV by comparing LON and non-LON matched controls. Although few LON and non-LON rheumatic controls displayed positive agglutination and granulocyte immunofluorescence test, none of the patients with LON or matched controls displayed positivity on MAIGA test for specific antineutrophil antibodies. Moreover, there was no significance difference regarding the number of patients between these groups (Paper II). Furthermore, we did not find anti-neutrophil antibodies in LON or non-LON matched controls in our prospective lymphoma study (Paper IV). Thus, the development of LON in our studies was not associated with production of anti-neutrophil antibodies.

4.4 CLINICAL FEATURES AND IMPACT OF LON

4.4.1 INFECTIONS (Paper I, II and IV)

In the majority of cases of lymphoma patients in our studies, LON was self-limited and did not have serious infectious complications (Paper I and Paper IV). Patients with severe neutropenia needed G-CSF treatment and a few received antibiotics (Table 1, Paper I and Table 2, Paper IV). However, in our study of rheumatic patients (Table 1, Paper III), the majority of LON patients developed severe infections and received G-CSF. Thus, 7 of 11 patients were hospitalized because of infections, 6 of them with sepsis and 1 with febrile neutropenia, and all required intravenous antibiotics; 6 also received G-CSF. The majority of cases had concomitant immunosuppressive treatment. This was suggestive of an increased vulnerability in rheumatic patients to develop infections during LON period. Hence, the clinical course of LON in patients with concomitant immunosuppressive treatment seems to differ from that of lymphoma patients.

4.4.2 CLINICAL OUTCOME (Paper III)

Although LON was associated with considerable direct morbidity in rheumatic patients (Paper II) we asked if LON might confer later benefits with regard to disease remission duration, based on the assumption that LON might be a sign of a more pronounced rituximab effect i.e. a more pronounced B-lymphocyte depletion. We defined the time to flare of the rheumatic disease as the time period between the day of rituximab initiation and the recurrence of symptoms that warranted therapy escalation beyond a temporary increase in the glucocorticoid dosage. LON occurrence was positively correlated to a longer time to flare at 12 month assessment ($r= 0.27$, $p=0.043$). Also in logistic regression analyses, the presence of LON was associated with longer time to flare independently of gender and previous treatment at 12 month assessment (OR 0.10, $P=0.028$), but not at later time points. Thus, LON was associated with a short-term reduction in flares shown by Kaplan-Meier curve in Figure 4b, Paper III.

Having shown a relation between the *FCGR3A* V/V genotype and occurrence of LON, we asked if the here assessed *FCGR* and *BAFF* genotypes related to the time to flare after rituximab therapy. The results are presented in Figure 4c, Paper III. There was a significant positive correlation between the *FCGR3*A 176 V/V (vs. V/F or F/F), and a longer time to flare ($r=0.29$, $P=0.039$). Also in logistic regression, possession of the V-allele was negatively

associated with flare at 12-month assessment, OR 0.10, p=0.034, but not at later time-points. This relation is also represented by the Kaplan-Meier curve in Figure 4c, Paper III. The presence of LON and the possession of the *FCGR3*A 176 V allele were together stronger predictive factors for longer time to flare independently of gender and previous treatment, OR 0.20, p=0.021.

No association was found between other tested *FCGR* and *BAFF* promoter polymorphisms and clinical outcomes (Figure 4d, Paper III). Thus, the high affinity *FCGR3*A 176 V/V genotype, as well as the presence of LON, was related to a longer time to flare of the rheumatic disease.

4.4.3 MATURATION ARREST (Paper I and IV)

Maturation arrest at (pro)myelocyte stages of granulopoiesis is an interesting clinical feature of LON (Paper I and IV). Four additional LON patients with severe neutropenia in Paper I, where the morphology was evaluated during LON, showed a selective depletion of granulopoiesis with an inverted granulopoiesis-erytropoiesis (G/E) ratio. They exhibited maturation arrest at (pro)myelocyte stages of granulopoiesis (Figure 1, Paper I). We were intrigued by the similarity of this maturation arrest often seen in patients with SCN. We assumed these to be heterozygous carriers of e.g. *HAX-1* mutations as they had normal ANC values prior to lymphoma treatment and might be at risk of developing neutropenia as an idiosyncratic drug-reaction. And hence, we performed *HAX-1* mutation analysis. However, all four cases were homozygous for wild-type *HAX-1*. Moreover, BMs were performed upon neutrophil recovery and did not show any residual abnormalities.

