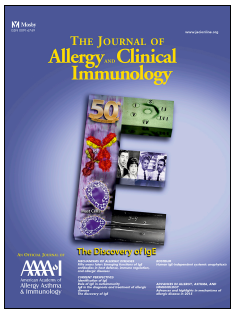
Accepted Manuscript



CD40 ligand deficiency causes functional defects of peripheral neutrophils that are improved by exogenous IFN-γ

Otavio Cabral-Marques, PhD, Tabata Takahashi França, MSc, Ashraf Al-Sbiei, PhD, Lena Friederike Schimke, MD, Taj Ali Khan, PhD, Claudia Feriotti, PhD, Tania Alves da Costa, PhD, Osvaldo Reis Junior, PhD, Cristina Worm Weber, MD, Janaíra Fernandes Ferreira, MD, Fabiola Scancetti Tavares, MD, Claudia Valente, MD, Regina Sumiko Watanabe Di Gesu, MD, Asif Iqbal, PhD, Gabriela Riemekasten, MD, Gustavo Pessini Amarante-Mendes, PhD, José Alexandre Marzagão Barbuto, PhD, Beatriz Tavares Costa-Carvalho, MD, PhD, Paulo Vitor Soeiro Pereira, PhD, Maria J. Fernandez-Cabezudo, PhD, Vera Lucia Garcia Calich, PhD, Luigi D. Notarangelo, MD, Troy R. Torgerson, MD, PhD, Basel K. al-Ramadi, PhD, Hans D. Ochs, MD, Antonio Condino-Neto, MD, PhD

PII: S0091-6749(18)30318-X

DOI: 10.1016/j.jaci.2018.02.026

Reference: YMAI 13334

To appear in: *Journal of Allergy and Clinical Immunology*

Received Date: 10 January 2017

Revised Date: 4 February 2018

Accepted Date: 13 February 2018

Please cite this article as: Cabral-Marques O, Takahashi França T, Al-Sbiei A, Friederike Schimke L, Khan TA, Feriotti C, da Costa TA, Junior OR, Weber CW, Ferreira JF, Tavares FS, Valente C, Di Gesu RSW, Iqbal A, Riemekasten G, Amarante-Mendes GP, Marzagão Barbuto JA, Costa-Carvalho BT, Pereira PVS, Fernandez-Cabezudo MJ, Calich VLG, Notarangelo LD, Torgerson TR, al-Ramadi BK, Ochs HD, Condino-Neto A, CD40 ligand deficiency causes functional defects of peripheral neutrophils that are improved by exogenous IFN-γ, *Journal of Allergy and Clinical Immunology* (2018), doi: 10.1016/j.jaci.2018.02.026.

This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final form. Please

note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

ACCEPTED MANUSCRIPT

CD40 ligand deficiency causes functional defects of peripheral neutrophils that are improved by exogenous IFN-γ

Otavio Cabral-Marques[a,b,*] PhD, Tabata Takahashi França[a,*] MSc, Ashraf Al-Sbiei[c] PhD, Lena Friederike Schimke[a,b] MD, Taj Ali Khan[a,d] PhD, Claudia Feriotti[a] PhD, Tania Alves da Costa[a] PhD, Osvaldo Reis Junior[e] PhD, Cristina Worm Weber[f] MD, Janaíra Fernandes Ferreira[g] MD, Fabiola Scancetti Tavares[h] MD, Claudia Valente[h] MD, Regina Sumiko Watanabe Di Gesu[i] MD, Asif Iqbal[j] PhD, Gabriela Riemekasten[b] MD, Gustavo Pessini Amarante-Mendes[k] PhD, José Alexandre Marzagão Barbuto[a,l] PhD, Beatriz Tavares Costa-Carvalho[m] MD, PhD, Paulo Vitor Soeiro Pereira[a,n] PhD, Maria J. Fernandez-Cabezudo[o] PhD, Vera Lucia Garcia Calich[a] PhD, Luigi D Notarangelo[p] MD, Troy R Torgerson[q] MD, PhD, Basel K. al-Ramadi[c] PhD, Hans D Ochs[q] MD, Antonio Condino-Neto[a] MD, PhD.

[a]Department of Immunology, Institute of Biomedical Sciences, University of São Paulo, São Paulo.
[b]Department of Rheumatology, University Lübeck, Lübeck, Germany;
cDepartment of Medical Microbiology & Immunology, College of Medicine and Health Sciences, UAE University, Al Ain, United Arab Emirates
[d]Department of Microbiology, Kohat University of Science and Technology, Kohat, Pakistan.
[e]The Central Laboratory of High Performance Technologies (LaCTAD), State University of Campinas, Campinas.
[f]Pediatric Allergy & Immunology Clinic, Caxias do Sul.
[g]Albert Sabin Hospital. Fortaleza.
[h]Pediatric Immunology Clínic, Unit of Pediatrics, Hospital de Base do Distrito Federal.
[i]Division of Allergy and Immunolgy, Department of Pediatrics, Conceicão Children Hospital, Porto Alegre.
[j]Department of Biochemistry, Institute of Chemistry, University of São Paulo, São Paulo, SP
[k]Department of Parasitology, Institute of Biomedical Sciences, University of São Paulo, São Paulo.
[l]Cell and Molecular Therapy Center, NETCEM, University of São Paulo, São Paulo.
[m]Division of Allergy-Immunology and Rheumatology, Department of Pediatrics, Federal University of São Paulo, São Paulo.
[n]Department of Pathology, Federal University of Maranhao, São Luis.
[o]Department of Biochemistry, College of Medicine and Health Sciences, UAE University, Al Ain, United Arab Emirates
[p]Laboratory of Clinical Immunology and Microbiology, Division of Intramural Research, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, MD.
[q]Department of Pediatrics, University of Washington School of Medicine, and Seattle Children's Research Institute, Seattle, WA.
*Contributed equally

Correspondence to:

Antonio Condino-Neto, MD, PhD

Department of Immunology - Institute of Biomedical Sciences

University of São Paulo

1730 Lineu Prestes Avenue, São Paulo, SP, ZIP 05508-000. Brazil

Tel (55) (11) 3091-7435 . Fax (55) (11) 3091-7224

E-mail: antoniocondino@gmail.com

ACCEPTED MANUSCRIPT

ABSTRACT

Background: Patients with X-linked Hyper-IgM syndrome (X-HIGM) due to CD40 ligand (CD40L) deficiency often present episodic, cyclic or chronic neutropenia, suggesting abnormal neutrophil development in the absence of CD40L-CD40 interaction. However, even when not neutropenic and despite immunoglobulin (Ig) replacement therapy, CD40L-deficient patients are susceptible to life-threatening infections caused by opportunistic pathogens, suggesting impaired function of phagocytes and the need of novel therapeutic approaches.

Objectives: To analyze whether peripheral neutrophils from CD40L-deficient patients display functional defects and to explore the *in vitro* effects of recombinant human interferon (rhIFN)-γ on neutrophil function.

Methods: We investigated the microbicidal activity, respiratory burst and transcriptome profile of neutrophils from CD40L-deficient patients. In addition, we evaluated whether the lack of CD40L in mice also affects neutrophil function.

Results: Neutrophils from CD40L-deficient patients exhibited defective respiratory burst and microbicidal activity which were improved *in vitro* by rhIFN-γ, but not soluble CD40L (sCD40L). Moreover, neutrophils from patients showed reduced CD16 protein expression and a dysregulated transcriptome suggestive of impaired differentiation. Similar to CD40L-deficient patients, CD40L-knockout mice were found to have impaired neutrophil responses. In parallel, we demonstrated that sCD40L induces the promyelocytic cell line HL-60 to proliferate and mature by regulating the expression of genes of the same gene ontology categories (e.g. cell differentiation) when compared to those dysregulated in peripheral blood neutrophils from CD40L-deficient patients.

Conclusion: Our data suggest a non-redundant role of CD40L-CD40 interaction in neutrophil development and function that could be improved *in vitro* by rhIFN-γ, indicating a potential novel therapeutic application for this cytokine.

ACCEPTED MANUSCRIPT

Clinical implications

Neutrophils from CD40L-deficient patients display defective development and function that are not corrected by therapies currently available. The improved function of CD40L-deficient neutrophils achieved by rhIFN-γ suggests a new potential therapeutic application for this cytokine.

ACCEPTED MANUSCRIPT

ACCEPTED MANUSCRIPT

78 **Capsule Summary**

79 Neutrophils from CD40L-deficient patients exhibit defective development and function that suggest an
80 important role of CD40L-CD40 interaction for neutrophil maturation. These defects improve following
81 *in vitro* exposure to rhIFN-γ.

82

83 **Grant support information**

84 This work has been supported by Fundação de Amparo à Pesquisa do Estado de São Paulo: Grant
85 2012/50515-4 to OCM, Grants 2012/51745-3 and 2016/22158-3 to ACN, and by the Jeffrey Modell
86 Foundation. The animal studies were supported by a UAEU Program for Advanced Research grant (No.
87 31M193) from the Office of Research and Sponsored Projects, UAE University (to BKa-R).

88

ACCEPTED MANUSCRIPT

ACCEPTED MANUSCRIPT

89 **Keywords: N**eutrophils, CD40 ligand; cell development, IFN-γ

90

91 **Running title:** Impaired neutrophil function in CD40L deficiency.

92

ACCEPTED MANUSCRIPT

ACCEPTED MANUSCRIPT

Abbreviations

CD40L: CD40 ligand

CD40LG: *CD40L* gene

CFU: colony forming units

CGD: chronic granulomatous disease

DEGs: differentially expressed genes

DHR: dihydrorhodamine

DMSO: dimethyl sulfoxide

FITC: fluorescein isothiocyanate

fMLP: N-formylmethionyl-leucyl-phenylalanine

G-CSF: granulocyte-colony stimulating factor

GO: Gene Ontology

Ig: immunoglobulin

IFN-γ: interferon-γ

NADPH: nicotinamide adenine dinucleotide phosphate

PMA: phorbol-12-myristate-13-acetate

Q-VD-OPh: carboxy terminal phenoxy group conjugated to the amino acids valine and aspartate

rhIFN-γ: recombinant human interferon-γ

RLU: relative light units

RNAseq: ribonucleic acid sequencing

ROS: reactive oxygen species

sCD40L: soluble CD40L

TPM: transcripts per million

X-HIGM: X-linked hyper-IgM syndrome

ACCEPTED MANUSCRIPT

ACCEPTED MANUSCRIPT

INTRODUCTION

Neutrophils are myeloid cells that play a major role in innate immunity through their microbicidal activity, thus representing the first line of defense against invading pathogens[1–5]. The neutrophil development program involves a variety of factors that direct myeloid specific gene expression, which leads to completion of cell differentiation[6,7]. The CD40 ligand (CD40L)/CD40 interaction, mediated locally by membrane/membrane contact and systemically by soluble CD40L (sCD40L) produced by T cells[8] and platelets[9] has been implicated in different biological processes and pathologies ranging from cancer to infectious and autoimmune diseases[10–12]. Of note, studies of healthy donor cells[13,14], patients with autoimmune diseases and high serum levels of sCD40L[15,16], and mouse models[17] demonstrate that the CD40L-CD40 interaction plays an important role during the inflammatory response and in the development of myeloid cells. This latter function is underlined by the fact that bone marrow stromal cells and myeloid progenitors express CD40, suggesting a direct or indirect involvement of the CD40L-CD40 interaction in the control of hematopoiesis by influencing the cytokine and growth factor network in the bone marrow[14,18,19].

Patients with X-linked Hyper-IgM syndrome (X-HIGM) or CD40L deficiency, which occurs due to loss of function mutations in the CD40L gene (*CD40LG*) can develop episodic, cyclic or chronic neutropenia[20,21]. Previous reports indicated a maturation arrest of the myeloid lineage at the promyelocyte-myelocyte stage in the bone marrow of CD40L-deficient patients[21,22], thus suggesting a pivotal role of CD40L-CD40 interaction during neutrophil development. Nonetheless, even when neutrophil counts are normal or CD40L-deficient patients are on granulocyte colony-stimulating factor (G-CSF) and immunoglobulin (Ig) replacement therapy,[23,24] these subjects are susceptible to life-threatening infections caused by opportunistic infectious agents such as fungi and intracellular bacteria. These infections cause high rates of morbidity and mortality[25], pointing to the need of complementary therapeutic options for patients with CD40L deficiency.

ACCEPTED MANUSCRIPT

Here, we aimed to investigate whether peripheral blood neutrophils from CD40L-deficient patients present functional defects. In addition, considering the impaired production of pro-inflammatory cytokines such as interferon (IFN)-γ by cells from CD40L- and CD40-deficient patients[12,26–28] and mice[29] and IFN-γ´s priming effect (*i.e.* effects that do not increase cell responses *per se*, but prepare them for markedly enhanced responses upon a secondary stimulation) on neutrophils[30–36], we hypothesize that *in vitro* IFN-γ treatment could improve presumptive impaired immune responses of peripheral neutrophils from patients with CD40L deficiency. Although sCD40L was also shown to possess the ability to prime peripheral blood neutrophils[13], this molecule is not a therapeutic option for CD40L-deficient patients because of severe side effects[37–39]. We therefore explored IFN-γ as a potential new therapy for patients with CD40L deficiency, which could improve disease-related morbidity and mortality rates[25].

ACCEPTED MANUSCRIPT

ACCEPTED MANUSCRIPT

MATERIAL AND METHODS

Subjects

We enrolled six CD40L-deficient patients (age range: 8-22 years) from six unrelated families. The clinical, immunological and genetic characteristics of the patients have been previously described[20,40] and are summarized in **Table S1**. The study was initiated in 2009 and completed in 2017; the patients were not neutropenic at the time of blood collection. However, all of them had developed episodes of intermittent neutropenia during their lives, which improved after G-CSF therapy. At the moment of blood collection, only P5 was still on G-CSF treatment. For each experiment, a healthy control (age range: 23-30) was included for comparison. Informed consent was obtained from the patients or their parents and from healthy controls. The blood was collected under institutional guidelines, and approval for the study was obtained from the Ethics Committee of the Institute of Biomedical Sciences, University of São Paulo, according to the Helsinki Convention.

Analysis of human neutrophil function

Neutrophils from healthy controls and CD40L-deficient patients were obtained from heparinized blood by dextran sedimentation followed by Ficoll-Hypaque centrifugation as previously described[41]. Viability and purity of fresh-isolated neutrophils were consistently >96% as determined by Trypan blue exclusion. When indicated, isolated neutrophils were incubated for 2 hours in the presence or absence of 100 U/ml recombinant human interferon-γ (rhIFN-γ) (Immukine, Boehringer Ingelheim, Germany) or 500 ng/ml trimeric sCD40L (Life Technologies, Frederick, MD). Neutrophil apoptosis at different time points was analyzed by flow cytometry after Annexin V- fluorescein isothiocyanate (FITC) staining as previously described[42,43] in the presence or absence of a carboxy terminal phenoxy group conjugated to the amino acids valine and aspartate (Q-VD-OPh) (10 µM; MP Biomedicals, Illkirch-Cedex, France), which is a broad spectrum caspase inhibitor with potent antiapoptotic properties[44].

ACCEPTED MANUSCRIPT

Neutrophil functions were analyzed as previously described: phagocytic capacity was assessed in whole blood by flow cytometry using propidium iodide (PI)-labeled *P. brasiliensis*[45]; respiratory burst (or production of reactive oxygen species, ROS) was measured by dihydrorhodamine (DHR) and luminol-dependent chemiluminescence[46,47]; neutrophil extracellular traps (NETs) release was investigated by fluorescence microscopy and quantified using Sytox Orange[48,49]; microbicidal activity was determined by counting colony forming units (CFU)[50]; neutrophil phenotype (expression of maturation and activation markers) was evaluated by flow cytometry[51–53]; and transcriptome profile was studied using RNA sequencing (RNAseq)[54]. Details of each method used to investigate neutrophils from patients with CD40L deficiency as well as studies performed using the promyelocytic leukemia cell line HL-60 cells are found in **Supplemental material and methods**.

Neutrophil responses in CD40L-deficient mice

CD40L-deficient ($CD40L^{-/-}$) mice were generously provided by Dr. Richard Flavell (Department of Immunobiology, Yale University School of Medicine, New Haven, CT) and have been described previously[55]. Female C57BL/6 mice were purchased from Harlan Olac (Bicester, UK). All strains were bred at the animal facility in the Faculty of Medicine and Health Sciences (UAE University), received rodent chow and water *ad libitum*, and were investigated at 8–12 weeks of age. All studies involving animals were carried out in accordance with, and after approval of, the animal research ethics committee of the Faculty of Medicine and Health Sciences, UAE University. Details of each method used to investigate neutrophil responses in CD40L-deficient mice can be found in Supplemental material and methods.

ACCEPTED MANUSCRIPT

Statistical analysis

Statistical significance was assessed by the nonparametric Mann-Whitney test. Data were expressed as median ± SD with 25^{th}, and 75^{th} percentiles or mean and standard deviation. The statistical analyses were performed using the GraphPad PRISM 5.01 software (GraphPad Software, San Diego, CA), and differences with a $p \leq 0.05$ were considered significant.

ACCEPTED MANUSCRIPT

ACCEPTED MANUSCRIPT

RESULTS

ROS production by neutrophils from CD40L-deficient patients is reduced with possible implications for their killing activity

In spite of normal phagocytic capacity (**Figure 1A**), neutrophils from patients with CD40L-deficiency displayed a reduced respiratory burst in response to *P. brasiliensis* (*Pb18*, a highly virulent isolate[50]) when compared to healthy controls (**Figure 1B**). The defect exhibited by neutrophils from CD40L-deficient patients was not restricted to this pathogen since they also showed a lower respiratory burst response upon activation with phorbol-12-myristate-13-acetate (PMA), N-formylmethionyl-leucyl-phenylalanine (fMLP; but not statistically significant) and *S. aureus* when compared to healthy controls (**Figure 1C)**. Of note, neutrophils from CD40L-deficient patients displayed sometimes a more vigorous respiratory burst following fMLP-stimulation than those of healthy controls, although this was statistically not significant. This fact can be explained by previously reported observations suggesting that blood neutrophils respond variably to stimuli such as fMLP, because of the existence of at least 2 distinct subsets of peripheral blood neutrophils that are differentially primed *in vivo*, and exhibit a diverse pattern of responses at sites of infection[56–59].

Notably, a partially affected respiratory burst, which is an essential mechanism that confers host resistance against opportunistic pathogens[60], possibly affects the killing activity of neutrophils , since a dose-dependent inhibition of ROS production induced by catalase (a hydrogen peroxide scavenger) abolished the capacity of healthy control neutrophils to kill *P. brasiliensis* (**Figure 1D and 1E**). A strong correlation between the respiratory burst and the inhibition of the microbicidal activity was observed (**Figure 1F**). Moreover, NET release, which is a mechanism often associated with ROS production[48,49], was impaired in human CD40L deficiency when neutrophils from patients were stimulated with PMA in comparison to those from healthy subjects (**Figure 1G and Figure S1)**. However, neither neutrophils from healthy controls nor those from CD40L-deficient patients released NETs in response to *P. brasiliensis* (**Figure S1**). The reasons for the differences between our finding

ACCEPTED MANUSCRIPT

and previous reports describing that *P. brasiliensis* is able to induce NETs[61,62] remain to be investigated. Of note is a previous report demonstrating that variations in the pattern of NET release occur when induced by distinct *P. brasiliensis* strains[63]; in addition, differences in antigenicity have been associated with distinct isolates of *P. brasiliensis*[64–66]. Therefore, these observations could explain the apparent opposite results.

rhIFN-γ improves defective repiratory burst and microbicidal activity of neutrophils from CD40L-deficient patients

We observed that rhIFN-γ alone was unable to significantly change the respiratory burst capacity of neutrophils from patients and healthy controls. However, rhIFN-γ enabled neutrophils from both CD40L-deficient patients and healthy controls to increase the PMA-induced respiratory burst (**Figure 2A**). While still showing a reduced respiratory burst compared to rhIFN-γ-primed neutrophils from healthy subjects, rhIFN-γ priming significantly elevated the PMA-induced respiratory burst of neutrophils from CD40L-deficient patients when compared to their unprimed neutrophils (**Figure 2A**). We also incubated neutrophils in the presence of sCD40L, although it is not a therapeutic option for CD40L-deficient patients, since the CD40L-CD40 interaction needs an endogenous tight regulation *in vivo* in order to avoid the development of severe side effects[37–39]. While increasing the neutrophil responses of both CD40L-deficient patients and healthy controls, sCD40L priming did not significantly elevate the PMA-induced respiratory burst of neutrophils from patients when compared to their unprimed neutrophils.

Of note, neutrophils from CD40L-deficient patients showed a reduced microbicidal activity when challenged with *P. brasiliensis* (**Figure 2B**). Similarly to the effects on the respiratory burst, both rhIFN-γ and sCD40L increased the microbicidal activity of neutrophils from patients and healthy controls. However, only rhIFN-γ-primed neutrophils from CD40L-deficient patients showed microbicidal activity comparable to that from untreated neutrophils from healthy controls (**Figure 2B**).

