Functional and phenotypical alterations of polymorphonuclear cells in Sézary syndrome patients [*MT. Fierro and AMC Cuffini contributed equally to this work]

Eur J Dermatol. 2011 Nov-Dec;21(6):921-9.

doi: 10.1684/ejd.2011.1544.

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Article accepted on 8/24/2011

Functional and phenotypical alterations of polymorphonuclear cells in Sézary syndrome patients Sézary syndrome (SS), the leukemic variant of cutaneous T-cell lymphoma (CTCL), has a poor prognosis and infections represent the most frequent cause of death. Polymorphonucleate granulocytes (PMNs) constitute an essential part of the innate immune system: their phagocytic and killing activity against pathogens is mediated by the interactions betweenToll-like receptors (TLRs) and the Pathogen-associated molecular patterns (PAMPs). The aim of this study was to investigate PMN functional activity and phenotype in SS patients and their correlation with he onset of infectious complications. This prospective study enrolled 18 consecutive SS patients; PMN functional activity was evaluated by phagocytosis and intracellular killing tests towards Klebsiella pneumoniae.

Flow-cytometry was applied to analyze PMN phenotype. PMNs from SS patients displayed a reduced phagocytic activity and intracellular killing against K. pneumoniae at 30 min and 60 min, more pronounced in SS patients with recurrent infections. CD11b and CD66b median fluorescence intensity (MFI) was significantly higher in SS than in healthy subjects, whereas CD62L MFI was decreased. No significant differences in TLR2, 4, 8 and 9 percentage expression or MFI were found. An increased TLR5 percentage expressionwas documented.

The impairment in PMN functional activities in SS could favour the immune-suppression and raise infection risk.

Key words: Sézary syndrome, cutaneous T cell lymphomas, human PMNs, intracellular killing, phagocytosis, infections

Primary Cutaneous T-cell lymphomas (CTCLs) are extranodal, non-Hodgkin's lymphomas characterized by a primary localization of atypical CD4+ T lymphocytes [1] in the skin. Mycosis fungoides (MF), the most common form of CTCL, displays a stepwise evolution with a sequential appearance of patches, plaques and

tumours and an indolent course; Sézary syndrome (SS) is a rare CTCL, characterized by erythroderma, peripheral adenopathies and blood involvement by atypical cerebriform lymphocytes. SS is included among the aggressive cutaneous lymphomas in the new WHO-EORTC classification [2] and its prognosis is poor with a 24% 5-year survival rate and a median survival varying from 2.5 up to 5 years [2]. The immune-suppression related both to the functional impairment of neoplastic T cells and to chemotherapeutic treatments, determines the development of severe infectious complications (bacterial, viral and fungal) which represent the most frequent cause of death [3]. Malignant Sézary cells are memory skin-homing CD4+CLA+CCR4+ cells and exhibit a Th2 profile with production of IL4 and IL10
and consequent impairment of the host adaptive immune response [4]. In advanced disease, expansion of the malignant clone in peripheral blood lymphocytes (PBL) results in a concomitant decrease of normal cytotoxic CD8+ lymphocytes and CD56+ NK cells, associated with a deterioration of both antitumoral immunity and immune surveillance against microbial organisms.
On the contrary, little is known in the literature about the quality of the innate immune response in CTCL, apart from the observation of hypodense activated neutrophils in CTCL compared with healthy subjects [5]. PMNs constitute an essential part of the innate immune system and are the first among inflammatory cells to migrate towards the site of inflammation [6]. The role of the innate immune system is to discriminate between self and non-self as well as to distinguish between pathogenic and non-pathogenic microorganisms; moreover, it is involved in triggering the adaptive immune response. The cornerstone of the innate immune system is represented by a set of pattern-recognition receptors (PRRs), to which the Toll-like receptors (TLRs) belong. These PRRs are activated upon recognition of Pathogen-associated molecular patterns (PAMPs) [7]. PAMPs are shared by large groups of pathogens and include cell wall constituents such as lipopolysaccharide (LPS), peptidoglycan (PGN), single or double-stranded RNA. TLRs are transmembrane proteins which differ from each other in the ligand specificities, expression patterns and target genes they induce [8]. At least 11 TLRs have been identified in humans, expressed predominantly but not exclusively in antigen presenting cells such as macrophages, neutrophils (PMNs) and dendritic cells [9-11]. PMN phagocytosis and killing activity against pathogens is mediated by the interactions between TLRs expressed on the PMN surface and PAMPs. Activation of TLRs facilitates phagocyte recruitment by upregulation of adhesion molecules and alteration of PMN trafficking, in order to localize neutrophils at the site of infection. PMNsalso respond toTLRactivation with the generation of reactive oxygen species [12], increased phagocytosis, and secretion of cytokines. In this prospective cohort study, the phenotype and functional activity ofPMNsfrom 18 SS patients were investigated and compared with those of a sex- and age-matched control group. In addition, we aimed at investigating the role of TRLs in mediating PMN disfunctions in CTCL.
Materials and methods

