



# Phenotype and Function of Myeloid-Derived Suppressor Cells Induced by *Porphyromonas gingivalis* Infection

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**ABSTRACT** *Porphyromonas gingivalis*, a major etiologic agent of periodontitis, has been reported to induce the expansion of myeloid-derived suppressor cells (MDSC); however, little is known regarding the subpopulations of MDSC expanded by *P. gingivalis* infection. Flow cytometry was used to evaluate bone marrow and spleen cells from mice infected with *P. gingivalis* and controls for surface expression of CD11b, Ly6G, and Ly6C. To characterize the phenotype of MDSC subpopulations induced by infection, cells were sorted based on the differential expression of Ly6G and Ly6C. Moreover, since MDSC are suppressors of T cell immune activity, we determined the effect of the induced subpopulations of MDSC on the proliferative response of OVA-specific CD4<sup>+</sup> T cells. Lastly, the plasticity of MDSC to differentiate into osteoclasts was assessed by staining for tartrate-resistant acid phosphatase activity. *P. gingivalis* infection induced the expansion of three subpopulations of MDSC (Ly6G<sup>++</sup> Ly6C<sup>+</sup>, Ly6G<sup>+</sup> Ly6C<sup>++</sup>, and Ly6G<sup>+</sup> Ly6C<sup>+</sup>); however, only CD11b<sup>+</sup> Ly6G<sup>+</sup> Ly6C<sup>++</sup>-expressing cells exerted a significant suppressive effect on T cell proliferation. Inhibition of proliferative responses required T cell-MDSC contact and was mediated by inducible nitric oxide synthase and cationic amino acid transporter 2 via gamma interferon. Furthermore, only the CD11b<sup>+</sup> Ly6G<sup>+</sup> Ly6C<sup>++</sup> subpopulation of MDSC induced by *P. gingivalis* infection was able to differentiate into osteoclasts. Thus, the inflammatory response induced by *P. gingivalis* infection promotes the expansion of immune-suppressive cells and consequently the development of regulatory inhibitors that curtail the host response. Moreover, monocytic MDSC have the plasticity to differentiate into OC, thus perhaps contributing to the OC pool in states of periodontal disease.

**KEYWORDS** periodontitis, *Porphyromonas gingivalis*, MDSC

Periodontitis is an infectious inflammatory process that affects the periodontium. The continuous inflammation characteristic of this disease underlies its chronic inflammatory nature, ultimately destroying the supportive tissues of teeth (1, 2). Current thought suggests that the development of periodontitis is associated with a shift from a balanced microbial community to an imbalanced one, with the ability to undermine the host response and allowing the persistence of pathogens in the inflammatory milieu (3, 4). Within the oral microflora, *Porphyromonas gingivalis* is considered a key pathogen able to exert an influence on the microbial environment and, consequently, in concert with other periodontal microorganisms, initiate and promote periodontitis (5, 6). *P. gingivalis* has a number of virulence factors by which it can escape or dampen host immunity, alter cytokine production, and affect the cell signaling mechanisms (7–10). Interestingly, studies in mice infected with *P. gingivalis*

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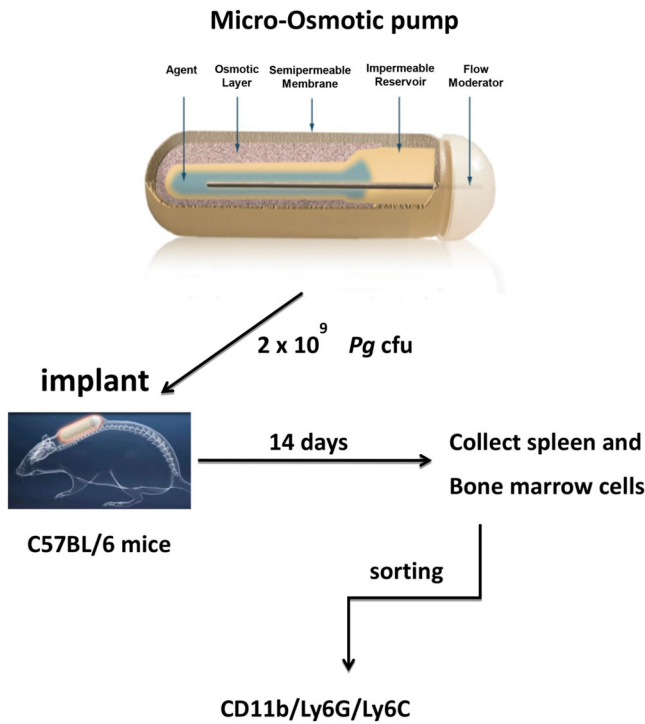
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have shown a downregulation of more than 1,000 genes modulating CD4 and CD8 activation and function, suggesting the suppression of these cells (11). Thus, although *P. gingivalis* can induce an inflammatory response, the resulting inflammation likely contributes to the characteristic chronicity of infection. While most studies have focused on understanding interactions of immune cells and *P. gingivalis* in the periodontium, little information is available on the effect *P. gingivalis* exerts systemically on the host immune response. This is most relevant, as *P. gingivalis* is able to disseminate from local sites of infection to the circulation and to distal sites (12, 13). Furthermore, purified T and B cells from infected human periodontal tissues express mainly memory phenotype (14, 15), and antigen-specific T cells can migrate from the circulation to the periodontium (16). Thus, exposure and priming of T cells and other immune cells likely occur systemically in the blood and/or in secondary lymphoid organs. Moreover, there is significant epidemiological evidence of associations between this bacterium and systemic disorders, where *P. gingivalis* infection does not cause the pathological condition but aggravates the severity of systemic diseases (17–21).

Cytokines released systemically in states of immune stress induce the expansion of myeloid-derived suppressor cells (MDSC) generated from bone marrow (BM) hematopoietic precursors. Under healthy conditions, the majority of MDSC reside in the BM and differentiate into mature granulocytes, macrophages, or dendritic cells involved in regulating hyperactive or autoimmune responses, whereas a small proportion of MDSC are found in blood and spleen. In response to inflammation and infection, MDSC rapidly expand without differentiating into mature cells and enter lymphoid organs and peripheral tissues (22). Upon constant antigenic stimulation, as in chronic infections, expansion and accumulation of MDSC are significantly increased. This has been observed in parasitic (23), bacterial (24), and viral (25) infections. Systemic infection of mice with *P. gingivalis* using a chamber model of chronic periodontitis has also been shown to induce the expansion of MDSC (26). MDSC have a striking ability to suppress immune responses. They can suppress effector T cells directly by depriving them of essential nutrients or indirectly via the recruitment of T regulatory cells (22, 27, 28). MDSC can also modulate the response of other immune cells (29, 30). Thus, increasing evidence suggests that MDSC postpone host pathogen clearance and contribute to the critical balance between pathogen eradication and pathogenicity (23, 25). It is currently known that MDSC represent a heterogeneous population of cells, and that the diverse subpopulations have differential biological functions; however, characterization of these subpopulations is required to enable precise therapeutic targeting (22, 31). MDSC not only exert powerful immune regulation on immune cells but also differentiate into osteoclast (OC) progenitors (32, 33). This cell plasticity has biological significance; hence, MDSC may contribute not only to the immune inhibition observed in periodontal disease and with *P. gingivalis* infection but also to the increased number and activity of OC seen in chronic periodontitis (34).

In mice, MDSC are identified by the expression of the myeloid markers CD11b and Gr-1. Gr-1<sup>+</sup> cells are granulocytic and monocytic cells characterized by the expression of CD11b<sup>+</sup> Ly6G<sup>+</sup> Ly6C<sup>low</sup> or CD11b<sup>+</sup> Ly6G<sup>-</sup> Ly6C<sup>hi</sup> phenotype, respectively (35). Interestingly, it has been reported that monocytic MDSC are the main type of cells involved in chronic infections (36). Here, we report the phenotype of MDSC subpopulations induced by *P. gingivalis* systemic infection, the ability of MDSC to suppress T cell-proliferative responses, the mechanisms mediating such an effect, and the plasticity of MDSC to differentiate into OC. Our findings reveal, for the first time, that systemic infection with *P. gingivalis* induces the expansion of three subpopulations of MDSC, as suggested by the differential expression of Ly6G and Ly6C. Of these, only one subpopulation was significantly suppressive of CD4<sup>+</sup> T cell-proliferative responses and was able to differentiate into OC. Furthermore, nitric oxide synthase (NOS2; iNOS) and gamma interferon (IFN- $\gamma$ ), induced upon infection with *P. gingivalis*, are critical inflammatory factors conducive to the inhibition of T cell activity.