ACCEPTED MANUSCRIPT

Dysregulated transcriptome of neutrophils from CD40L-deficient patients is improved by *in vitro* treatment with rhIFN-γ

To gain insights into mechanisms by which IFN-γ alters immune responses of neutrophils derived from CD40L-deficient patients, we used a high-throughput approach. The transcriptome of patient-derived neutrophils was evaluated in the absence and presence of rhFN-γ by RNAseq[67–69]. Because, RNAseq analysis demands a large volume of blood to obtain sufficient neutrophil-derived mRNA[54] we had to consider ethical rules and limited the amount of blood that can be safely collected from the patients. We therefore were only able to compare rhIFN-γ-treated (for 2 hours), but not sCD40L-treated neutrophils, to resting neutrophils. RNAseq analysis of untreated neutrophils from CD40L-deficient patients demonstrated a functional network of 164 differentially expressed genes (DEGs; 24 downregulated and 140 upregulated) when compared to those from healthy controls (**Figure S2**). Among others, the subsets of DEGs in neutrophils from CD40L-deficient patients include genes directly linked to several immune response aspects such as innate immune responses (GO0045087), oxidation-reduction process (GO:0055114) and genes regulating cell/myeloid differentiation (GO:0030154/GO:0030099) (**Table 1**). Sub-pathway analysis of DEGs involved in the innate immune responses using the Reactome Knowledgebase[70,71], revealed that the IFN-γ signaling pathway is severely impaired in the absence of CD40L (**Figure 2C**), suggesting an explanation why the function of peripheral neutrophils from CD40L-deficient patients improved when exposed to rhIFN-γ. Furthermore, in agreement with previous reports indicating that IFN-γ priming triggers *de novo* mRNA synthesis[33,72], the addition of rhIFN-γ to neutrophil cultures improved broadly the pattern of gene expression in neutrophils from CD40L-deficient patients, reducing the number of DEGs to 21 genes (**Figure 2D and 2E**). Taken together, our data suggest that rhIFN-γ improves the dysregulation of a network of molecular interactions in peripheral neutrophils from CD40L-deficient patients.

ACCEPTED MANUSCRIPT

Neutrophils from CD40L-deficient patients exhibit an immature phenotype

Functional defects of neutrophils from CD40L-deficient patients are associated with DEGs involved in cell/myeloid differentiation, thus indicating the presence of immature neutrophils in the blood circulation. In accordance, despite normal expression of molecules associated with the myeloid cell lineage (CD15, CD18, CD11b and CD11c, CD35, CD63, and CD66b), neutrophils from CD40L-deficient patients demonstrated reduced expression of CD16 (**Figure 3A and 3B**), a typical marker of neutrophil maturation[73]. Of note, neither rhIFN-γ nor sCD40L were able to increase CD16 expression by neutrophils from CD40L-deficient patients (data not shown), indicating a non-redundant role of the CD40L-CD40 interaction for early stages of myeloid cells differentiation. On the other hand, neutrophils from CD40L-deficient patients displayed normal kinetics of the expression of phosphatidylserine on the outer leaflet of their cell membrane in a caspase-dependent manner (**Figure 3C, 3D, and S3)** as well as normal expression of the activation marker CD62L in resting state when compared to those from healthy controls (**Figure 3E**). These findings indicate that the reduced CD16 expression on patients´ neutrophils did not reflect the influence of neutrophils undergoing apoptosis or activation[74–76]. However, despite the normal expression of several TLRs (TLR1, TLR2, TLR4 and TLR6) (**Figure 3F**), the shedding of CD62L from neutrophils from CD40L-deficient patients was slightly decreased in response to PMA and LPS, but statistically significant only when activated by the TLR1/TLR2 agonist Pam3CSK in comparison with healthy control subjects (**Figure 3E**). Neither rhIFN-γ nor sCD40L were able to improve the shedding of CD62L molecule by neutrophils from CD40L-deficient patients (data not shown). Thus, suggesting that the absence of CD40L results in multiple neutrophil functional defects.

At the latest stage of the study, patients P1-P3 had undergone hematopoietic stem cell transplantation, patient P4 had died in a cachectic state after recurrent diarrhea caused by the concomitant occurrence of refractory *Cryptosporidium parvum* and *Mycobacterial tuberculosis* infection, while patient P6 had developed severe and refractory Leishmaniosis and has been

ACCEPTED MANUSCRIPT

hospitalized for more than 2 months. Therefore, experiments to evaluate the simultaneous expression of CD62L and CD11b on neutrophils, which are both adhesion molecules well known to be down- and up-regulated respectively by cell activation[77,78], could only be performed with neutrophils from P5. While CD62L shedding in response to PMA was robust by neutrophils from both the control and patient P5, CD62L shedding in response to fMLP was less profound (Fig S4A). CD11b, on the other hand, was downregulated by exposure to PMA, but upregulated by fMLP (Fig S4B). These results do not support a clear correlation between CD62L shedding and CD11b upregulation. A possible explanation for this abnormal pattern of CD62L shedding and CD11b upregulation could be provided by the neutrophil isolation method we used, since washing steps and erythrocyte lysis are known to up-regulate the expression of CD11b by neutrophils[77].

Impaired neutrophil immune responses in CD40L$^{-/-}$ mice resemble defects shown by neutrophils of CD40L-deficient patients

In order to exclude the possibility that differences shown by neutrophils from CD40L-deficient patients are age-related or secondary to infections and/or therapies to which the patients are being subjected, we evaluated whether CD40L-deficient (CD40L$^{-/-}$) mice also present neutrophil defects in response to infection with attenuated *Salmonella enterica* Serovar *Typhimurium* (BRD509). CD40L$^{-/-}$ mice showed a significantly reduced number of neutrophils in the peritoneal cavity in response to intraperitoneal injection of thioglycollate or BRD509 in comparison to wild-type mice (**Figure 4A)**. In parallel, CD40L$^{-/-}$ mice were unable to control bacterial proliferation in the peritoneal cavity (**Figure 4B)**, and their neutrophils displayed reduced respiratory burst (**Figure 4C),** while maintaining normal phagocytic capacity (**Figure 4D).**

ACCEPTED MANUSCRIPT

sCD40L accelerates the differentiation of the human promyelocytic cell line HL-60

Our findings suggesting defects in maturation and function of neutrophils in the absence of CD40L-CD40 signaling are in agreement with previous reports demonstrating that the CD40L-CD40 interaction modulates the development of myeloid cells by regulating the milieu of cytokines in the bone marrow microenvironment[14,18,19]**.** Therefore, we hypothesized that the defects we observed could, at least in part, be explained by defective CD40L-CD40 signaling required for the maturation of myeloid cells. To investigate this possibility, because bone marrow-derived cells from CD40L-deficient patients could not be obtained for investigation, we assessed the effects of CD40L-CD40 interaction on the maturation of the promyelocytic leukemia cell line HL-60, an often used *in vitro* model to study the development of myeloid cells[79–81]. Cell viability in the presence of dimethyl sulfoxide (DMSO), a chemical agent traditionally used to induce granulocyte differentiation[79], and/or sCD40L was not different than the viability of untreated HL-60 cells during 6 days of culture (**Figure S5**). RNAseq analysis of HL-60 cells demonstrated that sCD40L induced the upregulation of 62 genes and downregulation of 19 genes compared to untreated HL-60 cells. Likewise, sCD40L induced upregulation of 19 genes and downregulation of 26 genes in HL-60 cells treated with DMSO plus sCD40L when compared to those cultured in the presence of DMSO alone (**Figure 5A and Table 1**). The functional association network of HL-60 genes affected by sCD40L is demonstrated in **Figure S6**. Considering the malignant features of HL-60 cells, it is not surprising that the sCD40L-induced genes were not the same DEGs displayed by neutrophils from CD40L-deficient patients. Nevertheless, the HL-60 genes controlled by sCD40L belong to the same Gene Ontology categories (e.g. innate immune responses GO0045087, oxidation-reduction process GO:0055114, and genes regulating cell/myeloid cell differentiation GO:0030154/GO:0030099) when compared to DEGs of peripheral blood neutrophils from CD40L-deficient patients (**Figure 5A and Table 1**).

Except for a slight reduction in the expression of CD40, when HL-60 cells were cultured in the presence of DMSO and/or sCD40L, we did not observe phenotypic changes (*e.g*. alterations in the

ACCEPTED MANUSCRIPT

expression of CD15 and CD16) by HL-60 cells in the presence of sCD40L (**Figure 5B**). This suggests that the direct effects of sCD40L on the regulation of CD16 (and consequently cell maturation) occur at early stages of hematopoietic cell differentiation or alternatively, that additional factors modulate CD16 expression. On the other hand, while DMSO inhibited HL-60 cell proliferation, sCD40L accelerated HL-60 proliferation significantly (**Figure 5C**). Moreover, as previously demonstrated[80,82], DMSO-induced differentiation enabled HL-60 cells to migrate toward a N-formylmethionyl-leucyl-phenylalanine (fMLP) gradient. Addition of sCD40L to the culture alone did not influence the migratory capacity of HL-60 cells. However, sCD40L stimulation in conjunction with DMSO increased both the spontaneous (without chemoattractant) and the fMLP-induced migration of HL-60 cells **(Figure 5D).**

Of note, sCD40L in the presence of DMSO significantly increased the PMA-stimulated respiratory burst of HL-60 cells (**Figure 5E**) when compared to cells culture only in the presence of DMSO. However, PMA-stimulated HL-60 cells cultured in both conditions, DMSO alone or DMSO plus sCD40, showed a very low response in their respiratory burst, which was comparable to the response of resting peripheral blood neutrophils from healthy controls (**Figure 5F**). This fact is in agreement with previous reports showing that, although HL-60 cells acquire certain characteristics of mature neutrophils, the magnitude of their respiratory burst response is significantly lower compared to primary blood-derived neutrophils[83,84]. This may be the reason for not observing even a slight effect of sCD40L on the microbicidal activity when comparing DMSO-differentiated with sCD40L plus DMSO-differentiated HL-60 cells (**Figure 5G**). However, although sCD40L alone did not increase the respiratory burst of HL-60 cells, it was able to enhance their killing capacity in comparison to untreated cells, suggesting that ROS-independent microbicidal mechanisms are also affected by sCD40L during cell development, a possibility requiring future investigation. As previously reported[85], another peculiarity observed was the fact that 100 U/ml IFN-γ added to the culture of malignant hematologic HL-60 cells induced rapid cell death with only 10% or less of cells remaining alive after three days of culture (data not shown).

ACCEPTED MANUSCRIPT

DISCUSSION

The present work suggests that in addition to the often reported episodic, cyclic or chronic neutropenia[20,21], CD40L-deficient patients also have functional defects of peripheral blood neutrophils due to abnormalities in myeloid development (**Figure 6**). This is not due to the age difference between our CD40L-deficient patients (8 – 22 years of age when studied) and controls (age range 23-30 years) since CD40L-deficient mice challenged with the attenuated Salmonella strain BRD509 showed similar neutrophil defects. Furthermore, as we routinely observe in our diagnostic lab, infants suspected wrongly to have chronic granulomatous disease (CGD), when tested for phagocyte defects (as we assessed in this study), had normal phagocyte responses that were comparable to those of healthy young adult controls[40]. In addition, after the first year of life infants have consistently normal neutrophil counts and function (e.g. chemotaxis, migration, phagocytosis, respiratory burst, expression of maturation markers such as CD16 and microbicidal activity) when compared to adults[86–88]. Of note, different to the constant B cell defects identified in X-HIGM, we hypothesize that the defects we report here might follow the intermittent pattern of neutrophil counts characterized by episodic, cyclic or chronic neutropenia frequently associated to this immune defect[89]. If that is the case, future studies clarifying the periodicity and rhythm of these neutrophil functional defects as well as which factors determine their occurrence and at what age do they become more frequent will be relevant to improve the treatment of CD40L deficiency.

Respiratory burst defects are associated with markedly reduced production of ROS by phagocytes due to intrinsic defects of the nicotinamide adenine dinucleotide phosphate (NADPH) oxidase components. These defects commonly cause CGD[90], a severe innate immune deficiency rendering affected patients susceptible to recurrent life-threatening infections[90–92]. Notably, CGD patients and female carriers of X-linked CGD with only slightly affected respiratory burst may also develop recurrent and severe infections caused by opportunistic intracellular bacteria and fungi[91,93]. In agreement, our findings suggest that even partially impaired respiratory burst shown by neutrophils and

ACCEPTED MANUSCRIPT

previously by macrophages[40] from CD40L-deficient patients contributes to their increased susceptibility to infections. Further, correlating the dose-dependent inhibition of the respiratory burst by catalase with reduced killing of *P.brasiliensis* killing by neutrophils from healthy subjects, is in line with the existence of a ROS dependent mechanism of microbial killing[94].

The high morbidity and mortality rates of insufficiently treated CD40L-deficient patients[20,21,23,25], emphasize the need for novel therapeutic options. Although sCD40L priming improved the response of neutrophils from CD40L-deficient patients[13], the improvement of neutrophil responses was far less than that shown by rhIFN-γ, which is known to have priming effect on the repiratory burst and fungicidal activity of peripheral blood neutrophils[33,95]. Similarly, *in vivo* treatment with recombinant sCD40L is insufficient to completely correct the effect of CD40L deficiency on adaptive immune responses[96]. In line with previous reports demonstrating a role of CD40L-CD40 stimulation during myelopoiesis[14,18,97,98], our finding of significant effects of sCD40L on the maturation of HL-60 cells suggests that sCD40L not only affects mature immune cells, but that the CD40L-CD40 interaction is also required for the early development and maturation of hematopoietic cells in primary lymphoid organs. In accordance, neither sCD40L nor rhIFN-γ increased the CD16 (a glycosylphosphatidylinositol (GPI)-anchored protein serving as maturation marker for neutrophils) expression on the surface of neutrophils from CD40L-deficient patients and addition of sCD40L failed to enhance the expression of CD16 by HL60 cells, suggesting that CD40L-CD40 signaling is most important during the earliest stages of hematopoiesis.

A recent study of bone marrow from CD40L-deficient mice suggested that CD40L controls hematopoietic stem and progenitor cell biology by inducing adhesion-mediated quiescence[99], which is an actively maintained state in which signaling pathways are involved in maintaining a poised state that allows rapid activation[100]. Other reports have described the effects of CD40L-CD40 interaction on hematopoietic progenitor cell proliferation and differentiation[101,102]. Multidimensional flow cytometric studies of normal human bone marrow aspirates demonstrate that expression of the Fcγ receptor IIIB

ACCEPTED MANUSCRIPT

(FcγRIIIB, also known as CD16), correlates with maturation stages of neutrophils. CD16 expressed on promyelocytic cells is upregulated during the different stages of neutrophil development, reaching its highest expression at late maturation stages (metamyelocytes and mature neutrophils)[51,103]. Mechanistically, the downregulation of CD16 expression by neutrophils from CD40L-deficient patients is likely not due to impaired synthesis of the GPI anchor moiety, since their neutrophils expressed CD66b (another GPI-anchored protein)[104] normally when compared to healthy controls. Of note, CD40L stimulation was shown to enhance the proliferation and differentiation of $CD34^+$ progenitor cells toward the myeloid lineage by regulating the network of several pleiotropic hematopoietic growth factors that are present in the bone marrow, such as the production of fms-like tyrosine kinase 3 (flt3)-ligand, granulocyte colony-stimulating factor (G-CSF), and granulocyte/macrophage colony stimulating factor (GM-CSF) by bone marrow stromal cells [14,18,105,106]. Therefore, multiple cytokine/growth factor pathways remain to be investigated in order to better understand the neutrophil functional and maturation defects in CD40L deficiency reported here.

Historically, peripheral blood neutrophils have been thought not to be able to significantly change their transcriptome profile. However, this concept has been revised when a significant change in the gene expression profile of peripheral neutrophils was observed in response to priming cytokines[33,43]. In agreement, we found that rhIFN-γ shifted the transcriptome of peripheral blood neutrophils from CD40L-deficient patients towards that of normal neutrophils, thus improving the responses of neutrophils deprived of CD40L-CD40 interaction during development[14,18,97,98]. These observations are in line with the essential need of proper control of gene expression for normal myeloid cell development and function[79,107,108]. In concurrence with the network view of the immune system orchestrated by a system-wide intercommunication between a variety of cells and soluble molecules[109–112], the study of CD40L-deficient patients demonstrates that CD40L-CD40 interaction, which was initially thought to be predominantly involved in the regulation of B cell maturation and Ig class switch recombination, plays a broader role and is essential for the development and maturation of the adaptive and innate immune

ACCEPTED MANUSCRIPT

458 system. An important question raised by the data presented here relates to the previously reported
459 defects in the function of T cells[26], dendritic cells[27,28] and macrophages[40] in the absence of CD40L-
460 CD40 signaling. Are these abnormalities caused by the absence of CD40L-CD40 interaction in the
461 peripheral immune system or does the absence of this interaction affect hematopoietic cell development
462 in central lymphoid organs?

463 Taken together, the data presented here provide new insights into the role played by
464 CD40L/CD40 interaction in innate immunity. We have extended our previously reported findings
465 obtained from studying macrophages[40] to neutrophils suggesting that the defective function of
466 phagocytes in CD40L deficiency can be improved by rhIFN-γ. Such adjuvant therapy could be
467 considered as a potential option for CD40L-deficient patients in cases of life-threatening refractory
468 infections or even as a prophylactic therapy given in a similar way as it is administrated to CGD
469 patients.

470

471

ACCEPTED MANUSCRIPT

ACCEPTED MANUSCRIPT

ACKNOWLEDGMENTS

The authors thank all patients and their families for their participation in this study. We acknowledge FAPESP (Grant 2012/50515-4 to OCM and Grants 2012/51745-3 and 2016/22158-3 to ACN), CNPq, and the Jeffrey Modell Foundation for financial support. We thank Dr. Antje Muller from the Department of Rheumatology, University of Lübeck, and Dr. Jing Sun from the University of Cincinnati College of Medicine; Cincinnati, Ohio, USA, for critical reading of the manuscript.

ACCEPTED MANUSCRIPT

ACCEPTED MANUSCRIPT

REFERENCES

1. Uhl B, Vadlau Y, Zuchtriegel G, et al. Aged neutrophils contribute to the first line of defense in the acute inflammatory response. *Blood*. 2016;128(19):2327–2337.
2. Kolaczkowska E, Kubes P. Neutrophil recruitment and function in health and inflammation. *Nat. Rev. Immunol.* 2013;13(3):159–75.
3. Mayadas TN, Cullere X, Lowell CA. The Multifaceted Functions of Neutrophils. *Annu. Rev. Pathol. Mech. Dis.* 2014;9(1):181–218.
4. Soehnlein O, Lindbom L. Phagocyte partnership during the onset and resolution of inflammation. *Nat. Rev. Immunol.* 2010;10(6):427–439.
5. Nauseef WM, Borregaard N. Neutrophils at work. *Nat. Immunol.* 2014;15(7):602–11.
6. Lian Z, Kluger Y, Greenbaum DS, et al. Genomic and proteomic analysis of the myeloid differentiation program: Global analysis of gene expression during induced differentiation in the MPRO cell line. *Blood*. 2002;100(9):3209–3220.
7. Newburger PE, Subrahmanyam Y V, Weissman SM. Global analysis of neutrophil gene expression. *Curr. Opin. Hematol.* 2000;7(1):16–20.
8. Mazzei GJ, Edgerton MD, Losberger C, et al. Recombinant soluble trimeric CD40 ligand is biologically active. *J. Biol. Chem.* 1995;270(13):7025–7028.
9. Henn V, Slupsky JR, Gr a fe M, et al. CD40 ligand on activated platelets triggers an inflammatory reaction of endothelial cells. *Nature*. 1998;391(6667):591–594.
10. Kato K, Santana-Sahagún E, Rassenti LZ, et al. The soluble CD40 ligand sCD154 in systemic lupus erythematosus. *J. Clin. Invest.* 1999;104(7):947–55.
11. Marigo I, Zilio S, Desantis G, et al. T Cell Cancer Therapy Requires CD40-CD40L Activation of Tumor Necrosis Factor and Inducible Nitric-Oxide-Synthase-Producing Dendritic Cells. *Cancer Cell*. 2016;30(4):651.
12. Subauste CS, Wessendarp M, Sorensen RU, Leiva LE. CD40-CD40 ligand interaction is central to cell-mediated immunity against Toxoplasma gondii: patients with hyper IgM syndrome have a defective type 1 immune response that can be restored by soluble CD40 ligand trimer. *J. Immunol.* 1999;162(11):6690–700.
13. Khan SY, Kelher MR, Heal JM, et al. Soluble CD40 ligand accumulates in stored blood components, primes neutrophils through CD40, and is a potential cofactor in the development of transfusion-related acute lung injury. *Blood*. 2006;108(7):2455–62.
14. Solanilla A, Dechanet J, El Andaloussi A, et al. CD40-ligand stimulates myelopoiesis by regulating flt3-ligand and thrombopoietin production in bone marrow stromal cells. *Blood*. 2000;95(12):3758–64.
15. Goules A, Tzioufas AG, Manousakis MN, et al. Elevated levels of soluble CD40 ligand (sCD40L) in serum of patients with systemic autoimmune diseases. *J. Autoimmun.* 2006;26(3):165–71.
16. Perazzio SF, Soeiro-Pereira PV, dos Santos VC, et al. Soluble CD40L is associated with increased oxidative burst and neutrophil extracellular trap release in Behçet's disease. *Arthritis Res. Ther.* 2017;19(1):235.
17. Song Z, Jin R, Yu S, et al. CD40 Is Essential in the Upregulation of TRAF Proteins and NF-KappaB-Dependent Proinflammatory Gene Expression after Arterial Injury. *PLoS One*. 2011;6(8):e23239.

ACCEPTED MANUSCRIPT

18. Mavroudi I, Papadaki V, Pyrovolaki K, et al. The CD40/CD40 ligand interactions exert pleiotropic effects on bone marrow granulopoiesis. *J. Leukoc. Biol.* 2011;89(5):771–83.

19. Saeland S, Duvert V, Caux C, et al. Distribution of surface-membrane molecules on bone marrow and cord blood CD34+ hematopoietic cells. *Exp. Hematol.* 1992;20(1):24–33.

20. Cabral-Marques O, Klaver S, Schimke LF, et al. First report of the hyper-IgM syndrome registry of the latin american society for immunodeficiencies: Novel mutations, unique infections, and outcomes. *J. Clin. Immunol.* 2014;34(2):146–156.

