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A Role for Immature Myeloid Cells in Immune Senescence

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The reduced efficiency of the mammalian immune system with aging increases host susceptibility to infectious and autoimmune diseases. However, the mechanisms responsible for these pathologic changes are not well understood. In this study, we demonstrate that the bone marrow, blood, and secondary lymphoid organs of healthy aged mice possess increased numbers of immature myeloid cells that are phenotypically similar to myeloid-derived suppressor cells found in lymphoid organs of mice with progressive tumors and other pathologic conditions associated with chronic inflammation. These cells are characterized by the presence of Gr1 and CD11b markers on their surfaces. Gr1⁺CD11b⁺ cells isolated from aged mice possess an ability to suppress T cell proliferation/activation and produce heightened levels of proinflammatory cytokines, both constitutively and upon activation, including IL-12, which promotes an excessive production of IFN-γ. IFN-γ priming is essential for excessive proinflammatory cytokine production and the suppressive activities by Gr1⁺CD11b⁺ cells from aged mice. These cells suppress T cell proliferation through an NO-dependent mechanism, as depletion of splenic Gr1⁺ cells reduces NO levels and restores T cell proliferation. Insights into mechanisms responsible for the proinflammatory and immune suppressive activities of Gr1⁺CD11b⁺ cells from aged mice have uncovered a defective PI3K–Akt signaling pathway, leading to a reduced Akt-dependent inactivation of GSK3β. Our data demonstrate that abnormal activities of the Gr1⁺CD11b⁺ myeloid cell population from aged mice could play a significant role in the mechanisms responsible for immune senescence. The Journal of Immunology, 2011, 186: 000–000.

Myeloid cells possessing immune suppressive activities have a long history. Almost two decades ago, it was reported that the vaccination of mice with an attenuated strain of Salmonella typhi-murium resulted in a transient state of immune suppression caused by the activities of myeloid cells that had infiltrated their secondary lymphoid organs (13). This nonspecific suppression of immune responsiveness was mediated through an NO-dependent mechanism and directly correlated with the magnitude of infection (12, 14).

An abnormal accumulation of immune suppressive immature myeloid cells in peripheral secondary lymphoid organs has now been observed to occur in both murine and human hosts harboring many types of progressing cancers, persistent bacterial or viral infections, or after surgical trauma or thermal injury (15–18). In mice, these cells are phenotypically heterogeneous, being composed of both immature granulocytic and mononuclear cell types (15). Currently, investigators collectively refer to Gr1⁺CD11b⁺ cells as myeloid-derived suppressor cells (MDSCs) (15, 19). The common characteristic of MDSCs is their ability to suppress the proliferation of both CD4⁺ and CD8⁺ T cells through mechanisms involving the increased activities of arginine metabolizing enzymes, inducible NO synthase (iNOS), and/or arginase 1 (ARG-1) (15, 20).

The primary objectives of our current investigation were to determine whether immune-compromising activities of Gr1⁺CD11b⁺ cells contribute to immune senescence and to better characterize the signaling defects responsible for their immune suppressive activities in aged hosts.

Materials and Methods

Laboratory animals

BALB/c mice (3–4 mo and 18–22 mo) of both sexes were purchased from Charles River Breeding Laboratory (Wilmington, MA). DO11.10 TCR transgenic mice on the BALB/c background were bred from animals originally purchased from The Jackson Laboratory (Bar Harbor, ME) and aged in our facility. The Institutional Animal Care and Use Committee and the Animal Resource Center at the University of Utah (Salt Lake City, UT) guarantee strict compliance with regulations established by the Animal Welfare Act.
Detection of Gr1⁺CD11b⁺ cells

Nucleated cells were isolated from the SLPs, peripheral lymph nodes, bone marrow (BM), and blood of young and aged DO11.10 or BALB/c mice and stained with anti-mouse Gr1-clone RB6-8C5-and anti-mouse CD11b-clone M1/70-monoclonal Abs (eBioscience, San Diego, CA). Some SLP cell samples were contaminated with anti-mouse CD11b, Ly-6G (clone 1A8), Ly-6C (clone AL-21), and CD31 (clone MEC13.3) (BD Pharmingen, San Jose CA) monoclonal Abs. The stained cell samples were analyzed by flow cytometry (FACS).

Intracellular cytokine analysis

Splenocytes were cultured in complete medium (RPMI 1640, 2-ME [50 μM], l-glutamine [2 mM] and gentamicin [10 μg/ml]) supplemented with 10% heat-inactivated FBS (HyClone, Logan, UT) at a density 2 × 10⁶ cells/ml in the presence or absence of Escherichia coli LPS (Sigma, St. Louis, MO; 10 or 100 ng/ml). Three hours prior to the cytokine staining, brefeldin A (3 μg/ml; eBioscience) was added to the cultures. At 6 (TNF-α) or 24 h (all other cytokines) postactivation, cells were stained with either anti-CD4 or with anti-Gr1 and anti-CD11b Abs, fixed, permeabilized, and additionally stained with anti-cytokine Abs (Miltenyi Biotec, Auburn, CA; and eBioscience). In the experiments where IFN-γ was evaluated, cell cultures were treated with neutralizing anti–IL-12 Abs (20 μg/ml; R&D Systems, Minneapolis, MN). Forty-eight hours later, cell culture supernatants were collected and analyzed by ELISA for the presence of secreted IFN-γ (21).

Isolation of Gr1⁺CD11b⁺ cells

Gr1⁺CD11b⁺ cells were purified from the SLPs, BM, and blood of young and aged DO11.10 or BALB/c donors by initial depletion of cells expressing Ly-6G, Ly-6C (clone AL-21), and CD31 (clone MEC13.3) (BD Pharmingen, San Jose CA) monoclonal Abs. The stained cell samples were analyzed by flow cytometry (FACS).