## Mouse infection Model



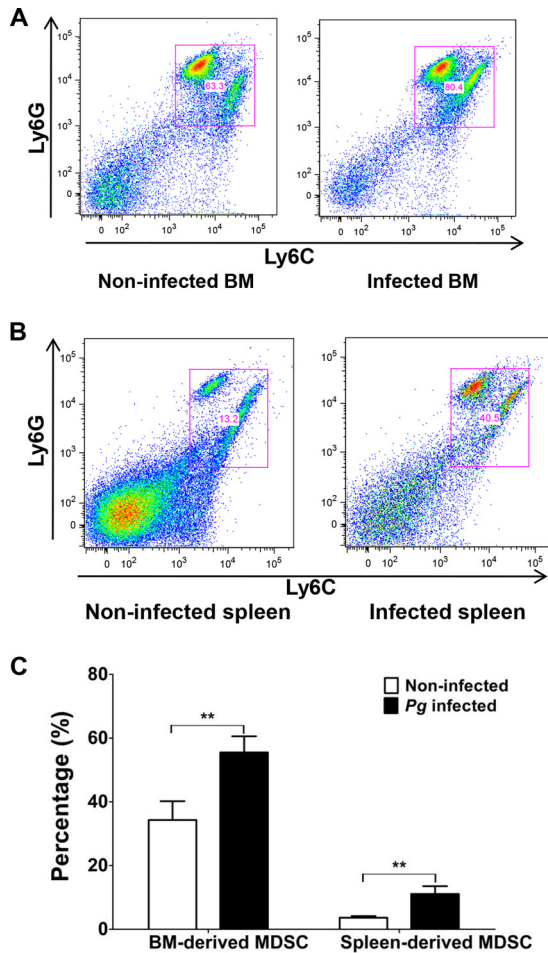
**FIG 1** Infection model. A suspension of freshly harvested *P. gingivalis* was loaded into the microosmotic pumps (100  $\mu$ l/pump) at a concentration of  $2 \times 10^{10}$  bacterial cells/ml ( $\sim 2 \times 10^9$  CFU). Micropumps filled with PBS served as controls. Pumps were then implanted into the dorsolumbar region of mice and left undisturbed for 14 days according to the manufacturer’s instructions. Microosmotic pumps delivered bacteria or PBS daily at a continuous and controlled rate (0.25  $\mu$ l/h).

### RESULTS

**Infection with *P. gingivalis* induces the expansion of MDSC.** Previous studies have shown that MDSC play a critical role in inflammatory immune responses resulting from bacterial infections (37, 38). Furthermore, infection with *P. gingivalis* has been shown to expand the Gr-1<sup>+</sup> Mac-1<sup>+</sup> (CD11b/CD18) cell population (26). The epitope Gr-1 is present on Ly6G and Ly6C glycoproteins, encoded by separate genes and expressed on granulocytic and monocytic cells, and the differential expression of these molecules can differentiate MDSC into separate subpopulations (22, 39). However, it is not known if MDSC differentially express Ly6G and Ly6C as a result of *P. gingivalis* infection. Therefore, C57BL/6 mice were *P. gingivalis* infected or left uninfected (i.e., phosphate-buffered saline [PBS] treatment) using microosmotic pumps, which allowed the daily delivery of bacteria or PBS for 14 days (Fig. 1). On day 14, mice were sacrificed and BM and spleen cells from infected and noninfected control mice were assessed for the expression of Ly6G and Ly6C by flow cytometry. The cells were gated on CD11b<sup>+</sup> expression and then further delineated based on the expression of Ly6G and Ly6C surface antigens (Fig. 2A and B). A significant increase in the percentage of MDSC expressing these surface molecules was observed in the BM and spleen cells derived from infected mice compared to the noninfected control (Fig. 2C).

Since Toll-like receptors are critical for the induction of innate and adaptive host immune responses (40, 41), we next assessed the roles of TLR2, TLR4, and MyD88 in the expansion of MDSC induced by *P. gingivalis* infection. The induced expansion of MDSC in infected wild-type (WT) mice was similarly observed in infected TLR2<sup>-/-</sup>, TLR4<sup>-/-</sup>, and MyD88<sup>-/-</sup> mice (not shown). These results suggest that the expansion of MDSC is not contingent on TLR2, TLR4, or MyD88 signaling.

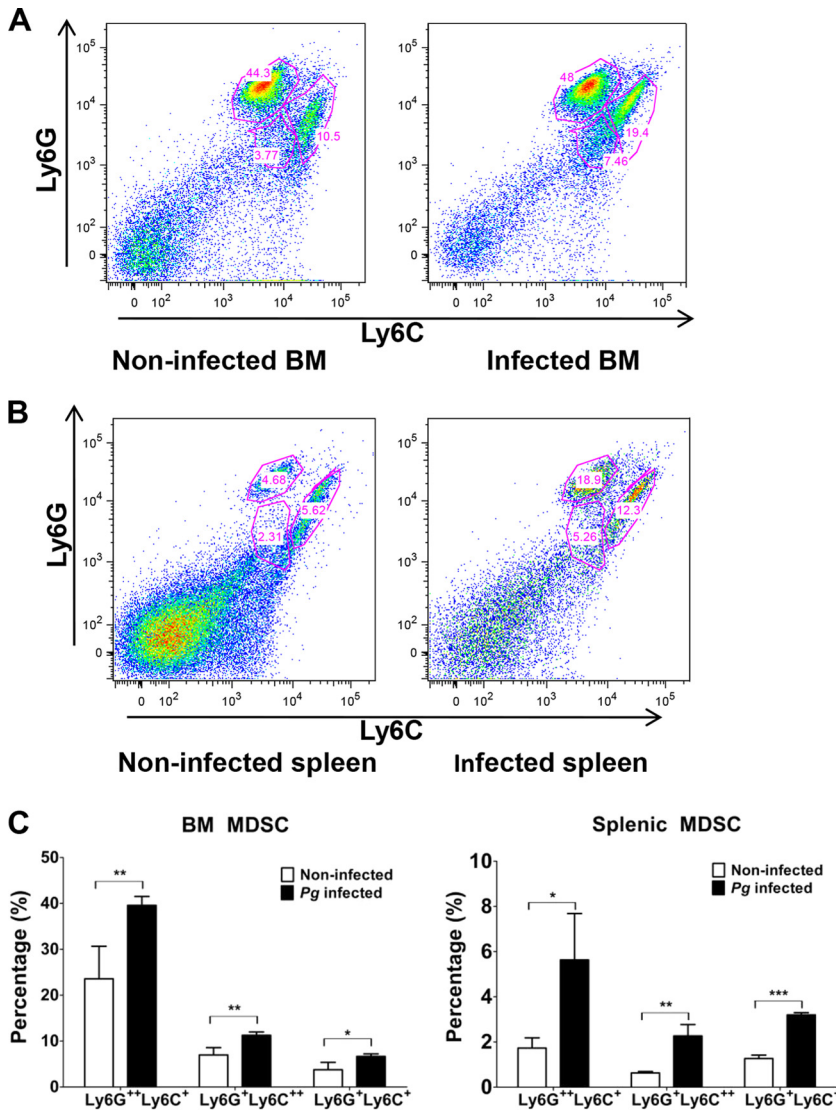
We next determined the phenotype of the subpopulations of MDSC expanded upon *P. gingivalis* infection. Using the infection model described above, BM and spleen cells