21. Levy J, Espanol-Boren T, Thomas C, et al. Clinical spectrum of X-linked hyper-IgM syndrome. *J. Pediatr.* 1997;131(1 I):47–54.

22. Jasinska A, Kalwak K, Trelinska J, et al. Successful haploidentical PBSCT with subsequent T-cell addbacks in a boy with HyperIgM syndrome presenting as severe congenital neutropenia. *Pediatr. Transplant.* 2013;17(1):E37-40.

23. Cabral-Marques O, Schimke L-F, Pereira PVS, et al. Expanding the clinical and genetic spectrum of human CD40L deficiency: the occurrence of paracoccidioidomycosis and other unusual infections in Brazilian patients. *J. Clin. Immunol.* 2012;32(2):212–20.

24. Winkelstein JA, Marino MC, Ochs H, et al. The X-linked hyper-IgM syndrome: clinical and immunologic features of 79 patients. *Medicine (Baltimore).* 2003;82(6):373–84.

25. de la Morena MT, Leonard D, Torgerson TR, et al. Long term outcomes of 176 patients with X-linked hyper IgM syndrome treated with or without hematopoietic cell transplantation. *J. Allergy Clin. Immunol.* 2016;

26. Jain A, Atkinson TP, Lipsky PE, et al. Defects of T-cell effector function and post-thymic maturation in X- linked hyper-IgM syndrome. *J. Clin. Invest.* 1999;103(8):1151–1158.

27. Fontana S, Moratto D, Mangal S, et al. Functional defects of dendritic cells in patients with CD40 deficiency. *Blood.* 2003;102(12):4099–106.

28. Cabral-Marques O, Arslanian C, Ramos RN, et al. Dendritic cells from X-linked hyper-IgM patients present impaired responses to Candida albicans and Paracoccidioides brasiliensis. *J. Allergy Clin. Immunol.* 2012;129(3):778–86.

29. Al-Ojali SM, Moore CBT, Fernandez-Cabezudo MJ, al-Ramadi BK. Enhancement of the anti-Salmonella immune response in CD154-deficient mice by an attenuated, IFN-γ-expressing, strain of Salmonella enterica serovar Typhimurium. *Microb. Pathog.* 2012;52(6):326–335.

30. Miralda I, Uriarte SM, McLeish KR. Multiple Phenotypic Changes Define Neutrophil Priming. *Front. Cell. Infect. Microbiol.* 2017;7:217.

31. Yao Y, Matsushima H, Ohtola JA, et al. Neutrophil priming occurs in a sequential manner and can be visualized in living animals by monitoring IL-1β promoter activation. *J. Immunol.* 2015;194(3):1211–24.

32. Tennenberg SD, Fey DE, Lieser MJ. Oxidative priming of neutrophils by interferon-gamma. *J. Leukoc. Biol.* 1993;53(3):301–8.

33. Ellis TN, Beaman BL. Interferon-gamma activation of polymorphonuclear neutrophil function. *Immunology.* 2004;112(1):2–12.

34. AAS V, LARSEN K, IVERSEN J-G. IFN-γ Induces Calcium Transients and Increases the Capacitative Calcium Entry in Human Neutrophils. *J. Interf. Cytokine Res.* 1998;18(3):197–205.

35. Gougerot-Podicalo MA, Elbim C, Chollet-Martin S. [Modulation of the oxidative burst of human neutrophils by pro- and anti-inflammatory cytokines]. *Pathol. Biol. (Paris).* 1996;44(1):36–41.

ACCEPTED MANUSCRIPT

36. Jabłońska E, Kiluk M, Markiewicz W, Jabłoński J. Priming effects of GM-CSF, IFN-gamma and TNF-alpha on human neutrophil inflammatory cytokine production. *Melanoma Res.* 2002;12(2):123–8.

37. Sacco MG, Ungari M, Catò EM, et al. Lymphoid abnormalities in CD40 ligand transgenic mice suggest the need for tight regulation in gene therapy approaches to hyper immunoglobulin M (IgM) syndrome. *Cancer Gene Ther.* 2000;7(10):1299–1306.

38. André P, Nannizzi-Alaimo L, Prasad SK, Phillips DR. Platelet-derived CD40L: the switch-hitting player of cardiovascular disease. *Circulation*. 2002;106(8):896–9.

39. Slupsky JR, Kalbas M, Willuweit A, et al. Activated platelets induce tissue factor expression on human umbilical vein endothelial cells by ligation of CD40. *Thromb. Haemost.* 1998;80(6):1008–14.

40. Cabral-Marques O, Ramos RN, Schimke LF, et al. Human CD40L deficiency dysregulates the macrophage transcriptome causing functional defects that are improved by exogenous IFN-γ. *J. Allergy Clin. Immunol.* 2016;

41. Kotz KT, Xiao W, Miller-Graziano C, et al. Clinical microfluidics for neutrophil genomics and proteomics. *Nat. Med.* 2010;16(9):1042–7.

42. Amarante-Mendes GP, Finucane DM, Martin SJ, et al. Anti-apoptotic oncogenes prevent caspase-dependent and independent commitment for cell death. *Cell Death Differ.* 1998;5(4):298–306.

43. Wright HL, Thomas HB, Moots RJ, Edwards SW. RNA-Seq Reveals Activation of Both Common and Cytokine-Specific Pathways following Neutrophil Priming. *PLoS One*. 2013;8(3):.

44. Caserta TM, Smith AN, Gultice AD, Reedy MA, Brown TL. Q-VD-OPh, a broad spectrum caspase inhibitor with potent antiapoptotic properties. *Apoptosis*. 2003;8(4):345–52.

45. Feriotti C, Loures F V., Frank de Araújo E, da Costa TA, Calich VLG. Mannosyl-Recognizing Receptors Induce an M1-Like Phenotype in Macrophages of Susceptible Mice but an M2-Like Phenotype in Mice Resistant to a Fungal Infection. *PLoS One*. 2013;8(1):.

46. Soeiro-Pereira P V, Falcai A, Kubo CA, et al. BAY 41-2272, a soluble guanylate cyclase agonist, activates human mononuclear phagocytes. *Br. J. Pharmacol.* 2012;166(5):1617–1630.

47. Bustamante J, Arias AA, Vogt G, et al. Germline CYBB mutations that selectively affect macrophages in kindreds with X-linked predisposition to tuberculous mycobacterial disease. *Nat. Immunol.* 2011;12(3):213–21.

48. Brinkmann V, Reichard U, Goosmann C, et al. Neutrophil extracellular traps kill bacteria. *Science (80-.).* 2004;303(5663):1532–1535.

49. Metzler KD, Fuchs TA, Nauseef WM, et al. Myeloperoxidase is required for neutrophil extracellular trap formation: Implications for innate immunity. *Blood*. 2011;117(3):953–959.

50. Pina A, Saldiva PHN, Restrepo LEC, Calich VLG. Neutrophil role in pulmonary paracoccidioidomycosis depends on the resistance pattern of hosts. *J. Leukoc. Biol.* 2006;79:1202–1213.

51. Terstappen LW, Safford M, Loken MR. Flow cytometric analysis of human bone marrow. III. Neutrophil maturation. *Leukemia*. 1990;4(9):657–63.

52. Moulding DA, Hart CA, Edwards SW. Regulation of neutrophil FcgammaRIIIb (CD16) surface expression following delayed apoptosis in response to GM-CSF and sodium butyrate. *J. Leukoc. Biol.* 1999;65(6):875–82.

ACCEPTED MANUSCRIPT

53. von Bernuth H, Ku C-L, Rodriguez-Gallego C, et al. A fast procedure for the detection of defects in Toll-like receptor signaling. *Pediatrics*. 2006;118(6):2498–2503.

54. Wright HL, Thomas HB, Moots RJ, Edwards SW. RNA-seq reveals activation of both common and cytokine-specific pathways following neutrophil priming. *PLoS One*. 2013;8(3):e58598.

55. al-Ramadi BK, Fernandez-Cabezudo MJ, Ullah A, El-Hasasna H, Flavell RA. CD154 is essential for protective immunity in experimental salmonella infection: evidence for a dual role in innate and adaptive immune responses. *J. Immunol.* 2006;176(1):496–506.

56. Elbim C, Chollet-Martin S, Bailly S, Hakim J, Gougerot-Pocidalo MA. Priming of polymorphonuclear neutrophils by tumor necrosis factor alpha in whole blood: identification of two polymorphonuclear neutrophil subpopulations in response to formyl-peptides. *Blood*. 1993;82(2):633–40.

57. Daniels RH, Elmore MA, Hill ME, et al. Priming of the oxidative burst in human neutrophils by physiological agonists or cytochalasin B results from the recruitment of previously non-responsive cells. *Immunology*. 1994;82(3):465–72.

58. Eggleton P, Fisher D, Crawford N. Heterogeneity in the circulating neutrophil pool: studies on subpopulations separated by continuous flow electrophoresis. *J. Leukoc. Biol.* 1992;51(6):617–25.

59. Eggleton P, Wang L, Penhallow J, Crawford N, Brown KA. Differences in oxidative response of subpopulations of neutrophils from healthy subjects and patients with rheumatoid arthritis. *Ann. Rheum. Dis.* 1995;54(11):916–23.

60. Meloni-Bruneri LH, Campa a, Abdalla DS, et al. Neutrophil oxidative metabolism and killing of P. brasiliensis after air pouch infection of susceptible and resistant mice. *J. Leukoc. Biol.* 1996;59(4):526–533.

61. Bachiega TF, Dias-Melicio LA, Fernandes RK, et al. Participation of dectin-1 receptor on NETs release against Paracoccidioides brasiliensis : Role on extracellular killing. *Immunobiology*. 2016;221(2):228–235.

62. Cano LE, López JA, Hernandez O, González Á, Mejía SP. Human neutrophils produce extracellular traps against Paracoccidioides brasiliensis. *Microbiology*. 2015;161(5):1008–1017.

63. Della Coletta AM, Bachiega TF, de Quaglia e Silva JC, et al. Neutrophil Extracellular Traps Identification in Tegumentary Lesions of Patients with Paracoccidioidomycosis and Different Patterns of NETs Generation In Vitro. *PLoS Negl. Trop. Dis.* 2015;9(9):e0004037.

64. Biagioni LM, Sadatsune T, Franco MF, Mattos MC. A comparative study of the immunoantigenicity of eight Paracoccidioides brasiliensis isolates. *Rev. Inst. Med. Trop. Sao Paulo*. 28(5):281–6.

65. Hahn RC, Macedo AM, Fontes CJF, et al. Randomly amplified polymorphic DNA as a valuable tool for epidemiological studies of Paracoccidioides brasiliensis. *J. Clin. Microbiol.* 2003;41(7):2849–54.

66. Macoris S, Sugizaki M, Peraçoli M, et al. Virulence attenuation and phenotypic variation of Paracoccidioides brasiliensis isolates obtained from armadillos and patients. *Mem. Inst. Oswaldo Cruz*. 2006;101(3):331–334.

67. Moyerbrailean GA, Davis GO, Harvey CT, et al. A high-throughput RNA-seq approach to profile transcriptional responses. *Sci. Rep.* 2015;5:14976.

68. Ozsolak F, Milos PM. RNA sequencing: advances, challenges and opportunities. *Nat. Rev. Genet.* 2011;12(2):87–98.

ACCEPTED MANUSCRIPT

69. Wang Z, Gerstein M, Snyder M. RNA-Seq: a revolutionary tool for transcriptomics. *Nat. Rev. Genet.* 2009;10(1):57–63.

70. Croft D, O'Kelly G, Wu G, et al. Reactome: a database of reactions, pathways and biological processes. *Nucleic Acids Res.* 2011;39(Database issue):D691-7.

71. Fabregat A, Sidiropoulos K, Garapati P, et al. The Reactome pathway Knowledgebase. *Nucleic Acids Res.* 2016;44(D1):D481–D487.

72. Worku M, Paape MJ, Marquardt WW. Modulation of Fc receptors for IgG on bovine polymorphonuclear neutrophils by interferon-gamma through de novo RNA transcription and protein synthesis. *Am. J. Vet. Res.* 1994;55(2):234–8.

73. Elghetany MT, Ge Y, Patel J, Martinez J, Uhrova H. Flow Cytometric Study of Neutrophilic Granulopoiesis in Normal Bone Marrow Using an Expanded Panel of Antibodies: Correlation with Morphologic Assessments. *J. Clin. Lab. Anal.* 2004;18(1):36–41.

74. Fortunati E, Kazemier KM, Grutters JC, Koenderman L, Van Den Bosch VJMM. Human neutrophils switch to an activated phenotype after homing to the lung irrespective of inflammatory disease. *Clin. Exp. Immunol.* 2009;155(3):559–566.

75. Berg M, James SP. Human neutrophils release the Leu-8 lymph node homing receptor during cell activation. *Blood*. 1990;76(11):2381–8.

76. Jones J, Morgan BP. Apoptosis is associated with reduced expression of complement regulatory molecules, adhesion molecules and other receptors on polymorphonuclear leucocytes: functional relevance and role in inflammation. *Immunology*. 1995;86(4):651–60.

77. Macey MG, McCarthy DA, Vordermeier S, Newland AC, Brown KA. Effects of cell purification methods on CD11b and L-selectin expression as well as the adherence and activation of leucocytes. *J. Immunol. Methods*. 1995;181(2):211–9.

78. Orr Y, Taylor JM, Cartland S, et al. Conformational activation of CD11b without shedding of L-selectin on circulating human neutrophils. *J. Leukoc. Biol.* 2007;82(5):1115–25.

79. Newburger PE, Chovaniec ME, Greenberger JS, Cohen HJ. Functional changes in human leukemic cell line HL-60. A model for myeloid differentiation. *J. Cell Biol.* 1979;82(2):315–22.

80. Millius A, Weiner OD. Manipulation of neutrophil-like HL-60 cells for the study of directed cell migration. *Methods Mol. Biol.* 2010;591:147–58.

81. Fleit HB, Wright SD, Durie CJ, Valinsky JE, Unkeless JC. Ontogeny of Fc receptors and complement receptor (CR3) during human myeloid differentiation. *J. Clin. Invest.* 1984;73(2):516–525.

82. Hauert AB, Martinelli S, Marone C, Niggli V. Differentiated HL-60 cells are a valid model system for the analysis of human neutrophil migration and chemotaxis. *Int. J. Biochem. Cell Biol.* 2002;34(7):838–54.

83. Yaseen R, Blodkamp S, Lüthje P, et al. Antimicrobial activity of HL-60 cells compared to primary blood-derived neutrophils against Staphylococcus aureus. *J. Negat. Results Biomed.* 2017;16(1):2.

84. Pullen GR, Hosking CS. Differentiated HL60 promyelocytic leukaemia cells have a deficient myeloperoxidase/halide killing system. *Clin. Exp. Immunol.* 1985;62(2):304–9.

85. Zhou Y, Weyman CM, Liu H, Almasan A, Zhou A. IFN- γ Induces Apoptosis in HL-60 Cells Through Decreased Bcl-2 and Increased Bak Expression. *J. Interf. Cytokine Res.* 2008;28(2):65–72.

ACCEPTED MANUSCRIPT

86. Melvan JN, Bagby GJ, Welsh DA, Nelson S, Zhang P. Neonatal sepsis and neutrophil insufficiencies. *Int. Rev. Immunol.* 2010;29(3):315–48.

87. Carr R. Neutrophil Production and Function in Newborn Infants. *Br. J. Haematol.* 2000;110(1):18–28.

88. Carr R, Davies JM. Abnormal FcRIII expression by neutrophils from very preterm neonates. *Blood*. 1990;76(3):607–11.

89. Leven EA, Maffucci P, Ochs HD, et al. Hyper IgM Syndrome: a Report from the USIDNET Registry. *J. Clin. Immunol.* 2016;36(5):490–501.

90. Kuhns DB, Alvord WG, Heller T, et al. Residual NADPH oxidase and survival in chronic granulomatous disease. *N. Engl. J. Med.* 2010;363(27):2600–10.

91. Borregaard N, Cross AR, Herlin T, et al. A variant form of X-linked chronic granulomatous disease with normal nitroblue tetrazolium slide test and cytochrome b. *Eur. J. Clin. Invest.* 1983;13(3):243–8.

92. Woodman RC, Newburger PE, Anklesaria P, et al. A new X-linked variant of chronic granulomatous disease characterized by the existence of a normal clone of respiratory burst-competent phagocytic cells. 1995.

93. Battersby AC, Cale CM, Goldblatt D, Gennery AR. Clinical manifestations of disease in X-linked carriers of chronic granulomatous disease. *J. Clin. Immunol.* 2013;33(8):1276–1284.

94. Perry FE, Elson CJ, Greenham LW, Catterall JR. Interference with the oxidative response of neutrophils by Streptococcus pneumoniae. *Thorax*. 1993;48(4):364–9.

95. Tennenberg SD, Fey DE, Lieser MJ. Oxidative priming of neutrophils by interferon-gamma. *J. Leukoc. Biol.* 1993;53(3):301–8.

96. Jain A, Kovacs JA, Nelson DL, et al. Partial immune reconstitution of X-linked hyper IgM syndrome with recombinant CD40 ligand. *Blood*. 2011;118(14):3811–7.

97. Ezekowitz RA, Sieff CA, Dinauer MC, et al. Restoration of phagocyte function by interferon-gamma in X-linked chronic granulomatous disease occurs at the level of a progenitor cell. *Blood*. 1990;76(12):2443–8.

98. Conti F, Aragão Filho WC, Prando C, et al. Phagocyte nicotinamide adenine dinucleotide phosphate oxidase activity in patients with inherited IFN-γR1 or IFN-γR2 deficiency. *J. Allergy Clin. Immunol.* 2015;135(5):1393–5.e1.

99. Seijkens T, Beckers L, Christ A, et al. Abstract 158: CD40L Regulates Hematopoietic Stem and Progenitor Cell Biology by Inducing Adhesion-Mediated Quiescence. *Arterioscler. Thromb. Vasc. Biol.* 2015;32(Suppl 1):

100. Cheung TH, Rando TA. Molecular regulation of stem cell quiescence. *Nat. Rev. Mol. Cell Biol.* 2013;14(6):329–340.

101. Flores-Romo L, Björck P, Duvert V, et al. CD40 ligation on human cord blood CD34+ hematopoietic progenitors induces their proliferation and differentiation into functional dendritic cells. *J. Exp. Med.* 1997;185(2):341–9.

102. B. C, S. C, M. Y, J.C. G. IL-4 and CD40 ligation affect differently the differentiation, maturation, and function of human CD34+ cell-derived CD1a+CD14- and CD1a- CD14+ dendritic cell precursors in vitro. *J. Leukoc. Biol.* 1998;64(2):235–244.

103. van Lochem EG, van der Velden VHJ, Wind HK, et al. Immunophenotypic differentiation patterns of normal hematopoiesis in human bone marrow: Reference patterns for age-related

ACCEPTED MANUSCRIPT

changes and disease-induced shifts. *Cytometry*. 2004;60B(1):1–13.

104. Hernández-Campo PM, Almeida J, Matarraz S, et al. Quantitative analysis of the expression of glycosylphosphatidylinositol-anchored proteins during the maturation of different hematopoietic cell compartments of normal bone marrow. *Cytometry B. Clin. Cytom.* 2007;72(1):34–42.

105. Vadhan-Raj S, Buescher S, LeMaistre A, et al. Stimulation of hematopoiesis in patients with bone marrow failure and in patients with malignancy by recombinant human granulocyte-macrophage colony-stimulating factor. *Blood*. 1988;72(1):134–41.

106. Anderlini P, Przepiorka D, Champlin R, Körbling M. Biologic and clinical effects of granulocyte colony-stimulating factor in normal individuals. *Blood*. 1996;88(8):2819–25.

107. De Kleer I, Willems F, Lambrecht B, Goriely S. Ontogeny of myeloid cells. *Front. Immunol.* 2014;5:423.

108. Skalnik DG. Transcriptional mechanisms regulating myeloid-specific genes. *Gene*. 2002;284(1–2):1–21.

109. Jerne NK. The immune system. *Sci. Am.* 1973;229(1):52–60.

110. Bergthaler A, Menche J. The immune system as a social network. *Nat. Immunol.* 2017;18(5):481–482.

111. Menche J, Sharma A, Kitsak M, et al. Disease networks. Uncovering disease-disease relationships through the incomplete interactome. *Science*. 2015;347(6224):1257601.

112. Rieckmann JC, Geiger R, Hornburg D, et al. Social network architecture of human immune cells unveiled by quantitative proteomics. *Nat. Immunol.* 2017;18(5):583–593.

113. Reinhardt TA, Sacco RE, Nonnecke BJ, Lippolis JD. Bovine milk proteome: quantitative changes in normal milk exosomes, milk fat globule membranes and whey proteomes resulting from Staphylococcus aureus mastitis. *J. Proteomics*. 2013;82:141–54.

114. Szklarczyk D, Franceschini A, Wyder S, et al. STRING v10: protein-protein interaction networks, integrated over the tree of life. *Nucleic Acids Res.* 2015;43(Database issue):D447-52.

115. Huang DW, Sherman BT, Lempicki RA. Bioinformatics enrichment tools: Paths toward the comprehensive functional analysis of large gene lists. *Nucleic Acids Res.* 2009;37(1):1–13.

116. Huang DW, Lempicki RA, Sherman BT. Systematic and integrative analysis of large gene lists using DAVID bioinformatics resources. *Nat. Protoc.* 2009;4(1):44–57.

117. Ashburner M, Ball CA, Blake JA, et al. The Gene Ontology Consortium. Gene ontology: tool for the unification of biology. *Nat. Genet.* 2011;25:25–29.