Study design

This was a prospective cohort study aimed at investigating PMNactivity in SS patients. Due to the rarity of the disease, we decided to include all the consecutive SS patients seen at the Section of Dermatology, Dept of Biomedical Sciences and Human Oncology, University of Turin, Italy, either at first diagnosis or already in treatment. The first patient was enrolled in April 2007 and the last in December 2008; 18 SS patients, 8 males and 10 females (median age 69 years, range 40-82) were included. Inclusion criteria were: histologically confirmed diagnosis of SS and absence of documented clinical or laboratory signs of infections. The SS diagnostic criteria [13, 14] included : a) erythroderma and peripheral lymphadenopathies; b) peripheral blood involvement by circulating Sézary cells (SCs) ; c) cutaneous biopsy proven CTCL. Peripheral blood involvement was defined on the basis of the criteria reported by the International Society of Cutaneous Lymphomas [15]. Peripheral blood immunophenotype and clonal V-beta-TCR (TCRV □) expression was also assessed to better characterize the pathological population as previously described [16, 17]. Fifteen healthy donors sex and age matched and negative for the presence of microbial and viral diseases, were used as controls. All patients participating in this study gave their informed written consent. The time of blood collection (i.e. at first diagnosis or during follow-up) and the

treatment received by the patients were recorded for study purposes. The study design, performed according to the principles of good clinical practice and to the Declaration of Helsinki Principles, included peripheral blood analysis with PMN phenotypical and functional determinations as well as 3 years clinical follow-up; during this period, information on infectious complications was recorded in order to distinguish patients with or without recurrent infections. The study protocol was approved by theTurin University thical Committee.

PMN functional activity

Bacteria

A clinical strain of Klebsiella pneumoniae was cultured on MacConkey Agar (Oxoid S.p.A., Garbagnate Milanese, Milan, Italy). Young colonies (18–24 h) were picked up to approximately 3–4 McFarland standard and inoculated into cryovials containing both cryopreservative fluid and porous beads to allow bacteria to adhere (Microbank, Biomérieux; Rome, Italy). After inoculation, cryovials were kept at - 80◦C for extended storage.

PMNs

PMNs from SS and healthy donors were separated from lithium heparinized venous blood using Ficoll–Paque (Pharmacia S.p.A., Milan, Italy), as previously described in detail [18, 19]. The PMNs were incubated at 37◦C in a shaking water bath before the addition of K.pneumoniae (107 CFU/ml). The PMN viability before and after each experiment was greater than 95%.

Phagocytosis and intracellular killing assays

The phagocytosis of radiolabelled klebsiellae [3H-uracil (specific activity: 1.27 TBq/mmol; NEN Products, Milan, Italy)] or the intracellular K. pneumoniae killing by PMNs were investigated by incubating the bacteria and the phagocytes (bacterium:PMN ratio was 10:1) at 37 ◦C for periods of 30, 60 or 90 min. Phagocytosis and intracellular killing were assessed by the methods previously described in detail [18-20]. Briefly, aliquots of 1 ml of K.pneumoniae in RPMI 1640 with 10% FCS were added to PMNs (106 cells) and incubated at 37◦C in a shaking water bath. After incubation for a period of 30, 60 or 90 minutes, the PMNs were twice centrifuged at 1,200 rpm for 5 minutes to remove free bacteria. 100 ?L of suspended cells were then placed in scintillation fluid (Atomlight, NEN) and counted by liquid scintillation spectrophotometry. Radioactivity was expressed as the counts per min/sample. The percentage of phagocytosis at a given sampling time was calculated as follows: percent phagocytosis=[(cpm inPMNspellet)/(cpm in total bacterial pellet)] ×100. In order to measure PMN antimicrobial activity, an aliquot of the cells containing bacteria was lysed by adding sterile water, and a viable count of intracellular K.pneumoniae was performed (time zero). The cells were then incubated further, and at intervals (30, 60, 90 minutes), the viable counts of the surviving intracellular bacteria were measured in the same way. The PMN killing values were expressed as survival index (SI), whichwas calculated by adding the number of surviving bacteria at time zero to the number of survivors at time x, and dividing by the number of survivors at time zero. According to this formula, if bacterial killing was 100% effective, the SI would be 1.