**FIG 2** Expansion of MDSC by *P. gingivalis* infection. A suspension of *P. gingivalis* was loaded into the microosmotic pumps (100  $\mu$ l/pump) at a concentration of  $2 \times 10^{10}$  bacterial cells/ml. Micropumps filled with PBS served as controls. Pumps were then implanted into the dorsolumbar region of mice and left undisturbed for 14 days according to the manufacturer's instructions. At the end of 14 days, mice were sacrificed and BM and spleen cells from infected and noninfected mice were harvested and stained for CD11b (FITC), Ly6G (APC), and Ly6C (PE), characteristic markers of MDSC. For flow cytometry, cells were gated on the CD11b<sup>+</sup> population and then analyzed for Ly6G<sup>+</sup> and Ly6C<sup>+</sup> cells. (A and B) Data expressed as dot plots showing expression of Ly6G<sup>+</sup> and Ly6C<sup>+</sup> cells in BM (A) and spleens (B) of infected and noninfected control mice. (C) Graph representing percentages of Ly6G<sup>+</sup> and Ly6C<sup>+</sup> cells. Data are expressed as the means  $\pm$  standard errors of the means (SEM) from three independent experiments. *Pg*, *P. gingivalis*. \*\*,  $P < 0.01$ .

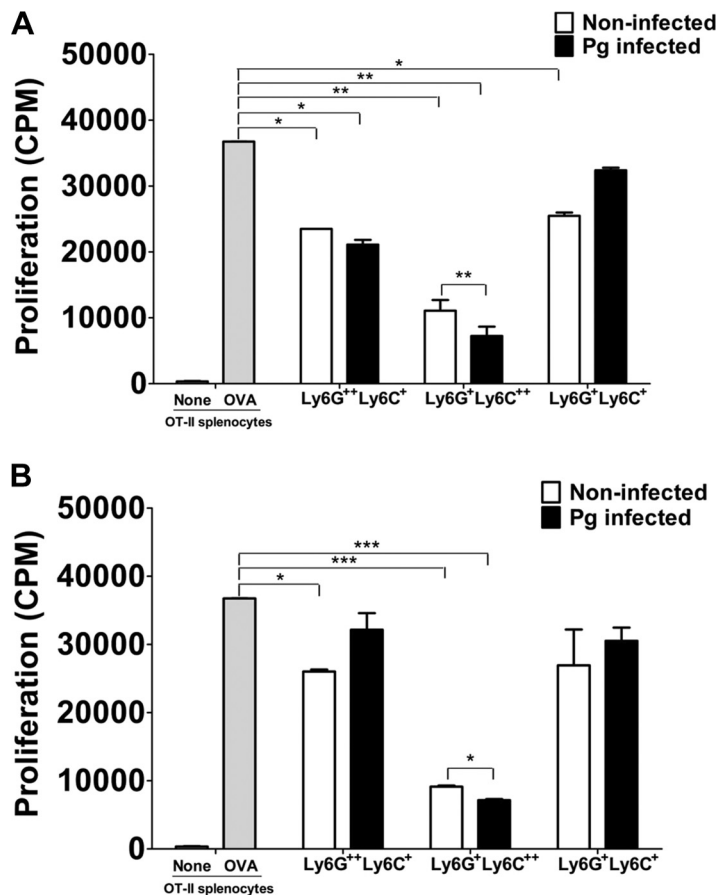
were obtained from infected and noninfected control mice, and the gated CD11b<sup>+</sup> cells were sorted into different subpopulations of MDSC based on their differential expression of Ly6G and Ly6C. Infection with *P. gingivalis* resulted in the expansion of three subpopulations of MDSC, specifically, Ly6G<sup>++</sup> Ly6C<sup>+</sup>, Ly6G<sup>+</sup> Ly6C<sup>++</sup>, and Ly6G<sup>+</sup> Ly6C<sup>+</sup> (Fig. 3A and B). It is worth noting that the percentages of the three subpopulations of MDSC identified by sorting and derived from either the BM or spleen of *P. gingivalis*-infected mice were significantly higher than those seen with cells derived from noninfected control mice (Fig. 3C), suggesting that infection with *P. gingivalis* induces the overall expansion of MDSC.

**MDSC inhibit CD4<sup>+</sup> T cell proliferation.** MDSC are well known for their ability to inhibit T-cell proliferation under pathological conditions, especially in chronic infections where expansion of MDSC is most notable (22, 36). Therefore, we wanted to determine if the sorted subpopulation of MDSC induced by *P. gingivalis* infection exerted an immunosuppressive effect on CD4<sup>+</sup> T cell-proliferative responses. Each of the three subpopulations of MDSC derived from the BM and spleens of infected and noninfected



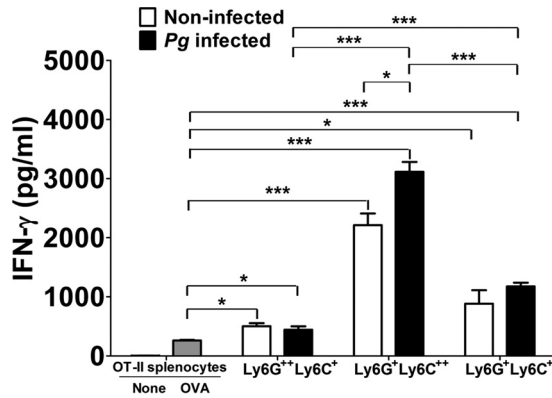
**FIG 3** Subpopulations of *P. gingivalis*-induced MDSC differentially express Ly6G and Ly6C surface antigens. A suspension of *P. gingivalis* was loaded into the microosmotic pumps (100  $\mu$ l/pump) at a concentration of  $2 \times 10^{10}$  bacterial cells/ml. Micropumps filled with PBS served as controls. Pumps were then implanted into the dorsolumbar region of mice and left undisturbed for 14 days according to the manufacturer's instructions. At the end of 14 days, mice were sacrificed and BM and spleen cells from infected and noninfected mice were harvested and stained for CD11b (FITC), Ly6G (APC), and Ly6C (PE). For flow cytometry, cells were gated on the CD11b<sup>+</sup> population and then sorted into subpopulations. Data are expressed as dot plots of cells showing Ly6G<sup>++</sup>Ly6C<sup>+</sup>, Ly6G<sup>+</sup>Ly6C<sup>++</sup>, and Ly6G<sup>+</sup>Ly6C<sup>+</sup> subpopulations of cells from the BM (A) and spleens (B) of infected and noninfected control mice. (C) Graph showing the percentages of Ly6G<sup>++</sup>Ly6C<sup>+</sup>, Ly6G<sup>+</sup>Ly6C<sup>++</sup>, and Ly6G<sup>+</sup>Ly6C<sup>+</sup> MDSC subpopulations derived from the BM and spleens of infected and noninfected control mice. Data are expressed as the means  $\pm$  SEM from three independent experiments. \*,  $P < 0.05$ ; \*\*,  $P < 0.01$ ; \*\*\*,  $P < 0.001$ .

control mice were cultured with T cell receptor-transgenic OT-II spleen cells and stimulated with OVA<sub>323-339</sub> peptide for 72 h. The cultures were pulsed for the last 24 h with [<sup>3</sup>H]thymidine, and the proliferative activity was expressed as the amount of [<sup>3</sup>H]thymidine uptake by CD4<sup>+</sup> proliferating T cells (counts per minute). Our results showed that CD11b<sup>+</sup> Ly6G<sup>+</sup> Ly6C<sup>++</sup>-expressing cells derived from BM ( $P < 0.01$ ) and from spleen ( $P < 0.001$ ) significantly inhibited the proliferation of CD4<sup>+</sup> T cells compared to controls, i.e., OT-II spleen cells stimulated with OVA peptide (Fig. 4A and B). However, when the inhibitory effect of this subpopulation of MDSC from *P. gingivalis*-infected mice was compared to that from noninfected mice, BM-derived MDSC (Fig. 4A) obtained from infected mice showed a significantly ( $P < 0.01$ ) greater



