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Original Contribution

Role of IL-23 in mobilization of immunoregulatory nitric oxide- or superoxide-producing Gr-1$^+$ cells from bone marrow

Therese A. Dietlin [a], Daniel J. Cua [b], Kathleen A. Burke [a], Brett T. Lund [a], Roel C. van der Veen [a,*]

[a] Department of Neurology, University of Southern California Keck School of Medicine, Los Angeles, CA 90033, USA
[b] Department of Discovery Research, Schering–Plough Biopharma, Palo Alto, CA 94304-1104, USA

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ABSTRACT

Spleens of mice injected with heat-killed *Mycobacterium tuberculosis* increase their Gr-1$^+$ cell content and develop a system of interactive Ly-6G$^+$ and Ly-6G$^-$Gr-1$^+$ populations or "G_{reg}" subsets, which, upon stimulation by activated T cells, produce immunoregulatory superoxide (O_2^-) and nitric oxide (NO), respectively. The balance between immunosuppressive NO and its antagonist O_2^- regulates T cell expansion, similar to regulation of vasodilation. Reduction of NO levels by O_2^- is required for efficient T cell expansion and development of autoimmunity. We studied the source of Gr-1$^+$ cells in bone marrow (BM), where their levels were higher than in spleen, with both G_{reg} subsets expressing strong activity. In the spleens of primed IL-23$^{-/-}$ mice, Ly-6G$^+$ cells remained at naïve levels and produced no O_2^-. The complementary Ly-6G$^-$Gr-1$^+$ splenocytes and their suppressive activity were partially reduced. Surprisingly, Gr-1$^+$ cell levels in BM of IL-23$^{-/-}$ mice were increased, as were their O_2^- and NO production. Transfer of primed BM cells partially restored regulatory function in the spleen of IL-23$^{-/-}$ recipients. The results suggest that IL-23 is involved in mobilization of O_2^-- and NO-producing Gr-1$^+$ cells from BM, which may contribute to its widely studied role in (auto)immunity.

Nitric oxide (NO) has been identified as a mediator in several classical immunosuppressive strategies [1–4] and is an important mediator of immunosuppression in a variety of models for autoimmune disorders [5–10] and parasitic infection [11]. NO induction during T cell activation inhibits subsequent T cell expansion, which has been implicated in NO-mediated immunosuppression in vivo [2,5,12–15]. Work in this laboratory and others has indicated that NO and superoxide anion (O_2^-) cooperatively interact to regulate the inflammatory immune response by regulating the bioavailability of NO [16–18], similar to their interactive regulation of blood pressure in the vascular system [19,20]. The modulating effect of simultaneously produced O_2^- and NO on inflammation has been demonstrated ex vivo in cultures of spleen cells from mice challenged with heat-killed *Mycobacterium tuberculosis* (Mtub)[1], a widely used component of adjuvants. In vivo, the effects of endogenously produced O_2^- on NO activity and consequently on inflammation have been shown with the passive induction of experimental allergic encephalomyelitis (EAE) by adoptive transfer in NADPH oxidase-knockout mice, which are resistant to *active* EAE induction [21]. This reversal in EAE susceptibility, which depended on ex vivo antigen-specific T cell activation in the presence of an NO synthase inhibitor, demonstrates the significance of NO–O_2^- interaction for immunoregulation. Furthermore, it shows that severe EAE development requires O_2^- production for the early development of an optimal immune response during peripheral immune induction, whereas the pathogenic inflammatory effector stage proceeds unimpeded in its absence.

The sources of both free radicals are likely to be generated and/or activated as part of the innate immune response to injected bacterial products. For instance, Gr-1$^+$ cells, which arise from of a variety of insults, were widely identified as the source of suppressive NO [22–28]. We use exposure of mice to Mtub as a model to study regulatory Gr-1 cells. We determined that O_2^- and NO were produced by distinct subsets of Gr-1$^+$ myeloid cells, induced by complete Freund's adjuvant (CFA), including Mtub, but not by incomplete Freund's adjuvant without Mtub. Overall, Gr-1$^+$ cells comprise as much as 45% of the leukocyte population recovered from the spleens of mice that were immunized with CFA [26]. When stimulated by activated T cells, enriched granulocytic Ly-6G-expressing cells uniquely produce O_2^-. These cells resemble immature granulocytes predominantly, with only a small number of mature neutrophils. O_2^- produced by these Ly-6G$^+$ cells interacts with NO, induced simultaneously in the complementary Ly-6G-depleted monocytic Gr-1-expressing subset [29]. Its interaction with O_2^- determines the bioavailability of suppressive NO and modulates T cell expansion and the intensity of subsequent immune responses and autoimmunity. Gr-1-