Ex vivo and in vivo depletion of Gr1⁺ cells

In some experiments, OV A-activated splenocytes from young and aged DO11.10 mice were added to the splenocyte culture well (2 × 10⁶/ml) and cultured in the presence of 100 μg/ml OVA. OV A-activated splenocytes from young or aged DO11.10 donors served as controls. Cell culture supernatants were collected, 2, 4, and 6 d later and analyzed for the presence of IL-2 and IFN-γ by ELISA and for nitrites using Griess reagent. Parallel cell culture supernatants were analyzed for their ability to incorporate [3H]thymidine. In vivo depletion of cells bearing Gr1 markers was performed by the i.p. injection of 100 μg anti-Gr1 mAb (clone RB6.8C5-18, a kind gift of Dr. Gerald Spangrude, University of Utah) into aged DO11.10 mice on days −6, −3, and 0. The quality of cell depletion was monitored by FACS analysis. The percentages of Gr1⁺CD11b⁺ cells were analyzed in the SLPs of representative naive mice and mice pretreated with anti-Gr1 Abs. Mice were then s.c. immunized with 50 μl of a vaccine formulation containing 50 μg OVA in Alum. Blood samples were collected at various time points postimmunization and analyzed for the levels of OVA-specific IgG Abs by ELISA as previously described (24, 25). Mice that were immunized after the initial Gr1⁺ cell depletion received additional injections of anti-Gr1 Abs on a weekly basis (50 μg/mouse/wk).

Western blot analysis

Gr1⁺CD11b⁺ cells isolated from SLPs of young or aged BALB/c mice were plated at density 1 × 10⁶ cells/ml in complete medium containing 1% FBS and stimulated with 10 ng/ml LPS. Western blot analysis was performed as previously described (26) using specific Abs directed against murine phosphorlated Ser473-Akt (Cell Signaling Technology), total Akt (eBioscience), SHIP1 (Santa Cruz Biotechnology), phosphorylated Ser9-GSK3β, and total GSK3β (Cell Signaling Technology).

Statistical analysis

The statistical significance of differences observed between different experimental and control groups was determined by Student t test. All data are expressed as the mean ± SD. Differences were considered as statistically significant when p values were <0.05.

Results

The blood, secondary lymphoid organs, and BM of aged mice possess elevated numbers of myeloid cells bearing the Gr1⁺CD11b⁺ phenotype

We have recently reported that the numbers of Gr1⁺CD11b⁺ cells were increased in the SLPs of normal aged BALB/c and C57BL/6 mice (12). Elevated numbers of Gr1⁺CD11b⁺ cells were also present in the SLPs of aged (18–22 mo) mice of other strains, including DBA/2, C57/HeN, and the TCR transgenic DO11.10 (Fig. 1A and data not shown). Although the increased percentages varied between individual aged mice (150–800% increase over young animals), the data presented in Fig. 1A reflects the most commonly observed changes in Gr1⁺CD11b⁺ cells that occur as a consequence of aging in our animal colony.

High numbers of Gr1⁺CD11b⁺ cells were also found in the peripheral lymph nodes of aged animals, whereas Gr1⁺CD11b⁺ cells were virtually undetectable in the peripheral lymph nodes of young mice (Fig. 1B). The BM of young mice normally contained high numbers of Gr1⁺CD11b⁺ cells (15). The percentage of these cells was greatly increased in the BM compartment of aged mice (Fig. 1C). The blood of aged mice was found to contain ~50% Gr1⁺CD11b⁺ cells, which represented a 4.5-fold increase compared with numbers of Gr1⁺CD11b⁺ cells present in the blood of young animals (Fig. 1D).

Cytological analysis of Gr1⁺CD11b⁺ cells purified from the SLPs of aged mice revealed similarities to what has been previously reported for the Gr1⁺ myeloid cells isolated from lymphoid tissues of septic mice (27). Gr1⁺CD11b⁺ cells isolated from either young or aged mice represented a heterogeneous cell population, consisting of four morphologically distinct subgroups: immature or mature polymorphonuclear leukocytes, and cells with immature or mature monocyte characteristics (27). The Gr1⁺ CD11b⁺ cells from the SLPs of aged DO11.10 or BALB/c mice...
The numbers of Gr1\(^+\)CD11b\(^+\) cells residing in multiple lymphoid organs is increased in aged mice. A–D. Cells isolated from SPLs, peripheral lymph nodes, BM, and blood of young (3 mo) and aged (18–22 mo) BALB/c or DO11.10 strain mice were stained with monoclonal Abs against CD11b and Gr1 and analyzed by FACS for Gr1\(^+\)CD11b\(^+\) cells. Percentages of Gr1\(^+\)CD11b\(^+\) cells in the dot plots are presented as mean \(\pm\) SD, \(n=4\) mice/group from one representative experiment of four independent experiments. E and F. Cytospins of Gr1\(^+\)CD11b\(^+\) cells isolated from SPLs and BM of young (3 mo) or aged (18 mo) DO11.10 donors were stained using buffered Wright–Giemsa stain solution. Pooled Gr1\(^+\)CD11b\(^+\) cells isolated from SPLs or BM of three young and three aged mice were used for staining. The data presented are representative of one of two independent experiments. Original magnification \(\times 100\). G and H. Splenocytes and BM cells isolated from young and aged BALB/c mice were stained with monoclonal Abs against CD11b, Ly6C, and Ly6G and analyzed by FACS. CD11b\(^+\) cells expressing Ly6C and Ly6G markers are presented in the dot plots. Percentages of CD11b\(^+\)Ly6ClowLy6G\(^+\) cells are presented as mean \(\pm\) SD, \(n=3\) mice/group from one representative experiment of three independent experiments.

demonstrated a 5-fold increase in the percentage of monocytes with a ring-shaped nucleus (immature cells) compared with that of cells from the SPLs of young donors (Fig. 1E and Ref. 12). The Gr1\(^+\)CD11b\(^+\) cells residing in the BM of young and aged donors were determined to be morphologically similar to one another (Fig. 1F).

Recent studies have determined that Gr1\(^+\)CD11b\(^+\) cells from the SPLs of tumor-bearing mice can be divided into distinct subpopulations based on their expression of the two Gr1 epitopes, Ly6C and Ly6G (28). When CD11b\(^+\) cells present in the secondary lymphoid organs of aged animals were analyzed for expression levels of Ly6G and Ly6C, we found that SPLs from aged mice contained increased numbers of cells expressing a CD11b\(^+\)Ly6C\(^{hi}\)Ly6G\(^{int}\) phenotype (a 9-fold increase) compared with that of SPLs from young mice (Fig. 1G). The vast majority of these CD11b\(^+\)Ly6C\(^{hi}\)Ly6G\(^{int}\) cells also expressed CD31 (data not shown). Additionally, the Gr1\(^+\)CD11b\(^+\) cells in the blood of aged mice contained twice as many Ly6C\(^{hi}\)Ly6G\(^{int}\) cells compared with that of the blood isolated from young donors (data not shown). Although aged animals accumulated this unique subset of immature myeloid cells in SPLs and blood, their BM contained a comparable percentage of cells expressing CD11b\(^+\)Ly6C\(^{hi}\)Ly6G\(^{int}\) phenotype to that present in young BM (Fig. 1H).