**FIG 4** Immunosuppressive activity of T cell proliferation by sorted MDSC. A suspension of *P. gingivalis* was loaded into the microosmotic pumps (100  $\mu$ l/pump) at a concentration of  $2 \times 10^{10}$  bacterial cells/ml. Micropumps filled with PBS served as controls. Pumps were then implanted into the dorsolumbar region of mice and left undisturbed for 14 days according to the manufacturer's instructions. At the end of 14 days, mice were sacrificed and BM and spleen cells from infected and noninfected mice were harvested and sorted into subpopulations of MDSC expressing the Ly6G<sup>++</sup> Ly6C<sup>+</sup>, Ly6G<sup>+</sup> Ly6C<sup>++</sup>, and Ly6G<sup>+</sup> Ly6C<sup>+</sup> phenotypes. The subpopulations of MDSC from BM (A) and spleens (B) of *P. gingivalis*-infected or noninfected control mice were then cocultured with spleen cells from OT-II mice and stimulated with the OVA<sub>323-339</sub> peptide for 72 h. The cultures were pulsed with [<sup>3</sup>H]thymidine during the last 24 h to assess CD4<sup>+</sup> T cell proliferation. Control cultures included OT-II spleen cells with or without the OVA<sub>323-339</sub> peptide. Proliferative responses are a measure of [<sup>3</sup>H]thymidine uptake and are expressed as counts per minute (CPM). Data represent the means  $\pm$  SEM from three independent experiments. \*,  $P < 0.05$ ; \*\*,  $P < 0.01$ ; \*\*\*,  $P < 0.001$ .

inhibition of CD4<sup>+</sup> T cell proliferation than that observed with splenic MDSC ( $P < 0.05$ ) (Fig. 4B). These results suggest that while naturally occurring MDSC expressing CD11b<sup>+</sup> Ly6G<sup>+</sup> Ly6C<sup>++</sup> have an ability to inhibit T cell proliferation, infection with *P. gingivalis* exacerbates the inhibitory effect exerted by this specific subpopulation. Interestingly, BM CD11b<sup>+</sup> Ly6G<sup>++</sup> Ly6C<sup>+</sup> MDSC derived from infected or noninfected mice (Fig. 4A) showed significant ( $P < 0.05$ ) inhibitory activity compared to controls, but no difference was seen between the infected and noninfected groups. A similar significant inhibitory effect was observed with splenic CD11b<sup>+</sup> Ly6G<sup>++</sup> Ly6C<sup>+</sup> MDSC obtained from noninfected mice but not from infected mice compared to controls, and no difference was seen in the inhibitory activity between the infected and noninfected groups (Fig. 4B). Furthermore, the subpopulation of MDSC expressing CD11b<sup>+</sup> Ly6G<sup>+</sup> Ly6C<sup>+</sup> derived from BM, but not from the spleen of noninfected mice, significantly (\*,  $P < 0.05$ ) inhibited CD4<sup>+</sup> T cell-proliferative responses. As seen with CD11b<sup>+</sup> Ly6G<sup>++</sup> Ly6C<sup>+</sup> cells, no statistical difference was observed between the infected and noninfected subpopulation of CD11b<sup>+</sup> Ly6G<sup>+</sup> Ly6C<sup>+</sup> MDSC derived from BM and spleen (Fig. 4A and B). These data suggest that there is a differential regulation in the inhibitory activity



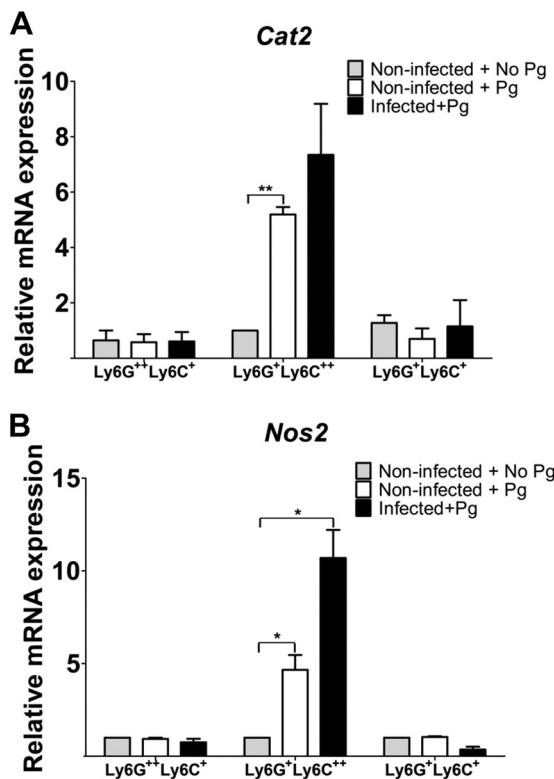
**FIG 5** Cytokine analysis. Supernatants from cocultures of spleen cells from OT-II mice and sorted subpopulations of MDSC obtained from the BM of infected and noninfected mice were harvested after 72 h of incubation in the presence or absence of the OVA<sub>323-339</sub> peptide. The level of IFN-γ in the culture supernatants was determined by ELISA according to the manufacturer’s instructions. Data represent the means ± SEM from three independent experiments. \*, *P* < 0.05; \*\*, *P* < 0.01; \*\*\*, *P* < 0.001.

of MDSC expressing the phenotype CD11b<sup>+</sup> Ly6G<sup>++</sup> Ly6C<sup>+</sup> or CD11b<sup>+</sup> Ly6G<sup>+</sup> Ly6C<sup>+</sup> compared to the subpopulation of MDSC expressing CD11b<sup>+</sup> Ly6G<sup>+</sup> Ly6C<sup>++</sup> surface molecules. Our findings further suggest that the three subpopulations of MDSC can have a suppressive effect on CD4<sup>+</sup> T cell proliferation regardless of *P. gingivalis* infection.

Since inflammation is critical for the suppressive effect of MDSC (42, 43), we next determined the level of the inflammatory cytokine IFN-γ. Spleen cells from OT-II mice were cocultured with subpopulations of BM-derived MDSC, and the level of IFN-γ produced was assessed by enzyme-linked immunosorbent assay (ELISA). Significantly higher levels of IFN-γ were detected in supernatants of cocultures of OT-II spleen cells with each of the subpopulations of MDSC than in supernatants from cultures of OT-II spleen cells alone stimulated with the OVA peptide (Fig. 5). However, supernatants from cocultures containing CD11b<sup>+</sup> Ly6G<sup>+</sup> Ly6C<sup>++</sup> MDSC contained significantly (*P* < 0.05) higher levels of IFN-γ when the cells were derived from *P. gingivalis*-infected mice compared to noninfected controls (Fig. 5). Moreover, supernatants from cultures with CD11b<sup>+</sup> Ly6G<sup>+</sup> Ly6C<sup>++</sup> MDSC had significantly (*P* < 0.001) higher levels of IFN-γ than supernatants from cultures containing the other subpopulations of MDSC derived from *P. gingivalis*-infected mice (Fig. 5). It is noteworthy that although CD11b<sup>+</sup> Ly6G<sup>+</sup> Ly6C<sup>++</sup> MDSC were the main players in the inhibition of CD4<sup>+</sup> T cell proliferation, this subpopulation of MDSC was also able to promote the induction of the highest level of IFN-γ, especially when these cells were obtained from *P. gingivalis*-infected mice. Results with spleen-derived MDSC were similar to those seen with BM-derived MDSC (not shown).

