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Neutrophils Confer T Cell Resistance to Myeloid-Derived Suppressor Cell–Mediated Suppression To Promote Chronic Inflammation

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Low-grade chronic inflammation can persist in aging humans unnoticed for years or even decades, inflicting continuous damage that can culminate later in life as organ dysfunction, physical frailty, and some of the most prominent debilitating and deadly age-associated diseases, including rheumatoid arthritis, diabetes, heart disease, and cancer. Despite the near universal acceptance of these associations, the mechanisms underlying unresolved inflammation remain poorly understood. In this study, we describe a novel inducible method to examine systemic chronic inflammation using susceptible animal models. Induced inflammation results in unresolved innate cellular responses and persistence of the same serum proinflammatory molecules used as diagnostic biomarkers and therapeutic targets for chronic inflammation in humans. Surprisingly, we found long-term persistence of an inflammation-associated neutrophil cell population constitutively producing the proinflammatory IFN-γ cytokine, which until now has only been detected transiently in acute inflammatory responses. Interestingly, these cells appear to confer T cell resistance to the otherwise potent anti-inflammatory function of myeloid-derived suppressor cells, revealing a novel mechanism for the maintenance of chronic inflammatory responses over time. This discovery represents an attractive target to resolve inflammation and prevent the inflammation-induced pathologies that are of critical concern for the well-being of the aging population. The Journal of Immunology, 2013, 190: 5037–5047.

The primary role of the inflammatory response is to protect the host from harmful insults such as infectious pathogens. Inflammation is mediated early by innate immune responses, which are followed later by adaptive responses, and can be further defined as acute or chronic. Acute inflammation involves an initial insult that triggers a cascade of soluble immune mediators, cell expansion, and cellular trafficking, which together clear the offending agent. This is followed by a contraction phase in which the system returns to homeostatic levels. Alternatively, chronic inflammation is characterized as a long-term immune response that evolves because of continuous stimulation and/or a dysregulated immune system and that continues to persist long after the stimulus is cleared. Low-grade chronic inflammation can continue unnoticed in humans for years or even decades, inflicting continuous damage that can culminate later in life as organ dysfunction, physical frailty, and some of the most prominent debilitating and deadly age-associated diseases, including rheumatoid arthritis, diabetes, heart disease, and cancer (1–3). Understanding the dysregulated immune system during chronic inflammation and thus identifying targets to resolve the response is of increasing interest for treatment of inflammatory disorders and prevention of pathological complications.

Development of chronic inflammation is commonly associated with the aging process and has been linked to both genetic and environmental risk factors (4–6); however, the mechanisms that perpetuate established chronic response remain unclear. Persistent innate immune activity beyond the acute phase suggests its potential role in the dysregulated response (7, 8). The innate immune system responds rapidly to pathologic insults, typically led by the recruitment and activation of polymorphonuclear neutrophils. Although a critical component of host protection, neutrophil activity must be tightly controlled to limit collateral tissue damage. This is evident in inflammatory diseases such as chronic obstructive pulmonary disease and rheumatoid arthritis where the innate neutrophil response persists at elevated levels and leads to significant tissue damage and organ dysfunction (5, 7). To counterbalance activation of the innate immune system, there are multiple mechanisms that can control the response. Myeloid-derived suppressor cells (MDSCs) are an innate cell population with strong immunosuppressive activity. Unlike the well-studied adaptive cell mediators of inflammation, regulatory T cells, the anti-inflammatory role of MDSCs is much less clear. MDSCs are commonly studied in cancer, where, like regulatory T cells (9), their function can be exploited as a tumor-induced immunoevasion mechanism to suppress antitumor T cell responses and innate immunity (10). MDSC expansion is seen in response to multiple infectious and noninfectious immune stimulants (11); however, their continued presence during chronic inflammation (12) suggests that MDSC function may be compromised in the dysfunctional immune response.

Two important molecular mediators associated with inflammation are IL-10 and reactive oxygen species (ROS). The anti-
inflammatory role for IL-10 has been clearly demonstrated using IL-10–deficient mice (IL-10−/−), which are susceptible to a numerous local and systemic inflammatory conditions (13–15). Furthermore, human genetic polymorphisms linked to decreased IL-10 activity are associated with chronic inflammation and age-associated inflammatory diseases (16–18), and conversely, enhanced IL-10 activity is positively associated with increased human longevity (19). Although critical for antimicrobial defense, human and animal studies have indicated that NADPH oxidase–produced ROS also play an independent role in regulating inflammation (20–22). This dual role was originally observed in patients with chronic granulomatous disease (CGD), a condition caused by genetic mutations in one of the essential subunits of the phagocyte NADPH oxidase complex (i.e., p47phox, gp91phox [NOX2], p22phox, and p67phox), the most common of which affects NOX2 (23). Interestingly, in addition to problems controlling microbial infections (24), CGD patients frequently present with noninfectious inflammatory phenomena including granuloma and abscess formation, Crohn’s-like disease, and pulmonary fibroses (25, 26).

