SCIENTIFIC REPORT: ESF-Exchange Grant (Reference number 2985)

ESF activity: The Identification of Novel Genes and Biomarkers for Systemic Lupus Erythematosus (BIOLUPUS)

Project title: *BLyS production by neutrophils in SLE*

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1. Purpose of the visit

Systemic lupus erythematosus (SLE) is an autoimmune disease characterized by B lymphocyte hyper-reactivity and formation of pathogenic auto-antibodies. B-lymphocyte stimulator (BLyS, also termed B cell activating factor, BAFF) and a proliferation-inducing ligand (APRIL) are closely related members of the TNF ligand superfamily that individually regulate B-cell maturation, survival, and function. BLyS-transgenic mice develop autoimmune disorders with SLE-like symptoms, indicating that the level of BLyS needs to be tightly regulated to maintain B-cell survival without triggering autoimmunity (*Groom J et al, J Clin Invest 2002; Zhang J et al, J Immunol 2001*). In addition, circulating levels of BLyS have been found elevated in SLE patients (*Cambridge G, Arthritis Rheum 2006*). For these reasons BLyS is already recognized as a clinical target for the treatment of SLE. This view has been confirmed by phase III trials using Lymphostat-B (belimumab), a fully human monoclonal antibody that specifically recognizes and inhibits the biological activity of BLyS for the treatment of SLE. However, the role of APRIL in SLE is less well understood and contradictory data about serum levels in patients compared to healthy volunteers and their correlation with disease state have been published. Also only few studies have analyzed the expression of this cytokine by different cell populations in SLE patients. Interestingly, while BLyS is produced as a membrane bound pro-form in most myeloid cell types (*Schneider P et al, J Exp Med 1999; Moore P et al, Science 1999*) previous data from our laboratory have revealed that freshly isolated neutrophils from healthy donors constitutively express BLyS on the surface as well as in intracellular pools being release from these stores by different pro-inflammatory cytokines (*Assi L et al, J Exp Med 2003*).

In this project, we investigated the relative surface and intracellular protein levels of BLyS and APRIL in isolated neutrophils from SLE patients and controls. Moreover, also we investigate both extra and intracellular BLyS expression from different subsets of fresh peripheral blood cells from SLE patients and healthy donors. Finally, we determined correlation with disease activity by the classic BILAG (British Isles Lupus Assessment Group) index.

2. Description of the work carried out during the visit

1) Quantification the protein surface and intracellular expression of BLyS by isolated neutrophils in SLE patients and age and sex matched controls.

Highly purified neutrophils from 33 SLE patients (excluded patients on B-cell targeting therapies) and 16 healthy donors were isolated by Percoll gradient separation as described previously (*de Boer M et al. J Immunol 1986*). Purity of the neutrophil preparations was routinely >97%, being the major contaminant eosinophils. Extracellular expression of BLyS was analysed by indirect immunofluorescence staining using mouse anti BLyS monoclonal antibody (Peprotech London, UK) and fluorescein isothiocyanate (FITC)–labeled goat anti mouse IgG 1 antibody (Southern Biotech, Birmingham, AL). To analyze APRIL extracellular, neutrophils were stained with rabbit anti APRIL polyclonal antibody (ProSci, California, USA) and FITC–labeled goat anti rabbit IgG 1 antibody (Southern Biotech). Intracellular expression was assessed after fixation and permeabilization using the *Fix & Perm Kit* (Caltag Laboratories, California, USA) and subsequent staining using the same antibodies as in the surface staining. All experiments were controlled using species-, isotype-, and concentration-matched irrelevant antibodies. Samples were read using a Cyan^{TM} flow cytometer (Beckman Coulter, Fullerton, CA). Samples were subsequently analyzed using FlowJo software (Tree Star Inc., Ashland, USA).

2) Determination of the membrane bound expression and intracellular BLyS by blood cell populations in SLE patients and healthy donors.

Peripheral blood samples from 16 controls and 24 SLE patients were collected in EDTA anticoagulant. Specific cell population cells were gated by staining of specific markers. For characterization of pDCs, blood cells were stained with anti-BDCA-2 and anti-CD123. At the same time, mDCs were defined as CD1c^{+} BDCA-1^{+}, and lymphocytes B and monocytes were gated as CD19^{+} or CD14^{+} cells, respectively. Neutrophils were gated according to forward and side-scattered properties. For intracellular measurement of BLyS, cells were fixed and permeabilized after staining for extracellular markers or the isotype control and incubated with anti-BLyS isothiocyanate (FITC)-labelled monoclonal antibodies according to the manufacturer's instructions (Fix & Perm Kit; Caltag). Analyses were carried out by acquisition of 200,000 events/tube on a Cyan^{TM} flow cytometer (Beckman Coulter) and subsequently analyzed using FlowJo software (Tree Star Inc.).