Flow cytometry

PBL were analysed by six-colour immunofluorescence using a FACSCantoII cytometer (Becton Dickinson, San José, CA) equipped with a 488 and a 633 laser. At least 3×104 leukocytes were collected for each

antibody combination and isotype-matched negative controls conjugated to each fluorochrome were used. A wide panel of monoclonal tested [16].

For this study purpose, the following mouse anti-human antibodies were used: CD11b APC (clone D12) and CD45 PerCP-Cy5.5 (clone 2D1) from BD Biosciences, San Jose, CA, USA, CD62L PE (clone DREG56) from Invitrogen Ltd, Paisley,UKand CD66b FITC (clone 80H3) from Beckman Coulter, Brea, CA, USA, TLR2/CD282 APC (clone TL2.1), TLR4/CD284 Alexa Fluor 647 (clone HTA125) and TLR5 PE (clone 85B152.5) from IMGENEX, San Diego, CA, USA. TLR9 and TLR8/CD288 cytoplasmic expression was analysed using whole blood samples. Cells were intracellulary stained with mouse anti-human TLR8 PE (clone 44C143) from IMGENEX, and rat anti-human TLR9/CD289 PE (clone eB72-1665) from eBioscience; San Diego, CA according to the manufacturer's protocol, using Fix&Perm reagents (Invitrogen Ltd, Paisley, UK). Antigen fluorescence intensity was expressed as median fluorescence intensity (MFI).

Statistical analysis

Statistical analyses were applied using the Graphpad Prism version 3.00 forWindows (Graphpad Software, San Diego, Calif., USA). Each phagocytosis and killing test was performed in triplicate, data were expressed as median and range. The Mann-Whitney U-test and the Kruskal-Wallis with Dunn post hoc test were used to compare the data among the two subgroups of SS patients and healthy controls. $P<0.05$ was considered statistically significant.

Results

All 18 patients enrolled in this study reported a SS diagnosis. An identical clone was evidenced in all patients by means of GeneScan analysis both in blood and skin. At initial diagnosis, the absolute SC number was <1,000 cells/mm3 in 7 patients and the percentage ranged from 11 % to 98 % of total lymphocytes. Blood was collected at diagnosis time in two SS patients and at different times during follow-up in the remaining 16 patients.

PMN functional activity against K. pneumoniae

in SS patients and healthy donors In all the experiments the viability of PMNs remained unchanged throughout. The pattern of phagocytosis and intracellular killing against K. pneumoniae by PMNs harvested both from healthy donors and SS patients is shown in table 1A, B. According to our results, PMNs from SS patients displayed reduced phagocytic activity when compared with that of controls. Indeed, PMNs harvested from healthy donors were able to engulf K. pneumoniae at a percentage ranging from 19.1% to 15.5% during 90 min of incubation, whereas PMNs from SS patients phagocytosed bacteria at a significantly decreased rate compared to that observed in healthy donors at 30 and 60 min (17.2%, $p=0.049$ and 15.6%, $p=0.0067$). Interestingly, the reduction of phagocytic activity was more pronounced in the group of SS patients with recurrent infections ($p<0.01$ at 30 min and $p<0.01$ at 60 min), whereas patients without complications showed values similar to those recorded for healthy donors (table 1). This reduced phagocytic activity in SS patients was also accompanied by a reduced PMN efficiency to kill ingested bacteria. The bactericidal activity in SS patients was significantly lower than observed in healthy donors at 30 and 60 min, with SI values of 1.64 vs 1.43 of controls ($p=0.0001$) and >2 vs 1.74 of controls ($p=0.013$) respectively; at 60 and 90 min ; the intracellular killing was totally absent, with SIs higher than 2. In this case, the killing defect was evident in all SS patients, including those without infections. Correlation between functional activity of PMNs and SS clinical course Laboratory characteristics of SS patients are shown in Table 2: all hematologic values refer to the time of diagnosis. Anyway, median PMN value at the

