Abstract
Release of NAD⁺ during preparation of murine lymphocytes causes enzymatic ADP-ribosylation of cell-surface proteins on T cells, catalyzed by toxin-related ecto-ADP-ribosyltransferase, ARTC2. ADP-ribosylation activates the cytolitic P2X7 ion channel and affects, in particular, the vitality and function of Tregs and NKT cells. Here, we describe a simple method— injection of an ARTC2-blocking nanobody—to greatly improve Treg and NKT cell vitality and to preserve their function during in vitro assays and in adoptive-transfer experiments. Moreover, we present a method for the sorting of functional, primary NKT cells, based on coexpression of ARTC2 and NK1.1. Our results pave the way for the efficient ex vivo proliferation of Tregs and NKT cells and for new experimental and therapeutic uses of these important regulatory cells.

Introduction
The recovery of functional Treg subsets from lymphatic tissues is notoriously difficult. Impaired function of freshly prepared Tregs (CD4⁺CD25⁺foxp3⁺ Tregs) and NKT cells (CD3⁺NK1.1⁺ NKT cells) can be attributed to enzymatic, NAD⁺-dependent ADP-ribosylation of cell-surface proteins in response to NAD⁺ released from cells during isolation [1, 2]. Treg subsets express high levels of the toxin-related ARTC2 cell-surface enzyme and its major target, the cytolitic P2X7 ion channel [2, 3]. ADP-ribosylation of Arg125 induces gating of P2X7, causing influx of calcium and efflux of potassium ions (Fig. 1A) [4]. This activates a cascade of downstream effects, resulting in shedding of the protease-sensitive ecto-domains of CD27 and CD62L, externalization of phosphatidylserine, uptake of the DNA-binding dye PI, and ultimately, cell death [5–8]. Preparation of cells from lymphatic organs is routinely carried out on ice (4°C) in an attempt to preserve the functional vitality of the isolated cells. Yet, ARTC2 is highly active at 4°C, and ADP-ribosylates cell-surface proteins even when cells are prepared on ice. Activation of P2X7, in contrast, requires a temperature range of 22°C–37°C [1]. Thus, the phenotypic and functional changes induced by ADP-ribosylation of P2X7 become visible only when cells are returned to 37°C, e.g., in vitro for functional assays or in vivo for adoptive-transfer studies.

Nanobodies are the smallest antigen-binding domains derived from heavy-chain antibodies naturally occurring in llamas [9–11]. Their propensity to bind to functional crevices on proteins accounts for their capacity to act as potent enzyme inhibitors [12]. Their small size (15 kD) accounts for their excellent tissue penetration in vivo [13]. Here, we present a simple nanobody-based approach that prevents ADP-ribosylation of cell-surface proteins during preparation of primary T cells. We show that injecting 50 µg of an ARTC2-blocking nanobody i.v. into mice, 15 min prior to death, greatly improves the recovery of functional Tregs and NKT cells from spleen and liver, respectively.

Materials and Methods

Mice
ARTC2−/− mice [14] and P2X7−/− mice [15] (kindly provided by Chris Gabel, Pfizer, Ann Arbor, MI, USA) were backcrossed onto the C57BL/6J background for at least 12 generations. The diphtheria toxin receptor-GFP transgene from DEREG mice [16] (kindly provided by Tim Sparwasser, TWINCORE, Hannover, Germany) was backcrossed onto ARTC2−/− and P2X7−/− mice, as described previously [2]. Mice were kept at the animal facility of the University Medical Center (UKE), and all experiments were performed with approval of the responsible regulatory committee.

Production and purification of nanobody s+16a
Nanobody s+16a was selected from a phage-display library, generated from a llama, immunized with a cDNA expression vector for ARTC2, as...
described previously [17]. The coding region of sH11001 16a was subcloned into the pCSE2.5 vector (kindly provided by Thomas Schirrmann, Technische Universität Braunschweig, Germany). Supernatants were harvested 5 days after transfection of this construct into HEK-6E cells (kindly provided by Ives Durocher, NRC, Ottawa, Ontario, Canada). His6x-tagged nanobodies were affinity-purified by immobilized metal-affinity chromatography on nickel-nitrilotriacetic acid columns (Sigma, St. Louis, MO, USA). Buffer was exchanged by gel filtration, and nanobodies were adjusted to a concentration of 1 mg/ml in PBS and stored at 4°C.

