Blocking ADP-ribosylation of cell surface proteins during cell preparation with a Nanobody against ART2 enhances chemotaxis of T cells during *in vitro* migration assays.

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Abstract

ADP-ribosylation is the enzymatic transfer of the ADP-ribose moiety from NAD^+ to a protein, a posttranslational modification that regulates the function of the targeted proteins. Although in normal conditions there is minimal extracellular NAD^+, it is released from cells in inflammation and during preparation of primary lymphocytes.

ART2 (CD296) is an ADP-ribosyltransferase expressed by most mature T cells, including NKT, γδ and regulatory T cells. A major substrate for ART2 is P2X7, an ion channel that mediates shedding of CD62L, and NAD^+-induced cell death (NICD). In this manner, NAD^+ released during cell preparation affects the phenotype and survival of cells expressing ART2.

In this study, we used a recombinant variable domain of the llama heavy-chain antibody S^+16a to block the ADP-ribosyltransferase activity of ART2. The recombinant antibody was administered i.v. to mice and 1-2 h later, the $CD4^+$ T cells from spleen and lymph nodes were isolated. The cell phenotype and functional state was analyzed by flow cytometry and chemotaxis assay respectively.

A single dose of S^+16a blocked the shedding of CD62L and reduced the number of apoptotic cells about 80%. The higher cell viability resulted in a 50-100% increase in the cell number responding to the chemokines CCL22 and CXCL12.

We conclude that S^+16a is a useful tool to protect mature T cells from NICD induced by the released NAD^+ from cells during purification, thereby enhancing the efficiency of *in vitro* migration assays.

Introduction

The release of NAD^+ from dead cells during cell purification results in the ADP-ribosylation of several proteins on the cell surface, catalyzed by ART2. Among the targets of ART2 are CD25, CD11a, CD18 and P2X7. P2X7 is an ion channel which opens when ribosylated, causing the downstream activation of caspases and metalloproteases. These, in turn, produce the shedding of CD62L and CD27, and at the end, apoptosis, in a process known as NICD.

ART2 remains active at 4°C. This means that even if the cells are kept cold at all times during their purification, P2X7 will still be ribosylated, and when the cells are left in culture or are transferred into a recipient, P2X7 will open, affecting the cell's phenotype and viability. This phenomena is particularly important during the isolation of regulatory T cells (Treg), as these cells express high levels of ART2 and P2X7.

Camelid antibodies (Abs) differ from conventional Abs because they lack the light chain and the first constant domain of the heavy chain (CH1); also, its IgG_3 is characterized by having a long hinge (Fig. 1A). In these Abs, the variable heavy chain domain (VH) is the only structure involved in the antigen (Ag) recognition, making them the smallest Ag-binding unit produced by an immune system. For this reason, the VH domain is also referred as "single domain antibody" or "Nanobody". S^+16a is a llama-derived nanobody that specifically and reversibly blocks the ectoenzyme ADP-ribosyltransferase 2 (ART2) (Koch-Nolte, F., *et al.* 2007. *FASEB J.* 21:3490). We decided to test if this tool can protect Tregs and mature T cells from NICD without negatively affecting their response to chemokines.

Materials and Methods

Nanobody: Production of recombinant VH domain S^+16a or the fusion protein S^+16a-Fc were described elsewhere (Koch-Nolte, F., *et al.* 2007. *FASEB J.* 21:3490. Scheuplein, A., *et al.* 2010. *J Autoimmun.* 34:145). C57BL/c mice were injected with 100µg of nanobody S^+16a (i.v.) 1-2 hours before start of cell isolation. Control animals received PBS only.