Collectively, our data indicate that an increased accumulation of immature myeloid cells occurs in multiple secondary lymphoid organs of aged mice and may orchestrate some of the immune dysfunctions associated with advanced age.

**Stimulation of aged splenocytes with TLR4 ligand results in a significant increase in the percentage of Gr1\(^+\)CD11b\(^+\) and CD4\(^+\) T cells producing proinflammatory cytokines**

We and others have previously reported that splenic cells from aged mice constitutively produce low levels of proinflammatory cytokines (e.g., IL-6 and IL-12) (22, 29–32). Intracellular staining of naive myeloid cells residing in SPLs of aged BALB/c mice (22 mo) for proinflammatory cytokines revealed that a higher percentage of splenic Gr1\(^+\)CD11b\(^+\) cells were positive for IL-6 and IL-12(p40) compared with that of Gr1\(^+\)CD11b\(^+\) cells present in SPLs of young mice (Fig. 2A). LPS stimulation of splenocytes showed that increased percentages of activated Gr1\(^+\)CD11b\(^+\) cells from aged donors produced TNF-\(\alpha\), IL-6, or IL-12(p40) and a lower percentage of activated Gr1\(^+\)CD11b\(^+\) cells generated anti-inflammatory IL-10 compared with those of LPS-stimulated splenocytes from young mice (Fig. 2A).

The studies presented above demonstrated that a greater percentage of Gr1\(^+\)CD11b\(^+\) cells in the SPLs of aged mice produce IL-12(p40). IL-12 is an important cytokine involved in IFN-\(\gamma\) production by T cells and NK cells (33, 34). We have previously reported that CD4\(^+\) T cells isolated from aged donors produce enhanced levels of IFN-\(\gamma\) after their activation in vitro (6, 35). Therefore, we questioned whether the heightened levels of IL-12 produced by splenic Gr1\(^+\)CD11b\(^+\) cells from aged mice (or any other cells present in SPLs of aged mice) might contribute to the observed increases of IFN-\(\gamma\). Splenocytes isolated from young (3 mo) and aged (20 mo) DO11.10 mice were stimulated in vitro with various doses of LPS (10–100 ng/ml), and the percentages of CD4\(^+\)IFN-\(\gamma\) T cells were determined after 24 h. As presented in Fig. 2B, ~4% of naive CD4\(^+\) T cells from aged DO11.10 mice were IFN-\(\gamma\)-, whereas in young mice only 1.4% of naive CD4\(^+\) cells expressed IFN-\(\gamma\). Stimulation of splenocytes from young and aged mice with LPS increased the percentage of CD4\(^+\)IFN-\(\gamma\)- T cells in dose-dependent manner (Fig. 2B). A parallel set of splenocytes was activated with LPS in the presence or absence of neutralizing anti–IL-12 Abs. Cell culture supernatants were collected 48 h later and analyzed for IFN-\(\gamma\) by ELISA. Similar to what we have observed in CD4\(^+\) T cells stained for IFN-\(\gamma\),
Gr1+CD11b+ cells from aged mice suppress CD4+ T cell activation/proliferation of T cells (18, 19, 36). Gr1+CD11b+ cells are characteristic of these cells is their ability to suppress the under many clinically relevant conditions that associate with a BM of aged mice possessed greater suppressive activity on CD4+ CD4+ T cell proliferation compared with the T cell responses from the SPLs of aged donors significantly suppressed Ag-induced T cell activation, and IL-12(p40), IL-6, and IL-10 or IFN-γ. Three hours prior to cell collection, cultures were treated with 3 μg/ml brefeldin A to prevent cytokine secretion. A, TNF-α–producing Gr1+CD11b+ cells were analyzed 6 h after LPS activation, and IL-12(p40), IL-6, and IL-10–producing Gr1+CD11b+ cells were detected 24 h postactivation. B, Splenocytes isolated from three young (3 mo) and three aged DO11.10 (20 mo) mice were activated with LPS for 24 h and analyzed for the presence of CD4+IFN-γ+ cells. C, A parallel set of splenocytes was activated with 10 or 100 ng/ml LPS in the presence or absence of neutralizing anti–IL-12 Abs (20 μg/ml). Supernatants were collected 48 h later and analyzed for IFN-γ production by ELISA. The data in A–C are presented as the mean ± SD, n = 3 mice/group from one representative experiment of two independent experiments. **p < 0.005.

**FIGURE 2.** Gr1+CD11b+ cells and CD4+ T cells in SPLs of aged mice produce proinflammatory cytokines. Splenocytes isolated from young (3 mo) and aged (22 mo) BALB/c mice were cultured at a density of 1 × 10^6 cells/ml in the presence of 0, 10, or 100 ng/ml LPS. Six or twenty-four hours post-activation, cells were collected and stained with anti-Gr1 and anti-CD11b Abs or with anti-CD4 Ab, fixed, permeabilized, and stained for TNF-α, IL-12 (p40), IL-6, and IL-10 or IFN-γ. Three hours prior to cell collection, cultures were treated with 3 μg/ml brefeldin A to prevent cytokine secretion. A, TNF-α–producing Gr1+CD11b+ cells were analyzed 6 h after LPS activation, and IL-12(p40), IL-6, and IL-10–producing Gr1+CD11b+ cells were detected 24 h postactivation. B, Splenocytes isolated from three young (3 mo) and three aged DO11.10 (20 mo) mice were activated with LPS for 24 h and analyzed for the presence of CD4+IFN-γ+ cells. C, A parallel set of splenocytes was activated with 10 or 100 ng/ml LPS in the presence or absence of neutralizing anti–IL-12 Abs (20 μg/ml). Supernatants were collected 48 h later and analyzed for IFN-γ production by ELISA. The data in A–C are presented as the mean ± SD, n = 3 mice/group from one representative experiment of two independent experiments. **p < 0.005.