**Nos2 and Cat2 mRNA are highly expressed in CD11b<sup>+</sup> Ly6G<sup>+</sup> Ly6C<sup>++</sup> MDSC, and inhibition of CD4<sup>+</sup> T cell proliferation is dependent on NOS2 and IFN-γ.**

An important factor involved in the regulation of immunity is the cationic amino acid L-arginine. MDSC can affect T cell-proliferative responses by L-arginine depletion through NOS2 and Arg1 enzymatic activity (44, 45). In the presence of IFN-γ, MDSC metabolize L-arginine by increasing nitric oxide (NO) production via NOS2 induction (46, 47), and in the presence of interleukin-4 (IL-4) or IL-13, MDSC regulate L-arginine depletion via Arg1 activity (48, 49). Importantly, regardless of the cytokine environment resulting in the corresponding regulation of L-arginine metabolism that consequently affects T cell-proliferative responses, the suppressive activity of MDSC also requires contact between T cells and MDSC (44). L-Arginine is present in the extracellular microenvironment, and thus transport systems are critical for L-arginine uptake. Studies have shown that CAT2 is the main L-arginine transporter in various cells, including MDSC (50). Moreover, CAT2 has been shown to be important in the establishment of



**FIG 6** Expression of *Nos2* and *Cat2* mRNA in MDSC. Sorted subpopulations of MDSC from the BM of infected and noninfected mice were cultured in the presence or absence of *P. gingivalis* for 48 h. Total RNA was isolated, and the presence of mRNA for *Cat2* (A) and *Nos2* (B) was assessed by real-time PCR using SYBR green. HPRT was used as the endogenous control. Data represent the means  $\pm$  SEM from three independent experiments. \*,  $P < 0.05$ ; \*\*,  $P < 0.01$ .

efficient cell-mediated immunity (51), and MDSC recruited to sites of inflammation and tumor growth express increased amounts of *Cat2* (50). Production of IFN- $\gamma$  is characteristic of a number of microbial infections, including infection with *P. gingivalis* (52–55), and an inflammatory milieu has been shown to be important for the suppressive activity of MDSC (42, 43). Therefore, we wanted to determine if *P. gingivalis*-induced MDSC exhibited increased expression of *Cat2* and *NOS2* mRNA. Since we had detected IFN- $\gamma$  in cocultures of MDSC and OT-II spleen cells, we also wanted to determine if IFN- $\gamma$  played a role in the observed suppressive effect of MDSC on T cell proliferation. Due to the low yield of splenic MDSC, we only used sorted subpopulations of MDSC from the BM of infected and noninfected mice to carry out these quantitative, real-time PCR studies. For these studies, subpopulations of MDSC were cultured in the presence or absence of *P. gingivalis* for 48 h and then assessed for *Nos2* and *Cat2* mRNA expression. The greatest increase in *Cat2* mRNA expression occurred in BM-derived CD11b<sup>+</sup> Ly6G<sup>+</sup> Ly6C<sup>++</sup> cells obtained from infected mice and restimulated *in vitro* with *P. gingivalis* (Fig. 6A). Interestingly, the level of *Cat2* mRNA was low in CD11b<sup>+</sup> Ly6G<sup>++</sup> Ly6C<sup>+</sup> cells under all experimental conditions compared with the other two subpopulations of MDSC (Fig. 6A). While an increased level of *Cat2* mRNA expression was observed in CD11b<sup>+</sup> Ly6G<sup>+</sup> Ly6C<sup>+</sup> MDSC obtained from infected mice restimulated *in vitro*, the increase was only slightly higher than the level seen in cells from noninfected mice that were not stimulated *in vitro* with *P. gingivalis* (Fig. 6A). These results suggest that *Cat2* mRNA expression is differentially regulated in MDSC subpopulations, and that antigen exposure is important for the increased expression of *Cat2* mRNA only in CD11b<sup>+</sup> Ly6G<sup>+</sup> Ly6C<sup>++</sup> MDSC, even when cells were derived from noninfected mice but exposed to *P. gingivalis* *in vitro*.

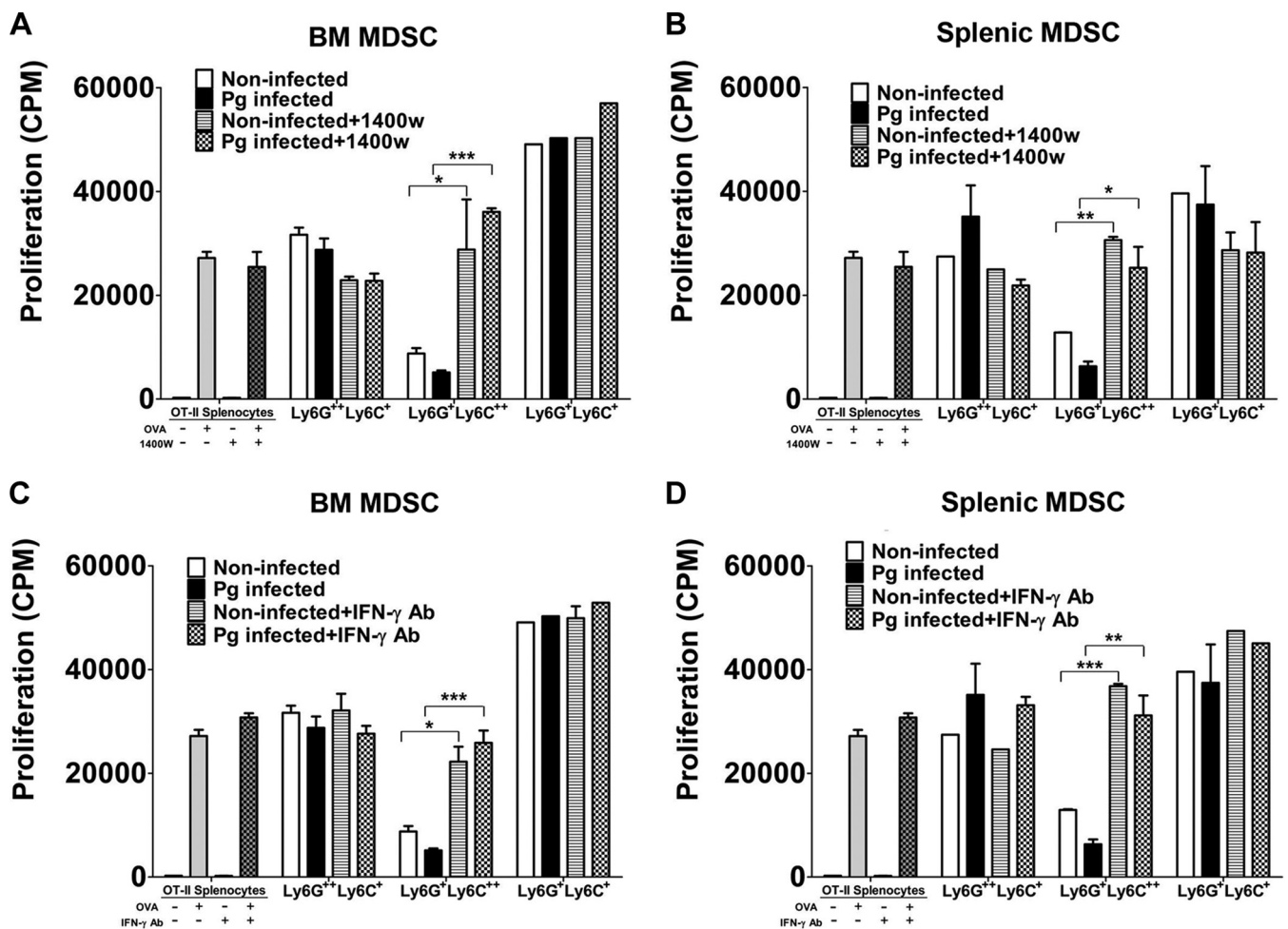
Assessment of *Nos2* mRNA also revealed that CD11b<sup>+</sup> Ly6G<sup>+</sup> Ly6C<sup>++</sup> MDSC exhibited the highest level of *Nos2* expression when cells were derived from infected mice



and restimulated with *P. gingivalis* *in vitro* (Fig. 6B). Some increase in the level of *Nos2* mRNA expression was seen in CD11b<sup>+</sup> Ly6G<sup>+</sup> Ly6C<sup>++</sup> MDSC from noninfected mice stimulated *in vitro* with *P. gingivalis* compared to those not stimulated *in vitro*, although the increase was about 2-fold lower than that seen in CD11b<sup>+</sup> Ly6G<sup>+</sup> Ly6C<sup>++</sup> MDSC obtained from infected mice and restimulated *in vitro* (Fig. 6B). Only a low level of *Nos2* mRNA expression was detected in CD11b<sup>+</sup> Ly6G<sup>+</sup> Ly6C<sup>+</sup> and CD11b<sup>+</sup> Ly6G<sup>++</sup> Ly6C<sup>+</sup> MDSC under all experimental conditions. These subpopulations of MDSC showed a slight decrease in the level of *Nos2* mRNA expression in cells derived from infected mice and restimulated with *P. gingivalis* *in vitro* compared to that seen with cells from noninfected mice (Fig. 6B). Although the relative level of *Cat2* mRNA expression is lower than that of *Nos2* expression, the results suggest that there is a correspondence in the increased expression of *Nos2* and *Cat2* mRNA mainly in CD11b<sup>+</sup> Ly6G<sup>+</sup> Ly6C<sup>++</sup> MDSC obtained from *P. gingivalis*-infected mice and restimulated with *P. gingivalis* *in vitro* (Fig. 6B).