118. Warde-Farley D, Donaldson SL, Comes O, et al. The GeneMANIA prediction server: biological network integration for gene prioritization and predicting gene function. *Nucleic Acids Res.* 2010;38(Web Server):W214–W220.

119. Den Dunnen JT, Antonarakis SE. Mutation nomenclature extensions and suggestions to describe complex mutations: A discussion. *Hum. Mutat.* 2000;15(1):7–12.

120. Taschner PEM, den Dunnen JT. Describing structural changes by extending HGVS sequence variation nomenclature. *Hum. Mutat.* 2011;32(5):507–511.

ACCEPTED MANUSCRIPT

FIGURES AND TABLE LEGENDS

Figure 1. Defective respiratory burst in CD40L deficiency and its relation to neutrophil microbicidal activity. (**A**) Neutrophils from CD40L-deficient patients (n = 6) exhibit normal phagocytic capacity compared to healthy controls. The neutrophil respiratory burst from CD40L-deficient patients was assessed by dihydrorhodamine analysis in response to (**B**) *P. brasiliensis* as well as (**C**) PMA, fMLP, or *S. aureus* from six independent experiments comparing each CD40L-deficient patients to different healthy controls. Results were expressed as mean fluorescence intensity (MFI) ($p \leq 0.05$; n = 6, Mann-Whitney test). **(D**) Respiratory burst from healthy control neutrophils treated with various concentrations of catalase were analyzed using luminol-enhanced chemiluminescence; the values were expressed as relative light units (RLU). (**E**) After challenging neutrophils with *P. brasiliensis* (ratio of 2 neutrophils:1 fungus), microbicidal activity was assessed by counting colony forming units (CFU) from recovering internalized fungi. The CFU values (% of controls) were determined in relation to the CFU number of untreated neutrophils from healthy controls. Both the respiratory burst and microbicidal activity of healthy control neutrophils were assessed in the presence of different doses of catalase (100, 300 and 1000 U/ml). (**F**) The correlation between respiratory burst (data set from D) and microbicidal activity (data set from E) reduction was assessed using Pearson's correlation analysis. (n = 3). (**G**)**.** Quantification of neutrophil extracellular traps (NETs) release by isolated neutrophils from CD40L-deficient patients in comparison to healthy controls. After incubation for 4 hours in the presence of PMA NETs release (n = 5) was analyzed using Sytox Orange and results expressed in relative fluorescence units (RFU) as previously described[113]. Patient 4 died before NET analysis was available. Significant difference is denoted by asterisk when $p \leq 0.05$.(Mann-Whitney test).

Figure 2. rhIFN-γ improves defects of neutrophil microbicidal activity and respiratory burst, as well as differences in the transcriptome observed in CD40L deficiency. (A) Neutrophil respiratory burst was analyzed using a luminol-enhanced chemiluminescence assay, values are expressed as relative light units (RLU). The respiratory burst of PMA-stimulated neutrophils from healthy controls was considered as 100%. ($p \leq 0.05$; n = 6, Mann-Whitney test). (**B**) After challenging neutrophils with *P. brasiliensis* (ratio of 2 neutrophils:1 fungus), microbicidal activity was assessed by counting CFU from recovering internalized fungi. The CFU values (% of controls) were determined in relation to the CFU number of untreated neutrophils from healthy controls. Prior to the analysis, neutrophils remained without (-) or with (+) sCD40L (500 ng/mL) or rhIFN-γ (100 U/mL) for 2h. Untreated neutrophils from healthy control subjects were considered to have microbicidal activity of 100%. (**C**) Functional network analysis of differentially expressed genes (DEGs) in neutrophils from CD40L-deficient patients was

ACCEPTED MANUSCRIPT

performed using Reactome Knowledgebase (http://reactome.org/), which indicated that IFN-γ signaling is significantly (p = 1.11 x 10^{-16}; FDR = 8.44 x 10^{-15}) impaired in the absence of CD40L-CD40 interaction. The figure shown is a zoomed image of the Genome-wide, hierarchical visualization of Reactome pathways in a space filling graphic obtained from the analysis of DEGs in human CD40L deficiency. **(D)** Heat maps of dysregulated genes of neutrophils from CD40L-deficient patients and the effect of rhIFN-γ. mRNA from neutrophils was sequenced by RNAseq. Values in transcripts per million (TPM) are represented in the heat maps on a Log2 scale where yellow shows low expression and blue high expression. Results of untreated (left panel) and *in vitro* treatment with rhIFN-γ (100 U/ml) for 2 hours (right panel) are shown in the heat map. DEGs in patients´ neutrophils are compared with those from healthy controls; the addition of rhIFN-γ to neutrophil cultures improved the pattern of gene expression by CD40L-deficient neutrophils reducing the number of DEGs from 164 to 21 genes. (**E**) Principle component analysis (PCA) of all DEGs observed in neutrophils from three CD40L-deficient patients was performed using Perseus (MaxQuant, v1.11, Martinsried, Germany) showing that rhIFN-γ regulates the transcriptome of neutrophils from healthy controls and patients, thus reducing differences in the pattern of gene expression of both groups.

Figure 3. Reduced CD16 expression suggests abnormal development of neutrophils in CD40L deficiency. (A) Histograms obtained by flow cytometric analysis showing the expression of different molecules on neutrophils from CD40L-deficient patients and healthy controls. The selectively reduced CD16 expression is identified with an arrow. (**B**) Graphics displaying the reduced CD16 expression by neutrophils from CD40L-deficient patients compared to healthy controls. **(C)** Dot plots displaying the kinetic measurement of phosphatidylserine on the outer leaflet of cell membrane using an annexin V-fluorescein isothiocyanate (FITC) single staining assay as previously described[42,43], suggesting no alterations in patient neutrophil apoptosis over a short (0 to 8 hours) or (**D**) after a longer period (18 hours) in the presence of IFN-γ or sCD40L when compared to healthy controls. Neutrophils were analyzed in the presence or absence of the broad spectrum caspase inhibitor Q-VD-OPh**.** Graphic data are shown in **Figure 3S.** (**E**) Graphics showing the levels of CD62L expression on resting and following activated neutrophils by PMA and TLRs agonist (Pam3CSK4 - TLR1/2 agonist; LPS – TLR4 agonist). (**F**) Expression of TLRs on neutrophils from patients and heathy controls. The cells were analyzed by flow cytometry. Significant difference is denoted by asterisk ($p < 0.05$; n = 5, Mann-Whitney test). MFI, median fluorescence intensity

ACCEPTED MANUSCRIPT

Figure 4. CD40L-deficient mice show defective neutrophil responses *in vivo*. (**A**) The recruitment of neutrophils to the peritoneal cavity from wilde-type and CD40L (CD154)-deficient ($CD154^{-/-}$) mice (3 per group) was assessed after 0, 24, 48 and 72 hours following inoculation with thioglycolate (TG), or challenging with BRD509, an attenuated *aroA*$^{-}$*/aroD*$^{-}$ mutant strain of *Salmonella enterica* serovar Typhimurium. All experimental procedures including (**B**) control of bacterial proliferation, (**C**) neutrophil respiratory burst and (**D**) phagocytic activity, are described in details in supplementary material and methods. Neutrophil phagocytic activity and respiratory burst capacity were analyzed 24 hours after challenging mice with BRD509.

Figure 5. Soluble CD40L regulates promyelocytic cell development. (**A**) Heatmap showing that sCD40L induces transcriptome alterations in the promyelocytic HL-60 cells. HL-60 cells were incubated for 48 hours in the absence and presence of 500ng/ml sCD40L and/or 1% DMSO prior RNAseq analysis. In the heatmap, the results obtained in transcripts per million (TPM) are represented in a Log2 scale where yellow shows low expression and blue high expression. Gene ontology analysis of genes up- or down-regulated by sCD40L compared to untreated cells or DMSO-treated *versus* DMSO plus sCD40L are listed in **Table 1**. A pool of three independent cultures of HL-60 cells, in which every experimental condition (Resting, DMSO, sCD40L, and DMSO plus sCD40L) has been performed in triplicates. (**B**) Histograms obtained by flow cytometric analysis showing the expression of CD15, CD16 and CD40 on HL-60 cells. (**C**) Equal numbers of promyelocytic HL-60 cells were distributed in 6 well plates and cultured in the presence or absence of 1% DMSO and/or 500ng/ml sCD40L. After 6 days, the cells were harvested, counted and their cell viability was assessed (**Figure S4)**. (**D**) HL-60 migration was assessed using a transwell migration assay. Microscopic images display cells that migrated. The data shown are representative of three independent experiments performed in triplicates. (**E**) The respiratory burst of HL-60 cells was induced by PMA (90 mM) and analyzed using a luminol-enhanced chemiluminescence assay. The values are expressed as relative light units (RLU). (**F**) Comparative graphics showing the kinetics of respiratory burst of HL-60 cells (upper left panel), neutrophils (upper right panel) and both overlapping (lower panel). (**G**) HL-60 cells were challenged with *P. brasiliensis* (ratio of 2 neutrophils:1 fungus) and their microbicidal activity was assessed by counting CFU from recovering internalized fungi. The CFU values (%) were determined in relation to the CFU number of untreated HL-60 cells.

Figure 6. Blood circulating neutrophils of CD40L-deficient patients presented defects in development and function that are improved by the presence of exogenous IFN-γ.

ACCEPTED MANUSCRIPT

876

877 **Table 1. Biological processes dysregulated in CD40L-deficiency.** Gene Ontology (GO) analysis of
878 DEGs in CD40L deficiency was performed using STRING [114] and DAVID [115,116] to categorize and
879 group the set of genes up- and down-regulated in neutrophils from CD40L-deficient patients and HL-60
880 cells based on a known functional association as defined by the Gene Ontology Consortium [117].

881

ACCEPTED MANUSCRIPT

ACCEPTED MANUSCRIPT

SUPLEMENTARY FIGURES AND TABLE LEGENDS

Figure S1. Reduced neutrophil extracellular trap release in CD40L deficiency. Neutrophil extracellular trap (NET) release by PMA- and *P. brasiliensis*-activated neutrophils from patients and healthy controls was assessed by fluorescence microscopy. Histones are shown in orange, neutrophil elastase in green and nucleus (DNA) in blue. One from 5 independent experiments is shown. Graphic data are shown in **Figure 1G.**

Figure S2. Interaction network of genes up- and down-regulated in neutrophils from CD40L-deficient patients compared with those from control neutrophils. Network is shown as predicted by GeneMania and visualized by Cytoscape[118]. Blue represents genes up-regulated and yellow down-regulated. The interaction network is based on features shared by genes, e.g. co-expression, co-localization, genetic interactions, signaling pathways, physical interactions. Red lines show connections among genes down-regulated or between genes down-regulated and up-regulated, while gray lines display connections only between genes up-regulated. The Gene Ontology categories are described in **Table 1**.

Figure S3. Normal phosphatidylserine levels on the outer leaflet of cell membrane of neutrophils from CD40L-deficient patients. Kinetic measurement of phosphatidylserine on the outer leaflet of the cell membrane using annexin V- fluorescein isothiocyanate (FITC) single staining assay as previously described[42,43]. The data suggest no alterations in patient neutrophil apoptosis over (**A**) a short (0 to 8 hours) or (**B**) a long period (18 hours) in the presence of IFN-γ or sCD40L when compared to healthy controls. Neutrophils were analyzed by flow cytometry at different time points in the presence or absence of a carboxy terminal phenoxy group conjugated to the amino acids valine and aspartate (Q-VD-OPh) (10 µM; MP Biomedicals, Illkirch-Cedex, France), which is a broad spectrum caspase inhibitor with potent antiapoptotic properties[44].

Figure S4. Expression of CD62L and CD11b by neutrophils from patient P4. Resting and (300ng/ml) PMA- or **(**50nM**)** fMLP-activated neutrophils were analyzed by flow cytometry.

Figure S5. sCD40L does not affect HL-60 cell viability. (A) Fluorescence microscopy images (using Axio Vert.A1) of HL-60 cells were obtained in the presence or absence of DMSO and/or sCD40L. After

ACCEPTED MANUSCRIPT

6 days of culture, HL-60 cells were harvested, counted and their viability analyzed after nuclear staining using Hoechst dye which is shown in blue. One of three individual experiments shown. The results were confirmed by trypan blue exclusion and by **(B)** the LIVE/DEAD Viability/Cytotoxicity Kit according to the manufacturer's instructions (Thermo Fisher Scientific, Waltham, MA, USA). The results are representative of three independent experiments performed in triplicates.

Figure S6. Genes up- or down-regulated by sCD40L in HL-60 cells. Interaction network of genes up- and down-regulated by sCD40L. Network is shown as predicted by GeneMania and visualized by Cytoscape[118]. Blue represents genes up-regulated and yellow down-regulated. The interaction network is based on features shared by the genes, e.g. co-expression, co-localization, genetic interactions, signaling pathways, physical interactions. Red lines show connections among genes down-regulated or between genes down-regulated and up-regulated, while gray lines display connections only between genes up-regulated. The Gene Ontology categories of sCD40L-inducible genes are described in **Table 1**. We evaluated the gene expression of (**A**) untreated HL-60 cells compared with those cultured in the presence of sCD40L, while (**B**) dimethyl sulfoxide (DMSO)-differentiated cells were evaluated in comparison to cells cultivated in the presence of DMSO + sCD40L. The gene expression profile was obtained by RNAseq from a pool of three independent cultures of HL-60 cells, in which every experimental condition (resting, DMSO, sCD40L, and DMSO plus sCD40L) was explored in triplicates.

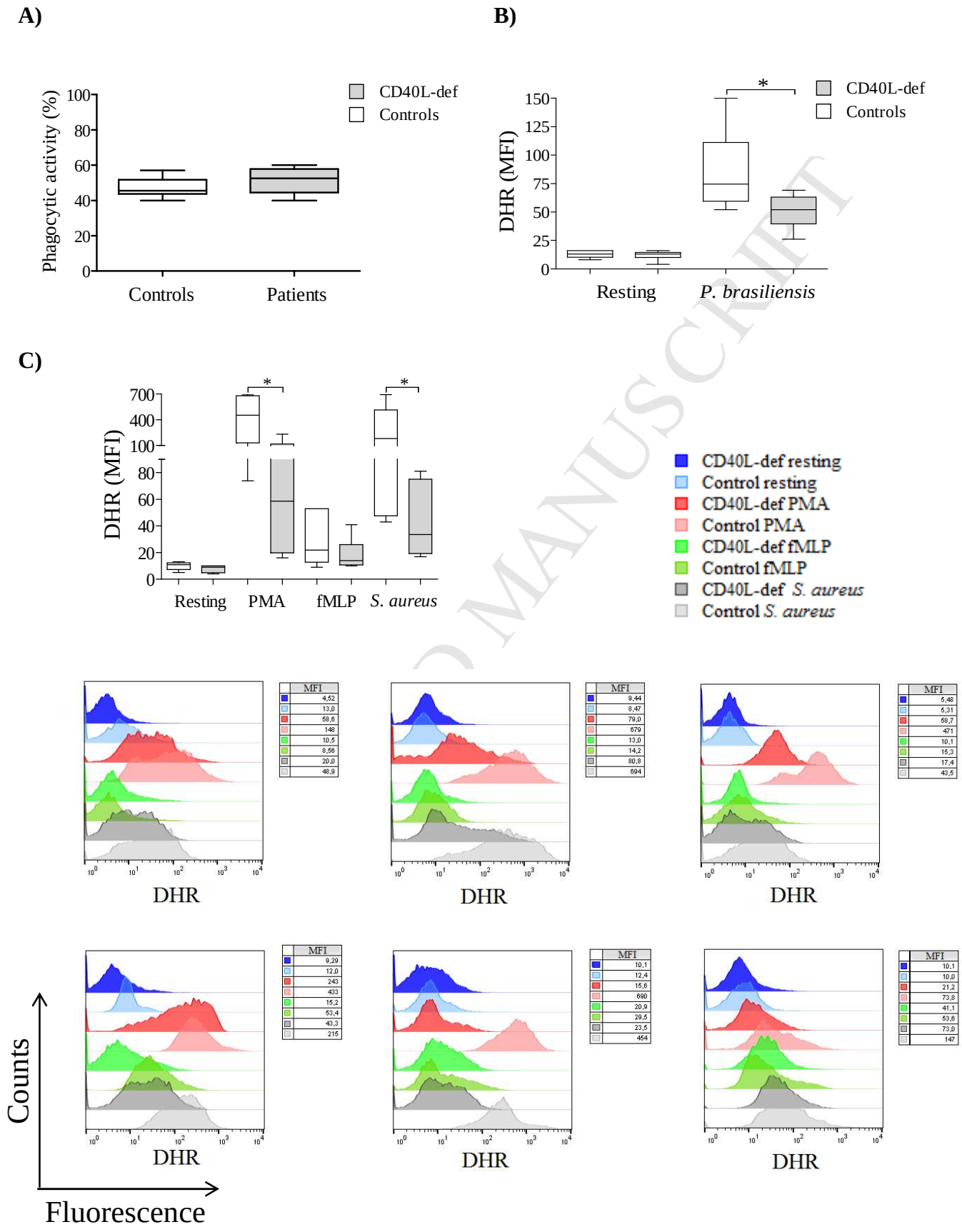
Table S1. Isolated pathogens and CD40L mutations identified in CD40L-deficient patients. [a]The nomenclature for the description of mutations are according to guidelines of Human Genome Variation Society[119,120] and Mutalyzer 2.0.26 — Name Checker (https://mutalyzer.nl/). [f]Patient P4 has recently died in a cachectic state after recurrent diarrhea caused by refractory *Cryptosporidium parvum* and concomitant severe *Mycobacterial tuberculosis* infection. Patient P6 developed severe refractory Leishmaniosis and is currently hospitalized. Patients P1-P3 underwent hematopoietic stem cell transplantation during the last trimester 2017. *, Stop codon.

ACCEPTED MANUSCRIPT

Table 1

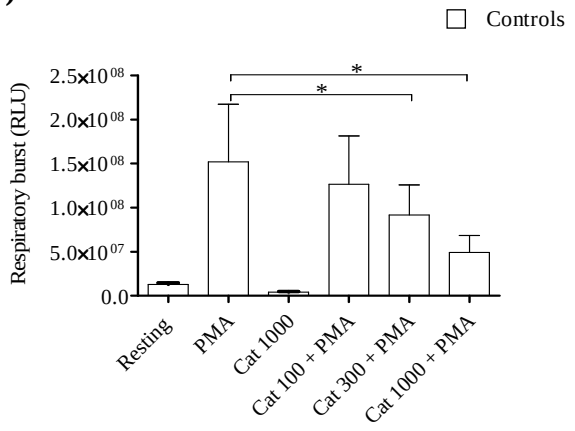
GO Term	GO Category	Neutrophils		HL-60			
				Untreated *versus* sCD40L treated		DMSO treated *versus* DMSO + sCD40L treated	
		Up-regulated	Down-regulated	Up-regulated	Down-regulated	Up-regulated	Down-regulated
GO:0002376	immune system process	*AGPAT5, AKT1S1, BNIP3, BST1, CLEC6A, CMTM7, HERC5, HERC6, HLA-C, IRAK1, IRF5, IRG1, LRRK1, PNP, PPIA, PSMA3, PSMC3, SLAMF7, ST6GAL1, TAB1, USP18, XAF1*	*BTN3A2, CSF1R, HLA-A,*	*C1QBP, CFL1, PPP3CA, PRKCSH, SYNCRIP*	*PSME3, RAB5B*	*CALCOCO2, CIAPIN1, CYLD, MSH2*	*CALM3, HCK, IFNGR2, MCM3AP, PTMS*
GO:0045087	innate immune response	*CLEC6A, HERC5, HLA-C, IRAK1,IRF5, IRG1, PSMA3, PSMC3, SLAMF7, TAB1, USP18, XAF1*	*AKT1S1, CSF1R, HLA-A,*	*C1QBP, CFL1, PPP3CA, PRKCSH, SYNCRIP*	*PSME3*	*CALCOCO2, CYLD*	*CALM3, HCK, IFNGR2, UNC93B1,*
GO:0030154	cell differentiation	*AGPAT5, ANGPTL6, ARID5B, BNIP3, CAPN2, CMTM7, CPNE1, EVL, FOXK1, HERC1, HERC6, IL6ST, ITGA7, LRRK1, PDLIM5, RBM38, STRBP, TNIK*	*CSF1R,*	*ARHGDIA, CASC5, CEP57, FOXN2, MSI2, OPA1, PLK4, PPP3CA, SEC23IP, SYNCRIP, TSNAX*	*CD63, FLNA, GLMN, HSPA8, LEF1*	*ACADVL, ELP3, MSH2, RAN, USP33*	*ANXA4, CDK16, EIF4G1, HCK, SDCCAG8*
GO:0030099	myeloid differentiation	*LRRK1*	*CSF1R*		*LEF1*		
GO:0048002	antigen processing and presentation of peptide antigen	*HLA-C, PSMA3, PSMC3*	*HLA-A,*		*PSME3, SEC61A1*		*UNC93B1*
GO:0080134	regulation of response to stress	*ACTL6A, CLEC6A, ERRFI1, F3, HERC5, IL6ST, IRAK1, IRG1, NPC1, NUP62, PFKP, PSMA3, PSMC3, TAB1, TNIK, USP18*	*AKT1S1, HLA-A, TNFRSF1A*	*C1QBP, DNAJC7, OPA1, RAE1, TMEM39B, TNFRSF1A*	*DDX5, HSPA8, NUP214, PSME3, SCFD1, UBL4A*	*ABR, CYLD, MAP4K4*	*CALM3, HCK, IFNGR2, UNC93B1*
GO:0007259	JAK-STAT cascade	*ELP2, IL6ST, TNFRSF1A*	*CSF1R,*	*TNFRSF1A*			
GO:0055114	**oxidation-reduction process**	*ABCD1, GSTT1, HBA2, HK3, HSD17B4, IL6ST, KMO, PFKP, PKM, SCP2*		*FAM54B, RRM1*		*ACADVL*	*CALM3*
GO:0006897	Endocytosis	*ADORA2A, FCHO1, HBA2, LMBR1L, NPC1*		*CFL1, RABEP1*	*SCFD1*	*USP33*	*HCK*
GO:0030334	regulation of cell migration	*EVL, F3, RTN4, STAP1*	*CSF1R*	*ARHGDIA, C1QBP*	*GNB2L1, LEF1*	*ABR, ELP3*	
GO:0007264	small GTPase mediated signal transduction	*ARHGEF3, EVL, LRRK1, RAB28, RHEB*	*GNGT2*	*ARHGDIA, CASC5, CFL1, ZWINT*	*FLNA, RAB5B*	*ABR, ARL1, RAN*	*CALM3*
GO:0016192	vesicle-mediated transport	*ADORA2A, CPNE1, DYSF, FCHO1, HBA2, LMBR1L, NPC1, PPIA, SYS1, TSNARE1*		*GGA3, RABEP1, COPZ1, CFL1*	*ARCN1, CD63, RAB5B, SEC16A, TUBA4A*	*USP33*	*CALM3, CDK16, HCK*
GO:0007599	Hemostasis	*ADORA2A, DGKA, F3, PPIA*		*C1QBP, CFL1*	*CD63, FLNA, TUBA4A*		*CALM3*
GO:0010628	positive regulation of gene expression	*ARID5B, CARD14, CIRBP, FOXK1, GPBP1L1, IRAK1, IRF5, MAML2, NUP62, PSMC3, PTBP1, RPS27L, STAP1, STK36, TAB1, YBX1*	*BRD7, FOSB, NRF1, PBX2, TNFRSF1A,*	*C1QBP, PPP3CA, TBP, TFDP1, TNFRSF1A*	*DDX5, HMGN1, HSPA8, LEF1*	*RAN*	*CDK4, NAMPT*
GO:0045892	negative regulation of transcription, DNA-templated	*ARID5B, FOXK1, IRAK1, IRG1, TAF1C, YBX1, ZFP161*	*BRD7, DEDD, FOSB, NFKBID,*	*AEBP2, C1QBP, HDGF, PA2G4, TBP*	*DDX5, FLNA, HSPA8, NFXL1, SMARCE1*	*CYLD*	*ANXA4*
GO:0010608	post transcriptional regulation of gene expression	*CIRBP, RBM38, RPS27L,YBX1*		*C1QBP, PA2G4, SYNCRIP*	*EIF3C, GNB2L1*	*RAN*	*CDK4, EIF3K, EIF4G1*
GO:0006417	regulation of translation	*CIRBP, RPS27L*		*C1QBP, PA2G4, SYNCRIP*	*EIF3C, GNB2L1*		*CDK4, EIF3K, EIF4G1*
GO:0031401	positive regulation of protein modification process	*CARD14, IL6ST, IRAK1, LRRK1, PPP2R4, PSMA3, PSMC3, STAP1, TAB1, TNIK*	*BRD7, CSF1R, TNFRSF1A*	*TNFRSF1A*	*FNTA, GNB2L1, PSME3*		*CALM3*
GO:0045859	regulation of protein kinase activity	*AKT1S1, CARD14, DUSP18, ERRFI1, HERC5, IRAK1, NUP62, STAP1, TAB1, TNIK*	*CSF1R,*		*GNB2L1*	*ELP3*	*CALM3, CDK4*