Gr1+CD11b+ cells from aged mice suppress CD4+ T cell responses, markedly increase iNOS or ARG-1 expression after activation, and produce elevated levels of proinflammatory cytokines

Increased numbers of Gr1+CD11b+ cells have now been observed under many clinically relevant conditions that associate with a state of generalized immune suppression (15, 18, 36). One common characteristic of these cells is their ability to suppress the activation/proliferation of T cells (18, 19, 36). Gr1+CD11b+ cells from the SPLs of aged donors significantly suppressed Ag-induced CD4+ T cell proliferation compared with the T cell responses observed after an addition of splenic Gr1+CD11b+ cells from young donors (Fig. 3A). The Gr1+CD11b+ cells isolated from the BM of aged mice possessed greater suppressive activity on CD4+ T cell proliferation compared with that of Gr1+CD11b+ cells isolated from the BM or SPLs of young donors (Fig. 3A). Gr1+CD11b+ cells from the blood of young and aged donors exhibited similar suppressive activities when analyzed at similar ratios to CD4+ T cells (data not shown), however the blood of aged mice contained ~4.5-fold more Gr1+CD11b+ cells than the blood of young donors (Fig. 1D).

It has been reported that suppression of T cell responses by MDSCs appears to be primarily mediated by their capacity to effectively metabolize arginine, either through the expression of iNOS or ARG-1 activities (18, 20, 37). We investigated whether resting and/or activated Gr1+CD11b+ cells isolated from aged donors express altered levels of iNOS and ARG-1. Because ARG-1 becomes maximally expressed in myeloid cells after their activation in the presence of IL-4 (38), Gr1+CD11b+ cells isolated from SPLs of young and aged donors were stimulated with LPS in the presence or absence of IL-4. Splenic Gr1+CD11b+ cells isolated from young animals had minimal ARG-1 gene expression and enzyme activity when stimulated with LPS alone, whereas Gr1+CD11b+ cells from SPLs of aged animals had higher ARG-1 gene expression/activity after stimulation with only LPS (Fig. 3B). Addition of IL-4 into cultures of LPS-activated Gr1+CD11b+ cells from aged donors considerably enhanced ARG-1 gene expression levels and activity compared with ARG-1 expression/activity in cells from young donors treated in a similar manner (Fig. 3B).

Next, we examined the ability of Gr1+CD11b+ cells isolated from aged animals to express iNOS and produce NO under resting conditions and upon activation. Unstimulated Gr1+CD11b+ cells isolated from SPLs of either young or aged donors did not express iNOS, and NO was not detected in cell culture supernatants from these cells (Fig. 3C). When activated with LPS or IFN-γ alone, the Gr1+CD11b+ cells from young animals failed to produce NO, whereas costimulation with LPS and IFN-γ resulted in detectible levels of NO (Fig. 3C). In contrast, NO was produced by Gr1+CD11b+ cells from aged mice after their exposure to IFN-γ alone (Fig. 3C). Combined treatment of Gr1+CD11b+ cells from aged donors with LPS and IFN-γ greatly potentiated iNOS expression and increased the levels of nitrates detected in the cell supernatants of costimulated cells, suggesting that IFN-γ plays a significant role in boosting LPS-induced responses in Gr1+CD11b+ cells from aged mice.

The data presented above demonstrate that the activities of two major arginine-metabolizing enzymes become greatly elevated in stimulated Gr1+CD11b+ cells from the SPLs of aged animals. Furthermore, when activated in a similar manner, Gr1+CD11b+ cells from the BM of aged mice also possessed increased iNOS or
ARG-1 enzyme activities (data not shown). This may provide an explanation of their suppressive activities on CD4+ T cells. An analysis of cytokines produced by Gr1+CD11b+ cells isolated from young and aged mice revealed that activated splenic Gr1+CD11b+ cells from aged animals produce greater levels of IFN-γ (Fig. 3D). Increases were observed after stimulation with LPS, or IFN-γ alone, although the greatest differences were observed in cells co-cultured with both LPS and IFN-γ (Fig. 3D). Activated Gr1+CD11b+ cells from BM of aged mice also produced excessive levels of IL-12(p40) and IL-6 compared with those of Gr1+CD11b+ cells isolated from young mice. These data are representative from two independent experiments. B–D. Another set of Gr1+CD11b+ cells isolated from the SPLs of three young (3 mo) and three aged (18–20 mo) BALB/c mice was cocultured in vitro with LPS (10 ng/ml), IFN-γ (10 ng/ml), or with LPS in combination with IFN-γ or IL-4 (10 ng/ml) cytokines. Resting Gr1+CD11b+ cells were used as controls. Cells were collected 24 h postactivation and subjected to RT-PCR analysis to assess iNOS and ARG-1 gene expressions. Supernatants were collected 48 h after cell activation and analyzed for nitrate (iNOS activity) and urea levels (ARG-1 activity). Additionally, 48 h postactivation, cell culture supernatants were analyzed for the presence of IL-12(p40) and IL-6 by ELISA. The data represent the mean ± SD, n = 4 mice/group from one representative experiment of three independent experiments conducted. *p < 0.05; **p < 0.005.

**FIGURE 3.** Gr1+CD11b+ cells isolated from the SPLs of aged mice produce excessive levels of proinflammatory cytokines, have increased activity of iNOS and ARG-1, and are capable of suppressing CD4+ T cell proliferation. A. Splenocytes isolated from young DO11.10 mice (3 mo) were cultured at a density of 2 × 10^6 cells/ml in the presence of 100 μg/ml OVA. Gr1+CD11b+ cells isolated from the SPLs or BM of three young (3 mo) or three aged (22 mo) BALB/c mice were added to the splenocyte cultures at ratio of 1:1, 0.5:1, and 0.25:1 (Gr1+CD11b+ cell/CD4+ T cell). Each sample was plated in triplicate. Four days later, OVA-specific CD4+ T cell proliferation was measured by assessing [3H]thymidine incorporation. Data are presented as the percentage of CD4+ T cell proliferation in cultures with added Gr1+CD11b+ cells isolated from young mice. These data are representative from two independent experiments. B–D. Another set of Gr1+CD11b+ cells isolated from the SPLs of three young (3 mo) and three aged (18–20 mo) BALB/c mice was cocultured in vitro with LPS (10 ng/ml), IFN-γ (10 ng/ml), or with LPS in combination with IFN-γ or IL-4 (10 ng/ml) cytokines. Resting Gr1+CD11b+ cells were used as controls. Cells were collected 24 h postactivation and subjected to RT-PCR analysis to assess iNOS and ARG-1 gene expressions. Supernatants were collected 48 h after cell activation and analyzed for nitrate (iNOS activity) and urea levels (ARG-1 activity). Additionally, 48 h postactivation, cell culture supernatants were analyzed for the presence of IL-12(p40) and IL-6 by ELISA. The data represent the mean ± SD, n = 4 mice/group from one representative experiment of three independent experiments conducted. *p < 0.05; **p < 0.005.