3) Correlation of extra and intracellular BLyS levels in isolated neutrophils and blood cell subsets from SLE patients with the disease activity (BILAG index).

3. Description of the main results obtained

Expression of BLyS and APRIL by isolated neutrophils from SLE patients

Initially, we analyzed the relative surface and intracellular protein levels of BLyS and APRIL by isolated neutrophils from SLE patients and healthy donors by flow cytometry. We could observe higher extracellular levels of BLyS in patients compared to controls (7.45 *vs* 3.29; p=0.0056), however we couldn't find significant differences in APRIL levels between patients and healthy donors. The analysis of patients with active and non-active disease did not show significant differences in BLyS and APRIL levels expressed in isolated neutrophils.

BLyS production by peripheral blood cell subsets from SLE patients

Next, we analyzed the relative surface and intracellular protein levels of BLyS by pDCs, mDCs, B cells, neutrophils and monocytes from whole blood samples from SLE patients and healthy donors by flow cytometry. To this end, blood cells were stained with antibodies subset-specific for CD19, CD14, BDCA-1, BDCA-2, CD123 and BLyS in order to identify cell populations. In SLE patients, increased expression of BLyS was detected in several peripheral blood cell subsets, including B cells both on their surface (0.23 *vs* 0.05; p=0.0067) and intracellularly (11.36 *vs* 7.68; p=0.0259), as well as intracellularly in mDCs (9.57 *vs* 7.45; p=0.0466) and pDCs (10.11 *vs* 6.27; p=0.0237). However, we did not detect significant differences between patients and controls in BLyS expression of monocytes and neutrophils.

Analysis of BLyS expression in patients with active versus inactive disease

Then, SLE patients were grouped by disease activity, and we could observe increased surface levels of BLyS in patients with active disease in mDCs (p=0.0416) and monocytes (p=0.0354), in addition of a reduced level of intracellular BLyS expression in the same cell types compared with minimal or non-active patients. These results suggest a mobilization of BLyS from intra to extracellular compartment during active disease. By this reason, we analyzed the ratio between surface bound and intracellular BLyS in active *vs* non-active SLE patients to get other fact supporting the possible mobilization of BLyS in function of the disease activity. Effectively, patients with active disease presented increased ratios between surface and intracellular BLyS compared with minimal or non-active patients, specifically in monocytes (0.20 *vs* 0.06; p=0.0125).

Statistical analysis

All data are represented by the median and differences between groups have been analyzed by non-parametric Mann Whitney test.

4. Future collaboration with host institution

In view of our results obtained from work performed in host institution of my Exchange Visit Grant, we have decided continue collaborating in this project, so Dr. Patricia Lopez (Autoimmunity Research Group managed by Dr. Ana Suarez, Oviedo University, Spain) in collaboration with Dr. Dagmar Scheel-Toellner and Prof. Caroline Gordon (Rheumatology Research Group, Birmingham University, United Kingdom) carry out next experiments in her Spanish laboratory.

Hypothesis and objective:

Our results show elevated BLyS levels of mDCs and monocytes on the surface from active patients as well as intracellularly in minimal or non-active patients, so we hypothesize that it exists a mobilization of BLyS from intra to extracellular compartment during active disease.

To test this hypothesis, we will study the regulation of BLyS expression and release from the intracellular and membrane bound pools in pro-inflammatory conditions.

To this end, we will use different isolated cell subsets (neutrophils, monocyes and monocyte derived DCs) from normal healthy donors and incubate them in a series of inflammatory conditions to test what factors would vary the intracellular and extracellular BLyS expression, the amount of released into the supernatant as well as the mRNA levels. The culture stimuli will range between using SLE sera, purified immune complexes and a comprehensive range of cytokines: IL-1β, IL-8, IL-17, IL-18, G-CSF, GM-CSF, TNFα, IFNα, IFNβ and immune complexes. If immune complexes induce a change in the production and release of BLyS, we will use an inhibitor of SYK, R406, to test if the effects of immune complexes can be blocked.

5. Projected publications/articles resulting or to result from the grant

All results obtained in this project are being compiled now to be published in an international indexed scientific journal (***BLyS/BAFF expression by peripheral blood cells subsets in systemic lupus erythematosus patients****. Lopez P, Gordon C, Scheel-Toellner D*). Of course, as it has been indicated in the guidelines for the present Exchange Visit Travel Grant, ESF will be acknowledged in publications resulting from the grantee's work.