test time (4,479/mm3) did not vary significantly from diagnosis (4,765/mm3) and no patient had active infection. Moreover, in most patients with recurrent infections (7/10) the phagocytosis and intracellular killing were evaluated before the onset of infections. At the time of PMN functional studies 4 patients out of 18 (22%, n◦ 4, 7, 12, 18) were under treatment with immunosuppressive drugs, consisting exclusively of low-doses alemtuzumab [21] (median cumulative dose administered to each patientwas 73 mg, range 13-137), whereas most of them (12/18) were undergoing extracorporeal photochemotherapy (ECP). The remaining two patients were treated with alpha-IFN and chlorambucil. Table 3 describes the infections we observed in SS patients: the most frequent bacterial infection was due to Staphylococcus aureus (39 episodes in 10/18 patients), which in50% of cases were methycillin-resistant (MRSA), followed by Streptococcus spp. and Pseudomonas aeruginosa. S. aureus was responsible for both disseminated cutaneous infections and sepsis. In patient n◦ 6, S. aureus was identified in blood during a sepsis complicated by spondilodiscitis and, one year later, in broncho-alveolar lavage as a responsible of pneumonia. The most frequent viral infection was cytomegalovirus (CMV; 4/18 cases, all in patients treated with alemtuzumab), followed by herpes simplex virus (HSV, 1 case) and varicella zoster virus (VZV, 2 cases) and hepatitis B virus (HBV; 1 case). No mycobacterial or fungal infections were recorded. Patients characterized by recurrent infections had a worse survival than those without infections, although the difference did not reach statistical significance (figure 1).

Cytometric detection of activation markers and toll-like receptors on PMN

Flow-cytometry did not reveal differences in the percentage values of CD11b, CD62L and CD66b, expressed by near all SS granulocytes, as in healthy donors. On the contrary CD11b (median: 686.4, range: 474.4-1846 vs 528.0, 160.0-983.8; p=0.0328) and CD66b (median: 533.2, range: 186.4-1,010.0 vs 292.0, 78.0-578.4; p=0.0104) MFI was significantly higher in SS than in healthy subjects, whereas CD62L MFI was decreased in SS patients (median: 808.6, range: 385.6-1,655.0 vs 2,012.0, 1,370.2-2,548.5; p=0.001) (figure 2). As for TLRs (figure 3), no significant differences in TLR2, 4, 8 and 9 percentage expression (TLR2: median 96.5%, range: 86.8-98.5 vs 94.0, 89.6-98.7; TLR4: median 95.3, range 91.2-98.8 vs 93.6, 90.3-97.7; TLR8: median 93.4%, range: 86.0-98.5 vs 96.8, 90.5-99.3; TLR9: median 79.1, range: 69.2-94.1 vs 85.2, range 62.0-97.6 n.s.) or MFI values were found TLR2: mean 98.7, range: 78.7-141.1 vs 74.0, 55.3-90.0; TLR4: mean 39.7, range 27.6-56.8 vs 59.9, 28.8-95.2; TLR8: mean 17.9, range: 12.0-21.5 vs 25.1, 13.6-45.9; TLR9: mean 44.1, range: 37.6-54.2 vs 47.5, range 38.9-60.6 n.s.). On the contrary, an increased percentage expression of TLR5 (median: 88.1%, range: 78.4-92.7 vs 75.6%, 62.5-81.5, p=0.0087) was documented on PMN surface (figures 3, 4), whereas the MFI was superimposable to that of normal controls (mean 57.6, range: 36.7-78.0 vs 63.6, range 44.5-91.2 n.s.).

PMN morphological evaluation by flow cytometry

The Side Scatter (SSC) parameter, a cytometric index of internal complexity determined by nucleus shape and cytoplasmic granule numbers and shapes, was analyzed. PMN from healthy donors had a median SSC of 506±435, whereas PMN from SS patients had a median SSC of 483±88 (p=0.149 n.s.). Patients characterized by recurrent infections had a significantly lower SSC than those without infections (473±48 vs 524±92, p = 0.035) (figure 5). Eosinophil count was balanced in the two groups (mean 404/mm3 in SS patients with recurrent infections vs 458/mm3 in those without infections, ns): we could therefore exclude a bias due to the high eosinophil side scatter. This cytometric finding was confirmed by the observation of May-Grünwald-Giemsa coloured smears: a pale and hypogranulated cytoplasm was detected in a high proportion of PMN from patients with infections (figure 5).

Discussion

Malignant T cells in CTCL are skin-homing, mature CD4+ T cells, characterized by a Th2-cytokine expression profile which may contribute to disease progression and host immune response impairment through production of IL- 4, IL-5 and IL-104. Bacterial sepsis is the most common infectious complication and is the cause of about 50% of deaths in CTCL [3, 22, 23]. Overall, the incidence of bacterial infections is greater than viral, fungal and parasitic infections and S .aureus is the most common bacterial pathogen [3, 22]. Infection risk is increased with advanced disease as well as combination chemotherapies [3, 23] and repeated hospitalizations also enhance the likelihood of infection with antibiotic-resistant microorganisms such as MRSA. The skin barrier disruption due to tumour infiltration facilitates local skin infections; these, coupled with intravenous catheter placement and biopsies, are frequent means of entry for bacteraemia [24].