We performed BM in all LON patients during LON period in case-controlled prospective NHL study (Paper IV). Likewise, maturation arrest at (pro)myelocyte stages of granulopoiesis was evident. All non-LON controls showed normal granulopoiesis and normal GE ratio except in 3 cases showing a tendency to GE inversion but this with consistent with a slight granulopoiesis suppression. Confirmatory flow cytometric comparisons between LON and controls was also done showing significant differences regarding numbers of myeloblasts and promyelocytes, (p=0.018) between LON and non-LON controls. Interestingly, the only

rheumatic LON patient with BM available showed also maturation arrest at (pro)myelocyte stages of granulopoiesis (Paper III). Thus, maturation arrest could be demonstrated in all LON patients depending on when BM examination was done. The occurrence of a maturation arrest is reminiscent of the findings in SCN, but the underlying molecular mechanism of this arrest in LON patients is unknown. Nonetheless, this observation suggests that profound B-lymphocyte depletion may affect selectively granulopoiesis.

4.5 B-CELL ACTIVATING FACTOR (BAFF) AND LON

4.5.1 BAFF AND B-LYMPHOCYTE DEPLETION (III and IV)

The pronounced B-lymphocyte depletion in LON patients during neutropenia and as well as regeneration of B-lymphocytes following a normalized ANC suggested that LON may be related to factors involved in the proliferation of B-lymphocytes, such as BAFF. BAFF, a cytokine mainly expressed by neutrophils and monocytes, plays a central role in the stimulation of B-lymphocyte proliferation, differentiation, immunoglobulin production and survival (Moore et al). Serum BAFF level increases following B-lymphocyte depletion after rituximab treatment and this rise precedes the return of B-lymphocytes to the PB [131].

Likewise, LON and non-LON controls showed increases of serum BAFF levels at 3 months and a decrease thereafter (Figure 3a, Paper III). However, few blood samples were available at baseline and at 3, 6 and 12 months follow-up. Nevertheless, since an association has been described previously between the *BAFF* -871 promotor T/T genotype and higher serum BAFF levels [129, 130], we analyzed such relationships. There was no significant association between serum BAFF levels (or changes in the levels from baseline) and the *BAFF* gene promoter -871 T/C polymorphism when LON and non-LON groups were compared. However, in the LON and non-LON group, we observed a trend-wise positive association between the T-allele (compared to C) and an increase of serum BAFF levels between 0 and 3 months ($r=0.27$; $p=0.073$). Blood samples were not available during LON period in this study for a correlative study of LON and BAFF.

In Paper IV, analysis was done on samples taken during and after the LON period for LON patients. Samples were analyzed from all 14 LON and 26 corresponding matched controls on PB. However, for non-LON matched controls, a sample was taken at the corresponding time of LON. Non-LON controls do not show any abnormalities in granulopoiesis and have normal granulocyte numbers, and we therefore assumed that cytokine levels should be comparable to

values seen after the LON period i.e. when LON patients normalized in ANC. In addition to BAFF, we have also analyzed other cytokines (APRIL, SDF-1 and G-CSF) involved in proliferation and hemostasis of lymphopoiesis and granulopoiesis (Paper IV). Serum BAFF values increased significantly in all LON patients (except one) during the LON period ($p=0.006$) and decreased after the LON period and reached the values measured for non-LON matched controls at a corresponding time for LON. Thus, serum values after the LON period and in matched controls were similar ($p=0.096$). However, there was significant difference between BAFF values during the LON period and controls (NHL patients with no LON) ($p=0.023$), as shown in Figure 2, Paper IV.

We did not observe significance differences regarding serum values for APRIL, SDF-1 and G- CSF between LON and controls or between values during and after LON for LON patients.