Figure 1

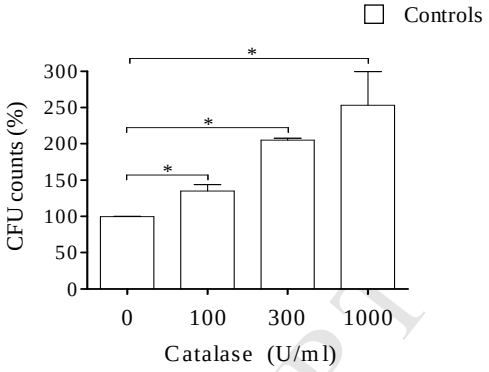

A)
Phagocytic activity (%)
100
80
60
40
20
0
Controls
Patients
CD40L-def
Controls
B)
DHR (MFI)
150
125
100
75
50
25
0
*
Resting
P. brasiliensis
CD40L-def
Controls
C)
DHR (MFI)
700
400
100
80
60
40
20
0
*
*
Resting
PMA
fMLP
S. aureus
CD40L-def resting
Control resting
CD40L-def PMA
Control PMA
CD40L-def fMLP
Control fMLP
CD40L-def S. aureus
Control S. aureus
MFI
DHR
Counts
Fluorescence

ACCEPTED MANUSCRIPT

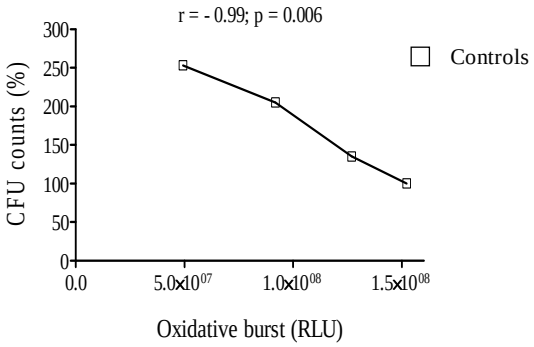
D)



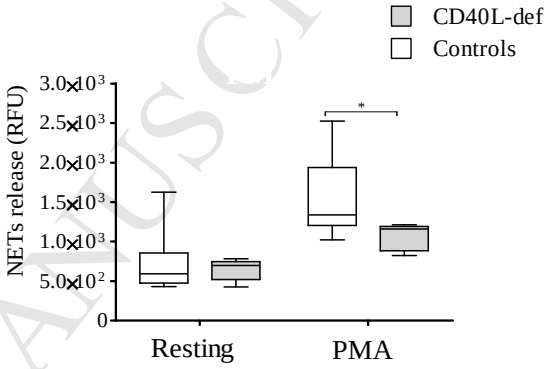
E)



F)



G)

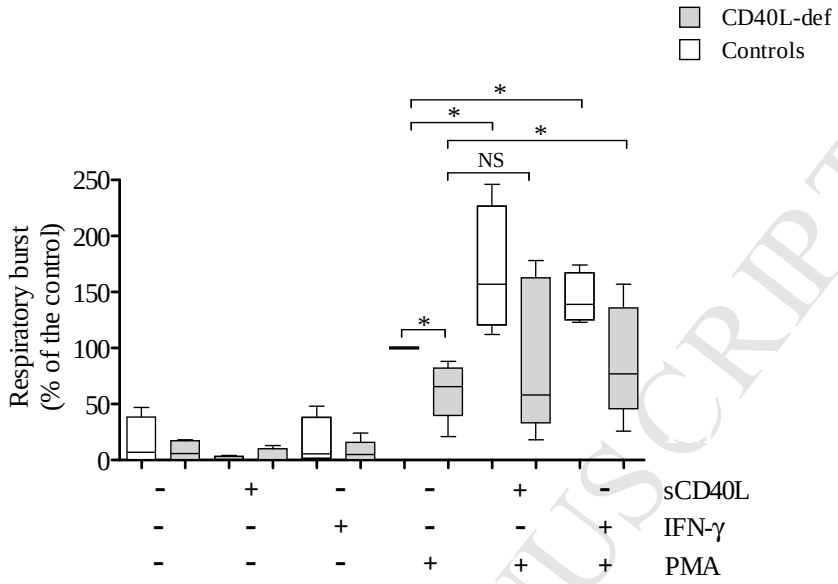


ACCEPTED MANUSCRIPT

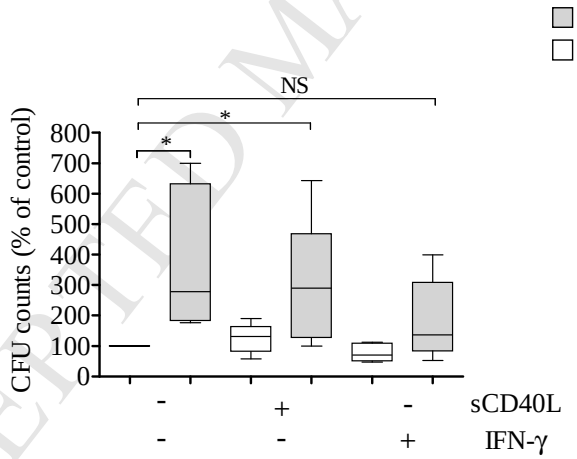
ACCEPTED MANUSCRIPT

Figure 2

A)



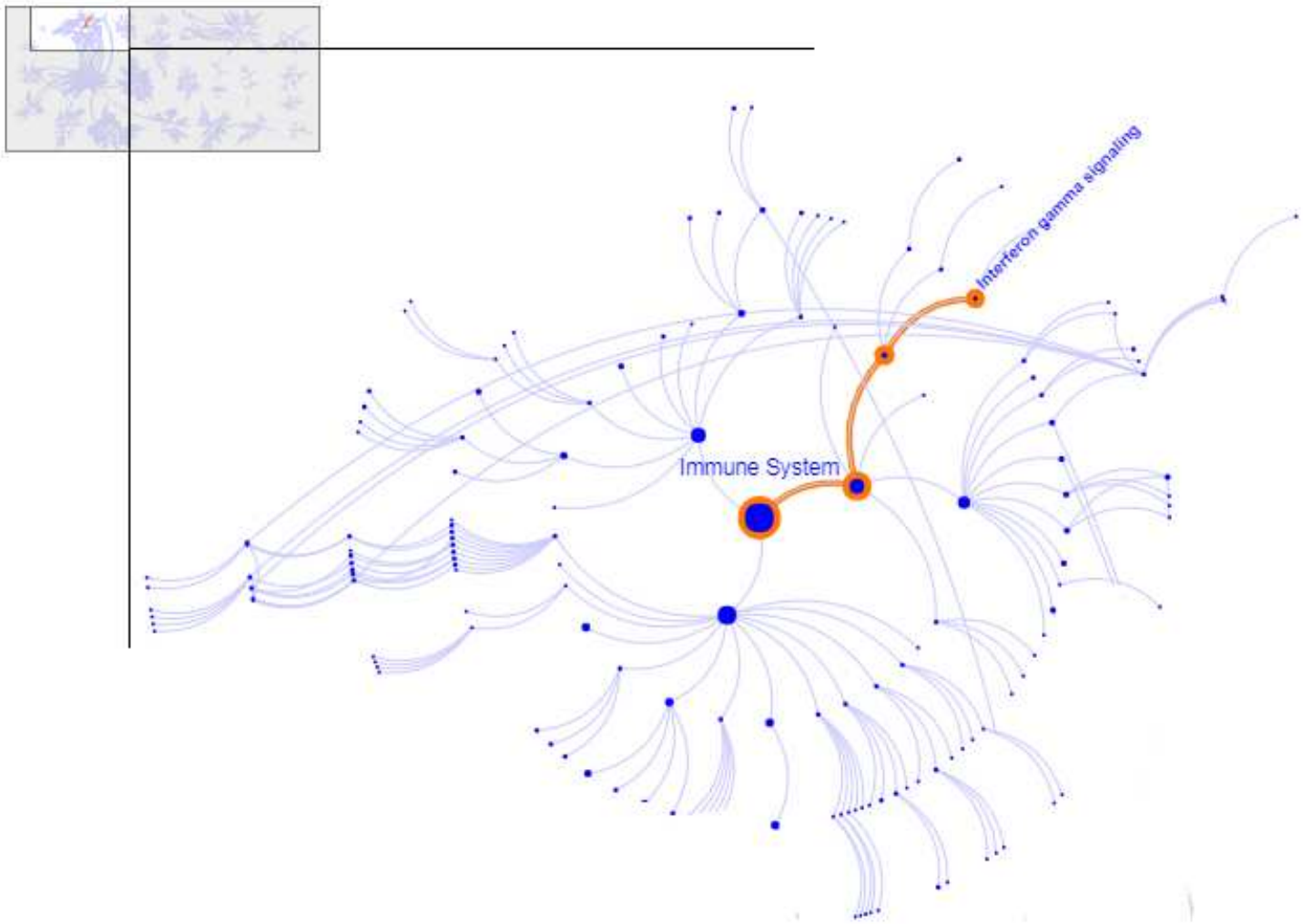
B)



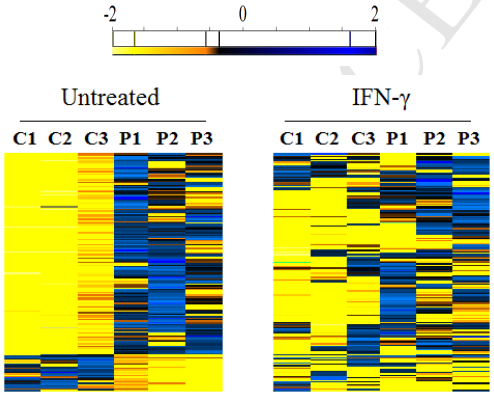
ACCEPTED MANUSCRIPT

Figure 2

C)


Interferon gamma signaling
Immune System

D)


-2
0
2
Untreated
IFN-γ
C1 C2 C3 P1 P2 P3
C1 C2 C3 P1 P2 P3

E)

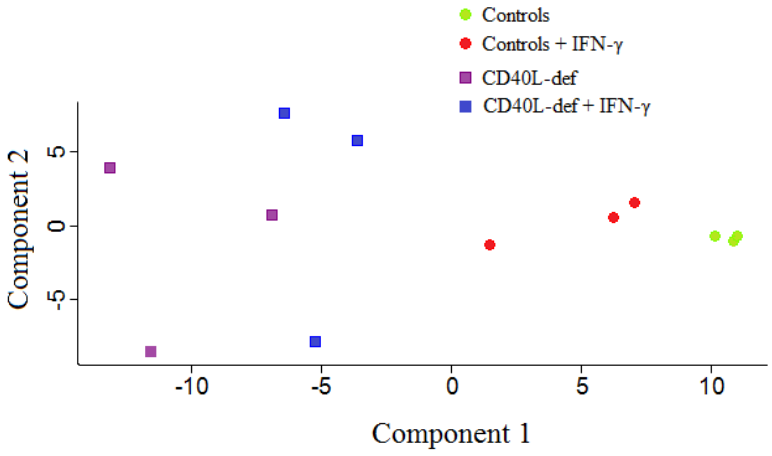
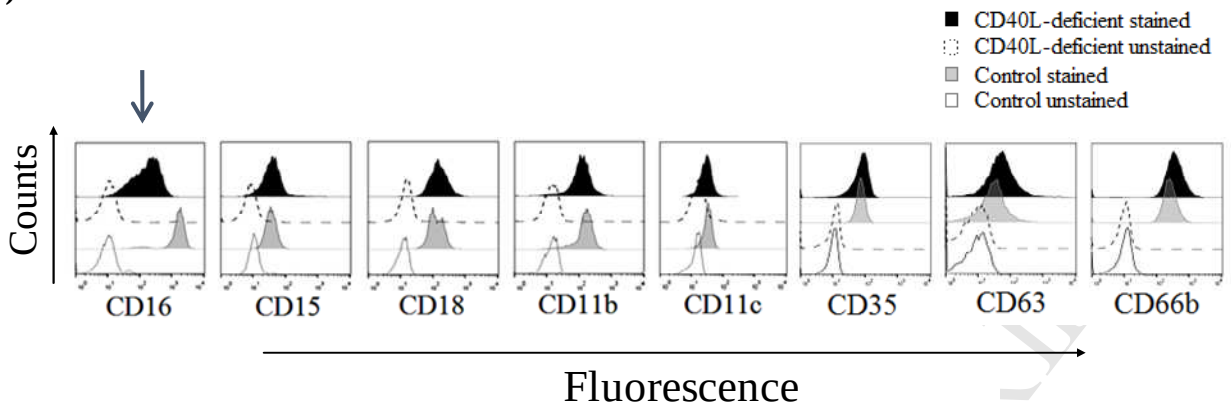
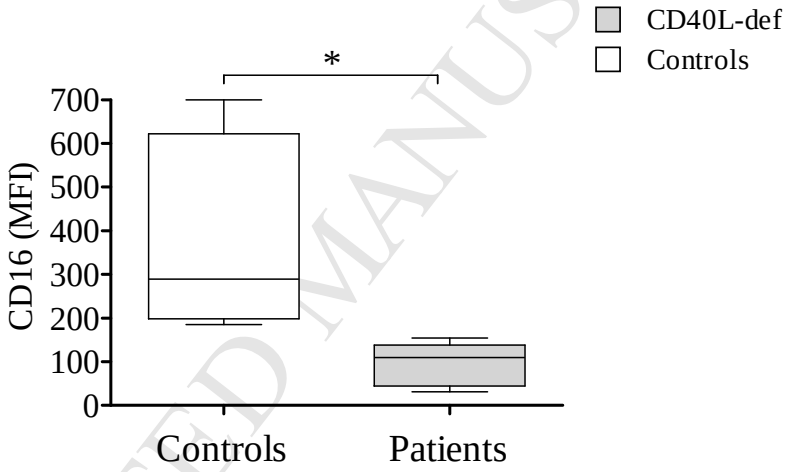

Controls
Controls + IFN-γ
CD40L-def
CD40L-def + IFN-γ
Component 2
5
0
-5
-10
-5
0
5
10
Component 1

Figure 3

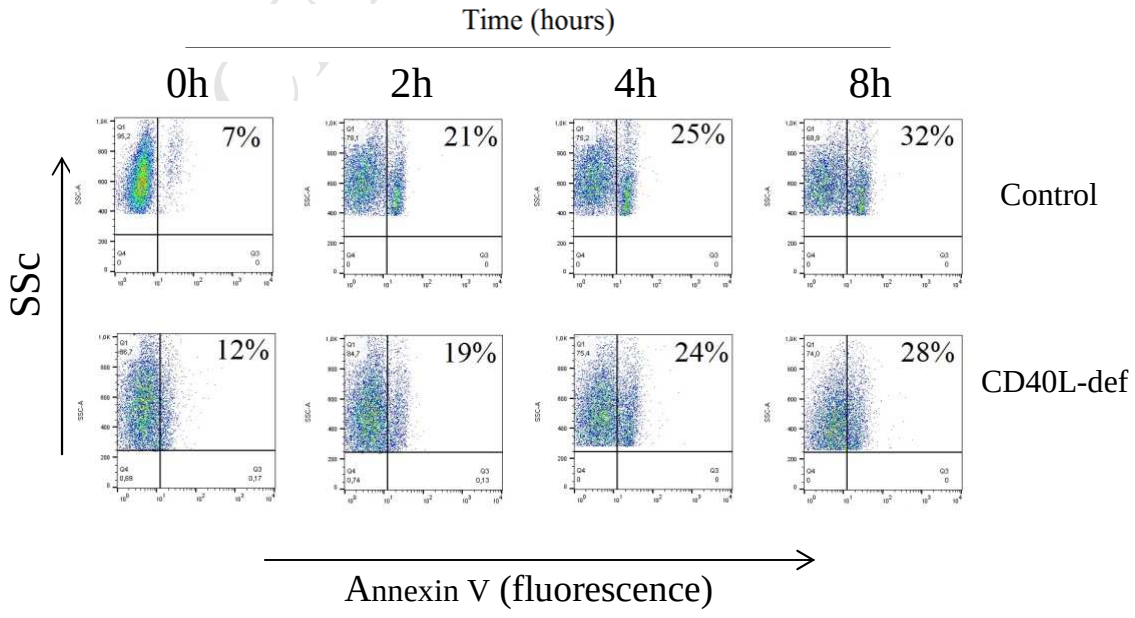
A)


CD40L-deficient stained
CD40L-deficient unstained
Control stained
Control unstained
Counts
CD16
CD15
CD18
CD11b
CD11c
CD35
CD63
CD66b
Fluorescence

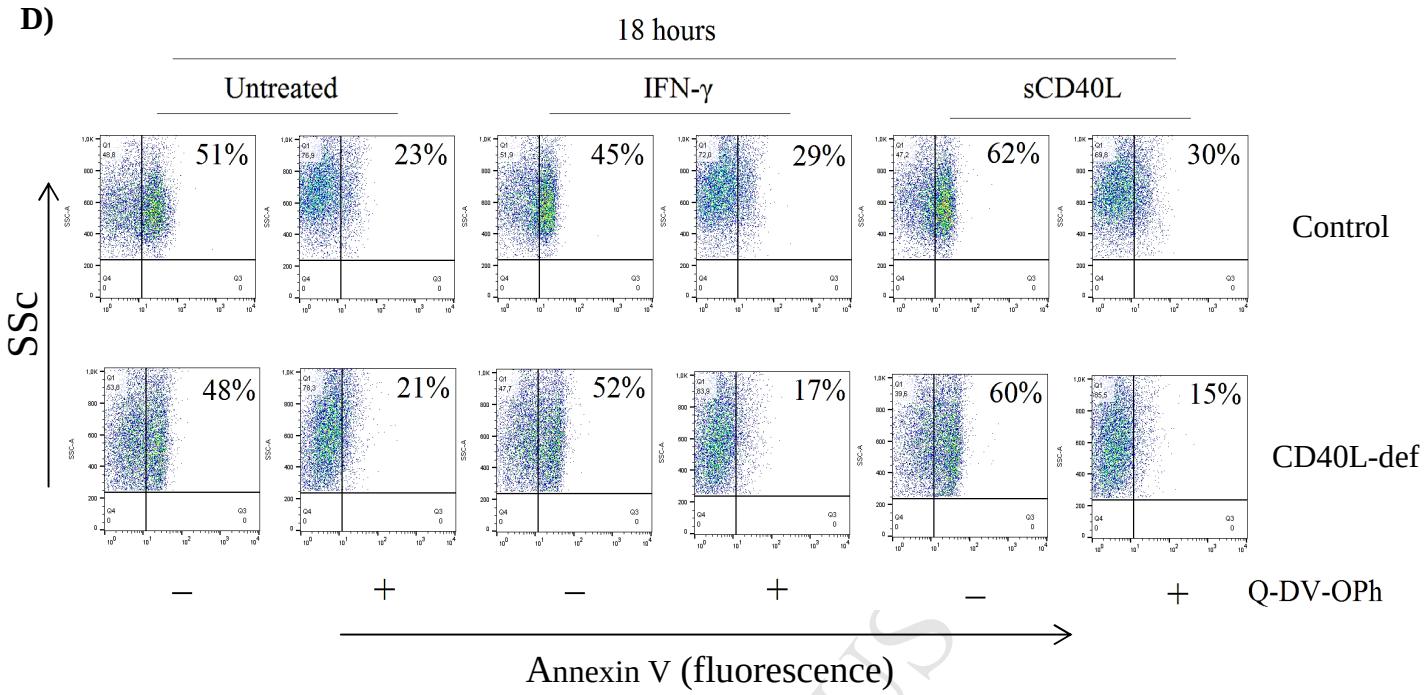
B)