Nanobody injections
Purified nanobodies (50 µg/200 µl sterile saline) were injected i.v. using a 27-G needle and a 1-ml tuberculine syringe (Omnifix; B. Braun, Melsungen, Germany). Two common methods for i.v. injection were found to work equally well. For injection into the tail vein, mice were placed under an infrared lamp for 3 min and then transferred to a fixation tube. For retro-orbital injections [18], mice were anesthetized briefly with isofluorane. Mice were killed by cervical dislocation, 15 min after nanobody injections.

Cell preparation
Single-cell suspensions were prepared from heparinized blood, spleen, pLNs, and liver. All preparation procedures were performed on ice. Blood was centrifuged, subjected to erythrocyte lysis (ACK lysis buffer, 155 mM NH4Cl, 10 mM KHCO3, 100 mM EDTA, pH 7.2), and washed once in PBS. Spleens and pLNs were gently minced through a Nytex membrane. Cells were filtered through a 70-µm cell strainer (Falcon) and washed once in PBS supplemented with 1% FCS. Spleen cells were resuspended in 5 mL ACK lysis buffer, incubated for 5 min on ice, centrifuged, and washed once in PBS containing 1% FCS. Liver lobes were gently mashed through a metal sieve using a syringe piston. Purification of liver leukocytes was achieved by running a Percoll gradient using Easycoll separation medium (Biochrom, Merck Millipore, Berlin, Germany). To this end, cells were resuspended in 5 mL 40% Percoll/RPMI, layered onto 3 mL 66% Percoll/RPMI in a 15-mL Falcon tube, and centrifuged at 1600 rpm, 16°C, for 20 min without breaks. The interphase was collected, and cells were washed once in PBS supplemented with 1% FCS. Cells were resuspended in 2 mL ACK lysis buffer for 2 min to lyse erythrocytes. The tube was filled with 10 mL PBS containing 1% FCS, and cells were centrifuged at 1600 rpm for 5 min. Cells were washed once in PBS, supplemented with 1% FCS, and resuspended in RPMI medium (Invitrogen, Carlsbad, CA, USA), containing 1% L-glutamine (Invitrogen), 1% HEPES (Invitrogen), 1% Na-pyruvate (Invitrogen), 0.1 mM -2-mercaptoethanol (Invitrogen), and 10% FCS.

Flow cytometry and FACS
Cells were analyzed using BD FACSComp II and BD Fortessa flow cytometers, following staining with fluorochrome-conjugated mAb directed against CD4 (Clone RM4-5; BioLegend, San Diego, CA, USA), CD8a (Clone 53-6.7; BioLegend), CD25 (Clone PC61.5; BioLegend), CD3 (Clone 145-2C11; BioLegend), NK1.1 (Clone PK136; BioLegend), and P2X7 (Clone P2X7.7) mice do not shed CD27 upon incubation at 37°C (sp; n=3 individual mice). (D) Splenic Tregs (CD4+GFP+) and liver NKT cells (CD3+NK1.1+) were sorted by FACS at 4°C. Incubation of purified cells at 37°C results in staining by PI of a large fraction of cells. Results are representative of two or three similar experiments.