In alemtuzumab-treated CTCL patients, the frequency of infections is even higher. Nevertheless, the modified protocol we applied allows the achievement of clinical and haematologic responses using subcutaneous administration of low doses (maximum 10 mg) alemtuzumab, with a more favourable toxicity profile [21]. CMV reactivation is the most common viral infection in alemtuzumab-treated patients [26], followed by HSV [25] and VZV. Phagocytes are the immune system's key defenders against bacterial infection, their primary function being to ingest and destroy invading pathogens. This is achieved by engulfing the pathogen into an intracellular compartment called a phagosome, and then subjecting it to an array of both oxygen-independent and oxygen-dependent (oxidative burst) killing mechanisms. These include the release of antimicrobial and proteolytic peptides into the phagosomal space, along with the production of reactive oxygen species by the NADPH oxidase complex that assembles in the membrane [27]. Despite the high incidence of infections, PMN functional activity has been poorly investigated in CTCL, apart from the observation [5] by Goddard et al. of PMN activation as measured by a shift in buoyant density, respiratory burst priming and surface marker expression. Goddard and collegues [5] not only assumed that the mechanism underlying eosinophil and neutrophil activation is a cytokine imbalance associated with a Th2 skewing, but also that PMN activation could be involved in the pathophysiology of CTCL. Considering lymphoproliferative diseases as a whole, functional abnormalities in PMNs have only been documented in multiple myeloma [28] and in a subgroup of B-CLL patients with frequent infections [29]. In the present study in SS patients, we analyzed PMN activities against K. pneumoniae, a pathogen which can cause severe problems in immunodepressed subjects. The results showed an impairment of phagocytic activity compared to that of healthy donors, which was more pronounced in SS patients who experienced frequent infection episodes.

Our data were obtained using a radioactive technique, which remains time consuming but accurately reflectsPMN in vivo behaviour, instead of a flow cytometric analysis, widely used to rapidly screen PMN function abnormalities [31]. In all SS patients, including those without infections, the reduced phagocytic activity was accompanied by a reduced PMN efficiency in killing ingested bacteria. It has to be underlined that patients characterized by recurrent infections had a worse survival than those without infections, although statistical significance was not reached due to a small sample size versus highly variable parameters. The reduced PMN efficiency detected is in apparent contrast with phenotypical data, which confirms the phagocyte activation described by Goddard [5], documented by an increase of CD66b and CD11b MFI and a decrease of CD62L MFI values. CD62L down-regulation has already been described both in elderly and long-term steroid treated patients and seems to induce an adhesion defect which enhances the susceptibility to infections [32]. As to Goddard's observation on the presence of hypodense neutrophils, we could observe such PMNs on May- Grunwald-Giemsa staining of PBL smears but we did not identify this population using Ficoll density gradient separation, maybe due to the different techniques used or a different patient population. In agreement with the phenotypical finding of hypogranular cytoplasm, cytometry established that PMN from SS patients with recurrent infections had a lower internal complexity (SSC) than that from normal subjects and patients without infections.

Microbial ligands activate the innate immune system to mount a defence response by binding to TLRs, and this process is suggested to be important for an effective presentation of antigens to the adaptive immune system. Surprisingly, most of TLRs we have analyzed were expressed normally on circulating PMNs from SS patients: particularly TLR2, which recognizes a wide spectrum of Gram-positive and Gram-negative bacteria including S. aureus, and TLR4, which recognizes Gram-negative bacteria trough its ligand LPS, known as the causative agent of Gram-negative sepsis. The intracellular expression of the receptor for unmethylatedCpGdinucleotides TLR9, known to be essential for pro-inflammatory cytokine production and the induction of a Th1 acquired immune response, did not differ from control subjects. Interestingly, TLR9 recognizes HSV and CMV, which are quite frequent infections in SS patients. An increased percentage in flagellin receptor TLR5 surface expression was the only abnormality we could detect: further studies, including an immunohistochemical evaluation of TLR expression in SS skin, are needed to ascertain whether this observation has clinical relevance.

In conclusion, to our knowledge, this is the first demonstration of a functional PMN defect in CTCL patients. The degree of functional PMN impairment was related to a major incidence of infectious complications as determined during follow-up: in particular, the phagocytic defect was significantly more pronounced in the group of patients who developed infections with respect to that of patients without complications ($p=0.01$). The possibility that PMN impairment may induce higher susceptibility to infections is further supported by the observation that, in most patients, tests were performed before the infection occurred. If confirmed, these tests could help in identifying a subset of patients who could benefit from antibiotic prophylaxis. On the basis of these interesting results, we are planning to extend our study to mycosis fungoides and other dermatological diseases, such as atopic dermatitis, in which microbial infections play an important role.