4.5.2 BAFF IN RELATION TO NEUTROPENIA

Our observation of a correlation between an increase in serum BAFF levels and neutropenia suggested that the reduction in granulocyte counts might be related to the levels of BAFF in serum. BAFF is produced by monocytes and neutrophils [26, 27] but our LON patients experienced severe neutropenia and a lack of granulopoiesis and we therefore turned our attention to other BAFF producing cells. Interestingly, we noted a significant increase in the number of monocytes in LON patients coinciding with neutropenia supporting the notion that this is a compensatory mechanism to fulfill the need of increased BAFF production to promote the proliferation of B-lymphocytes (Paper IV). Hence, we compared changes (Δ) in serum BAFF levels between the levels measured during and after the LON period and the lowest ANC reached during the LON period. We found that (Δ) BAFF levels, i.e. the increase in BAFF levels, were inversely correlated to the lowest ANC during the LON period ($R=-0.818$, $P=0.01$).

5. GENERAL DISCUSSIONS

5.1 INCIDENCE, TIME TO ONSET AND DURATION

It is difficult to determine the true incidence of LON. Most incidence reports are derived from studies on rituximab treated lymphoma patients and there are few reports from an autoimmune setting. Incidence reports are confounded by differences in inclusion criteria, i.e. time to neutropenia after rituximab treatment (varies from 1 to 2 months), grades of neutropenia (ranges from grade II to grade IV, i.e, ANC between 0.1 and 1.5 x 10^9/L), and follow-up after rituximab treatment (varying from 6 to 12 months). Moreover, most studies are of retrospective nature without appropriate controls. Thus, the incidence reports of LON in lymphoma patients range from 5.6% to 27.3% [6-13]. This is summarized in Table 1. Indeed, other studies have reported a much higher incidence of LON, but these studies lacked a clear definition of LON and included few patients for incidence calculation, 14 and 10, respectively [132, 133]. Furthermore, LON cases were detected at routine follow-up at most once a month and, hence the reported incidence data are probably underestimation since patients with a shorter period of neutropenia, being asymptomatic, could have been missed. Nevertheless, the incidence figures are the highest ever noted for idiosyncratic drug-induced neutropenia [1, 2]. Moreover, DIAG reports never revealed neutropenia >7 days after cessation of drug treatment.

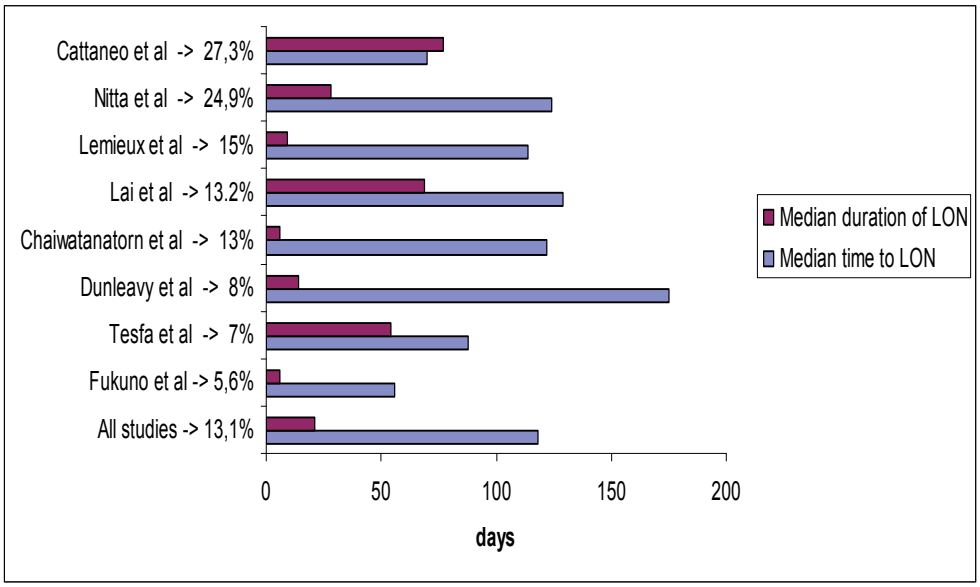
The median duration of LON reported ranged from 6 to 77 days depending on the ANC inclusion criteria, Table 1. Although, the reported median onset of LON after the termination of rituximab treatment varied between 56 and 175 days, all except one occurred approximately before 4 months, i.e. before B-lymphocyte recovery. This is in consistent with our studies. The reported incidence of LON in relation to median duration and time to onset of neutropenia is given in Figure 2. The median duration and time to onset of LON calculated from these studies is approximately 20 days and 120, respectively.