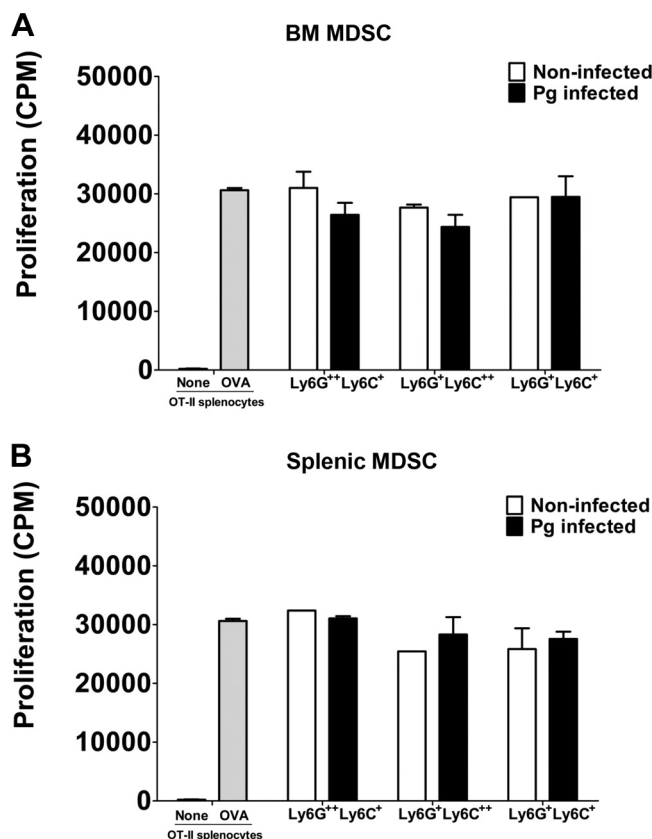
We next determined if MDSC-mediated inhibition of CD4<sup>+</sup> T cell-proliferative responses was dependent on NOS2 and IFN- $\gamma$ , especially that exerted by CD11b<sup>+</sup> Ly6G<sup>+</sup> Ly6C<sup>++</sup>-expressing cells. Subpopulations of MDSC obtained from the BM and spleens of infected and noninfected control mice were cocultured with spleen cells derived from OT-II mice to assess the proliferative response of CD4<sup>+</sup> T cells upon stimulation with OVA<sub>323–339</sub> peptide as described above. However, in this experimental series, inhibitors of NOS2 (1400W) and IFN- $\gamma$  (IFN- $\gamma$  receptor 1 blocking antibody) were added to or left out of cocultures of cells. Controls included OT-II spleen cells with or without OVA peptide and with or without the corresponding inhibitors. The inhibitory effect of the BM-derived CD11b<sup>+</sup> Ly6G<sup>+</sup> Ly6C<sup>++</sup> MDSC from *P. gingivalis*-infected mice on the proliferative activity of CD4<sup>+</sup> T cells was significantly ablated ( $P < 0.001$ ) in the presence of the NOS2 inhibitor (Fig. 7A). Moreover, this subpopulation of MDSC, when obtained from noninfected *P. gingivalis* mice, also showed a significant ( $P < 0.05$ ) reduction in its ability to suppress the proliferative activity of CD4<sup>+</sup> T cells in the presence of the NOS2 inhibitor (Fig. 7A). Furthermore, the ability of the spleen-derived CD11b<sup>+</sup> Ly6G<sup>+</sup> Ly6C<sup>++</sup> MDSC to suppress the proliferative responses of CD4 T cells was also ablated by the NOS2 inhibitor (Fig. 7B). Finally, no significant inhibitory effect was observed for the other subpopulations of MDSC (Fig. 7A and B). Interestingly, in the presence of the IFN- $\gamma$  blocking antibody, we observed results similar to those obtained in the presence of the NOS2 inhibitor (Fig. 7C and D). These results suggest that inhibition of CD4<sup>+</sup> T cell proliferation by CD11b<sup>+</sup> Ly6G<sup>+</sup> Ly6C<sup>++</sup> MDSC involves the presence of the inflammatory cytokine IFN- $\gamma$  and the consequent induction of NOS2 (45, 53).

**Cell-cell contact is necessary for the suppressive activity of MDSC.** Inhibition of T cell proliferation by MDSC often involves cell-cell contact not dependent on major histocompatibility complex (MHC) and mediated by mechanisms that involve, for instance, depletion of L-arginine (48, 53). Thus, we next determined if cell-cell contact played a role in the inhibition of CD4<sup>+</sup> T cell proliferation by MDSC subpopulations induced by *P. gingivalis*. Each of the three subpopulations of MDSC was cultured separately on top of the filter membranes of Transwell inserts (0.4- $\mu$ m pores) at a concentration of  $2 \times 10^5$  cells. The spleen cells derived from OT-II mice were cultured in the lower wells of the plates at a 1:1 ratio. The OVA<sub>323–339</sub> peptide was used to stimulate the proliferative response of CD4<sup>+</sup> T cells for 72 h, and the cells were pulsed with [<sup>3</sup>H]thymidine for the last 24 h for the assessment of CD4<sup>+</sup> T cell proliferation. Our findings revealed that the capacity to suppress CD4<sup>+</sup> T cell proliferation by all subpopulations of MDSC derived from BM (Fig. 8A) or spleen (Fig. 8B), and especially that exerted by CD11b<sup>+</sup> Ly6G<sup>+</sup> Ly6C<sup>++</sup>-expressing cells, was lost when T cell-MDSC contact was prevented (Fig. 8A and B). These studies suggest that cell-cell contact is critical for the suppression of CD4<sup>+</sup> T cell-proliferative responses by subpopulations of MDSC derived from noninfected and, in particular, *P. gingivalis*-infected mice.



**FIG 7** Inhibition of NOS2 and IFN- $\gamma$  prevents the suppression of OVA-specific CD4<sup>+</sup> T cell-proliferative responses by MDSC. Sorted subpopulations of MDSC from the BM and spleen of infected and noninfected mice were cocultured with spleen cells obtained from OT-II mice and stimulated with the OVA<sub>323-339</sub> peptide for 72 h. The inhibitor of NOS2 (1400W) was added to some cocultures of BM (A) or spleen (B) MDSC and OT-II-derived spleen cells. Controls included OT-II-derived spleen cells left unstimulated or stimulated with the OVA peptide in the presence or absence of the NOS2 inhibitor. The IFN- $\gamma$ -blocking antibody was added to other cocultures of BM (C) or spleen (D) MDSC and OT-II-derived spleen cells. Controls included OT-II-derived spleen cells stimulated with the OVA peptide or left unstimulated in the presence or absence of the IFN- $\gamma$  blocking antibody. [<sup>3</sup>H]thymidine was added to cocultures during the last 24 h of incubation for the assessment of proliferative responses. Proliferative responses are a measure of [<sup>3</sup>H]thymidine uptake and expressed as counts per minute (CPM). Data represent the means  $\pm$  SEM from three independent experiments. \*,  $P < 0.05$ ; \*\*,  $P < 0.01$ ; \*\*\*,  $P < 0.001$ .