∗ This study was presented in part at EORTC CLTF 2009, Cutaneous Lymphoma Clinical Meeting, October 23-25 2009, Athens, Greece.

Disclosure. *Financial support: This work was supported by grants from Ricerca Sanitaria Finalizzata 2007 n. 2074 (Regione Piemonte, Italy).*
Conflicts of interest: none.

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Table 1. Functional activity of PMNs from healthy donors and SS patients: A) K. pneumoniae percentage of phagocytosis (median; range), B) intracellular killing against K. pneumoniae (median; range).

A. Phagocytosis (%)			
	30 min	60 min	90 min
Healthy donors (HD)	19.1 (16.2-20.8)	19.7 (16.8-21.4)	15.5 (14.1-17.0)
SS patients	17.2 (11.4-26.7)	15.6 (10.0-22.8)	14.3 (10.0-21.5)
Mann Whitney test	p=0.049	p=0.0067	n.s.
SS patients without infections (A)	19.7 (14.6-26.7)	17.8 (14.4-22.8)	15.4 (11.0-21.5)
SS patients with infections (B)	15.7 (11.4-20.9)	14.1 (10.0-17.9)	13.5 (10.0-19.7)
Kruskal-Wallis with Dunn post hoc test	HD vs A n.s HD vs B p<0.05 A vs B p<0.01	HD vs A n.s HD vs B p<0.01 A vs B p<0.01	HD vs A n.s HD vs B n.s A vs B n.s
B. Killing (survival index)			
	30 min	60 min	90 min
Healthy donors (HD)	1.43 (1.3-1.5) (57%*)	1.74 (1.4-2.0) (26%*)	>2
SS patients	1.64 (1.4-1.8) (36%*)	>2	>2
Mann Whitney test	p=0.0001	p=0.013	n.s.
SS patients without infections (A)	1.61 (1.4-1.8) (39%*)	1.95 (1.5->2) (5%*)	> 2
SS patients with infections (B)	1.65 (1.4-1.9) (35%*)	> 2	> 2
Kruskal-Wallis with Dunn post hoc test	HD vs A p<0.01 HD vs B p<0.001 A vs B n.s	HD vs A p<0.01 HD vs B p<0.01 A vs B n.s	HD vs A n.s HD vs B n.s A vs B n.s

* % of initial bacterial population killed by PMNs.

Table 2. Phenotypical and haematological features in SS patients at diagnosis.

Patient	Immunophenotype	WBC/mm^3	PMN/mm^3	L/mm^3	SC/mm^3
1	CD3+dim CD4+ CD7- CD26-	36,620	4,761	630	30,863
2	CD3+ CD4+ CD2± CD7± CD26-	16,730	4,751	1,088	9,536
3	TCR-Vβ22+ CD3+dim CD7- CD26-	11,220	6,451	3,358	737
4	CD3+dim CD4+ CD7- CD26-	10,800	4,504	1,795	3,994
5	CD3+dim CD7+ CD26-	5,560	4,443	51	498
6	CD3+ CD4+ CD7+ CD26-	4,440	2,886	832	234
7	CD45+dim CD5+dim CD3- CD4- CD8+bright CD26+	14,170	5,866	2,004	2,261
8	TCR-Vβ22+ CD7- CD26±	15,320	10,418	613	3,830
9	CD3+ CD4+ CD7+ CD26-	3,740	1,560	942	116
10	TCR-Vβ21.3+ CD3+dim CD4+dim CD2± CD7± CD26-	9,570	7,197	1,633	358
11	TCR-Vβ1 CD3+dim CD4+dim CD2±dim CD7- CD26±	12,320	7,478	2,057	2,464
12	TCR-Vβ17+ CD2+dim CD3+dim CD4+dim CD7- CD26-	13,450	8,070	1,083	3,625
13	TCR-Vβ20+ CD3+dim CD4+ CD7- CD26-	24,260	7,399	638	15,301
14	TCR-Vβ3.1+ CD4+ CD7+ CD26±	9,170	4,768	1,908	1,751
15	TCR-Vβ1+dim CD3+dim CD4+ CD7± CD26-	7,410	5,557	519	741
16	TCR-Vβ2+ CD2+dim CD3+high CD4+ CD7- CD26-	4,600	3,671	308	511
17	CD3+dim CD4+ CD7- CD26-	6,750	2,848	2,160	1,269
18	TCR-Vβ6.7+ CD3+bright CD4+ CD7± CD26-	13,800	2,484	2,208	9,053

WBC/mm3: absolute white blood cell count; PMN/mm3: absolute white blood cell count; L/mm3: absolute normal lymphoid cell count; SC/mm3: absolute SC cell count.