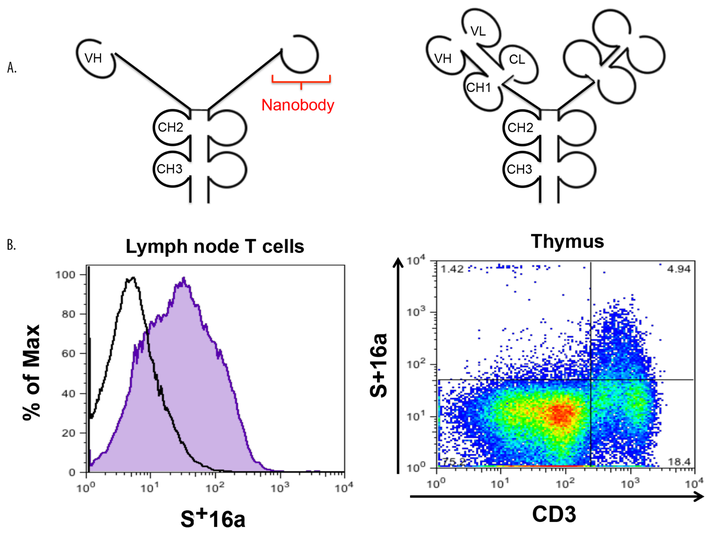
T cell purification: $CD4^+$ T cells were purified from splenocytes by magnetic separation using a CD4 negative isolation kit (Miltenyi Biotech), following the manufacturer's instructions.

Cell migration assay: $CD4^+$ T cells were suspended in RPMI 1640 plus 0.2% BSA at 5 x 10^6 cells/ml. Cell motility was quantified using transwell insert plates. The lower chambers were filled with 600 ul of the indicated chemokine and the upper chamber was loaded 100 µl of the cell suspension. The plates were incubated at 37°C for two hours and the migrated cells were collected and counted using a FACS Calibur flow cytometer (BD Biosciences).

Flow cytometric analysis: Cell were stained with the following monoclonal antibodies: CD3 (clone 17A2), CD4 (clone GK1.5), CD25 (clone PC61), CD27 (clone LG.3A10),CD62L (clone MEL-14), and Annexin V, all from BioLegend, and the samples were analyzed using a LSRFortessa flow cytometer (BD Biosciences). Data was analyzed using FlowJo (Tree Star Inc). Percentage of cell subsets is indicated within the quadrant or next to the gate. Results are representative of 3 different experiments.

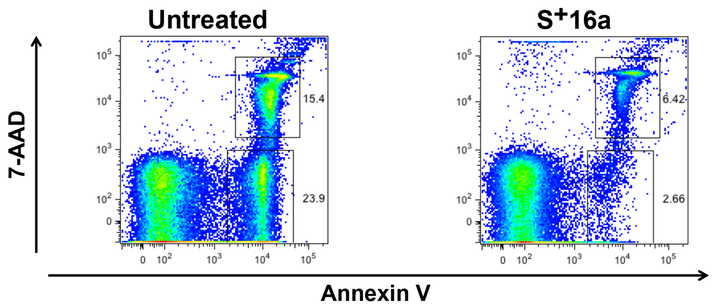
Results

Figure 1. Nanobody S^+16a recognizes ART2 on T cells



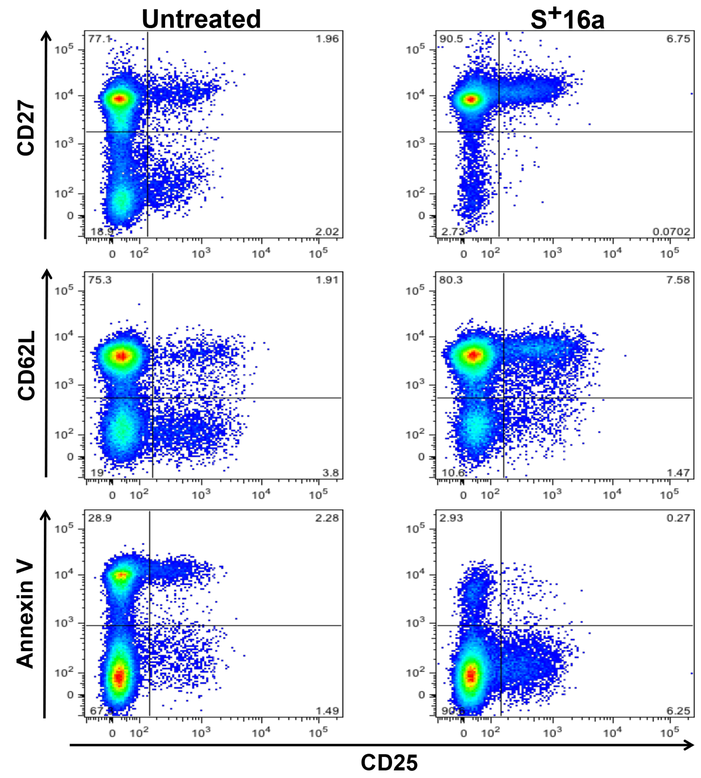
A) Diagram showing the structure of llama IgG3 (left). Note the absence of light chain, CH1 domain and the long hinge region compared with a conventional IgG (right). The VH domain is the minimal unit with Ag-binding activity and is named "Nanobody". B) A chimeric protein consisting of nanobody S^+16a fused to the constant domains of mouse IgG2c, was used to stain T cells from a Balb/c mouse. Lymph node was gated on $CD3^+$ cells, open histogram, isotype control.