**Differentiation of MDSC into OC.** Murine CD11b<sup>+</sup> cells from BM or spleen can become osteoclasts (OC), and human circulating CD14<sup>+</sup> monocytes can differentiate into OC (56). It has also been shown that MDSC can give rise to OC (33, 57). Therefore, in order to determine if the *P. gingivalis*-induced subpopulations of MDSC have the ability to differentiate into OC, sorted MDSC derived from the BM or spleen of *P. gingivalis*-infected and noninfected control mice were suspended in  $\alpha$ -10 medium and plated in 24-well plates at  $5 \times 10^4$  cells/well in the presence or absence of macrophage colony-stimulating factor (M-CSF) and RANKL. After 4 or 5 days, multinuclear OC were readily observed in cultures of CD11b<sup>+</sup> Ly6G<sup>+</sup> Ly6C<sup>++</sup> MDSC derived from the BM and spleen, respectively, whereas very few multinuclear OC were observed in cultures of CD11b<sup>+</sup> Ly6G<sup>+</sup> Ly6C<sup>+</sup> or CD11b<sup>+</sup> Ly6G<sup>++</sup> Ly6C<sup>+</sup> MDSC (Fig. 9A and B). To further confirm these observations, we next assessed the cells for the expression of tartrate-resistant acid phosphatase (TRAP), a specific marker of OC. As can be seen, CD11b<sup>+</sup> Ly6G<sup>+</sup> Ly6C<sup>++</sup>-expressing MDSC were indeed TRAP<sup>+</sup> (Fig. 9C and D). Importantly, a significantly higher number of TRAP<sup>+</sup> multinuclear osteoclasts differentiated from MDSC derived from *P. gingivalis*-infected mice compared to the number of TRAP<sup>+</sup> multinuclear osteoclasts in cultures of MDSC derived from noninfected controls (Fig. 9A



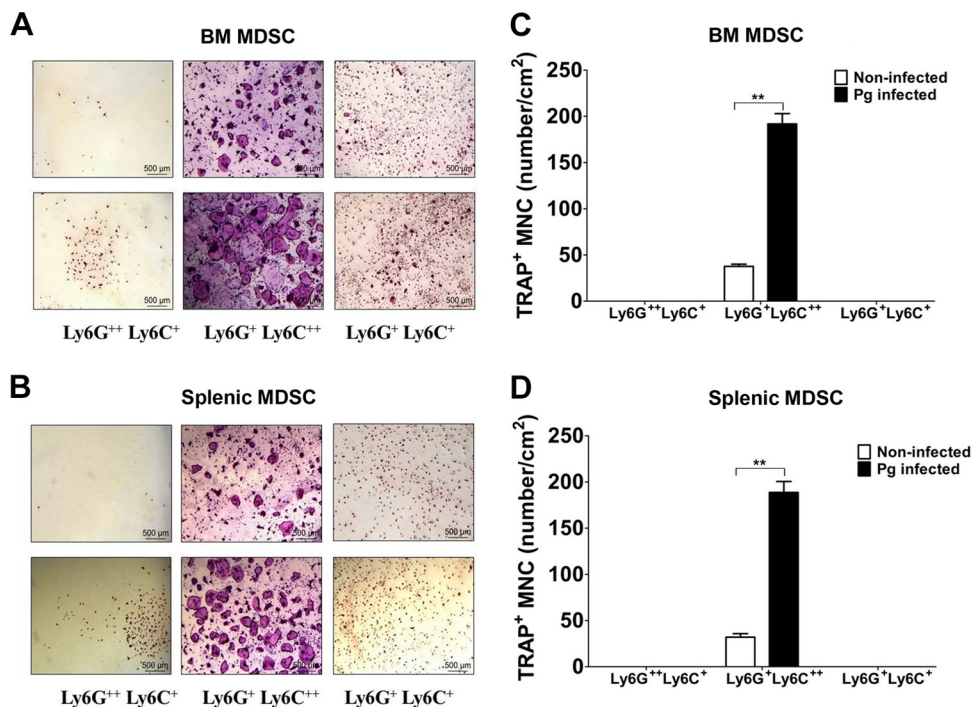
**FIG 8** MDSC-mediated suppression of T cell proliferation is contact dependent. Sorted subpopulations of MDSC were obtained from the BM (A) and spleens (B) of *P. gingivalis*-infected or noninfected mice and cultured in Transwell inserts, whereas OT-II-derived splenic cells were cultured in the corresponding plate wells at a 1:1 ratio in a modified MDSC suppression assay. Cultures were stimulated with the OVA<sub>323-339</sub> peptide for 72 h. Control cultures included OT-II spleen cells cultured with or without the OVA<sub>323-339</sub> peptide. [<sup>3</sup>H]thymidine was added to all cultures during the last 24 h of incubation. Proliferative responses are a measure of [<sup>3</sup>H]thymidine uptake and expressed as counts per minute (CPM). Data represent the means ± SEM from three independent experiments. \*, *P* < 0.05; \*\*, *P* < 0.01; \*\*\*, *P* < 0.001.

and B). These findings suggest that CD11b<sup>+</sup> Ly6G<sup>+</sup> Ly6C<sup>++</sup>-expressing MDSC have the plasticity to differentiate into OC, and as such they may constitute a source of OC involved in the destruction of bone upon *P. gingivalis* infection.

**DISCUSSION**

MDSC are one of the dominant immunosuppressive populations of cells that rapidly expand under infectious, inflammatory conditions, especially in chronic infections (23–26). Although *P. gingivalis* can induce inflammation, this inflammatory response is necessary and conducive to the eventual inhibition of immunity. By subverting the host response, *P. gingivalis* promotes a chronic, infectious, inflammatory state locally in the periodontium and the occurrence of pathological, systemic, inflammatory events (58–62). It is worth noting that *P. gingivalis* is able to disseminate from local sites of infection to the circulation and to distal sites (12, 13), and antigen-specific T cells can migrate from the circulation to the periodontium (16). Furthermore, T cells from infected human periodontal tissues express mainly a memory phenotype (14), suggesting that exposure and priming of T cells and other immune cells occur systemically in the blood and/or secondary lymphoid organs.

Since cytokines, induced systemically in states of immune stress such as infections, induce the expansion of MDSC, it is not surprising that chronic infection promoted by *P. gingivalis*, a key pathogen able to support the development of pathogenic microbiota by altering the equilibrium of commensal bacteria (3, 63), can result in the



**FIG 9** Subpopulation of MDSC has the ability to differentiate into OC. Sorted subpopulations of MDSC obtained from the BM and spleens of *P. gingivalis*-infected or noninfected mice were cultured in the presence of M-CSF. Cells were stimulated with RANKL for 4 to 5 days and then stained for TRAP activity. Osteoclasts derived from BM MDSC were detected by day 4 (A), whereas OC derived from splenic MDSC differentiated into OC by day 5 (B). A representative area of the cultures from each condition at  $\times 40$  magnification is shown. The number of TRAP-positive multinucleated cells (TRAP<sup>+</sup> MNC) in cultures of the different subpopulations of MDSC from BM (C) and spleen (D) was counted on days 4 and 5, respectively. Data are expressed as means  $\pm$  SEM from three independent experiments. \*\*,  $P < 0.01$ .