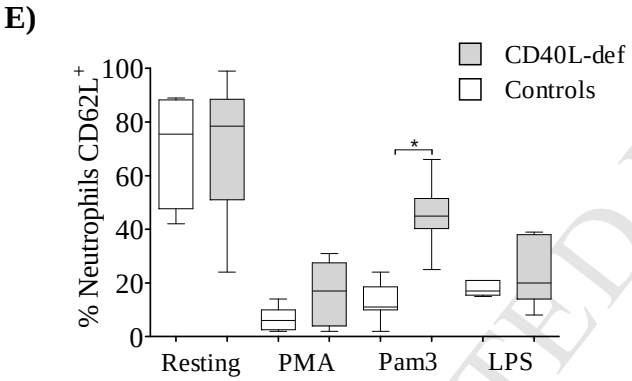

CD40L-def
Controls
*
CD16 (MFI)
700
600
500
400
300
200
100
0
Controls
Patients

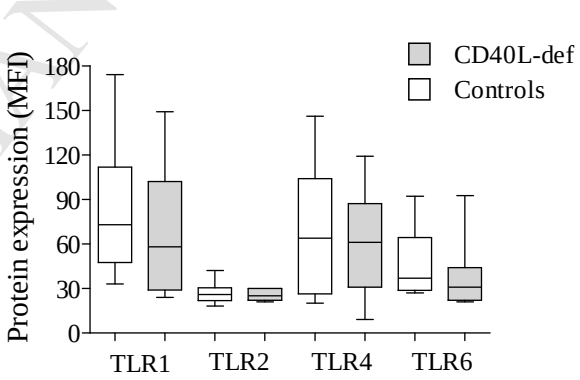
C)


Time (hours)
0h
2h
4h
8h
7%
21%
25%
32%
Control
12%
19%
24%
28%
CD40L-def
SSc
Annexin V (fluorescence)

ACCEPTED MANUSCRIPT



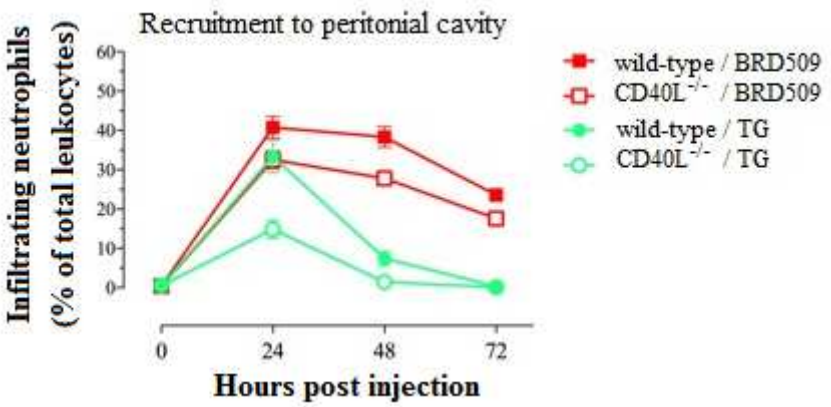




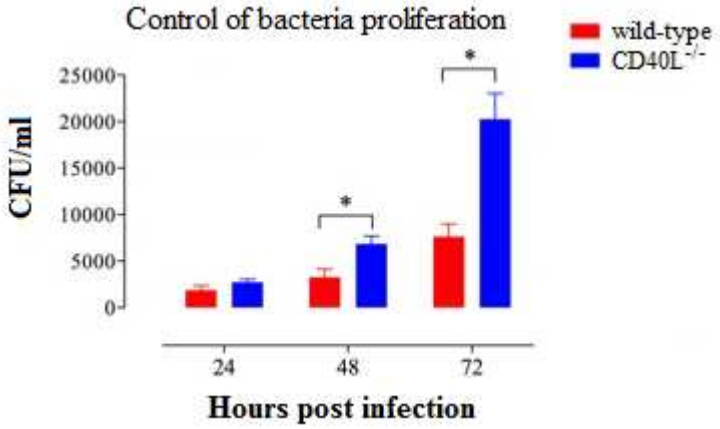
ACCEPTED MANUSCRIPT

Figure 4

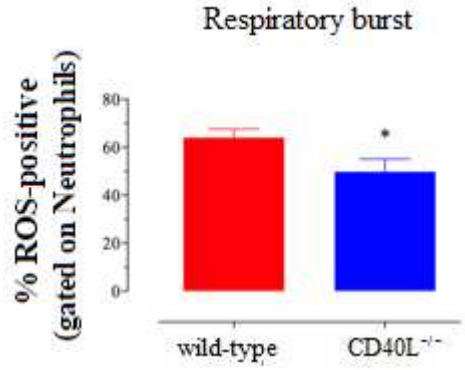
A)



B)



C)



D)

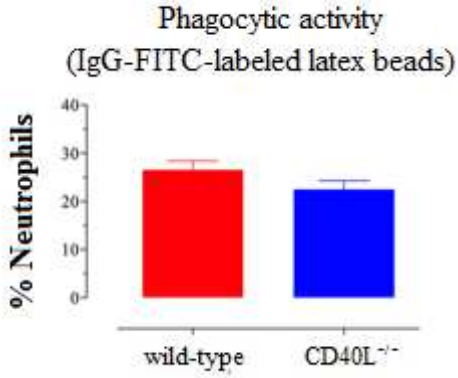
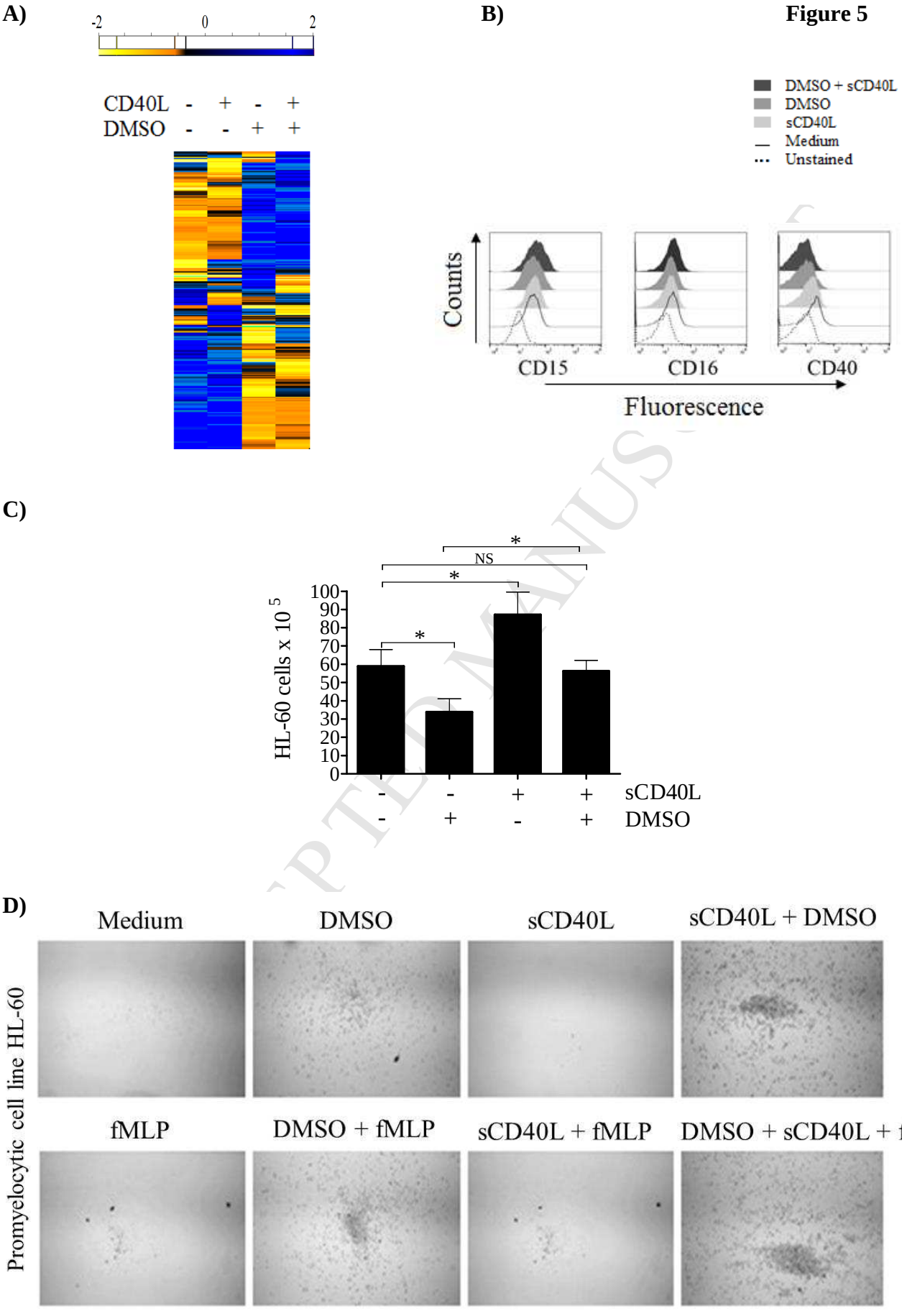
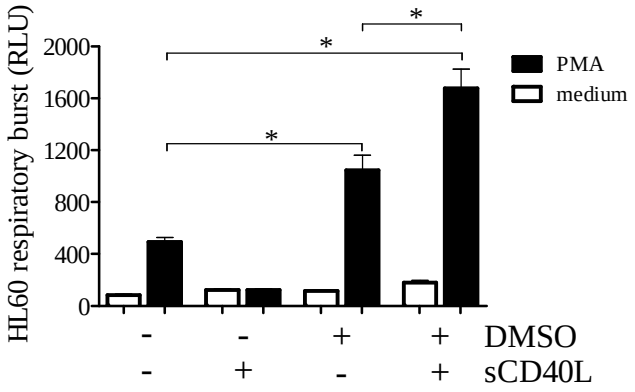


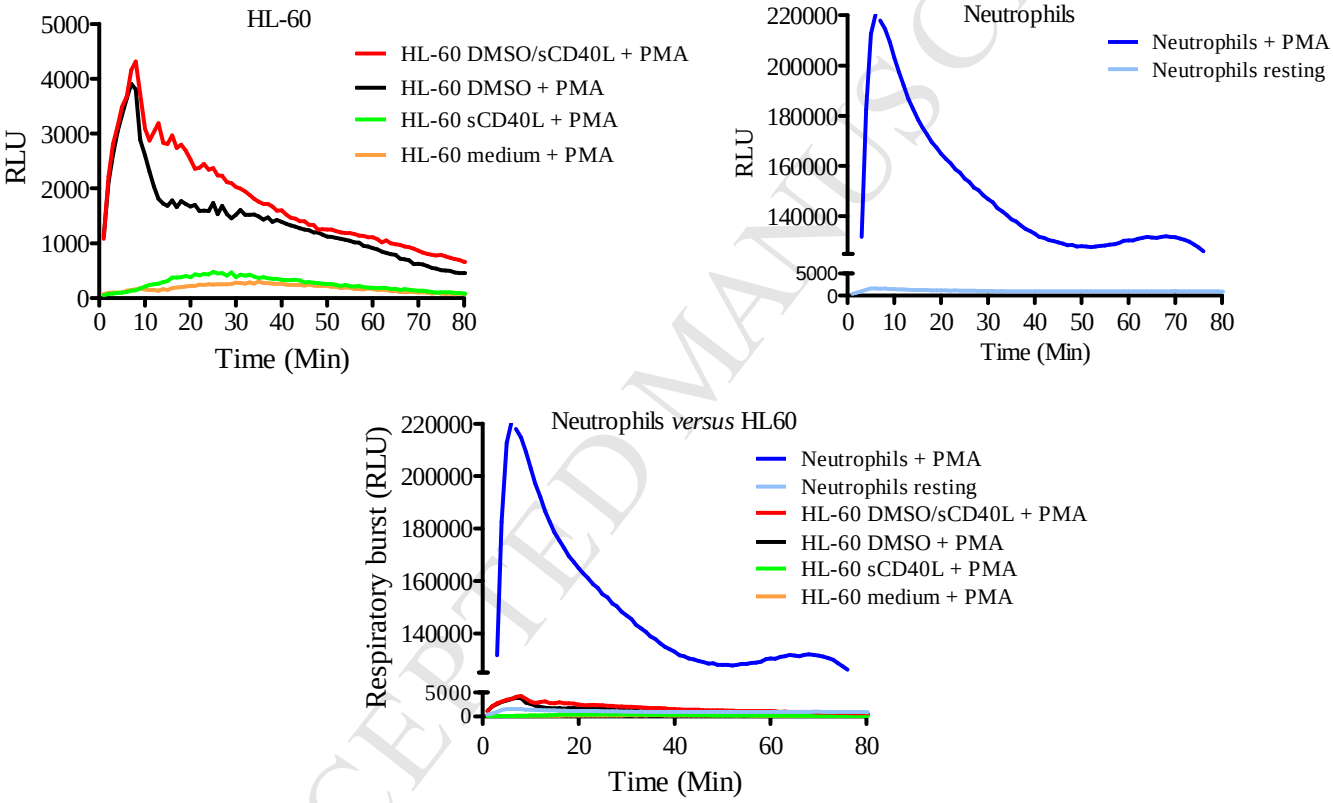
Figure 5


A)
-2
0
2
CD40L - + - +
DMSO - - + +
B)
DMSO + sCD40L
DMSO
sCD40L
Medium
Unstained
Counts
CD15
CD16
CD40
Fluorescence
C)
HL-60 cells x 10 5
100
90
80
70
60
50
40
30
20
10
0
*
NS
*
*
- - + + sCD40L
- + - + DMSO
D)
Medium
DMSO
sCD40L
sCD40L + DMSO
Promyelocytic cell line HL-60
fMLP
DMSO + fMLP
sCD40L + fMLP
DMSO + sCD40L + fMLP

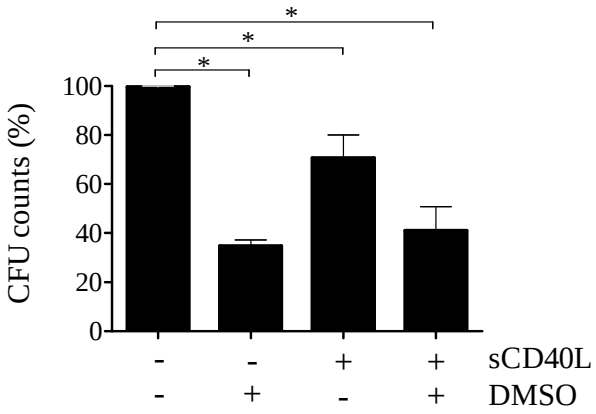
E)



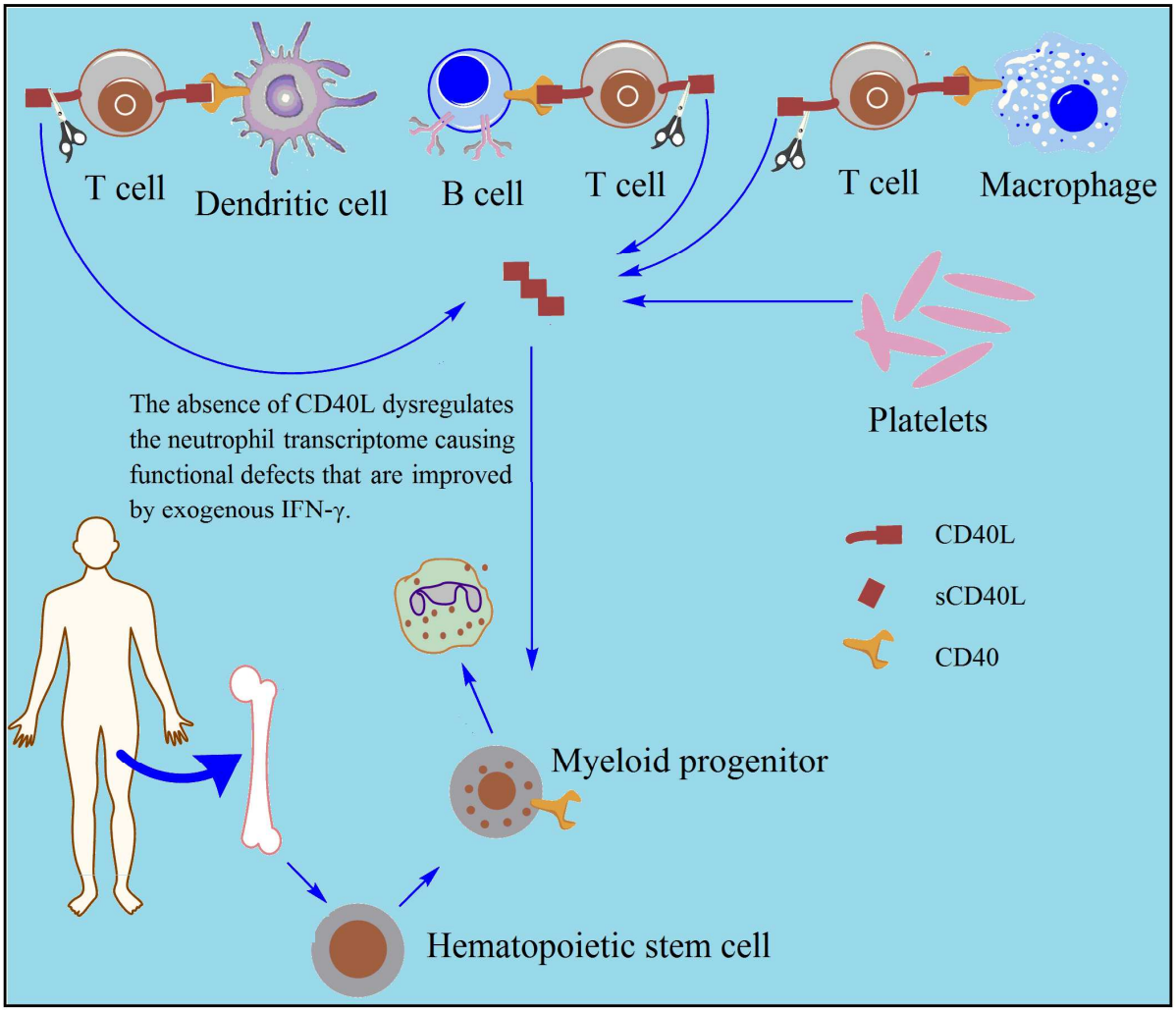
F)



G)



ACCEPTED MANUSCRIPT


T cell
Dendritic cell
B cell
T cell
T cell
Macrophage
Platelets
The absence of CD40L dysregulates the neutrophil transcriptome causing functional defects that are improved by exogenous IFN-γ.
CD40L
sCD40L
CD40
Myeloid progenitor
Hematopoietic stem cell