Figure 1. NAD+ affects the vitality of freshly prepared Tregs and NKT cells. (A) Extracellular NAD+ serves as a substrate for toxin-related ADP-ribosyltransferase ARTC2. ARTC2-catalyzed ADP-ribosylation of P2X7 at Arg125 results in gating of the P2X7 ion channel, shedding of the metalloprotease-sensitive ecto-domain of CD27, and cell death, visualized by staining with the DNA-binding dye PI. (B) Tregs and NKT cells show prominent expression of ARTC2 and P2X7. Single-cell suspensions were prepared from spleen and liver on ice and stained with fluorochrome-conjugated antibodies directed against CD4, CD3, CD25, NK1.1, ARTC2, and P2X7 before FACS analyses. Spleen cells were gated on CD3+ cells; Tregs are characterized by coexpression of CD4 and CD25 and NKT cells by coexpression of CD3 and NK1.1. (C) A 15-min incubation of primary cells from spleen and liver at 37°C but not at 4°C induces shedding of CD27 by the majority of GFP+ splenic Tregs and CD1d-tetramer+ liver NKT cells (all plots gated on CD4+ cells). Tregs and NKT cells from ARTC2−/− [ARTC2 knockout (ARTC2ko)] and P2X7−/− (P2X7ko) mice do not shed CD27 upon incubation at 37°C (sp; n=3 individual mice). (D) Splenic Tregs (CD4+GFP+) and liver NKT cells (CD3+NK1.1+) were sorted by FACS at 4°C. Incubation of purified cells at 37°C results in staining by PI of a large fraction of cells. Results are representative of two or three similar experiments.
USA). ARTC2 (Clone Nika109; UKE), TCRβ (Clone H57-597; BioLegend), IFN-γ (Clone XMG1.2; ebioscience), or a PE-conjugated α-GalCer-loaded CD1d tetramer (kindly provided by Dave Serreze, The Jackson Laboratory, Bar Harbor, ME, USA). Tregs were identified as CD4+CD25+ cells or when using DEREG mice, as CD4+GFp+ cells. NKTs were identified as CD3ε+ NK1.1+, CD5+CD1d-tetramer+, TCRβ+ NK1.1+, or ARTC2+ NK1.1+ cells. For flow cytometric cell sorting, spleen and LN cells from DEREG mice were depleted of B cells using sheep anti-mouse IgG Dynabeads (Invitrogen), according to the manufacturer’s instructions. Cells were then stained for 20 min with anti-IFN-γ, washed, and fixed with 2% PFA for 10 min. Cells were then stained for 20 min with anti-IFN-γ, washed, and fixed with 2% PFA for 10 min. Cells were then stained for 20 min with anti-IFN-γ, washed, and fixed with 2% PFA for 10 min. Cells were then stained for 20 min with anti-IFN-γ, washed, and fixed with 2% PFA for 10 min.

### RESULTS AND DISCUSSION

**Splenic Tregs and liver NKT cells respond to NAD⁺ released during cell preparation**

Previous studies have shown that NAD⁺ is released during cell preparation and serves as a substrate for ARTC2 that activates P2X7 by ADP-ribosylation [1]. Most splenic Tregs (CD4+CD25+) and liver NKT cells (CD3ε+NK1.1+) express high levels of ARTC2 and P2X7 (Fig. 1B). In contrast, thymic Tregs and NKT cells show little, if any, cell-surface expression of ARTC2 or P2X7 (Supplemental Fig. 1).

To assess the impact of NAD⁺ released during cell preparation on spleen Tregs and liver NKT cells, we monitored P2X7-dependent loss of CD27 from the cell surface and staining by PI of freshly prepared splenocytes and liver lymphocytes upon incubation at 4°C versus 37°C. To allow better visualization and sorting of Tregs, we used DEREG mice that express GFP under control of the foxp3 promoter for some experiments [16]. We found that the majority of splenic Tregs (CD4+GFp+) and liver NKT cells (CD4+CD1d-tetramer+) from WT mice shed CD27 within 15 min of incubation at 37°C (Fig. 1C). Analyses of cells from ARTC2−/− and P2X7−/− mice confirm that shedding of CD27 at 37°C depends on both of these proteins (Fig. 1C). Furthermore, incubation of FACS-sorted WT Tregs and NKT cells at 37°C, for up to 120 min, resulted in irreversible uptake of PI by the majority of these cells (Fig. 1D). These results are consistent with the results of an earlier report, showing a high proportion of apoptotic NKT cells obtained from livers of WT but not of ARTC2−/− or P2X7−/− mice [19].

**Injection of the ARTC2-blocking nanobody s+16a preserves the vitality of Tregs and NKT cells**

On the basis of these observations, we hypothesized that preventing ARTC2-mediated ADP-ribosylation of P2X7 during cell preparation could be a means to preserve the vitality and function of primary WT Tregs and NKT cells. Recently, we generated a nanobody, designated s+16a, from an immunized llama that specifically and effectively blocks the enzymatic activity of ARTC2 (Fig. 2A) [17]. The injection of 50 μg of this nanobody i.v. into WT mice, 15 min before death, indeed, effectively prevented shedding of CD27 (Fig. 2B) and uptake of PI (Fig. 2C) by splenic Tregs and liver NKT cells.