Table 1. Incidence of LON after rituximab treatment in lymphoma patients

Study	No. of pts.	Patient charac-teristics	Treatment	ANC inclusion	Median time to LON (days)	Median duration of LON (days)	Incidence of LON %
Cattaneo et al	77	Mixed	Mixed	Grade II	70	77	**27.3**
Nitta et al	107	Mixed	Mixed	Grade III	124	28	**24.9**
Liemeux et al	39	DLBCL	Chemotherapy + R + ASCT	Grade IV	114	9	**15**
Lai et al	121	DLBCL	Chemotherapy + R	Grade III	129	69	**13.2**
Chawatantorn et al	53	Mixed	Mixed	Grade IV	122	9	**13**
Dunleavy et al	76	Mixed	Chemotherapy + R	Grade IV	175	14	**8**
Tesfa et al	113	Mixed	Mixed	Grade III	88	54	**7**
Fukuno et al	54	Mixed	Mixed	Grade IV	56	6	**5.6**

LON=late-onset neutropenia, ANC=absolute neutrophil count, grade IV=ANC < 0.5 x 10^9/L, grad III=ANC < 1.0 x 10^9/L, grad II=ANC <1.5 x 10^9/L, DLBCL=diffuse large B-cells lymphoma, R=rituximab, ASCT= autologous stem cell transplantation.

Figure 2. Incidence in relation to median duration and time to LON in lymphoma patients



Reports on LON incidence in rituximab treated autoimmune-patients were scarce, except for a few case reports in patients with autoimmune diseases [14-17]. Hence, we performed a case-control analysis of retrospective cohort of 209 patients treated with rituximab for well-defined rheumatic diseases. We found 5% incidence of LON which is comparable to the reports in lymphoma patients, Paper II. The duration and time to onset of LON were similar too. Since then, a recent report showed an incidence of 6% in this patient group with similar clinical characteristics [18]. This emphasizes that LON is not unique for rituximab-treated hematology patients.

5.2 PREDISPOSING FACTORS

The risk factors for the development of LON are not fully understood. Higher rates are reported in NHL patients after SCT [7, 9, 13, 132, 133]. We reported similar results in our studies (Paper I and IV). In our recent study of LON on rheumatic patients, the incidence of LON appeared to vary with the autoimmune disease type (Paper II). In that, we found a higher rate in GPA and SLE patients compared to RA patients. However, GPA and SLE were heavily treated with myelotoxic and immunosuppressive drugs which could have resulted in

this observation. Moreover, higher rates are reported in lymphoma patients receiving previous chemotherapy, a high cumulative dose of rituximab, previous use of purine analogues or high-dose methotrexate containing regimens [6, 7]. However, we could not find a correlation between LON and cumulative doses of rituximab and methotrexate (Paper II and Paper IV). Nevertheless, the evidence points to myelotoxic treatments as a risk factor for LON development.

One of the difficulties in characterizing LON is that heterogeneous populations assessed in most studies, i.e. different diagnosis, disease stage, treatment intensity and history. In addition, most of the studies are of retrospective nature and lack matched control groups. Still, a recent study on a homogenous population of patients with DLBCL, treated according to R-CHOP protocol, could not identify any significant predisposing factor by analyzing patient age and performance status, disease stage, serum lactate dehydrogenase, BM lymphoma involvement, international prognostic index, blood counts and albumin levels [9].

The absence of a clearly discernible common risk factor profile in these studies may indicate that host genetics traits may also play a role in LON development. The impact of polymorphisms of immunoglobulin (Ig) G or FcγR genes on drug induced neutropenia/agranulocytosis have been described previously [134]. Two recent studies in rituximab treated lymphoma patients have reported a correlation between a higher rate of LON and a specific polymorphism in the IgG Fc receptor *FCGR3A* 176 V/F [135, 136]. We found also that this genotype was correlated to LON development in rheumatic patients (Paper III). Interestingly, we could demonstrate that each additional V allele was associated with a 4-fold increase in the odds ratio of neutropenia, identifying a risk factor for LON development in rheumatic patients.