ARG-1 enzyme activities (data not shown). This may provide an explanation of their suppressive activities on CD4+ T cells. An analysis of cytokines produced by Gr1+CD11b+ cells isolated from young and aged mice revealed that activated splenic Gr1+CD11b+ cells from aged animals produce greater levels of IL-12 (p40) and IL-6 compared with those of Gr1+CD11b+ cells isolated from young donors (Fig. 3D). Increases were observed after stimulation with LPS, or IFN-γ alone, although the greatest differences were observed in cells co-stimulated with both LPS and IFN-γ (Fig. 3D). Activated Gr1+CD11b+ cells from the BM of aged mice also produced excessive levels of IL-12(p40) and IL-6 (data not shown). These findings indicate that Gr1+CD11b+ cells from the SPLs and BM of aged animals elicit exaggerated responses after their stimulation.

**Gr1+ myeloid cells residing in the lymphoid organs of aged mice negatively affect Ag-induced CD4+ T cell proliferation in vitro and the ability to mount T cell-dependent Ab responses in vivo**

To evaluate whether the increased numbers/activities of Gr1+CD11b+ cells residing in SPLs of aged mice may affect their ability to generate Ag-specific immune responses, we aged a colony of DO11.10 TCR transgenic animals and evaluated their ability to produce low levels of IL-2 and high levels of IFN-γ, otherwise were not affected by Gr1+ cell depletion from the SPLs of aged animals (Fig. 4A). Although excesses in IFN-γ produced by aged CD4+ T cells may indirectly influence their ability to proliferate after activation,
our studies suggest that inhibition of T cell proliferative responses is actually being mediated by the excessive NO produced by IFN-γ~primed Gr1~CD11b~ myeloid cells. CD4~ T cells from aged DO11.10 donors were able to proliferate normally in response to OVA when either neutralizing anti–IFN-γ Abs or specific iNOS inhibitors were added to the cell cultures, adding further support to this possibility (data not shown).

Treatment of aged BALB/c mice with monoclonal anti-Gr1 Abs (41) reduced the numbers of Gr1~ cells present in SPLs of aged mice to levels comparable with those of young controls (Fig. 4B). This allowed us to question whether aged mice depleted of Gr1~ cells in vivo could be successfully immunized. Young, aged, and Gr1-depleted aged BALB/c mice were s.c. immunized with OVA in Alum. Aged mice depleted of Gr1~ cells mounted significantly higher Ab responses than those of untreated aged controls, achieving anti-OVA IgG responses similar to those of young immunized controls (Fig. 4C). It appears that suppressive Gr1~CD11b~ cells are also involved in compromising humoral immune responses in aged animals.

Activated Gr1~CD11b~ cells from aged donors express decreased levels of phosphorylated Akt and fail to inactivate GSK3β effectively

Depressions in activation-induced phosphorylation of Akt have recently been reported to occur in monocyte-derived dendritic cells from aged human donors, leading to their proinflammatory state (42). We therefore questioned whether activation of the PI3K–Akt pathway is altered in Gr1~CD11b~ cells from aged animals. This was strikingly different to what was observed in Gr1~CD11b~ cells isolated from young donors, indicating that aged cells retained more enzymatically active GSK3β after their stimulation (Fig. 5B).

We then questioned whether the expression levels of SHIP1 were altered in Gr1~CD11b~ cells from aged animals. SHIP1 is only expressed by hematopoietic cells and inhibits the downstream activation of Akt by enzymatically converting PI3K-induced phosphatidylinositol-3,4,5-triphosphate (PI[3,4,5]P3) to PI(3,4)P2 (49, 50). High levels of SHIP1 expression were observed in both resting and LPS-exposed splenic Gr1~CD11b~ cells from aged mice. This was strikingly different to what was observed in Gr1~CD11b~ cells isolated from young donors, where cell activation was necessary to induce a moderate upregulation of SHIP1 expression (Fig. 5C). These data support a hypothesis that the observed suppression of the PI3K–Akt signaling pathway in immature myeloid cells from aged animals may involve an abnormal expression of
SHIP1, similar to what was recently reported to occur after the glucocorticoid conditioning of differentiating myeloid cells in vitro (26).

Because the Gr1+CD11b+ cells isolated from SPLs of aged donors retain GSK3\(\beta\) activity after stimulation, we next questioned whether the treatment of these Gr1+CD11b+ cells with a highly specific GSK3 inhibitor would influence their proinflammatory properties subsequent to activation. Although inhibition of GSK3 activity in Gr1+CD11b+ cells from aged mice moderately reduced the levels of IL-6 and IL-12(p40) produced in response to LPS or IFN-\(\gamma\) stimulation alone (Fig. 6A, 6B), it was able to abrogate the capacity of these aged cells to produce NO abnormally in response to IFN-\(\gamma\) treatment (Fig. 6C). The maximum effects of GSK3 inhibition occurred with cells that were stimulated with both LPS and IFN-\(\gamma\) (Fig. 6A – C). The enhanced cytokine responses and iNOS activities in these cells were reduced to the levels found in young Gr1+CD11b+ cells treated in a similar manner. Exposure of Gr1+CD11b+ cells from aged donors to the SB216763 did not, however, correct the enhanced levels of ARG-1 in cells stimulated with LPS and/or IL-4 (Fig. 6D).