In our present study, we define a novel inducible model of systemic chronic inflammation in NOX2−/− mice that is independent of infectious pathogens (i.e., sterile) and similar to the inflammatory response induced in IL-10−/− mice. Sterile inflammation was induced using an established method (27), which in wild-type (WT) mice generated an acute (∼1 wk) inflammatory response characterized by transient peripheral expansion of innate Gr1+ cells and short-term development of peritoneal abscesses. However, in susceptible mice (i.e., IL-10−/− and NOX2−/−), we found a common hyperacute immune response to a single inflammatory challenge, which developed into a chronic (∼3 wk) condition characterized by sustained cellular and molecular innate responses and long-term persistence of peritoneal abscesses. Further characterization of NOX2−/− mice showed inflammation–induced expansion of inflammation–associated Gr1high (Ly6G+) neutrophils producing IFN-γ and a Gr1low (Ly6G−) MDSC subpopulation during the chronic inflammatory response with enhanced inducible NO synthase (iNOS)–dependent suppressive activity. Surprisingly, we found that in the chronic inflammatory environment, the Gr1high neutrophils confer T cell resistance to MDSC-mediated suppression, thereby preventing resolution of the response. This novel proinflammatory mechanism by which neutrophils actively influence dysregulated adaptive T cell responses reveals a mechanism for the maintenance of chronic inflammation and thus represents an attractive target for the prevention of age-associated inflammatory complications.

Materials and Methods

**Animals and common reagents**

WT (C57BL/6j; stock 000664), NOX2−/− (B6.129S6-Cybmm10tm4J/J, stock 002365), IL−10−/− (B6.129P2-I10tmC5J/J, stock 002251), and OTII (B6. Cg-Tg[Tcr(Tcrβ)425Cbn/J; stock 004194) breeders were purchased from The Jackson Laboratory. NOX2−/− and IL−10−/− mice were backcrossed to the C57BL/6j genetic background 13 generations prior to purchase (The Jackson Laboratory). Animal colonies were maintained in a specific environment, the Gr1high neutrophils confer T cell resistance to MDSC-mediated suppression, thereby preventing resolution of the response. This novel proinflammatory mechanism by which neutrophils actively influence dysregulated adaptive T cell responses reveals a mechanism for the maintenance of chronic inflammation and thus represents an attractive target for the prevention of age-associated inflammatory complications.

**Inflammatory challenge and abscess detection**

The inflammatory immune response was induced as described previously (27). Briefly, a 1:4 dilution of sterile cecal contents and 50 μg polysaccharide A (PSA) in PBS was delivered i.p. in a final volume of 200 μL. At indicated time points, mice were scored for the presence of intra-abdominal abscesses as determined by number and size (average diameter). Serum and lymphoid organs were also collected at these time points for further analysis.

**Cell analysis**

Isolated cells were surface labeled with Abs in staining buffer (PBS [pH 7.2], 3% FBS, and 0.05% sodium azide) for 30 min on ice before analysis on an Accuri C6 flow cytometer (BD Biosciences). For cytokine detection, cells were incubated for 5–6 h at 37°C in media supplemented with brefeldin A (eBioscience), followed by surface labeling and intracellular detection of cytokine using Fixation/Permeabilization reagents (eBioscience). Analyses of FACS data were performed using FCS Express (De Novo Software). The Abs used in this study were purchased from BioLegend or eBioscience to detect CD3 (clone 145-2C11), Gr1 (clone RB6-8C5), CD19 (clone 6D5), F4/80 (clone CI:A3-1), CD11c (clone N418), Ly6G (clone 1A8), CD11b (clone M1/70), IFN-γ (clone XMG1.2), MHCI (clone M5/114.15.2), and CD4 (clone RM4-5). The phospho-Stat5 (Tyr694) Ab was purchased from Cell Signaling Technology. Annexin V staining was performed following the manufacturer’s protocol (BD Biosciences). Cytology was performed after cytopsin of sorted Ly6G+ cells using the Hema-3 differential stain kit following the manufacturer’s protocol (Fisher Scientific).

**Serum cytokine analysis**

Serum was isolated from blood using Microtainer serum separator tubes (BD Medical) and stored at −80°C. Serum cytokine concentrations were determined by ELISA (BioLegend).