Abbreviations: Ab, antibody; APC, allophycocyanin; BM, bone marrow; CFA, complete Freund's adjuvant; EAE, experimental autoimmune encephalitis; FITC, fluorescein isothiocyanate; G-CSF, granulocyte colony-stimulating factor; iNOS, inducible nitric oxide synthase; L-NMA, *N*-monomethyl-L-arginine; mAb, monoclonal Ab; Mtub, *Mycobacterium tuberculosis*;NBT, nitroblue tetrazolium; SOD, superoxide dismutase; sfu, superoxide-forming unit; WT, wild type.

* Corresponding author. Fax: +1 323 442 1064.
E-mail address: vanderve@usc.edu (R.C. van der Veen).

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expressing cells in naïve spleen are present in low numbers and do not produce either free radical. We named the system of interactive Gr-1 subsets G_{reg} to distinguish these regulatory Gr-1 cells from nonregulatory Gr-1 cells, such as those present in the spleens of naïve mice or in draining lymph nodes [29]. We defined G_{reg} as a system of distinct innate Gr-1$^+$ myeloid subsets, capable of producing interactive NO or O_2^- upon stimulation by activated T cells, resulting in the regulation of subsequent T cell expansion [29].

IL-23, which has recently been studied intensely for its role in autoimmunity [30], is required for granulocyte maturation and neutrophil development, probably through the involvement of G-CSF [31]. Therefore, we reasoned that mice lacking IL-23 may have a deficit in Ly-6G-expressing cells and thus O_2^- production, possibly contributing to an overall immunosuppressive phenotype due to high NO levels. Indeed, splenocytes from CFA-primed mice lacking IL-23 have few if any Ly-6G-expressing cells and produce no detectable levels of O_2^- after T cell activation. Unexpectedly, they also have reduced capacity to produce NO and inhibit proliferation only modestly. Surprisingly, and in sharp contrast to the reduced levels found in their spleens, BM from IL-23$^{-/-}$ animals contained numbers of cells in both the Ly-6G$^+$ and the Ly-6G$^-$ Gr-1-expressing subsets equal to or higher than wild-type (WT) BM and had functional regulatory capacity generally exceeding that in WT mice. Transfer of BM cells resulted in increased Gr-1 levels in the spleen, demonstrating that mobilization or release from the BM is deficient in IL-23$^{-/-}$ mice. In conclusion, these studies suggest that G_{reg} develop and expand in BM and migrate to the spleen where they are immunoregulatory. They implicate IL-23 as an important agent for the mobilization of Gr-1$^+$ cells from the BM, because Gr-1 cell levels remained unchanged in spleens of primed IL-23$^{-/-}$ mice, but increased in their BM to the same or higher levels compared to primed WT mice.

Materials and methods

Mice and reagents

C57BL/6 mice were obtained from Harlan (Indianapolis, IN, USA). IL-23p19$^{-/-}$ mice were generated at DNAX Research, now part of Schering–Plough. DO11.10 ovalbumin (OVA)$^{323\text{-}339}$ T cell receptor (TCR) transgenic mice were originally obtained from Dr. A. O'Garra, DNAX Corp. (Palo Alto, CA, USA) and maintained in local facilities. All experiments were approved by the Institutional Animal Care and Use Committee. Peptide OVA$^{323\text{-}339}$ was synthesized by the Microchemical Core Facility at the University of Southern California Comprehensive Cancer Center. *N*-monomethyl-L-arginine (L-NMA) was purchased from Alexis (San Diego, CA, USA). All other reagents were from Sigma (St. Louis, MO, USA), unless indicated.

Gr-1$^+$ cell induction and enrichment

These procedures were described before [26]. Regulatory cells were induced in mice by subcutaneous immunization at two or three dorsal sites with 0.2 ml emulsion of buffered saline in Freund's adjuvant, supplemented with 500 μg *M. tuberculosis* H37RA (Difco, Detroit, MI, USA). After 6–8 days, Gr-1$^+$ cells were enriched from single-cell suspensions of splenocytes and from BM cells flushed from both femurs. Magnetic beads coated with RB6-8C5 mAb (IMag, BD PharMingen, San Diego, CA, USA) were used for the enrichment, according to the manufacturer's instructions. Positively selected populations were 95–98% pure, as determined by flow cytometry using allophycocyanin (APC)-labeled RB6-8C5 mAb.