ACCEPTED

Figure S1

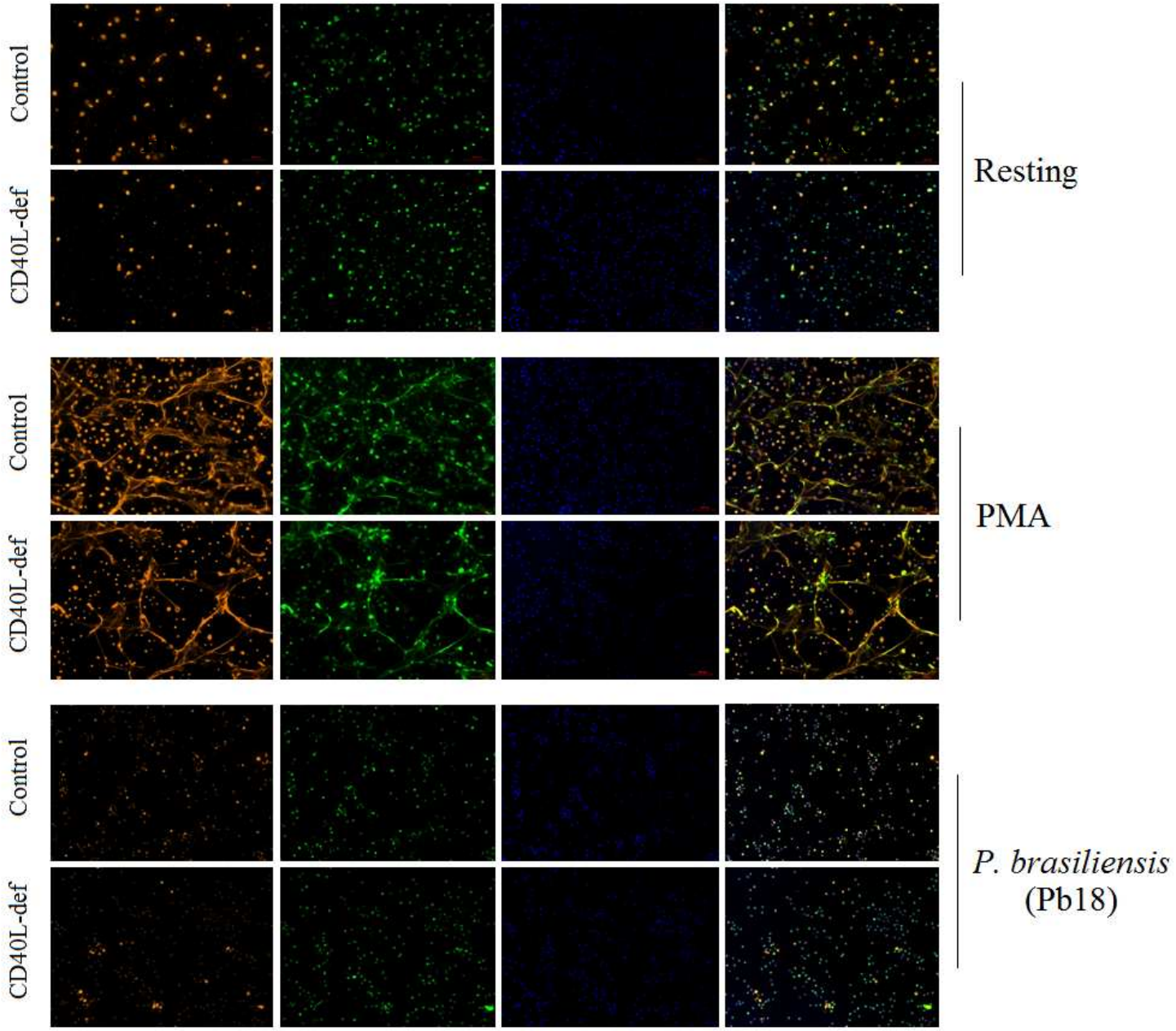
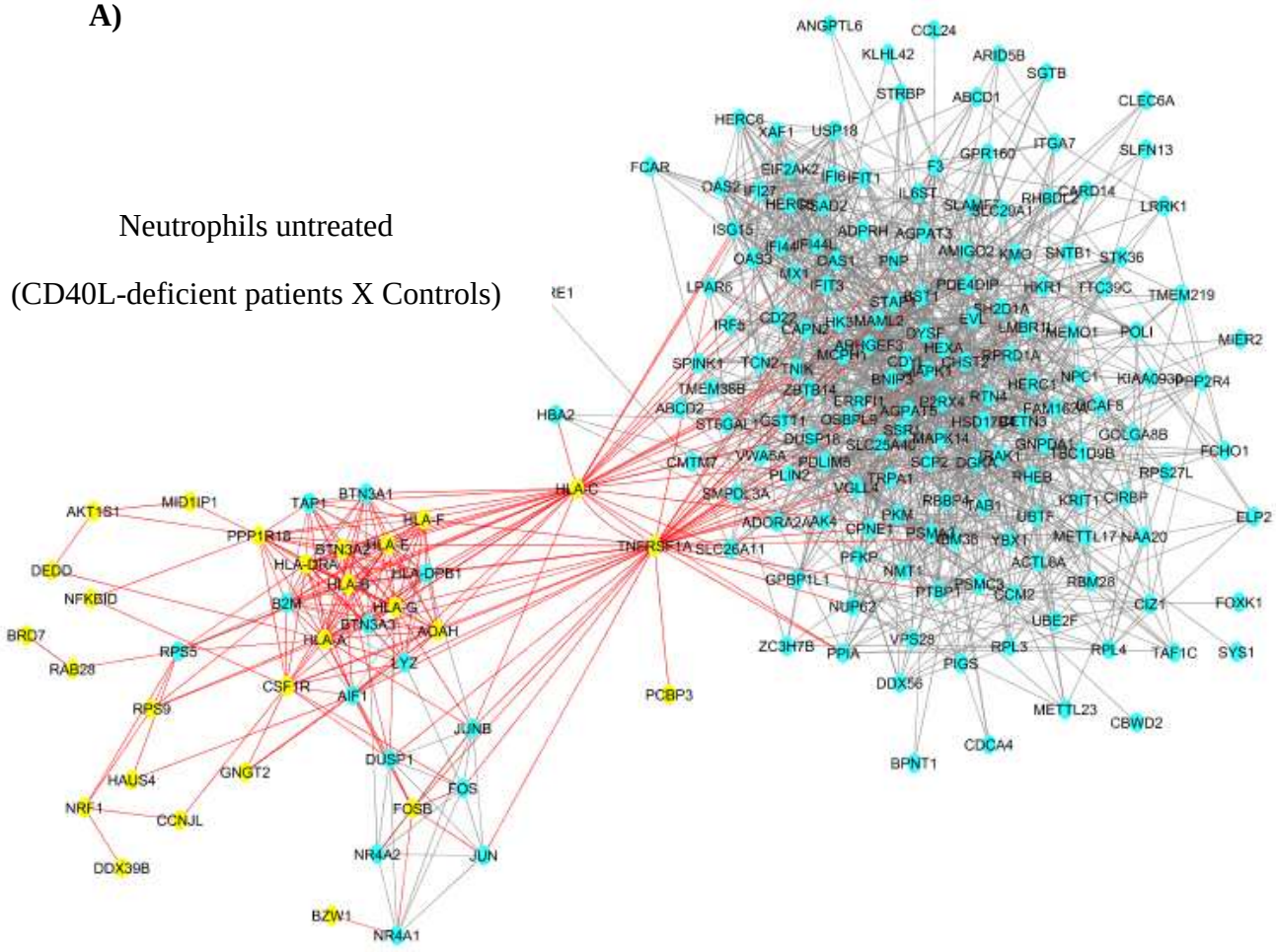

Control
CD40L-def
Resting
Control
CD40L-def
PMA
Control
CD40L-def
P. brasiliensis
(Pb18)

Figure S2

A)

Neutrophils untreated

(CD40L-deficient patients X Controls)



B)

Neutrophils+ IFN-γ

(CD40L-deficient patients X Controls)

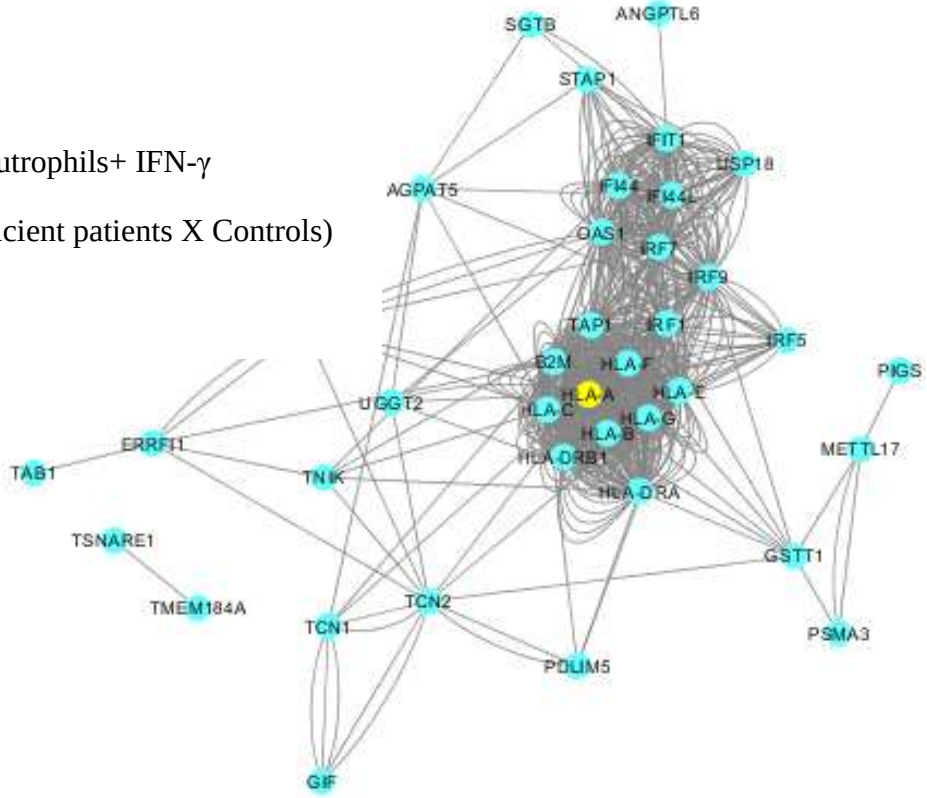
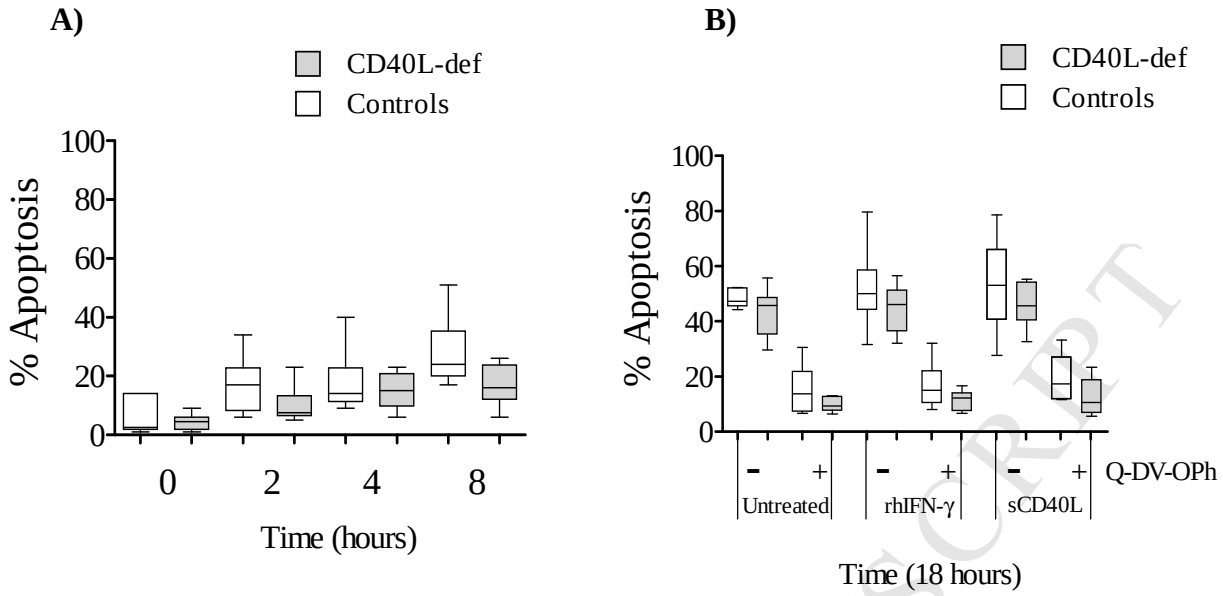
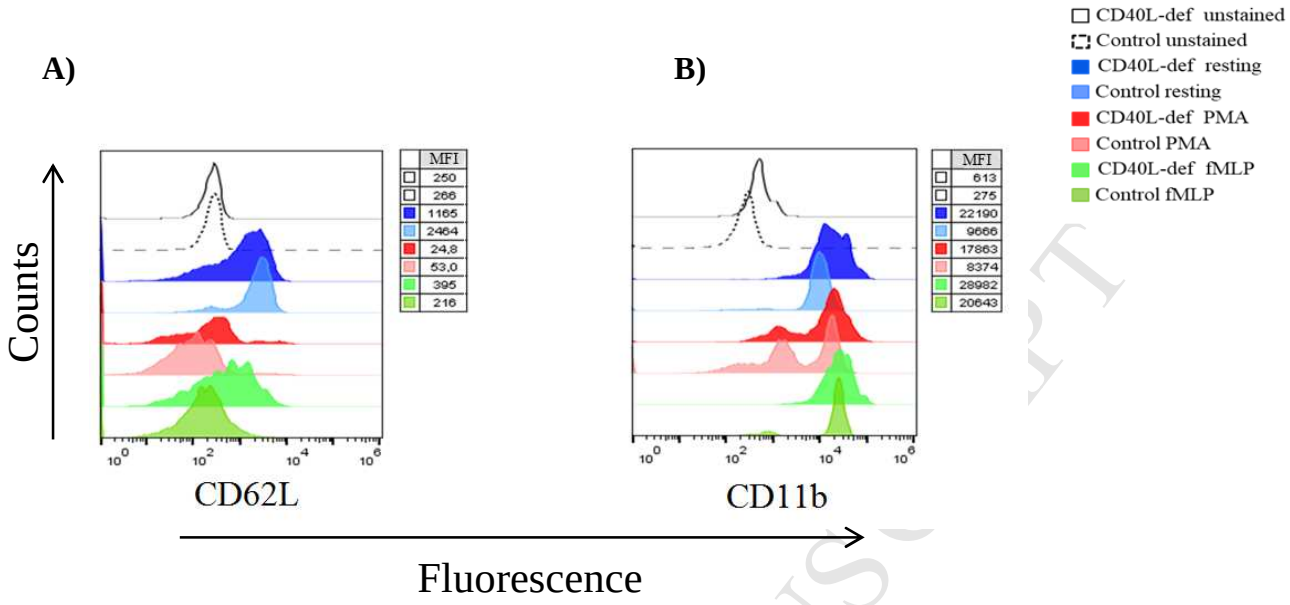


Figure S3



ACCEPTED MANUSCRIPT

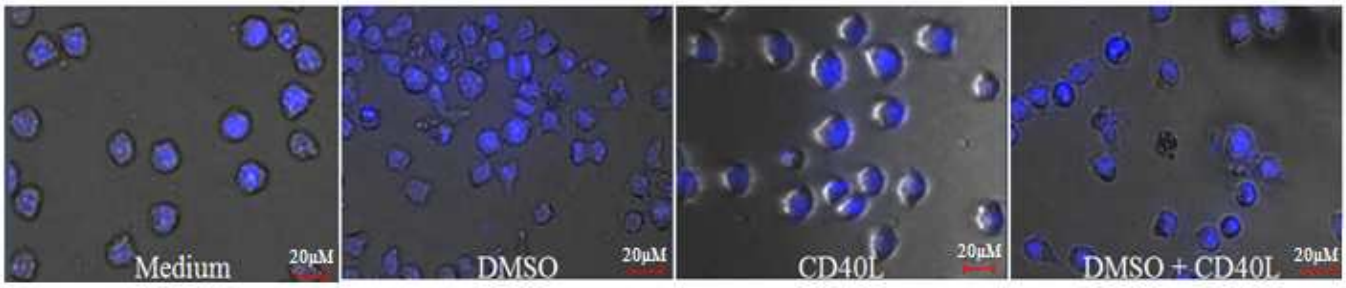
Figure S4



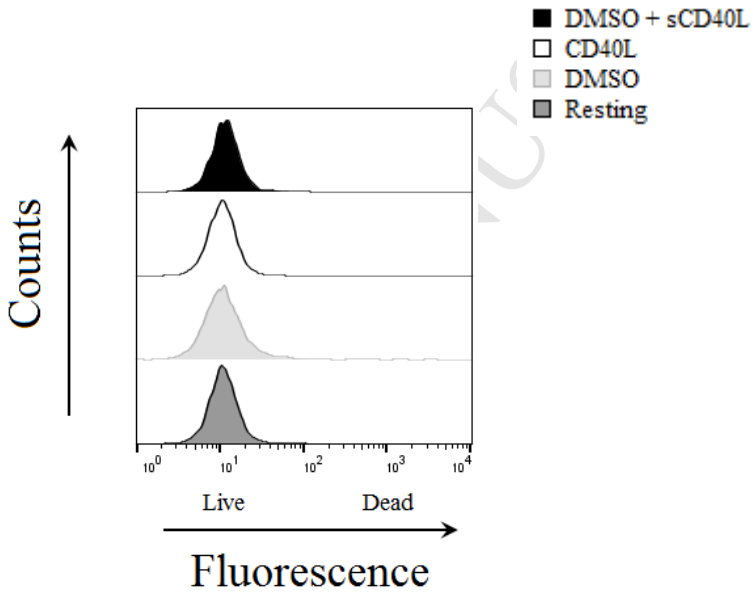
ACCEPTED MANUSCRIPT

Figure S5

A)

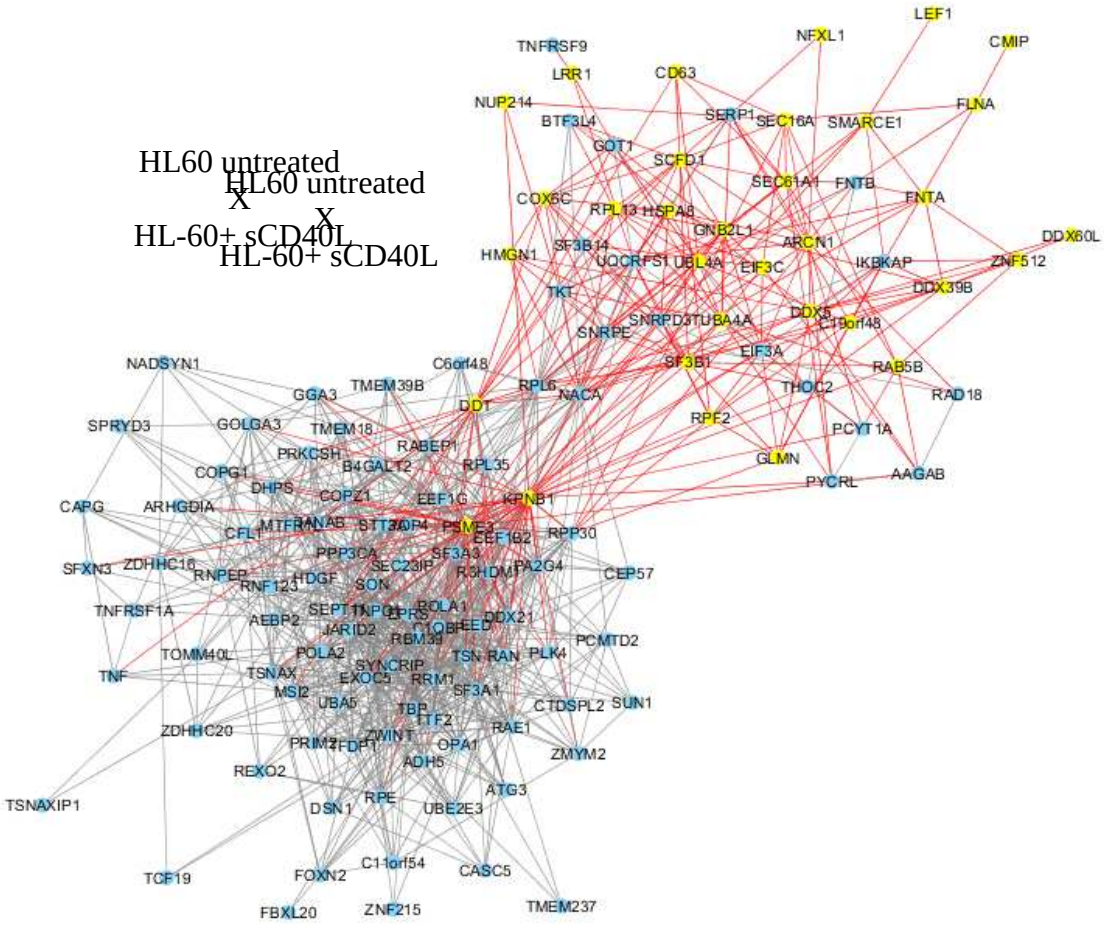


B)