**Accessory cell functional assays**

Accessory cells were derived using CD90.2 magnetic microbeads (Miltenyi Biotech) to deplete T cells from splenocyte preparations. In some cases, the accessory cell populations were further depleted using Ly6G magnetic microbeads (Miltenyi Biotech). CD4 T cells were isolated from OTII mice splenocytes by positive selection (Miltenyi Biotech). A sample of each accessory cell population was analyzed by flow cytometry to determine the respective percent MHCI+ cells to quantify APCs. The concentration of each accessory cell population was adjusted so that an equal 1 × 106 MHCI+ cells were present in each 96-well, along with a constant 3 × 104 OTI CD4+ T cells/well and either OVA Ag, plate-bound Abs to CD3 (clone 145-2C11; eBioscience) and CD28 (clone 37.51; eBioscience) or media alone. After 3-d incubation at 37°C, cell culture supernatants were collected for cytokine analysis by ELISA (BioLegend), Cell proliferation was measured by adding [3H]thymidine for the final 12 h of the activation assay.

Ly6G+ and Ly6G− cell functional assays

Isolation of cell populations was done using magnetic microbead selection following the manufacturer’s protocol (Miltenyi Biotech). For isolation of Gr1+CD11b+ subpopulations based on Ly6G expression, splenocytes were first depleted of MHCI+ cells. The remaining MHCI− cells were positively selected for Ly6G to isolate the Ly6G+ cell population. The remaining MHCI− Ly6G+ cells were then positively selected for CD11b+, which isolated the Ly6G+CD11b+ cell population. Sorted cell populations were analyzed by flow cytometry to confirm purity. Ly6G+ or Ly6G− cells were cultured at varying ratios with 1 × 106 OTI CD4+ T cells/well and either OVA Ag, plate-bound Abs to CD3 (clone 145-2C11; eBioscience) and CD28 (clone 37.51; eBioscience) or media alone. After 3-d incubation at 37°C, cell culture supernatants were collected for cytokine analysis by ELISA (BioLegend) to determine cytokine concentrations or by Griess reagent kit (Molecular Probes) to determine nitrite levels. Cell proliferation was measured by adding [3H]thymidine for the final 12 h of the activation assay.

**In vivo T cell functional assays**

On day −1, CD4 T cells were isolated from OTII mice splenocytes using magnetic microbead positive selection (Miltenyi Biotech). Cells were then labeled for 5 min at 37°C with 5 μM CFSE dye (Molecular Probes). WT-recipients mice each received 2 × 106 labeled cells i.v. in 200 μL PBS. On day 0, mice received an additional i.v. cell transfer of 5 × 106 Ly6G+ cells and/or 2 × 106 Ly6G− cells, followed by intranasal delivery of 100 μg OVA protein (Sigma-Aldrich) in 20 μL PBS or PBS alone. For intranasal delivery, mice were anesthetized with 3% isoflurane (Webster Veterinary) using a Laboratory Animal Anesthesia System (VetEquip). Lung-draining
response to inflammatory challenge were calculated using a Student T test. (proliferation index = 1/[MFI of experimental group/MFI of average PBS alone control]).

**Lung inflammation model**

On day −1, 2 × 10⁶ OTII CD4 T cells were transferred i.v. to recipient mice in 200 μl PBS. On day 0, some mice received an additional i.v. cell transfer of 5 × 10⁶ Ly6G+ cells and/or 2 × 10⁶ Ly6c− cells, followed by intranasal delivery of 40 μg OVA in 20 μl PBS or PBS alone. Mice received similar doses of OVA or PBS alone each day for a total of 6 d. For intranasal delivery, mice were anesthetized with 3% isoflurane (Webster Veterinary) using a Laboratory Animal Anesthesia System (VetEquip). On day 6, mice were anesthetized using i.p. injection of a ketamine (102 mg/kg), xylazine (20 mg/kg), and acepromazine (3.5 mg/kg) mixture, followed by exsanguination. Bronchoalveolar lavages were performed using 3 × 1 ml washes with PBS containing 0.6 mM EDTA. Cell differentials on lavage samples were performed using a HEMAVET analyzer (Drew Scientific). For tissue histology, lungs were inflated with OCT (Tissue-Tek) and preserved in 10% buffered formalin (Fisher Scientific). Paraffin embed-

**Results**

**Mice susceptible to inflammatory disease develop a chronic immune response to sterile inflammatory challenge**

Using two relevant mouse strains that are susceptible to inflammatory disease, NOX2−/− (28) and IL-10−/− (29), we applied an established method known to induce acute sterile inflammation in healthy WT mice (27). The inflammatory challenge consisted of a single i.p. delivery of sterilized cecal contents and purified PSA from the common intestinal commensal *Bacteroides fragilis*. The WT response to this sterile inflammation challenge is measured by the formation of peritoneal abscess(s) at 7 d (Fig. 1A). Within 2 wk, abscesses resolve and are no longer detectable in WT mice (Fig. 1A). The inflammatory abscess response is a common complication found in many inflammatory diseases. As we have shown previously (30), NOX2−/− mice produce a more exaggerated acute response detected by increased abscess numbers in the peritoneum (Fig. 1A). Remarkably, abscesses persisted for at least 3 wk after the initial insult in NOX2−/− mice (Fig. 1A) and IL-10−/− mice (Supplemental Fig. 1A). Rather than the transient increase in splenic Gr1+ cell numbers that peaks around day three in WT mice and returns to baseline within 1 wk (Fig. 1B), the expansion of Gr1+ cells in both NOX2−/− (Fig. 1B) and IL-10−/− (Supplemental Fig. 1B) mice continues to increase throughout the first week and is maintained at elevated levels at least 3 wk after the initial challenge. Increases were seen in both Gr1highCD11b+ and Gr1lowCD11b+ myeloid cell subsets (Supplemental Fig. 2), phenotypes used to differentiate granulocytic and monocytic cell lineages, respectively (31). Furthermore, levels of proinflammatory cytokines IL-1β and IL-6 remained elevated in the serum at 2 and 3 wk, indicating systemic inflammation and establishment of sterile chronic inflammation (Fig. 1C, Supplemental Fig. 1C), a phenomenon that has yet to be described in NOX2−/− model mice.