Figure 2. Administration of Nanobody S^+16a blocks ART2-mediated apoptosis derived from cell preparation



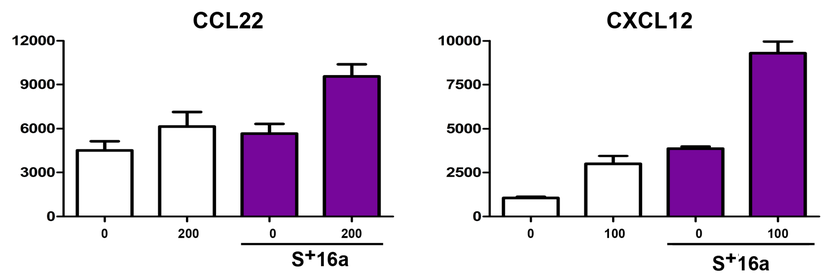
Purified $CD4^+$ T cells from a mouse treated with S^+16a (right) or PBS (left) were incubated 30 min at 37°C and then stained with Annexin V and 7-AAD. We found a marked reduction on apoptosis (7-AAD^-, Annexin V^+), on cells from the mouse treated with the nanobody (2.7%) vs the untreated preparation (23.9%). Moreover, the lower percentage of apoptosis was also reflected in a lower percentage of necrotic cells (7-AAD^+, Annexin V^+)

Figure 3. Nanobody S^+16a protects the phenotype and viability of Treg from the effects of NAD^+



ART2 is highly expressed on Tregs ($CD25^+$ $CD4^+$ T cells). The cells from the control mouse (left column) showed a high susceptibility to NICD (Annexin V) and shedding of CD27 and CD62L. In contrast, the cells from the mouse treated with S^+16a (right column) retained their phenotype and viability. Data shown gated on $CD4^+$ $CD3^+$ lymphocytes.

Figure 4. The protective activity of Nanobody S^+16a increased the number of $CD4^+$ T cells migrating toward chemoattractants



To test the effect of S^+16a on the migration of $CD4^+$ T cells, we assayed two different chemokines selected by the expression of receptors on Tregs. In both cases, we found a significant increase in the number of chemokine-responsive cells derived from the mice treated with S^+16a (filled bars) compared with the control mice (open bars).

DISCUSSION

We have shown that intravital injection of the Nanobody S^+16a, by blocking the enzymatic activity of ART2, protects Tregs and mature T cells from detrimental effects of NAD^+ released from dead cells during their preparation. This protection was reflected by i) a reduction of apoptotic cells and ii) a higher number of T cells responsive to the chemotactic action of CXCL12 and CCL22 as compared with the control populations.

Our results confirm and expand previous reports about the ability of S^+16a to confer protection on mature T cells, Tregs and iNKT cells (another cell type with a high expression of ART2 and P2X7) without detriment to their biological functions.

S^+16a is a novel tool that specifically and reversibly blocks ART2, and is useful in protecting Tregs and iNKTs from NICD during cell preparation.