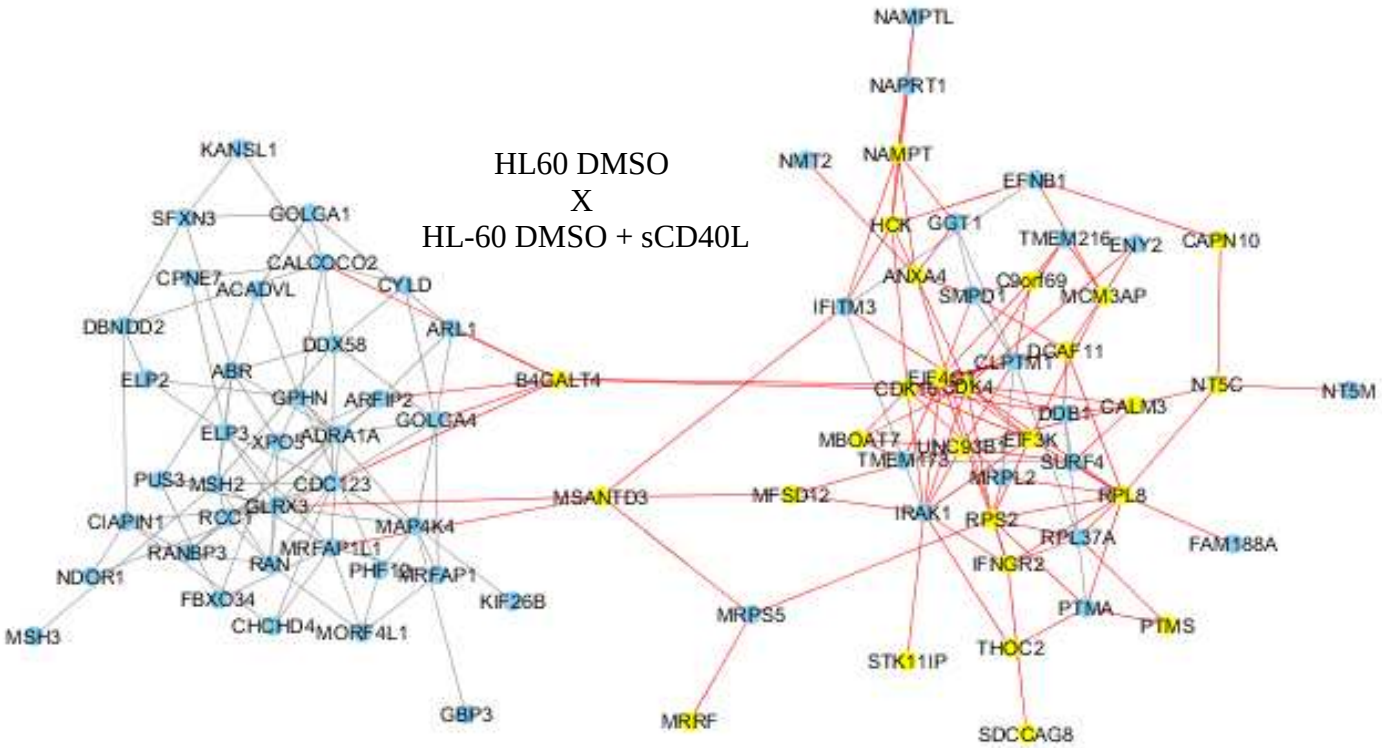


ACCEPTED MANUSCRIPT

A) **Figure S6**


HL60 untreated
X
HL-60+ sCD40L
LEF1
NFXL1
CMIP
TNFRSF9
LRR1
CD63
NUP214
FLNA
BTF3L4
SERP1
SEC16A
SMARCE1
GOT1
SCFD1
SEC61A1
FNTB
FNTA
COX6C
RPL13
HSPA8
GNB2L1
ARCN1
DDX60L
HMGN1
SF3B14
UQCRFS1
UBL4A
EIF3C
IKBKAP
ZNF512
TKT
DDX39B
SNRPD3
TUBA4A
DDX5
C19orf48
SNRPE
EIF3A
NADSYN1
C6orf48
SF3B1
RAB5B
TMEM39B
RPL6
NACA
THOC2
RAD18
GGA3
DDT
RPF2
SPRYD3
GOLGA3
TMEM18
PCYT1A
RABEP1
PRKCSH
GLMN
B4GALT2
RPL35
COPG1
PYCRL
AAGAB
DHPS
COPZ1
EEF1G
KPNB1
CAPG
ARHGDIA
MTFR1NAB
STT3A
PSME3
CFL1
EEF1B2
RPP30
PPP3CA
SF3A3
SFXN3
ZDHHC16
SEC23IP
PA2G4
CEP57
RNPEP
RNF123
HDGF
SON
TNFRSF1A
AEBP2
SEPT11
TNPO1
PPIS
ROLA1
DDX21
JARID2
C10orf6
EED
RBM39
PCMTD2
TOMM40L
POLA2
TSN
RAN
PLK4
TNF
TSNAX
SYNCRIP
EXOC5
RRM1
MSI2
SF3A1
UBA5
TBP
CTDSPL2
SUN1
ZDHHC20
ITF2
RAE1
PRIM2
FDPS
ZWINT
OPA1
ZMYM2
ADH5
REXO2
ATG3
TSNAXIP1
RPE
DSN1
UBE2E3
TCF19
FOXN2
C11orf54
CASC5
FBXL20
ZNF215
TMEM237

B)


HL60 DMSO
X
HL-60 DMSO + sCD40L
NAMPTL
NAPRT1
KANSL1
NMT2
NAMPT
EFNB1
SFXN3
GOLGA1
HCK
GGT1
TMEM216
ENY2
CAPN10
CALCOCO2
ANXA4
C9orf69
CPNE7
ACADVL
CYLD
IFITM3
SMPD1
MCM3AP
DBNDD2
ARL1
DDX58
DCAF11
CLPTM1
ELP2
ABR
B4GALT4
NT5C
NT5M
GPHN
ARFIP2
CDK6
CDK4
CALM3
DDB1
GOLGA4
ELP3
XPO5
ADRA1A
MBOAT7
UNC93B1
EIF3K
TMEM173
SURF4
PUS3
MSH2
CDC123
MRPL2
RPL8
MSANTD3
MFSD12
GLRX3
IRAK1
RPS2
CIAPIN1
RCC1
MAP4K4
RPL37A
FAM188A
RANBP3
MRFAP1L1
RAN
IFNGR2
NDOR1
PHF10
MRFAP1
FBXO34
KIF26B
PTMA
PTMS
MRPS5
MSH3
CHCHD4
MORF4L1
THOC2
STK11IP
GBP3
MRRF
SDCCAG8

ACCEPTED MANUSCRIPT

SUPPLEMENTAL MATERIAL AND METHODS

Analysis of neutrophils from CD40L-deficient patients

Phagocytosis

Neutrophil phagocytic capacity was analyzed in whole blood by flow cytometry using propidium iodide (PI)-labeled *P. brasiliensis* as previously described[1]. After one hour incubation at 37°C, red blood cells were lysed using a red blood cell (RBC) lysis solution (Qiagen, Santa Clarita, CA). Neutrophils were stained with anti-CD15, and phagocytosis was analyzed by flow cytometry.

Analysis of neutrophils respiratory burst

Neutrophil respiratory burst was evaluated as previously described[2]. The cells were stimulated with phorbol-12-myristate-13-acetate (PMA, 300ng/ml, Sigma-Aldrich, St. Louis, MO), N-formylmethionyl-leucyl-phenylalanine (fMLP, 50nM, Sigma-Aldrich) or heat killed *Staphylococcus aureus* for 1 hour at 37°C followed by dihydrorhodamine (DHR) 123 (Sigma-Aldrich) staining. Neutrophils were stained with anti-CD15 and analyzed by flow cytometry. Results were expressed as mean fluorescence intensity (MFI). Neutrophil respiratory burst was also measured by luminol-dependent chemiluminescence as previously described[3]. In brief, 2 x 10^5 neutrophils in the absence or presence of PMA (300 ng/mL) and luminol (1mM, Sigma-Aldrich) were monitored by chemiluminescence for 2 hours using a microplate luminometer reader (EG&G Berthold LB96V, Bad Wildbad, Germany). The results were expressed as relative light units (RLU) during the observation period and shown as % of the control cells in relation to the PMA-induced respiratory burst of neutrophils from healthy controls. When indicated, experiments where performed in the presence or absence of

ACCEPTED MANUSCRIPT

different catalase concentrations (100, 300 and 1000 U/ml, Sigma-Aldrich) and *Paracoccidioides brasiliensis* (Pb18, a highly virulent isolate) [4].

Neutrophil extracellular trap (NET) release by neutrophils

The release of NET was analyzed as previously described[5,6]. In brief, after PMA (100ng/ml) or *P. brasiliensis* (1 neutrophil:2 fungi) stimulation for 4 hours, neutrophils from controls were seeded on glass coverslips, were fixed with PBS plus 4% paraformaldehyde, permeabilized with 0.1% Triton X-100 and blocked by 3% bovine serum albumin. The samples were incubated with primary antibody to the histone complex H2A-H2B (kindly provided by Prof. Dr. Arturo Zychlinsky from the Department of Cellular Microbiology, Max Planck Institute for Infection Biology, Charitéplatz 1, Berlin, Germany) and with anti-neutrophil elastase monoclonal antibodies followed by exposure to a secondary antibody, and nuclear staining with Hoechst dye. The cells were analyzed by fluorescence microscopy. In addition, NET formation was quantified using Sytox Orange and results expressed in relative fluorescence units (RFU) as previously described[7].

Microbicidal activity

Neutrophil microbicidal activity was analyzed by challenging with the fungus *P. brasiliensis* and determined by counting colony forming units (CFU) as previously described with few modifications[4]. Briefly, neutrophils were suspended in 200 μL RPMI 1640 in a 96-well round bottom cell culture microplates and challenged with *P. brasiliensis* at a ratio of 2:1 (neutrophils/fungus). After a two hour incubation at 37^{o}C, the plates were centrifuged, washed, and the supernatants containing the non-ingested microorganisms were discarded and the plates washed. The neutrophils were lysed with chilled (4^{o}C) ultrapure water. *P. brasiliensis* CFU were counted 5 days after the lysates were cultured at 35^{o}C on brain heart infusion agar (Difco, Detroit, MI) in plates containing 4% (vol/vol) normal horse serum

(Instituto Butantan, Sao Paulo, Brazil) and 5% *P. brasiliensis* (Pb192) culture filtrate. CFU index was determined in relation to the CFU number of the unchallenged neutrophils from healthy control subjects.

Neutrophil phenotyping

The expression of neutrophil maturation markers (CD15, CD16, CD18, CD11b, CD11c, CD35, CD63, and CD66b all from BD Biosciences, San Jose, CA) was analyzed in whole blood after red blood cell lysis using RBC Lysis Solution (Qiagen), according to the manufacturer's instruction. After staining using monoclonal fluorophore-conjugated antibodies (Becton Dickinson, Mountain View, CA), cells were analyzed by flow cytometry on a BD FACS Canto II Cytometer, and neutrophils were gated according to size (forward scatter, FSC) and granularity (side scatter, SSC). The median fluorescence intensity (MFI) for the expression of each molecule was obtained using FlowJo software (Treestar, Inc, Ashland, OR).

RNA-seq and data processing

Neutrophil transcriptome profiles from three CD40L-deficient patients and three healthy controls were analyzed as described [8]. Total ribonucleic acid (RNA) was obtained using TRIZOL (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. RNA integrity and concentration were assessed using the Agilent 2100 Bioanalyser RNA Nano chip, and orthogonally validated by visualization of the integrity of the 28S and 18S band on an agarose gel. cDNA libraries were obtained using the Illumina CBot station, and HiScanSQ using the Illumina TruSeq RNA Sample Preparation Kit (Illumina Inc., San Diego, CA) according to each manufacturer´s instructions. Sequencing was carried out using the Illumina HiSeq 2000 paired-end 100-bp (PE 100) system. The bioinformatics analysis of data obtained

was performed as previously described [9]. The protocol of the transcriptome analysis and data obtained were submitted to ArrayExpress, and is public available at: http://www.ebi.ac.uk/arrayexpress/experiments/E-MTAB-5316.

Assessment of CD62L shedding

We evaluated expression and shedding of membrane-bound L-selectin (CD62L) by flow cytometry as previously described[33]. In brief, after neutrophil TLRs bind their ligands, signaling pathways are activated, triggering the shedding of CD62L. Briefly, 100 μL of whole blood was incubated for 1 hour without stimulation or with PMA (400 ng/mL), synthetic triacylayed lipopeptide Pam3CSK4 (1 μg/mL, a TLR1/2 agonist, Invivogen, Carlsbad, CA, USA) or lipopolysaccharide (LPS, 100 ng/mL, a TLR4 agonist, Sigma-Aldrich) at 37°C and 5% CO_2 . After erythrocyte lysis, the cells were washed, stained with anti-human CD62L antibody (BD Biosciences) and analyzed by flow cytometry. When indicated, the CD62L shedding was analyzed simultaneously with the CD11b expression The MFI of CD62L and CD11b expression from stimulated cells were compared to the MFI of unstimulated cells.

Analysis of HL-60 cell responses

HL-60 cells, a useful model for myeloid differentiation, were cultured for 6 days as previously described[10] in the presence or absence of 500 ng/mL sCD40L and/or 1,0 % dimethyl sulfoxide (DMSO). After 6 days of incubation, the cells were harvested, counted, and their viability determined by Trypan blue exclusion and the LIVE/DEAD Viability/Cytotoxicity Kit (Thermo Fisher Scientific, Waltham, MA) according to the manufacturer's instructions. To assess the HL-60 maturation state, we analyzed the respiratory burst, fungicidal activity, and transcriptome profile as described above for

neutrophils from CD40L-deficient patients. Their migration was analyzed by the transwell migration assay (24-well plate, Corning, Inc., Corning, NY) as previously described [11].

Neutrophil responses in CD40L-deficient mice

Bacterial strains and growth conditions

The characteristics of the bacterial strains used here have been published elsewhere [12–14]. BRD509 is an attenuated *aroA⁻/aroD⁻* mutant strain of *Salmonella enterica* serovar Typhimurium, which is ~10,000-fold less virulent than the parental SL1344 strain from which it was derived [15]. Aliquots of frozen bacteria were routinely plated on *Salmonella Shigella* (S.S) agar in the presence of ampicillin and grown overnight at 37°C. Log-phase bacterial suspensions were prepared in pyrogen-free saline and injected intraperitoneally in 0.5 ml volume. Bacterial doses were confirmed by colony forming unit (CFU) plate counts.

Enumeration of bacteria in peritoneal cavity

The quantification of bacterial loads in peritoneal cavity was carried out as previously described [16]. Mice (3 per group) were sacrificed at 24-72 hours after inoculation. Peritoneal lavage was performed using cold Ca^{2+}, Mg^{2+}-free saline and aliquots were centrifuged at 2500g for 5 min and re-suspended in sterile water. Aliquots (in 50-100 µl volume), or appropriate dilutions thereof, were plated on SS agar plates and viable CFUs were determined after an overnight incubation.

Flow cytometric analysis of peritoneal exudate cells

Peritoneal exudate cells were analysed as previously described [17]. Briefly, peritoneal exudate cells (PECs) were suspended to a concentration of $5x10^6$/ml and 100µl aliquots dispensed into wells of a round-bottom 96-well plate. Cells were pre-incubated with anti-CD16/CD32-

specific monoclonal antibody (mAb, clone 2.4G2, BD Biosciences) for 30 min on ice to block FcγIII/II receptor sites. Analysis of PECs was carried out using a six-color flow cytometric analysis, following a standard procedure[18] after staining cells with the following panel of conjugated mAbs: CD3-FITC, CD19-PE, CD8-APC, CD4-PE-Cy7, and CD11b-APC-Cy7 (all from Biolegend, San Diego, CA). Analysis of myeloid cell subpopulations was carried out using a second panel of mAbs: CD11b-APC-Cy7, F4/80-PE-Cy7, Ly6C-PE, Ly6G-APC and anti-MHC class II-FITC (Biolegend). The viability dye 7AAD (Biolegend) was used with each panel of mAbs to exclude non-viable cells from the analysis. Data was collected on 30,000 cells using FACSCanto II (BD Biosciences) and analyzed using FACSDiva software (BD Biosciences).

Measurement of neutrophil phagocytic activity

24 hours after challenging mice with BRD509, the phagocytic potential of PECs was assessed using a flow cytometric-based phagocytosis assay kit (cat# 500290) from Cayman Chemical (Cayman Pharma s.r.o., Neratovice, Czech Republic), according to manufacturer's instructions. 24 hours after challenging mice with BRD509, PECs were incubated with rabbit IgG-FITC-labeled latex beads for 3 hrs at 37°C, followed by surface staining with a panel of mAbs including CD11b-APC-Cy7, F4/80-PE-Cy7, Ly6C-PE and Ly6G-APC (Biolegend). The cells were then further processed and analyzed by flow cytometry as described above.

Measurement of intracellular reactive oxygen species (ROS)

Production of ROS was measured by flow cytometry using 2′,7′–dichlorofluorescein diacetate (DCF-DA; Sigma-Aldrich) as a fluorescent probe, following an established protocol [19]. Briefly, 24 hours after challenging mice with BRD509, PECs were stained with 5 µM DCF-DA for 30 min at 37°C in the dark. The cells were then washed, resuspended and surface

ACCEPTED MANUSCRIPT

147 stained with mAbs (CD11b-APC-Cy7, F4/80-PE-Cy7, Ly6C-PE and Ly6G-APC), followed

148 by analysis on FACSCanto II, as described above.

149

ACCEPTED MANUSCRIPT

ACCEPTED MANUSCRIPT

REFERENCES

1. Feriotti C, Loures F V., Frank de Araújo E, da Costa TA, Calich VLG. Mannosyl-Recognizing Receptors Induce an M1-Like Phenotype in Macrophages of Susceptible Mice but an M2-Like Phenotype in Mice Resistant to a Fungal Infection. *PLoS One*. 2013;8(1):.

2. Bustamante J, Arias AA, Vogt G, et al. Germline CYBB mutations that selectively affect macrophages in kindreds with X-linked predisposition to tuberculous mycobacterial disease. *Nat. Immunol.* 2011;12(3):213–21.

3. Soeiro-Pereira P V, Falcai A, Kubo CA, et al. BAY 41-2272, a soluble guanylate cyclase agonist, activates human mononuclear phagocytes. *Br. J. Pharmacol.* 2012;166(5):1617–1630.

4. Pina A, Saldiva PHN, Restrepo LEC, Calich VLG. Neutrophil role in pulmonary paracoccidioidomycosis depends on the resistance pattern of hosts. *J. Leukoc. Biol.* 2006;79:1202–1213.

5. Brinkmann V, Reichard U, Goosmann C, et al. Neutrophil extracellular traps kill bacteria. *Science (80-.).* 2004;303(5663):1532–1535.

6. Metzler KD, Fuchs TA, Nauseef WM, et al. Myeloperoxidase is required for neutrophil extracellular trap formation: Implications for innate immunity. *Blood*. 2011;117(3):953–959.

7. Reinhardt TA, Sacco RE, Nonnecke BJ, Lippolis JD. Bovine milk proteome: quantitative changes in normal milk exosomes, milk fat globule membranes and whey proteomes resulting from Staphylococcus aureus mastitis. *J. Proteomics*. 2013;82:141–54.

8. Wright HL, Thomas HB, Moots RJ, Edwards SW. RNA-seq reveals activation of both common and cytokine-specific pathways following neutrophil priming. *PLoS One*. 2013;8(3):e58598.

9. Cabral-Marques O, Ramos RN, Schimke LF, et al. Human CD40L deficiency dysregulates the macrophage transcriptome causing functional defects that are improved by exogenous IFN-γ. *J. Allergy Clin. Immunol.* 2016;

10. Newburger PE, Chovaniec ME, Greenberger JS, Cohen HJ. Functional changes in human leukemic cell line HL-60. A model for myeloid differentiation. *J. Cell Biol.* 1979;82(2):315–22.

11. Carrigan SO, Weppler AL, Issekutz AC, Stadnyk AW. Neutrophil differentiated HL-60 cells model Mac-1 (CD11b/CD18)-independent neutrophil transepithelial migration. *Immunology*. 2005;115(1):108–17.

12. Al-Ojali SM, Moore CBT, Fernandez-Cabezudo MJ, al-Ramadi BK. Enhancement of the anti-Salmonella immune response in CD154-deficient mice by an attenuated, IFN-γ-expressing, strain of Salmonella enterica serovar Typhimurium. *Microb. Pathog.* 2012;52(6):326–335.

13. Fernandez-Cabezudo MJ, Mechkarska M, Azimullah S, al-Ramadi BK. Modulation of macrophage proinflammatory functions by cytokine-expressing Salmonella vectors.

ACCEPTED MANUSCRIPT

Clin. Immunol. 2009;130(1):51–60.

14. al-Ramadi BK, Adeghate E, Mustafa N, Ponery AS, Fernandez-Cabezudo MJ. Cytokine expression by attenuated intracellular bacteria regulates the immune response to infection: the Salmonella model. *Mol. Immunol.* 2002;38(12–13):931–40.

15. al-Ramadi BK, Fernandez-Cabezudo MJ, Ullah A, El-Hasasna H, Flavell RA. CD154 is essential for protective immunity in experimental salmonella infection: evidence for a dual role in innate and adaptive immune responses. *J. Immunol.* 2006;176(1):496–506.

16. al-Ramadi BK, Mustafa N, AbouHaidar M, Fernandez-Cabezudo MJ. Induction of innate immunity by IL-2-expressing Salmonella confers protection against lethal infection. *Mol. Immunol.* 2003;39(13):763–70.

17. Issac JM, Sarawathiamma D, Al-Ketbi MI, et al. Differential outcome of infection with attenuated Salmonella in MyD88-deficient mice is dependent on the route of administration. *Immunobiology*. 2013;218(1):52–63.

18. Ramadi KB, Mohamed YA, Al-Sbiei A, et al. Acute systemic exposure to silver-based nanoparticles induces hepatotoxicity and NLRP3-dependent inflammation. *Nanotoxicology*. 2016;10(8):1061–74.

19. Aryappalli P, Al-Qubaisi SS, Attoub S, et al. The IL-6/STAT3 Signaling Pathway Is an Early Target of Manuka Honey-Induced Suppression of Human Breast Cancer Cells. *Front. Oncol.* 2017;7:167.

ACCEPTED MANUSCRIPT

ACCEPTED MANUSCRIPT

Table S1

Pt	Year of Birth	Isolated pathogens			Infections with unidentified pathogens	cDNA Mutation[a]	Predicted effect on protein
		Fungi	Intracelular bacteria and protozoa	Virus			
P1	2007	-	*M. tuberculosis*	-	pneumonia, otitis,	c.419G>A	p. W140*
P2	2005	*P. jirovecii*	-	HPV, Herpes simplex	otitis, sinusitis, pneumonia	c.157_160del	p.I53Kfs*13
P3	2007	*P. jirovecii, C. albicans*	*C. parvum*	-	otitis, sinusitis, pneumonia	c.157_160del	p.I53Kfs*13
P4[f]	1993	*P. brasiliensis, P. jirovecii*	*M. tuberculosis*		pneumonia, otitis, sinusitis,	c.289_346del	p.D97Vfs*12
P5	2004	*Aspergillus sp P. jirovecii*	*C. parvum*	-	diarrhea	c.del of 170 nucleotides in 5' UTR, affecting promoter region	no mRNA, no protein expression
P6	2006	-	*M. tuberculosis*	-	pneumonia, otitis urinary tract infection,	c.574_577dup	p.L193Qfs*9

ACCEPTED MANUSCRIPT