Functional analysis of regulatory Gr-1$^+$ cell activity in proliferation assays

To analyze suppressive NO production by regulatory myeloid cells, we routinely use a coculture system consisting of regulatory cells and splenic T cells from OVA$^{323\text{-}339}$ TCR transgenic DO11.10 mice. Upon activation with OVA$^{323\text{-}339}$, the T cells in turn stimulate the cocultured Gr-1$^+$ cells to produce NO or O_2^-. NO mediates the suppression by myeloid cells [29]. Thus, Gr-1$^+$-enriched cell populations (0.5×10^6 cells/ml) purified from CFA-primed donors' splenocytes or BM were cultured in triplicate with spleen cells from naïve DO11.10 mice (0.6×10^6 cells/ml) in standard 96-well plates at 0.2 ml/well at 37 °C. Before enrichment, splenic red blood cells were removed using Sigma's red blood cell lysing buffer, as directed. After 48 h, cultures were pulsed with tritiated thymidine and incubated for an additional 16 h, followed by analysis of tritium incorporation in individual wells. The extent of subsequent proliferation of the activated T cells is an indicator of G_{reg} activity, because the naïve transgenic spleen cells cannot produce NO or O_2^- themselves [26]. This heterogeneous culture system does not induce significant mixed lymphocyte reactions, because T cell proliferation is minimal in the absence of OVA, even in the presence of an NO synthase (NOS) inhibitor [26]. Furthermore, it restricts antigen (Ag)-specific T cell activation to the allogeneic DO11.10 splenocytes and enables us to limit the analysis of Gr-1$^+$ cell populations strictly to their regulatory activity, without confounding antigen presentation activity by the regulatory population. This technique is used primarily to analyze suppressive G_{reg} activity, which is defined as immunoregulatory NO activity induced by activated T cells.

To determine maximal proliferation, NO production was inhibited by the addition of L-NMA (0.15 mM) after 20 h of culture. To determine maximal NO-dependent inhibition, O_2^- was inactivated by addition of SOD (100 U/ml), also after 20 h. Data are presented either in bar graphs as tritium incorporation for a single representative assay or in dot plots as the ratio of maximal inhibition to maximal proliferation, expressed as percentage inhibition for individual splenocyte or BM populations.

To enrich CD4$^+$ lymphocytes, naïve splenocytes were lysed with red cell lysing buffer and incubated with magnetic beads coated with anti-CD4 antibody (BD Pharmingen), according to the recommended protocol. The enriched, washed cells (4×10^5 cells/ml) were activated in wells coated with 4 μg/ml anti-CD3ε antibody for 4 h at room temperature. Before use, the wells were washed twice with PBS. Total BM cells were used at 5×10^5 cells/ml.

Culture medium consisted of RPMI 1640 supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 1 mM sodium pyruvate, 0.05 μM 2-mercaptoethanol, penicillin (100 U/ml), streptomycin (100 U/ml), and gentamycin (10 μg/ml), all from Life Technologies (Gaithersburg, MD, USA), and 10 mM Hepes (Sigma).

Measurement of nitrite

Parallel cultures under conditions identical to proliferation assays were incubated in duplicate with and without OVA and supernatants taken for assay after 64 h. Nitrite was measured in 1:1 mixtures of culture supernatant and Griess reagent (Alexis Biochemicals). A standard curve with $NaNO_2$ was used to determine concentration.

Quantification of O_2^--producing cells

To analyze O_2^--producing cells, parallel cultures under conditions identical to proliferation assays in the presence of L-NMA described above were incubated in 96-well Multiscreen-HA plates (Millipore, Watertown, MA, USA) in triplicate in the presence or absence of Ag for 23 h, as described [21]. Nitroblue tetrazolium (NBT) (12.5 μg/ml; KPL, Gaithersburg, MD, USA) was added and incubation continued for 1 h, after which the wells were washed with PBS and dried. Spots were quantified on a KS ELISPOT Assay System (Zeiss, Thornwood, NY, USA) with parameters set to include spots of 15–100 μm and varying from 60 to 100% circular shape. An increase in the number and total area of spots in the presence of antigen was considered a measure of Ag-dependent increase in O_2^- production. Results are presented as