It is well documented that levels of various cytokines produced by T cells are altered as a consequence of the aging process (6, 51–54). It appears that the age-associated dysregulation of IL-2 and IFN-\(\gamma\) production by Ag-activated T cells was independent of the activities by Gr1+ cells. Because Akt activation is also depressed in T cells from aged donors (55, 56), we evaluated the effects of GSK3 inhibition on CD4+ T cell responses. Splenocytes from young and aged DO11.10 donors were cultured with OVA in the presence or absence of SB216763. Four days postactivation, CD4+ T cells were analyzed for their ability to proliferate and cell culture supernatants assayed for levels of IL-2, IFN-\(\gamma\), IL-10, and IL-17A. We found that CD4+ T cell proliferative responses from aged mice were significantly improved when SB216763 was present (Fig. 7A). In addition, Ag-induced IL-2 production was increased, and IFN-\(\gamma\) production was decreased to near young adult values when aged splenocytes were cultured in the presence of SB216763 (Fig. 7A). Additionally, OVA-activated splenocytes from aged mice treated with GSK3 inhibitor produced increased amounts of IL-10 and reduced levels of IL-17A. The cytokine pattern observed in the GSK3 inhibitor-treated cell cultures from aged mice is shown in Fig. 7B–D.

**FIGURE 5.** Purified Gr1+CD11b+cells from aged donors have a reduced ability to phosphorylate Akt and are incapable of effectively inactivating GSK3\(\beta\). Gr1+CD11b+ cells isolated from SPLs of young (3 mo) and aged (18–20 mo) BALB/c mice were cocultured with LPS (10 ng/ml) for 30 and 60 min. Total cellular proteins were prepared and then separated by SDS-PAGE for Western blot analysis. Membranes were probed with Abs specific for (A) murine pSer473-Akt, (B) pSer9-GSK3\(\beta\), and (C) SHIP1. All blots were then stripped and reprobed with appropriate Abs to determine equal protein loading. This experiment was repeated three times with similar results. Pooled samples from three mice per group were used in each independent experiment.

**FIGURE 6.** The inhibition of GSK3\(\beta\) activity in Gr1+CD11b+ cells from aged mice ameliorates the proinflammatory condition and reduces inducible iNOS activity. Gr1+CD11b+ cells (1 × 10^6 cells/ml) purified from the SPLs of young (3 mo) and aged (20 mo) BALB/c mice were cocultured with LPS alone (10 ng/ml), IFN-\(\gamma\) (10 ng/ml), IL-4 (10 ng/ml), or the combination of cytokine plus LPS. Additional groups of Gr1+CD11b+ cells from aged mice were pretreated with 10 \(\mu\)M SB216763 and activated in a similar manner. Forty-eight hours later, supernatants were collected and analyzed by ELISA for (A) IL-12(p40), (B) IL-6, (C) nitrate, and (D) urea levels. The data represent the mean ± SD, \(n = 3\) mice/group from one representative experiment of two independent experiments. **\(p < 0.005\).
levels of IL-2 and IL-10 and reduced levels of IFN-γ were capable of proliferating normally and produced increased supernatants were collected and analyzed for IL-2, IFN-γ, IL-10, and IL-17A. B, CD4+ T cells isolated from SPLs of young (3 mo) and aged (20 mo) DO11.10 mice (5 × 10^6 cells/ml) were placed into anti-CD3ε (2 μg/ml) coated tissue culture plates, and anti-CD28 Abs (2 μg/ml) were added to the culture. An additional group of CD4+ T cells isolated from aged mice was activated in the presence of 10 μM SB216763. Twenty-four hours later, CD4+ T cell proliferation was measured by assessing [3H]thymidine incorporation. At the same time, cell culture supernatants were collected and analyzed for the presence of IL-2, IFN-γ, IL-10, and IL-17A. The pooled data (for A and B) from two independent experiments with three mice/group are presented as mean ± SD. *p < 0.05; **p < 0.005.

FIGURE 7. Treatment of activated CD4+ T cells isolated from aged donors with a specific GSK3 inhibitor enhances T cell proliferation, increases IL-2, and reduces IFN-γ production by these cells. A, Splenocytes from young (3 mo) or aged (20 mo) DO11.10 strain mice were cultured in complete medium at a density of 5 × 10^6 cells/ml in the presence or absence of OVA (100 μg/ml). An additional group of splenocytes isolated from aged mice was treated with 10 μM SB216763. Four days later, OVA-specific CD4+ T cell proliferation was measured by assessing [3H]thymidine incorporation. Cell culture supernatants were collected and analyzed for IL-2, IFN-γ, IL-10, and IL-17A. B, CD4+ T cells isolated from SPLs of young (3 mo) and aged (20 mo) DO11.10 mice (5 × 10^6 cells/ml) were placed into anti-CD3ε (2 μg/ml) coated tissue culture plates, and anti-CD28 Abs (2 μg/ml) were added to the culture. An additional group of CD4+ T cells isolated from aged mice was activated in the presence of 10 μM SB216763. Twenty-four hours later, CD4+ T cell proliferation was measured by assessing [3H]thymidine incorporation. At the same time, cell culture supernatants were collected and analyzed for the presence of IL-2, IFN-γ, IL-10, and IL-17A. The pooled data (for A and B) from two independent experiments with three mice/group are presented as mean ± SD. *p < 0.05; **p < 0.005.

animals was similar to that found in activated splenocytes from young donors.

Because many cell types present in the SPLs could be affected by the GSK3 inhibitor, its effects were examined on purified CD4+ T cells from aged DO11.10 donors activated with immobilized anti-CD3ε and soluble anti-CD28. Activated CD4+ T cells from young DO11.10 mice served as controls. Polyclonally activated CD4+ T cells from aged DO11.10 donors treated with SB216763 were capable of proliferating normally and produced increased levels of IL-2 and IL-10 and reduced levels of IFN-γ and IL-17A in comparison with activated aged CD4+ T cells that were not treated with SB216763 (Fig. 7B). This finding suggests that many of the age-associated defects in CD4+ T cell cytokine production and proliferative responses may be linked to abnormal GSK3β activities postactivation.