**Inflammation-induced expansion of multiple myeloid lineages in NOX2−/− mice includes long-term persistence of IFN-γ-producing neutrophils**

We first examined the 1-wk time point, which appeared to be a transition phase where the response in WT mice is resolving and the hyperacute response in NOX2−/− and IL-10−/− mice plateaus and becomes chronic. Focusing on the relatively understudied NOX2−/− mouse model, we found inflammation-induced expansion in multiple myeloid lineages, including macrophages (F4/80+) and dendritic cells (CD11c+), in addition to neutrophils/monocytes (Gr1+) (Fig. 2A); however, the number of T (CD3+) and B (CD19+) lymphocytes remained similar to nonchallenged NOX2−/− mice (Fig. 2A). Alternatively, there was no difference in cell lineage

**FIGURE 1.** Mice susceptible to inflammatory disease develop a chronic immune response to sterile inflammatory challenge. Inflammation was induced in groups of NOX2−/− and WT mice by i.p. challenge with sterile cecal contents/PSA. (A) The presence of peritoneal abscesses was assessed prior to inflammatory challenge (time 0) or 1–3 wk after challenge. Data points represent the average diameter of individual abscesses. Above each time point is the average number of abscesses detected per mouse (n = 5). (B) As a measure of the innate immune response, total numbers of Gr1+ cells in the spleen were enumerated at time 0, 3 d, 1 wk, 2 wk, or 3 wk following inflammatory challenge (n > 4/time point). Statistical comparisons to WT mice were made using a Student t test. (C) Serum levels of the inflammatory cytokines IL-1β and IL-6 were determined by ELISA at time 0 (n = 4–6), 2 wk (n = 5–8), and 3 wk (n = 5–8) postinflammatory challenge. Results are cumulative from at least three independent experiments. Statistical increases in serum cytokine levels in response to inflammatory challenge were calculated using a Student t test (*p < 0.05, **p < 0.01).
numbers between WT challenged and nonchallenged mice 1 wk after administering the inflammatory insult (Fig. 2B). In addition to myeloid lineage expansion, we found that splenocytes from NOX2−/− mice were actively secreting IFN-γ (Fig. 2C), a cytokine commonly associated with a proinflammatory response (32). Surprisingly, the IFN-γ was being produced by Gr1high, CD11b+, and Ly6G+ cells (Fig. 2D). Increased numbers of Gr1highIFN-γ+ cells were detected in WT and NOX2−/− mice 3 d after the primary insult; however, whereas those numbers decreased to baseline levels in WT mice within 1 wk, there was an additional increase in Gr1highIFN-γ+ cell numbers in NOX2−/− mice after 1 wk that persisted at least 3 wk after the initial challenge (Fig. 2E). A similar persistence of Gr1highIFN-γ+ cells was seen in IL-10−/− mice following inflammatory challenge (Supplemental Fig. 3). Inflammation-associated Gr1high cells were further identified as neutrophils based on the high light side scatter of the cells (Fig. 2F), indicating granularity, in addition to their ringed, polymorphonuclear characteristics (Fig. 2G).

Unresolved inflammation induces a potent T cell suppressor population

Previous studies have shown that adaptive CD4 T cell responses are involved in the development of inflammatory abscesses (reviewed in Ref. 33); thus, the long-term persistence of abscesses (Fig. 1A) suggests a dysregulated T cell response. In addition, many inflammatory disorders such as arthritis, colitis, and multiple sclerosis have implicated dysregulated T cell responses in driving disease complications (34–36). We sought to determine whether the inflammation-associated changes in the myeloid cell compartment (Fig. 2) was directly enhancing T cell responses, thus promoting chronic inflammation. Surprisingly, we found exactly the opposite. T cells were depleted from splenocytes collected from the different inflammatory or noninflammatory environments. A constant number of OVA-specific (OTII) CD4 T cells were then added back to the T cell–depleted splenocytes (“accessory cells”) to serve as independent indicators of Ag-induced T cell stimulation. We found that Ag-induced T cell proliferation was significantly suppressed in the presence of NOX2−/−-derived accessory cells as compared with WT-derived accessory cells isolated 1 wk after inflammatory challenge (Fig. 3A). To confirm the decrease in T cell proliferation was not due to an indirect effect, such as decreased Ag presentation, we also stimulated T cells directly using plate-bound CD3 and CD28 Abs. Again, T cell proliferation was significantly suppressed in the presence of NOX2−/−-derived accessory cells but not similar WT-derived accessory cells (Fig. 3A). Alternatively, T cell proliferation in the presence of accessory cells from naive mice (time 0) was comparable (Fig. 3B), suggesting that the strong suppressive
activity of the accessory cells derived from NOX2−/− mice is related to the ongoing inflammatory response.