Table 3. Infections in SS patients during follow-up.

Patient	S. aureus (n. episodes)		Viral infections	Pneumonia	Therapy
	Skin	Sepsis			
1	5	3	VZV,HSV1	—	anti-CD52, ECP
2	—	—	—	—	anti-CD52
3	—	—	—	—	ECP, BEX
4	—	—	—	—	anti-CD52, ECP
5	3	—	VZV,HBV, CMV	—	ECP, anti-CD52
6	5	3*	—	1 (MRSA)	ECP
7	2	—	—	—	anti-CD52, ECP
8	2	—	—	—	ECP, anti-CD52
9	3	2	CMV	—	IFN, FAMP, ECP, anti-CD52
10	—	—	—	—	ECP, MTX basse dosi
11	—	—	—	—	ECP, anti-CD52
12	2	—	—	1 (n.d.)	antiCD52, ECP, CHL, BEX
13	—	—	—	—	CHL, anti-CD52
14	—	—	—	—	ECP
15	—	—	—	—	ECP, anti-CD52
16	2	1	CMV	—	anti-CD52, ECP, FAMP, HDAC
17	2	2	—	1 (n.d.)	ECP, anti-CD52
18	2	—	CMV	—	anti-CD52, BEX

ECP: extracorporeal photochemotherapy; anti-CD52: alemtuzumab; FAMP: fludarabine; monophosphate MTX: methotrexate; BEX: bexarotene; CHL: chlorambucil; HDAC: histone deacetilase inhibitors.

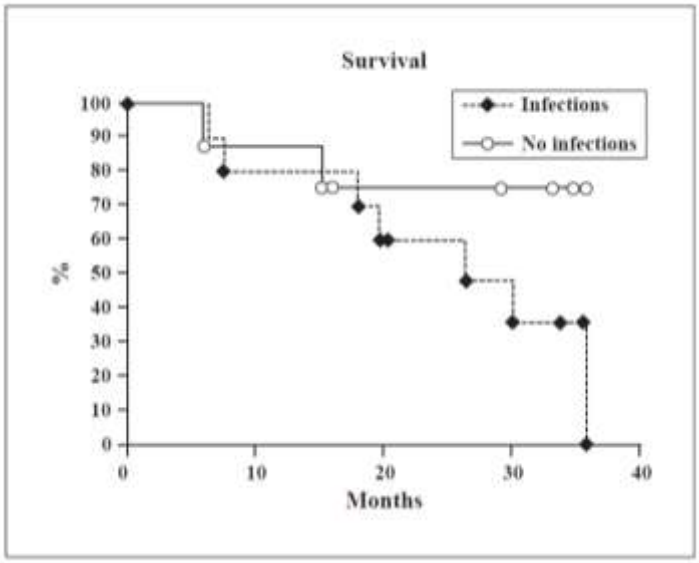

Figure 1. SS patient's survival according to infectious episodes.

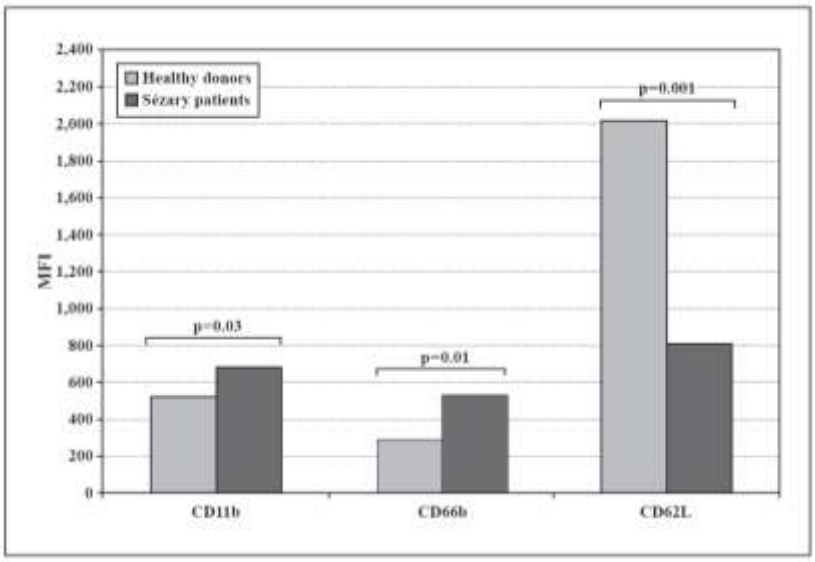

Figure 2. CD11b, CD66b and CD62L median fluorescence intensity values.