**Discussion**

Data presented in this article clearly demonstrate that multiple lymphoid organs (BM, blood, SPLs, and peripheral lymph nodes) of healthy aged mice harbor increased numbers of Gr1+CD11b+ cells. Cytological analysis of splenic Gr1+CD11b+ cells established that they are a heterogeneous population, with increased numbers/percentages of immature myeloid cells of monocytic origin. Excessive numbers of immature myeloid cells have been described in the secondary lymphoid organs and blood of mice and humans with cancer, trauma, sepsis, as well as various infectious and autoimmune diseases (15, 18). A blockade that occurs during terminal differentiation of myeloid progenitors into mature macrophages, dendritic cells, and granulocytes may lead to the efflux of immature myeloid cells into the bloodstream followed by their localization into the secondary lymphoid organs of affected animals (15, 57). The immature myeloid cells that express Gr1 and CD11b markers in mice and are capable of suppressing T cell responses have now been termed MDSCs (19).

A more detailed analysis revealed that the SPLs of aged mice contained a phenotypically unique subset of immature myeloid cells that was practically absent in SPLs of young mice. This subset was characterized by the expression of intermediate levels of both Ly6C and Ly6G (CD11b+Ly6CintLy6Gint). A 2-fold increase in the percentage of these cells was also observed in the blood of aged mice. The BM of young and aged mice contained approximately the same percentage of CD11b+Ly6CintLy6Gint cells, although the pool of Gr1+CD11b+ cells in the BM of aged mice was greatly elevated. We believe that activities of the CD11b+Ly6CintLy6Gint subset may be responsible for much of the suppressive activities of splenic Gr1+CD11b+ cells in aged mice because the treatment of aged mice with vitamin E-supplemented diets resulted in a complete elimination of this subset and enhanced the immune competence of the treated animals (E. Enioutina, D. Bareyan, and R. Daynes, unpublished observations) (24, 58, 59).

It has been previously demonstrated that MDSCs isolated from Salmonella-infected mice possess high levels of iNOS activity and strongly suppress T cell proliferation via a NO-mediated pathway, whereas the suppressive activities of MDSCs that are present in the secondary lymphoid organs of mice bearing transplantable tumors predominately depend upon ARG-1 overexpression (12, 14, 15, 60). Our studies show that “resting” Gr1+CD11b+ cells isolated from SPLs or BM of aged BALB/c mice do not constitutively express either arginine metabolizing enzyme, although are easily stimulated to express high levels of iNOS or ARG-1 after activation with LPS in the presence of IFN-γ or IL-4, respectively.

Monocytes undergo a microenvironment-dependent polarization process during their terminal maturation into macrophages (61). Monocytes stimulated with LPS in the presence of IFN-γ terminally differentiate into classical M1 macrophages that can effectively mediate resistance against intracellular microorganisms and tumors (61). The stimulation of monocytes in the presence of IL-4, IL-13, or IL-10, however, results in the generation of alternatively...
activated M2 macrophages, which facilitate tissue repair and angiogenesis (62). M1 macrophages express iNOS and actively produce NO, whereas M2 cells express ARG-1, causing a local depletion of arginine (61, 62). A similar type of polarization process has been observed in MDSCs (20). Our data indicate that the immature Gr1⁺CD11b⁺ cells residing in the secondary lymphoid organs of aged mice have not yet undergone a polarization process and can differentiate down to either M1-like or M2-like pathway. IFN-γ treatment of LPS-activated splenic Gr1⁺CD11b⁺ cells from aged mice resulted in an exaggerated level of iNOS gene expression, whereas their treatment with LPS plus IL-4 induced the marked overexpression of ARG-1 gene.

It has been demonstrated that aged humans have a significant expansion of activated monocytes, which constitutively produce IL-1β and IL-6 (63). We have previously reported that lymphoid cell populations from aged mice constitutively produce IL-12, IL-6, and IFN-γ through NF-κB-dependent process (9, 22, 59). The data presented in this study demonstrate that SPLs of healthy aged mice contain elevated numbers/percentages of Gr1⁺CD11b⁺ cells constitutively producing IL-12(p40) and IL-6, as well as CD4⁺ T cells constitutively producing IFN-γ. The numbers of Gr1⁺CD11b⁺ cells from aged mice constitutively producing the anti-inflammatory IL-10 were quite low.

Activation of splenocytes from aged mice with LPS increased the percentage of Gr1⁺CD11b⁺ cells producing IL-12(p40) and IL-6. IFN-γ treatment of LPS-activated cells greatly augmented this response, suggesting an important role for IFN-γ in creating the inflammatory phenotype in aged animals. The increased generation of inflammatory cytokines by aged immature myeloid cells may also represent an unrecognized contributing factor to the heightened susceptibility of aged mice to endotox shock (64).

An exposure of splenocytes from aged mice to LPS in vitro resulted in a greater percentage of their CD4⁺ T cells being induced to produce IFN-γ compared with that of the CD4⁺ T cells from young animals. This augmented response was determined to be IL-12 dependent. IFN-γ has already been implicated in the expansion and activation of MDSCs (15). An abnormal production of IFN-γ could be involved in the accumulation of suppressive Gr1⁺CD11b⁺ cells in the various lymphoid organs of aged mice. Many other substances (e.g., GM-CSF, G-CSF, PGs, S100A8/A9 proteins, IL-1β, IL-6, and IL-12) have been implicated in the expansion and activation of MDSCs (15). Inflammatory S100 proteins seem to play a critical role in the accumulation of MDSCs in peripheral lymphoid organs of tumor-bearing mice (65, 66). The S100 proteins are of particular interest to us because their production becomes upregulated during inflammation (67). We have found that plasma levels of S100A8 and A9 are increased 1.5- to 2-fold in healthy aged mice (E. Enioutina, D. Bareyan, and R. Daynes, unpublished observations). Others have reported that the expression levels of S100A8 and S100A8/A9 are elevated in skin and in prostate of aged humans (68, 69). The S100 proteins are small calcium-binding proteins involved in a large number of important cellular processes such as calcium homeostasis and cell growth and differentiation (70). Some S100 proteins are capable of activating cells through the receptor for advanced glycation end products and can also serve as a ligand for TLR4 (65, 71, 72). Activation through the receptor for advanced glycation end products results in the activation of NF-κB by inducing the sustained synthesis of p65 (71). We have reported that activated NF-κB is present in multiple cell types of aged animals, helping to facilitate constitutive proinflammatory cytokine production (9, 58).