Although T cell proliferation was significantly suppressed in the presence of accessory cells from the NOX2−/− mouse inflammatory environment, IFN-γ cytokine production by T cells was not significantly affected (Fig. 3C), indicating that the APCs derived from both mouse strains were comparable in processing and presenting Ag for T cell recognition. We have shown that Gr1+ cells can actively produce low levels of IFN-γ (Fig. 2C); however, the IFN-γ production induced by antigenic stimulation or direct TCR stimulation in these assays is attributed to T cells (Fig. 3E, 3F), rather than the Gr1+ cells (Fig. 3G, 3H). Annexin V analysis of cell viability showed increased apoptosis after CD4 T cells were stimulated with accessory cells sourced from the NOX2−/− mouse inflammatory environment (Fig. 3I). In the context of intact early T cell activation events (i.e., cytokine production) (Fig. 3C, 3F), this indicates that accessory cells may be selectively limiting the proliferative response through activation induced cell death.

**Inflammation-associated T cell suppression is mediated by enhanced monocytic MDSC activity**

Because endogenous T cells were depleted from the accessory cell populations, which would exclude regulatory T cells, we evaluated MDSCs as the source of enhanced suppressive activity. MDSCs are generally characterized as Gr1+CD11b+ and MHCII− but can be further subdivided into granulocytic (Gr1high,Ly6G+) or monocytic (Gr1low,Ly6G−) cell subsets (37). Potential MDSC populations were isolated based on differential Ly6G expression (Supplemental Fig. 4) at time 0, 1, or 2 wk following sterile inflammatory challenge. We found no difference in T cell proliferation (Fig. 4A).
suggests increased activity of monocytic MDSCs during inflammation. Results are representative of at least two independent experiments.

or nitrite levels (Fig. 4B), the latter being a measure of NO production, in the presence of Ly6G+ cells from NOX2−/− or WT mice. On a per cell basis, Ly6G− cells also did not influence T cell proliferation (Fig. 4C). Although some suppressive activity was detected by Ly6G+ cells from both NOX2−/− and WT mice isolated prior to inflammatory challenge, the lack of T cell suppression during the 1- and 2-wk time points (Fig. 4A) again indicates that these Ly6G+ cells are neutrophils (Fig. 2G), rather than granulocytic MDSCs. Alternatively, the monocytic Ly6G− MDSC population showed significant suppressive activity after inflammatory challenge in NOX2−/− mice (Fig. 4D), and are associated with elevated levels of NO production (Fig. 4E), as compared with WT mice. Two weeks after challenge (i.e., during the established chronic response), the Ly6G− MDSCs from NOX2−/− mice still possess potent suppressive activity, whereas the cells derived from WT mice are comparable to the noninflammatory environment (Fig. 4D). Even at a 4-fold lower ratio, the Ly6G− MDSCs from NOX2−/− mice are significantly more potent T cell suppressors compared with WT (Fig. 4E). Thus, although chronic inflammation develops in NOX2−/− mice, it appears not to be perpetuated by an intrinsic defect in MDSC function. The increased production of NO with Ly6G− MDSCs correlates with enhanced T cell suppression and is consistent with our previous study revealing increased NO production during inflammation in NOX2−/− mice (30) and suggests that MDSCs may be actively attempting to suppress the chronic inflammatory response in vivo.

Inflammation-associated MDSC suppressive activity is iNOS dependent

Previous studies have linked NO production as the primary mechanism used by monocytic MDSCs to mediate suppressive activity (38, 39). To confirm the causal relationship between NO and suppression, we examined MDSC suppressive activity using the iNOS inhibitor 1400W (40, 41). Directly inhibiting NO production (Fig. 5A) completely eliminated the suppressive activity of the MDSCs (Fig. 5B), thus identifying the mechanism of T cell suppression.