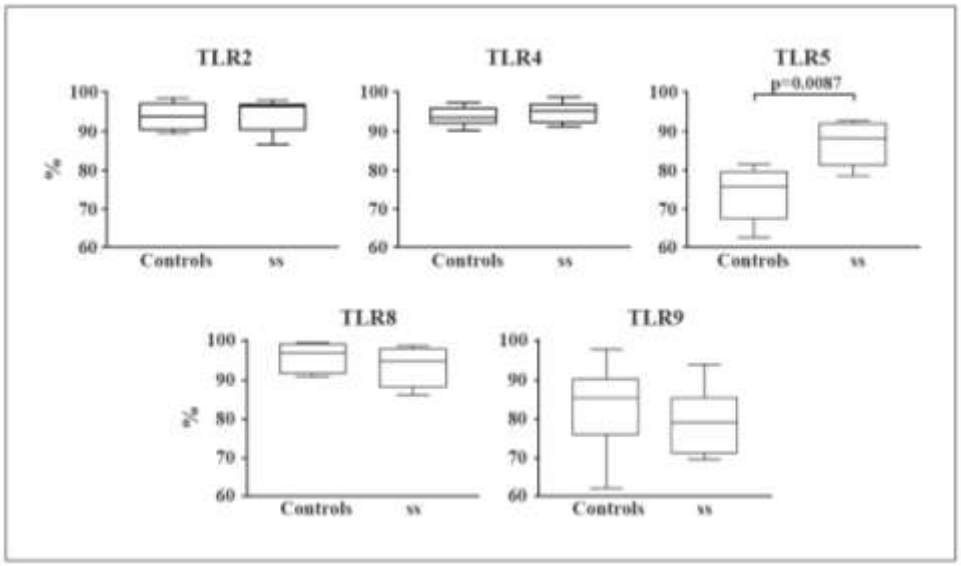

Figure 3. Percentage expression of TLRs on granulocytes in controls and SS patients. Data are shown as box plots. Box plots show minimum/maximum (range), median, 25th and 75th percentile. Only TLR5 shows a significant increase.

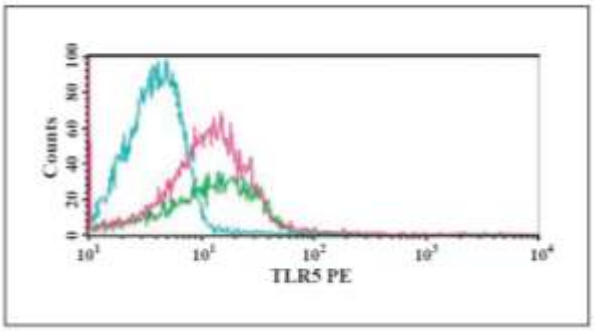

Figure 4. TLR5 representative histogram (sky-blue line: isotype control; green line: healthy donor; purple line: SS patient).

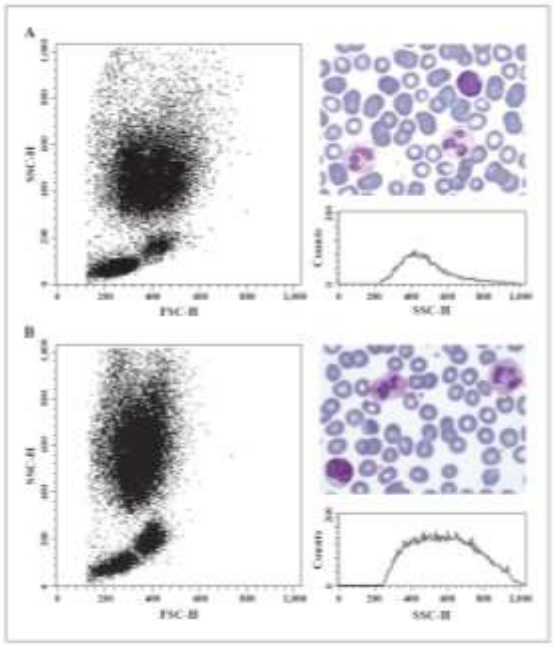

Figure 5. A) SS with recurrent infections: low Side Scatter distribution and few cytoplasmic granules in granulocytes. B) SS without infections: normal Side Scatter distribution and regular number of granules.