**Injection of the ARTC2-blocking nanobody s+16a preserves the function of splenic Tregs in vitro**

To assess how ADP-ribosylation of P2X7 during cell preparation affects the function of primary Tregs during in vitro experiments, we analyzed the suppressive function of splenic...
Tregs in a fluorescence-based T suppression assay. We cocultured Tregs with Tresp labeled with eFluor670 in the presence of a stimulatory anti-CD3 antibody and APCs and measured proliferation of Tresp (0.1 or WT mice treated with 50 μg/mL anti-CD3, 72 h) compared to mock-injected WT mice in an in vitro T suppression assay. Injection of nanobody s+16a-injected versus mock-injected WT mice revealed much weaker suppressive activity of WT Tregs compared with ARTC2−/− or P2X7−/− Tregs (Fig. 3), consistent with the notion that ARTC2-catalyzed ADP-ribosylation of P2X7 during cell preparation affects the function of Tregs. To assess whether injection of the ARTC2-blocking nanobody s+16a could preserve the function of WT Tregs in vitro, we compared Tregs derived from s+16a-injected versus mock-injected WT mice in an in vitro T suppression assay. Injection of nanobody s+16a, indeed, resulted in a markedly improved, suppressive activity of WT Tregs (Fig. 3), i.e., to a level comparable with that of ARTC2−/− or P2X7−/− Tregs. Thus, the simple injection of an ARTC2-blocking nanobody can preserve the inherent functionality of WT Tregs during ex vivo suppression assays.

Injection of the ARTC2-blocking nanobody s+16a preserves the function of liver NKT cells in vitro

To assess how ARTC2-catalyzed ADP-ribosylation of P2X7 during cell preparation affects the function of primary NKT cells during in vitro experiments, we analyzed the proliferation and IFN-γ production of liver NKT cells in response to stimulation with α-GalCer. Comparative analyses of NKT cells obtained from WT, ARTC2−/−, and P2X7−/− mice revealed much weaker suppressive activity of WT Tregs compared with ARTC2−/− or P2X7−/− Tregs (Fig. 3), consistent with the notion that ARTC2-catalyzed ADP-ribosylation of P2X7 during cell preparation affects the function of Tregs. To assess whether injection of the ARTC2-blocking nanobody s+16a could preserve proliferation of WT NKT cells in vitro. To this end, we compared α-GalCer-induced proliferation of NKT cells obtained from s+16a-treated versus mock-treated mice. Injection of nanobody s+16a, indeed, resulted in a markedly increased amount of proliferating WT NKT cells (Fig. 4A), i.e., to a level comparable with that of ARTC2−/− or P2X7−/− NKT cells.

Furthermore, we analyzed whether injection of nanobody s+16a could preserve proliferation of WT NKT cells in vitro. To this end, we compared α-GalCer-induced proliferation of NKT cells obtained from s+16a-treated versus mock-treated mice. Injection of nanobody s+16a, indeed, resulted in a markedly increased amount of proliferating WT NKT cells (Fig. 4A), i.e., to a level comparable with that of ARTC2−/− or P2X7−/− NKT cells.

To assess further the effect of nanobody treatment on the function of NKT cells, we investigated cytokine production by NKT cells in response to stimulation with α-GalCer. Intracellular staining for IFN-γ revealed a robust response of NKT cells derived from ARTC2−/− or P2X7−/− mice, as evidenced by dilution of the e-Fluor dye and an increase in the relative cell number (Fig. 4A). In contrast, NKT cells from WT mice showed some dilution of e-Fluor dye but little, if any, increase in the relative cell number. These results are consistent with the notion that ADP-ribosylation of P2X7 during cell preparation impairs the capacity of NKT cells to respond to α-GalCer.
cells (≤7% IFN-γ⁺ cells; Fig. 4B). Injection of nanobody s+16a markedly improved IFN-γ production by WT NKT cells (41% IFN-γ⁺ cells; Fig. 4B), i.e., comparable with levels seen with ARTC2⁻/⁻ or P2X7⁻/⁻ NKT cells.