Neutrophils antagonize MDSC suppressive activity during chronic inflammation

Although MDSCs derived during chronic inflammation in NOX2−/− mice have enhanced immunosuppressive activity (Fig. 4D), the long-term unresolved nature of the response suggests that an unknown mechanism could be antagonizing their function in vivo. This was addressed initially by evaluating Ag-induced T cell stimulation in the presence of accessory cells (i.e., T cell–depleted splenocytes) derived from naive WT and NOX2−/− mice (time 0) or 2 wk after inflammatory challenge. Interestingly, we found that T cell suppression induced from the established chronic response (+2 wk; Fig. 5B) was much less robust than the 1-wk “transition” phase (Fig. 3A). Because of the sustained increase in inflammation-associated neutrophils (Fig. 2E), we examined whether this cell population was influencing MDSC suppressive activity. Accessory cell populations were depleted of neutrophils based on Ly6G expression. The presence of neutrophils in the

FIGURE 4. Enhanced T cell suppression is mediated by monocytic MDSCs. WT and NOX2−/− mouse splenocytes were collected at either time 0, 1, or 2 wk postinflammatory challenge and used to isolate the two potential Gr1+CD11b+ MDSC subsets: granulocytic/Ly6G+CD11b+ or monocytic/Ly6G−CD11b+ cells. OTII splenocytes were cultured with OVA alone (cont.) or OVA and a 1:1 ratio of Ly6G+ or Ly6G− cells isolated at time 0, 1, or 2 wk postinflammatory challenge. Cell proliferation (A, D) and nitrite levels (B, E) were measured after 3.5 d. Constant numbers of OTII splenocytes cultured with OVA alone (0:1) or varying ratios of Ly6G+ (C) or Ly6G− (F) cells and OVA Ag for 3.5 d. T cell proliferation by [3H]thymidine incorporation and nitrite levels is shown as a stimulation index (S.I.), normalized to the OTII alone response. Results are representative of three independent experiments.

FIGURE 5. MDSC suppression of T cell proliferation is controlled by an iNOS-mediated mechanism. Ly6G− MDSCs isolated from NOX2−/− mice at 1 wk postinflammatory challenge were cultured with OVA Ag alone (0:1) or varying ratios Ly6G− MDSCs and OVA for 3.5 d in combination with a pharmacologic inhibitor of iNOS (1400W). We found that blocking NO production by 1400W, as measured by nitrite levels (A), was completely responsible for the observed suppression of T cell proliferation (B). T cell proliferation by [3H]thymidine incorporation is shown as a stimulation index (S.I.), normalized to the OTII alone response. Results are representative of three independent experiments.
completed of Ly6G+ neutrophils. T cell stimulation was from the NOX2 donor NOC-18. (42) In the presence of an IFN-γ-neutralizing Ab, there was no differential effect on T cell activity (Fig. 6C). Interestingly, neutrophils did not appear to block the inactivation of T cell STAT5, a reported method of NO-mediated suppression. We found that neutrophils isolated after inflammatory challenge in NOX2−/− and WT mice both influence T cell activity; however, it should be noted that the endogenous number of these cells in WT mice at 2 wk is significantly lower than that found in the chronic inflammatory environment (Fig. 1B, Supplemental Fig. 2). These results show for the first time, to our knowledge, that unresolved neutrophil activity can play a direct immunomodulatory role by decreasing T cell sensitivity to NO-mediated suppression.

We next determined the antagonistic activity of inflammation-associated neutrophils on MDSC function in vivo. Because the lung is a common site of inflammatory complications in many inflammatory disorders (5, 23, 44), we evaluated whether the isolated myeloid cell populations could independently control increasing concentrations of the soluble NO–donor molecule NOC-18, with or without Ly6G+ neutrophils derived from NOX2−/− or WT mice 2 wk after inflammatory challenge. The NOC-18 dose range was chosen to encompass the levels of NO known to occur in vivo in sites of acute infection and inflammation and thus routinely used in in vitro experiments (43). We found that with increased numbers of neutrophils, a 100 μM higher concentration of NOC-18 was needed to induce T cell suppression (38), because levels of phosphorylated STAT5 were downregulated in the presence of 250 μM NOC-18 in all conditions (Fig. 7B). We found that neutrophils isolated after inflammatory challenge in NOX2−/− and WT mice both influence T cell activity; however, it should be noted that the endogenous number of these cells in WT mice at 2 wk is significantly lower than that found in the chronic inflammatory environment (Fig. 1B, Supplemental Fig. 2). These results show for the first time, to our knowledge, that unresolved neutrophil activity can play a direct immunomodulatory role by decreasing T cell sensitivity to NO-mediated suppression.