A major characteristic of MDSCs is their ability to suppress T cell functions via ARG-1 or iNOS-dependent mechanisms (15, 18). On a per cell basis, splenic and BM Gr1⁺CD11b⁺ cells, but not Gr1⁺CD11b⁺ cells from blood of aged mice, had increased suppressive activity compared with that of Gr1⁺CD11b⁺ cells from young mice. It appears that blood Gr1⁺CD11b⁺ cells from young and aged donors possessed a similar suppressive activity on T cells. However, Gr1⁺CD11b⁺ cells in the blood of aged mice outnumbered Gr1⁺CD11b⁺ cells in the blood of young mice (~4.5-fold). This suggests that microenvironmental differences within the SPLs and BM of aged mice have an impact on the suppressive activity of Gr1⁺CD11b⁺ cells residing in these organs.

Because lymphoid cells from aged donors produce elevated levels of IFN-γ, it is reasonable to assume that their activated Gr1⁺CD11b⁺ cells inhibit T cell proliferation via the NO-dependent suppression. Indeed, CD4⁺ T cells present in splenocyte cultures from aged DO11.10 donors were able to proliferate normally in response to OVA stimulation, when either neutralizing anti–IFN-γ Abs or specific iNOS inhibitors were added to the cell cultures (E. Enioutina, D. Bareyan, and R. Daynes, unpublished observations). We have also established that the removal of Gr1⁺ cells from aged DO11.10 splenocyte cultures reduces NO levels and restores CD4⁺ T cell proliferation in response to OVA stimulation. These findings suggest that NO produced by splenic Gr1⁺ cells is responsible for suppressing CD4⁺ T cell activities with aging. It has been reported that NO-mediated suppression of T cell proliferation by MDSCs requires IFN-γ priming and results in a direct impairment of IL-2R signaling (73).

It is possible, however, that Gr1⁺CD11b⁺ cells in individuals, who have chronic conditions causing elevations in IL-4 or IL-13, would upregulate expression of ARG-1. A localized depletion of arginine would also be suppressive to T cell activation by altering effective signal transduction through the TCR (15). The MDSCs present in the SPLs of aged mice bearing transplantable tumors were able to suppress tumor-specific T cell cytotoxic activity through a process that appeared to be ARG-1 dependent (74).

Insight into the molecular mechanisms responsible for the suppression of adaptive immune responses in aged mice has revealed that the suppressive properties of Gr1⁺CD11b⁺ cells may be regulated through a modulation of the PI3K–Akt signaling pathway, resulting in a depressed Akt activation poststimulation. Akt plays important roles in many cellular processes such as glucose metabolism, cell proliferation, apoptosis, transcriptional regulation, cellular migration, and inflammation (75–79). We have found that stimulated Gr1⁺CD11b⁺ cells from the SPLs of aged animals had a diminished capacity to activate Akt, resulting in a reduced ability to inactivate the downstream enzyme, GSK3β. Under conditions where cellular GSK3β activity is sustained postactivation, increases in the magnitude and duration of inflammatory responses occur through compromises in important feedback mechanisms necessary for resolution of inflammation (46, 47). It has been recently shown that dendritic cells generated from CD14⁺ monocytes from aged human donors also exhibit an impaired Akt activation (42). This signaling defect has been implicated as being responsible for proinflammatory status “aged” monocytes (42).

Akt activity can be controlled in many ways (75, 80–82). We believe that the elevated basal expression of SHIP1 in Gr1⁺CD11b⁺ cells from the SPLs of aged mice may be responsible for the reduction in Akt activation in these cells subsequent to their stimulation through mechanisms that we have recently reported (26, 83). An increased expression of phosphatase and tensin homologue deleted on chromosome 10 (PTEN), another enzyme that inhibits the Akt activation by limiting inositol trisphosphate, appears to be responsible for the observed decreases in the phosphorylation of Akt in activated human dendritic cells, and cholesterol increases in the lipid rafts of lymphocyte membranes has...
been determined to be responsible for the decreases in Akt activity in aged lymphocytes (42, 56, 80).

Because GSK3β activities were elevated in stimulated Gr1+CD11b+ cells from aged mice, the addition of a specific GSK3 inhibitor to Gr1+CD11b+ cells from spls of aged mice significantly decreased the production of proinflammatory cytokines and iNOS activity postactivation but had no effect on inductive ARG-1 activity. Other investigators demonstrated that active GSK3 is necessary for proinflammatory cytokine production (44, 46). Inhibition of GSK3 activity resulted in up to 90% reduction in IL-12 (p40), IL-6, and NO production by activated cells (46, 84). Notably, the Gsk3 inhibitor had its maximal normalizing effects on TNF-α and NO production also depends upon increased activity of GSK3β (85). Our unpublished observations demonstrate that SHIP1 expression becomes markedly elevated in BM-derived macrophages from young animals treated with small doses of IFN-γ, suggesting that the IFN-γ constitutively produced by lymphocytes from aged animals could negatively influence the PI3K–Akt signaling pathway and GSK3β activity in hematopoietic cells via an upregulation of SHIP1 expression.

The dysregulation caused by maintenance of GSK3β activity postactivation is not restricted only to the myeloid lineage cells. It appears that abnormal GSK3β activity is also found in T cells (86). It is well known that simultaneous costimulation of CD3 and CD28 activity in T cells in their BM, blood, and secondary lymphoid organs. Investigations into the molecular mechanisms responsible for mediating these suppressive influences of immature myeloid cells from aged mice has uncovered a key role for the depressed activation of the PI3K–Akt signaling pathway. The data presented in this paper demonstrate that depressed activation of the PI3K-Akt signaling pathway could compromise normal myeloid and lymphoid cell functions during the aging process.

**Disclosures**

The authors have no financial conflicts of interest.

**References**


In summary, our studies have demonstrated that healthy aged mice harbor increased numbers of suppressive immature myeloid cells in their BM, blood, and secondary lymphoid organs. Investigations into the molecular mechanisms responsible for mediating these suppressive influences of immature myeloid cells from aged mice has uncovered a key role for the depressed activation of the PI3K–Akt signaling pathway followed by a sustained enzymatic activity of GSK3β. The data presented in this paper demonstrate that depressed activation of the PI3K-Akt signaling pathway could compromise normal myeloid and lymphoid cell functions during the aging process.