Coexpression of ARTC2 and NK1.1 allows sorting of functional NKT cells from nanobody-injected mice

NKT cells express TCRs that recognize glycolipids presented by the MHC I-like CD1d cell-surface protein [20]. They can be distinguished from conventional T cells on the basis of their expression of CD3 or TCR-δ chain and NK1.1. Use of antibodies directed against the TCR complex during cell sorting, however, may affect subsequent responses to TCR triggering. We noticed that NKT cells could also be distinguished from conventional T cells by their high expression of ARTC2. FACS analysis revealed that NK1.1⁺ARTC2⁺ cells correspond to NK1.1⁺CD3⁺ T cells (Supplemental Fig. 2A).

We hypothesized that functional NKT cells could be sorted from s+16a-treated mice using an anti-ARTC2 mAb that recognizes an ARTC2 epitope, independent of s+16a binding, thereby avoiding interference with TCR components. Flow cytometric cell sorting of NK1.1⁺ARTC2⁺ cells revealed that >90% of the purified cells were CD1d-tetramer⁺ (Supplemental Fig. 2B). When combined with an injection of nanobody s+16a before death, ARTC2⁺NK1.1⁺-purified NKT cells responded with robust IFN-γ production to α-GalCer stimulation (59% IFN-γ⁺ cells), whereas NKT cells from mock-treated mice showed little if any IFN-γ response (2% IFN-γ⁺ cells; Fig. 5A).

To determine whether the mode of sorting might affect the function of NKT cells, we compared the IFN-γ response of NKT cells sorted via ARTC2 versus NKT cells sorted using a nonactivating antibody against TCR-β. In both cases, a strong IFN-γ response was seen in NKT cells from s+16a-treated mice but not from control mice (Fig. 5B).

Tregs and NKT cells from nanobody-injected mice survive better than cells from untreated WT mice upon adoptive transfer into Rag⁻/⁻ mice

In addition to in vitro assays, purified Tregs and NKT cells are commonly used in adoptive-transfer experiments for functional analyses in vivo [21–23]. Considering our findings—that simply incubating primary Tregs and NKT cells at 37°C results in shedding of cell-surface proteins and impaired vitality in vitro—we inferred that such events probably also occur in vivo upon injection of purified Tregs or NKT cells into mice. Moreover, we hypothesized that Tregs and NKT cells purified from WT animals after injection of nanobody s+16a would exhibit enhanced vitality in vivo upon adoptive transfer into mice, as they do in vitro.

To test this hypothesis for Tregs, we coinjected Tregs purified from s+16a-treated and mock-treated mice in a 1:1 ratio into Rag1⁻/⁻ mice. Each Treg population was labeled differently with a fluorescent dye and could thus, readily be distinguished in vivo. Twenty-four hours after cotransfer of Tregs, mice were killed, and Treg frequencies in spleen, pLNs, blood,
and liver were determined. The recovered Tregs contained approximately fourfold more cells from s+16a-treated mice than from mock-treated mice at each of these sites (Fig. 6A). Therefore, we conclude that a simple injection of nanobody s+16a protects Tregs from the deleterious effects of NAD+-dependent ADP-ribosylation of P2X7, thereby improving Treg functionality in adoptive-transfer experiments.

To test our hypothesis for NKT cells, we cotransferred NKT cells purified from s+16a-treated and mock-treated mice in a 1:1 ratio into Rag1−/− mice. Twenty-four hours after injection, only NKT cells from s+16a-treated mice were detectable in blood and liver (Fig. 6B). Thus, injection of s+16a provides a novel, simple protocol to enable the survival of adoptively transferred, naive NKT cells in vivo.

In summary our findings demonstrate that the functional integrity of primary murine Tregs and NKT cells can be maintained by preventing ART2-mediated ADP-ribosylation of P2X7 during cell preparation. A single injection of nanobody s+16a, 15 min before death, greatly enhances the survival and function of Tregs and NKT cells in vitro and in adoptive-transfer experiments in vivo. These findings underscore the potential of nanobody s+16a as a valuable tool to improve and facilitate research on Tregs and NKT cells and pave the way for new experimental and therapeutic approaches using these cells.


KEY WORDS: NAD+ · cell preparation · ARTC2 · regulatory T cells · natural killer T cells
Technical Advance: A new cell preparation strategy that greatly improves the yield of vital and functional Tregs and NKT cells

Björn Rissiek, Welbeck Danquah, Friedrich Haag, et al.

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