The *FCGR3A* 176 V/V genotype enhances the ligation of rituximab to FcγRs. This suggested that patients with high affinity *FCGR3A* 176 V allele might develop maximum B-lymphocyte depletion. Indeed, we recently reported a more pronounced and sustained B-lymphocyte depletion and lower levels of IgM in LON patients compared to rituximab treated matched controls with rheumatic diseases (Paper II). Thus, the levels of B-lymphocyte depletion and

IgM, as well as the possession of *FCGR3A* 176 V/V genotype may help in identifying patients at risk for LON development.

5.3 POSSIBLE MECHANISMS OF LON

The mechanism of LON after rituximab treatment is still under investigation, and many theories have emerged. However, two major approaches are frequently advocated, i.e disordered immunological status and disturbance of hematopoiesis following rituximab treatment (Figure 3). Mechanisms of LON after rituximab therapy seem to differ from mechanisms of other DIAG/DINP. This is reviewed in the relevant publication added to this thesis, ***Idiosyncratic drug-induced agranulocytosis: possible mechanisms and management. Tesfa et al, Am J Hematol. 2009 jul;84(7):428-34. Review***

Direct cytotoxicity of rituximab is highly unlikely. First, CD 20 is not expressed on neutrophils or stem or progenitor cells. Secondly, there are no correlations either to rituximab pharmacokinetics and pharmacodynamics, since LON occurs long after disappearance of the rituximab. An infectious etiology, e.g. parvovirus B 19, has been purposed in a few case reports [137, 138], but this could not be confirmed by other studies [Paper IV, 6-13]. The early reports on LON have hypothesized the B-cell reconstitution of immune repertoire after rituximab induced B-lymphocyte depletion may favor the production of autoantibodies against neutrophils and their precursor [10, 139]. However, this finding has not been consistently demonstrated in further studies [Paper II, Paper IV, 6-9, 11-13].

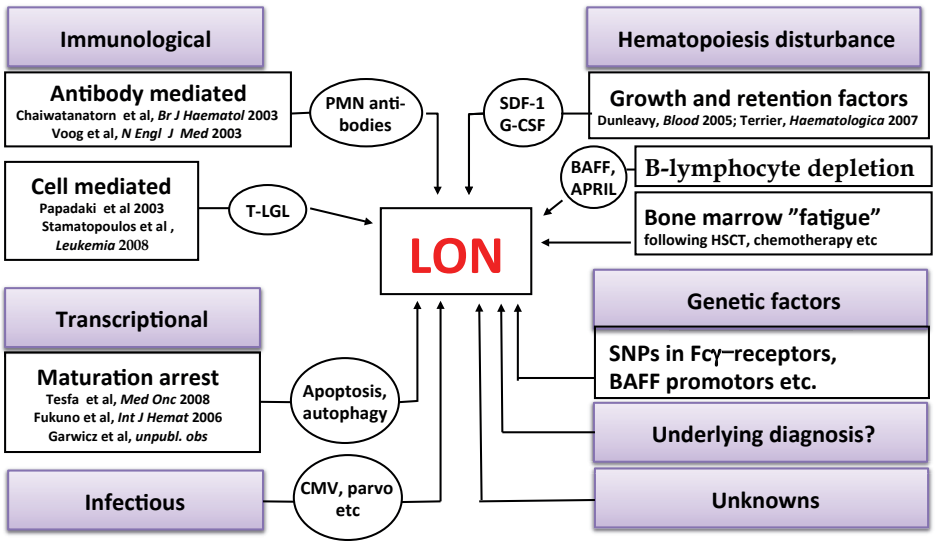
Some authors have postulated the proliferation of T-LGL due the lymphocyte subpopulation imbalance following rituximab therapy may lead to LON [140, 141]. They proposed T-LGL proliferation might lead to the secretion of FAS and FAS ligand, leading to apoptosis of mature neutrophils. However, this could not explain observations of development of excess T-LGL without neutropenia, seen in patients after non-myeloablative SCT and fludarabine treated B-CLL [142]. We and others did not find a proliferation of T-LGL in PB or BM [Paper I, Paper IV, 6-13].