Because enhanced NOX2−/−/MDSC function is mediated by NO production (Fig. 5B), we determined whether neutrophils could directly influence NO-mediated T cell suppression in the absence of MDSCs. T cells were stimulated in the presence of increasing concentrations of the soluble NO–donor molecule NOC-18, with or without Ly6G+ neutrophils isolated 2 wk after inflammatory challenge from NOX2−/− or WT mice, along with increasing concentrations of the soluble NO donor NOC-18. (A) Cell proliferation was determined by [3H]thymidine incorporation during the final 12 h of a 3.5-d stimulation period. T cell proliferation by [3H]thymidine incorporation is shown as a stimulation index (S.I.), normalized to the OTII response without NOC-18. (B) Levels of phospho-Stat5 (pStat5) in CD4+ OTII cells were determined after 36 h using intracellular flow cytometry. Each histogram shows pStat5 levels in nonstimulated (filled), Ag alone (solid line), or Ag plus 250 μM NOC-18 (dashed line). Results are representative of at least two independent experiments.
T cell activity and thus mediate lung inflammation. To directly measure T cell activity in vivo, we seeded OTII CD4⁺ T cells labeled with CFSE dye into WT-recipient mice. Recipient mice then received no cell transfer, Ly6G⁺ MDSCs alone, or Ly6G⁺ MDSCs and Ly6G⁺ neutrophils isolated from NOX2⁻/⁻ mice 2 wk post-inflammatory challenge. In addition, we also evaluated Ly6G⁺ neutrophils derived from WT mice. Because neutrophils alone did not appear to influence T cell activity in the absence of active MDSCs (compare Fig. 4A with Fig. 6B), we focused on experimental conditions that included MDSCs. T cell stimulation was induced by nasal delivery of OVA Ag. After 3 d, the mediastinal lymph nodes and spleen were collected, and T cell proliferation was measured as a function of CFSE dye dilution (Fig. 8A). As expected, there was robust Ag-induced T cell proliferation both locally (mediastinal lymph node; Fig. 8B) and peripherally (spleen; Fig. 8C); however, transfer of MDSCs significantly suppressed this response. Conversely, when MDSCs and Ly6G⁺ neutrophils (NOX2⁻/⁻ or WT) were cotransferred, there was no detectable T cell suppression locally (Fig. 8B) or peripherally (Fig. 8C).

In addition to suppressing Ag-induced T cell responses in vivo, we found that transfer of active MDSCs alone, sourced from the NOX2⁻/⁻ mouse chronic inflammatory environment, was sufficient to significantly suppress lung inflammation in recipient WT mice. Lung infiltration of neutrophils and eosinophils was significantly decreased, and macrophage numbers trended lower with transfer of MDSCs (Fig. 9A). However, the presence of inflammation-associated neutrophils again antagonized the suppressive activity of MDSCs, resulting in elevated inflammatory lung infiltrate numbers (Fig. 9A). Because lung inflammation is commonly associated with a Th2-type immune response, such as with allergic inflammation (45), we determined the Ag-induced IL-5 cytokine response from mediastinal lymph node CD4⁺ T cells. There was a significant reduction in IL-5 cytokine production from mice receiving MDSCs; however, cotransfer of inflammation-associated neutrophils reversed the effect (Fig. 9B). Furthermore, lung histology showed a decrease in inflammatory infiltrate lining the airspaces in mice receiving MDSCs alone but not when inflammation-associated neutrophils were present (Fig. 9C–F). Taken together, these data confirm the relevance of the in vitro findings showing that the chronically inflamed environment in NOX2⁻/⁻ mice contains fully competent MDSCs capable of suppressing inflammation; however, the concurrent increase in inflammation-associated neutrophil numbers antagonize their activity and thus represent a viable mechanism to perpetuate the chronic inflammatory response.

**Discussion**

Chronic inflammation is a common thread that ties together most pathological events linked to unsuccessful aging. Determining the mechanisms that control the dysregulated immune response early in the chronic inflammatory process, prior to the accumulation of clinical disease-inducing damage, will allow development of new anti-inflammatory targets with potentially broad disease treatment applications. In our present study, we describe a novel inducible model of sterile chronic inflammation. We show that induction of an inflammatory response, using two genetically susceptible mouse lines (NOX2⁻/⁻ and IL-10⁻/⁻), triggers an exaggerated expansion and long-term persistence (≥3 wk) of a myeloid Gr1⁺CD11b⁺ cell population that includes Gr1 high (Ly6G⁺) CD11b⁺ neutrophils actively producing the proinflammatory IFN-γ cytokine. In both mouse lines, we detected elevated levels of serum IL-1β and IL-6 biomarkers, two of the most common...
proinflammatory cytokines tested for in human studies of chronic inflammation. In fact, multiple human clinical trials have been initiated to evaluate anti-inflammatory therapies, including IL-1β and IL-6 cytokine inhibitors (46–48).

Somewhat surprisingly, when examining the chronic inflammatory response in NOX2−/− mice in more detail, we identified a population of monocytic MDSCs (Gr1lowLy6GCD11b+) with significantly enhanced NO-dependent immunosuppressive activity ex vivo, which suggests a viable mechanism of immune regulation present yet ineffective in vivo. In the competitive immune environment, we found that increased numbers of inflammation-associated neutrophils conferred T cell resistance to NO-mediated suppression, thereby antagonizing the suppressive activity of MDSCs. This indicates that removal of neutrophils during established chronic inflammation may promote resolution of the response by the functionally competent MDSCs.