Maturation arrest at the pro(myelocytes) stages of granulopoiesis in the BM of LON patients, a finding seen in patients with SCN such as Kostmann disease, is reported by our group and others [Paper I, Paper IV, 13, 140]. Maturation arrest at pro(myelocytes) stages of

granulopoiesis is a characterstic, but rarely well defined, morphological feature of SCN due to mutations in HAX-1 and *ELANE* genes [21, 22], but it is not found in many other severe chronic neutropenia states. It has been attributed to an excessive apoptosis of neutrophil progenitors in SCN patients [23, 24]. As stated above, we could not show such mutations in our LON patients (Paper I). Thus, the mechanism behind maturation arrest in LON patients is not understood yet. Nevertheless, this finding implies that a profound B-lymphocyte depletion selectively hampers granulopoiesis.

The median time to onset of LON coincided with the period of B-lymphocyte depletion. Hence, we turned our attention to the course of B-lymphocyte depletion in search for possible mechanism of LON. In deed, a recent study proposed that perturbations of SDF-1 during B-lymphocyte recovery retards neutrophil egress from the BM [11]. However, the median time to onset of LON is too early for B-lymphocyte recovery (Paper II and IV). Moreover, our LON patients presented with LON during complete B-lymphocyte depletion and, we found a pronounced and prolonged B-lymphocyte depletion in LON patients compared to non-LON rheumatic patients (Paper II). We confirmed this observation in a prospective study of lymphoma patients (Paper IV). The majority of our LON patients harbor *FCGR3A* V polymorphism (Paper III). This is in consistence with a previous report that B-lymphocyte depletion was better in patients with *FCGR3A* V polymorphism [49]. Hence, the depth of B-lymphocyte depletion might play an important role for mechanisms of LON.

Figure 3. Possible mechanisms for late-onset neutropenia



APRIL: a proliferation-inducing ligand; BAFF: B-cell activating factor; CMV: cytomegalovirus; HSCT: hematopoietic stem-cell transplantation; G-CSF: granulocyte colony stimulating factor; LON: late-onset neutropenia; SDF-1: stromal-derived factor; SNPs: single nucleotide polymorphisms; T-LGL: T-cell large granular lymphocyte leukemia/disorder

The pronounced B-lymphocyte depletion in LON patients coinciding with neutropenia and on the other hand regenerated B-lymphocytes showing a normalized ANC suggested that LON may be related to factors involved in the proliferation of B-lymphocytes, such as BAFF. In addition to BAFF, which plays a central role in B-lymphocyte proliferation, we have also analyzed other cytokines (APRIL, SDF-1 and G-CSF) involved in proliferation and hemostasis of lymphopoiesis and granulopoiesis. We did not observe significance differences regarding serum values for APRIL, SDF-1 and G- CSF. However, we found that serum BAFF levels increased significantly during the LON period and this increase differed significantly to BAFF levels measured in non-LON matched controls. This is in agreement with a previous report of extraordinary BAFF levels during LON in a lymphoma patient [118]. Since, we

found a correlation between an increase in serum BAFF levels and neutropenia, we hypothesized that the decrease in granulocyte counts might be related to the levels of serum BAFF. Interestingly, the granulocyte count was inversely correlated to the increase in serum BAFF levels. There was a corresponding significant increase in the number of monocytes during neutropenia, implying a compensating monocytosis to meet the need of increased BAFF production. Our results are suggestive of relation between perturbations of neutropoiesis, B-lymphocyte depletion and BAFF production.

5.4 CLINICAL IMPACT OF LON

Recent report indicates that LON posed serious, but not life-threatening, infections in the setting after autologous SCT [143]. Indeed, the majority of lymphoma patients treated with standard chemotherapy, including ours (Paper I and IV), did not develop serious infectious complications and LON was self-limited in most of the cases. Only few cases have been reported with concurrent drop in hemoglobin and platelets [6]. Few LON patients, with severe neutropenia, needed G-CSF and antibiotics. Most patients responded promptly with G-CSF treatment. However, the clinical course of LON in rheumatic patients with concomitant immunosuppressive treatment seems to differ (Paper II). However, more studies are needed before operational guidelines for LON can be suggested. Thus, although evidence is pointing towards infectious complications in patients with LON, it is still unclear which patients develop infections during the LON period. Nevertheless, an increased vigilance might be necessary.

A close clinical follow-up or CBC monitoring is good enough in most LON cases. BM evaluations should be carried in selected cases for information on neutrophil hypoplasia and maturation arrest, prior to treatment with G-CSF, as well as to detect a possible lymphoma relapse or myelodysplastic features.