The lack of a functional phagocyte NADPH oxidase complex in neutrophils of CGD patients is thought to be the central cause of recurrent microbial infections; however, the role of neutrophils in noninfectious inflammation and the potential chronic inflammatory response is less clear. A recent study found that NADPH oxidase activity in mononuclear phagocytes (e.g., macrophages and dendritic cells), but not neutrophils, was critical for limiting the acute hyperinflammatory response (49). Increased Gr1high cell infiltration was observed in skin lesions; however, the study stopped short of defining the potential establishment of chronic inflammation or a role for increased neutrophil numbers. Although it is important to understand initiating events leading to the establishment of chronic inflammation, the often overlooked development of subclinical chronic inflammatory responses and the heterogeneity of susceptibility factors highlight the need to define potential common mechanisms to resolve established chronic inflammation. We propose that the persistence of increased neutrophil numbers established during chronic inflammation is critical for maintaining the response by directly influencing dysregulated adaptive T cell activity.

Our data support the conclusion that NOX2 deficiency in neutrophils and monocytic MDSCs does not play a critical role in their immunoregulatory activity. In addition to numerous studies showing that monocytic MDSC suppressive activity is attributed to NO production rather than ROS production, the function of MDSCs from NOX2−/− mice was actually enhanced, which is contradictory to an expected decline in function if suppressive activity was ROS dependent (37). Furthermore, MDSCs from NOX2−/− mice had functional anti-inflammatory activity in vivo when transferred to WT-recipient mice, which in the context of the well-known short half-life of NO and the need for close proximity to exert its effects (38, 50), also indicates that the MDSCs successfully traffic to sites of immune activation.

Multiple inflammatory diseases are described as having overactive neutrophil and T cell activity (51, 52). For example, rheumatoid arthritis and intestinal bowel disease are characterized by persistent increases in neutrophil numbers and pathologic T cell responses that contribute to disease complications (8). Our findings support the hypothesis that the dysregulated response of adaptive T cells is linked to neutrophils (8). The functional relevance of this phenomenon is potentially related to low cytokine production per neutrophil compared with other immune cells (e.g., macrophages and lymphocytes), and thus, with increased neutrophil numbers during inflammation, a threshold of secreted cytokine could be reached to exert secondary signals to surrounding cells (8, 53). For example, neutrophils can influence T cell responses by producing the stimulatory cytokines IL-12 or TNF-α (8). During transient acute inflammation, this effect may be beneficial for T cell priming in part by holding off immunosuppressive mechanisms such as MDSCs until signals are received for response resolution. In established chronic inflammation, our model indicates that persistence of increased neutrophil numbers...
are directly promoting continued T cell activity by conferring resistance to NO-mediated suppression by MDSCs. Both NOX2/−/− and WT neutrophils had the same effect in the presence of physiologically relevant concentrations of NO (43), indicating a common mechanism; however, the relative in vivo cell numbers in WT mice is significantly lower than that found in the chronic inflammatory environment of NOX2/−/− mice. These results show for the first time, to our knowledge, that unresolved neutrophil activity during chronic inflammation can play a direct immunomodulatory role by decreasing T cell sensitivity to NO-mediated suppression.

IFN-γ–producing Gr1+ neutrophils have been detected in studies of transient acute inflammatory responses (54, 55); however, to our knowledge, the persistence of these cells during chronic inflammation is a novel finding. Similar to the WT inflammatory response in our system, the expansion of Gr1+IFNγ+ neutrophils occurs during the innate response and then numbers contract within days (54, 55). Multiple functional attributes have been ascribed to IFN-γ production by neutrophils. During acute bacterial infection, IFN-γ–competent neutrophils increased pathogen clearance compared with IFN-γ−/− neutrophils (54), which may be related to increased neutrophil extracellular traps production (56). Furthermore, IFN-γ has been shown to extend the life span of neutrophils (57), suggesting the cytokine may have autocrine activity contributing to neutrophil persistence. A recent study implicated NOX2 as a requirement for IFN-γ production by emigrating neutrophils in response to acute bacterial lung infection (56); however, this requirement was extrapolated from acute pathogen-infected lungs in NOX2/−/− mice without delineating a cell intrinsic mechanism from extrinsic factors in the dysregulated inflammatory environment. In contrast, we show that long-term persistence of IFN-γ–producing neutrophils can be induced in both the NOX2/−/− (Fig. 2E) and IL-10−/− inflammatory models. To this end, although IFN-γ–producing neutrophils are a useful biomarker of active inflammation, further studies are needed to clarify their functional role.

In conclusion, to our knowledge, our study is the first to ascribe novel drug targets for anti-ageing strategies and successful ageing achievement. Care. Pharm. Des. 16: 584–596.