A very important question is re-challenging patients who developed LON. This may affect treatment strategy and clinical outcome as retreatment with rituximab is a part of lymphoma and autoimmune protocols. Moreover, disease control by maintenance therapy with rituximab is used in low-grade lymphomas, e.g. follicular lymphoma. Recurrence of LON episodes have been reported upon repeated treatment with rituximab [Paper II, 144]. In addition, two large

prospective trials reported an increase in the incidence of neutropenia during maintenance therapy [145, 146] implying a recurrence of neutropenia. However, the current data does not differentiate between patients at risk during de novo or relapse treatments. Frequent monitoring of CBC after rituximab treatment may not be feasible. Furthermore, the risk of LON during retreatment for patients who developed LON at first exposition is not known either. There are no studies with regular CBCs monitoring to address the issue of shorter median duration of LON, as asymptomatic LONs could have been missed. Thus, vigilance for LON patients is advisable.

Although LON is associated with increased infections in rheumatic patients (Paper II), it conferred a later clinical benefit with regard to disease remission duration (Paper III). We found that LON was correlated to the possession of *FCG3RA* 176V/V genotype. Indeed, we also observed that the *FCG3RA* 176V/V genotype, as well as the presence of LON, has an unexpected positive clinical impact, leading to a longer time to flare of the rheumatic disease. This is in agreement with a previous report that patients with more prolonged B cell depletion were more likely to respond than those in whom B cell return was early [147]. Likewise, a previous report in rheumatic patient showed a short-lived alleviation of rheumatic symptoms during neutropenia after DIAG [148]. However, we could not show a significance positive effect of LON at a later point time i.e. one year after cessation of rituximab treatment. Nevertheless, the effect described herein was of a different nature since it postponed the need for renewed treatment. Finally, our observation, LON as a prognostic factor in rheumatic patients could have a clinical consequence. However, this conclusion needs to be confirmed in lymphoma patients and larger prospective studies.

6. CONCLUSIONS

LON is a clinical significant late-adverse event occurring both in lymphoma and rheumatic patients. It is unique since it appears long time after the cessation of rituximab therapy.

The incidence of LON in rheumatic patients is comparable to reports on lymphoma patients. However, patients treated with a highly myelotoxic regimen seem to have a higher incidence of LON.

Maturation arrest at the (pro)myelocyte stage of granulopoiesis during LON is a clinical feature implying a selective depletion of granulopoiesis.

The clinical course of LON in patients with immunosuppressive treatment seems to differ from lymphoma patients i. e. higher infection rates compared to those treated with standard cytoreductive treatment.

The level of B-lymphocyte depletion as well as IgM reductions may be risk factors for LON development.

LON patients do often display *FCGR3A* 176 V/V genotype which is also correlated with a profound B-lymphocyte depletion.

LON patients with rheumatic disease may have a better clinical outcome, that may be related to the *FCGR3A* genotype and as well as LON.

The perturbations of granulopoiesis in LON patients might be related to the profound B-lymphocyte depletion and the excessive BAFF production.

7. FUTURE DIRECTIONS

Rituximab and other anti-CD 20 mAbs usage is expanding into the fields of autoimmune disease and new indications are added every year. New mAbs, with enhanced affinity to the CD 20 antigen, have now been introduced in clinical practice. Hence, LON is anticipated to be a clinical problem encountered in many disciplines and probably with increased incidence compared to rituximab. Frequent monitoring of CBC after rituximab treatment may not be feasible. However, the correlation between LON and *FCGR* polymorphisms may be helpful in identifying patients at risk. Moreover, the relation between B-lymphocyte depletion and IgM levels can be additional accessible risk markers in a daily clinical setting. Although LON is associated with a higher rate of infection in patients with concomitant immunesuppressive treatment, we have found that LON patients may have a clinical advantage compared to non-LON patients. This is an important observation which has to be confirmed in future studies. Finally, the mechanisms of rituximab, leading to unexpected late-adverse effects, have highlighted the interdependence of lymphopoiesis and granulopoiesis. Thus, vigilance is advisable when introducing a drug with the ability of a long-term manipulation of hematopoiesis.

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