expansion of MDSC (26). Indeed, our findings are in line with those of Ezernitchi et al. (26), who demonstrated that *P. gingivalis* induced the expansion of Gr-1<sup>+</sup> Mac-1<sup>+</sup> (CD11b/CD18) cells. However, in the present study, using a micropump that constantly delivered *P. gingivalis*, thus reflecting a chronic infection, we show that *P. gingivalis* induces the expansion of three subpopulations of MDSC based on the differential expression of Ly6G and Ly6C. These two glycoproteins have the Gr-1 epitope, are encoded by separate genes, and are expressed in granulocytic and monocytic cells, respectively (22, 64). In mice, MDSC have been divided into granulocytic (Ly6G<sup>high</sup> Ly6C<sup>low</sup>) and monocytic (Ly6G<sup>low</sup> Ly6C<sup>high</sup>) cells based on the coexpression of CD11b, Ly6G, and Ly6C, nuclear morphology, and T cell-suppressive functions. Our findings revealed that infection with *P. gingivalis* induced the expansion of CD11b<sup>+</sup> Ly6G<sup>++</sup> Ly6C<sup>+</sup>, CD11b<sup>+</sup> Ly6G<sup>+</sup> Ly6C<sup>++</sup>, and CD11b<sup>+</sup> Ly6G<sup>+</sup> Ly6C<sup>+</sup> MDSC. Surprisingly, this expansion was not dependent on TLR2, TLR4, or MyD88 signaling. Studies by Delano et al. (65) demonstrated that expansion of CD11b<sup>+</sup> Gr-1<sup>+</sup> cells early in sepsis was dependent on MyD88 but not TLR4. However, if sepsis was prolonged, some expansion was observed in the absence of MyD88 signaling. It is known that *P. gingivalis* is a pathogen that can signal via TLR4 and TLR2, hence, perhaps the difference in the results of these studies can be explained by the longer infection period used in our studies and by the participation of compensatory mechanisms, thus involving signaling via different molecules.

Under healthy conditions, the majority of MDSC reside in the BM and differentiate into mature granulocytes, macrophages, or dendritic cells, but in response to inflammation, MDSC rapidly expand without differentiating into mature cells and enter lymphoid organs and peripheral tissues (42, 43, 53). Interestingly, although in this study all subpopulations of MDSC exerted an inhibitory effect on the proliferative activity of OVA-specific CD4<sup>+</sup> T cells derived from OT-II mice, the most significant inhibition of T

cell proliferation was seen with the subpopulation of MDSC expressing a CD11b<sup>+</sup> Ly6G<sup>+</sup> Ly6C<sup>++</sup> phenotype, especially those derived from *P. gingivalis*-infected mice. These findings suggest that *P. gingivalis* infection affects, in particular, the monocytic subpopulation of MDSC. In the case of CD11b<sup>+</sup> Ly6G<sup>++</sup> Ly6C<sup>+</sup> and CD11b<sup>+</sup> Ly6G<sup>+</sup> Ly6C<sup>+</sup> subpopulations, the MDSC from infected mice did not exhibit more inhibitory activity than those from noninfected mice. In fact, for these two subpopulations, it was the MDSC from the noninfected mice that exhibited slightly more inhibition of CD4<sup>+</sup> T cell proliferation. Furthermore, the same subpopulation of MDSC from different tissues did not necessarily show the same inhibitory effect, suggesting that MDSC with the same phenotype have different properties, perhaps due to the influence of the specific environment of the BM or spleen. While the granulocytic population of MDSC is characterized by the surface expression of Ly6G<sup>high</sup> Ly6C<sup>low</sup>, cells expressing CD11b<sup>+</sup> Ly6G<sup>+</sup> Ly6C<sup>+</sup> surface molecules detected in the present work may represent MDSC that are in transition to become granulocytic or monocytic cells. Whether further exposure to bacteria could push the cells to express a particular phenotype will have to be determined in future studies.

Surprisingly, cocultures of OT-II-derived spleen cells and CD11b<sup>+</sup> Ly6G<sup>+</sup> Ly6C<sup>++</sup> MDSC showed a highly significant increase in the production of IFN- $\gamma$  compared to the cocultures with the other MDSC subpopulations, and the increase was higher when cells were derived from *P. gingivalis*-infected than noninfected mice. It should be noted that the IFN- $\gamma$  detected was not produced by the MDSC based on *P. gingivalis* stimulation of the MDSC (not shown). However, one of the main cytokines produced by the MDSC in our hands (not shown) and those of others (66, 67) was IL-1, a cytokine shown to act directly on CD4<sup>+</sup> T cells and enhance their activation (68). IFN- $\gamma$  stimulates and enhances the production of IL-1 (69, 70), thus possibly creating a self-feeding activation circle between MDSC and the OVA-specific CD4<sup>+</sup> T cells. Strikingly, the monocytic MDSC were specifically affected, suggesting that the CD11b<sup>+</sup> Ly6G<sup>+</sup> Ly6C<sup>++</sup> MDSC were the main effector cells within the expanded MDSC subpopulations resulting from *P. gingivalis* infection.

It is well known that IFN- $\gamma$  induces the upregulation of NOS2 (iNOS), which catalyzes the synthesis of NO, a factor that is necessary and sufficient to inhibit T cell-proliferative responses. In line with findings of other investigators (53, 71, 72), our studies showed that inhibition of NOS2 and of IFN- $\gamma$  signaling abrogated the suppressive effect of *P. gingivalis*-induced MDSC on CD4<sup>+</sup> T cell proliferation. These observations indirectly suggest that secretion of NO requires surface and soluble signals, such as NOS2 and IFN- $\gamma$  from activated T cells, factors that contribute to immune suppression. Our results further demonstrated the importance of cell-to-cell contact for the inhibition of OVA-specific CD4<sup>+</sup> T cell-proliferative responses. Indeed, different suppressive mechanisms seem to be used by mouse and human MDSC, including cell-to-cell contact (67, 73). Thus, *P. gingivalis*-induced MDSC can inhibit T cell-proliferative responses by cell-to-cell contact in a manner that does not involve MHC antigens and also can use NOS2 induced by IFN- $\gamma$  to produce NO and consequently suppress T cell activity (44, 53). It is important to keep in mind that while in the present study we showed the importance of IFN- $\gamma$  in the indirect inhibition of T cell-proliferative responses, other cytokines, such as IL-13, granulocyte-macrophage colony-stimulating factor (GM-CSF), and IL-6, also have been shown to induce MDSC to suppress T cell activity (67, 74). Whether these cytokines also play a role in the inhibitory mechanisms generated as a result of *P. gingivalis* infection needs to be addressed in future studies.

In addition to the suppressive effect of MDSC on immune cells, we have demonstrated that MDSC can differentiate into OC cells, which is in line with findings of others (32, 33). However, it is noteworthy that in our investigations, only CD11b<sup>+</sup> Ly6G<sup>+</sup> Ly6C<sup>++</sup> MDSC had the ability to differentiate into TRAP<sup>+</sup> cells. Studies by Gao et al. (75) have demonstrated that in an inflammatory milieu, IFN- $\gamma$  indirectly promotes OC differentiation. Although in our studies OC differentiation involved only purified MDSC cultured in the presence of M-CSF and stimulated with RANKL, it would be interesting in future studies to determine if blocking IFN- $\gamma$  signaling in cocultures of CD4<sup>+</sup> T cells

and CD11b<sup>+</sup> Ly6G<sup>+</sup> Ly6C<sup>++</sup> MDSC would cause OC differentiation to be ablated. The plasticity of MDSC has biological significance, especially in the context of *P. gingivalis* infection, due to its association with periodontal disease, since its hallmark is the loss of alveolar bone. Hence, MDSC may contribute to the development and establishment of the disease process not only by exerting immune suppression but also by contributing to the increased number and activity of OC seen in chronic periodontitis (34).

## MATERIALS AND METHODS

**Mice.** C57BL/6 wild-type (WT) mice, OT-II transgenic mice, and TLR2<sup>-/-</sup>, TLR4<sup>-/-</sup>, and MyD88<sup>-/-</sup> mice (on the C57BL/6 background) were bred and maintained within an environmentally controlled, pathogen-free animal facility at the University of Alabama at Birmingham (UAB). Female mice (8 to 10 weeks of age) were used in this study. All studies were done according to National Institutes of Health guidelines, and all protocols were approved by the UAB Institutional Animal Care and Use Committee.

**Bacterial cultures.** *P. gingivalis* ATCC 33277 was cultured and maintained on enriched Trypticase soy agar plates containing Trypticase soy agar, 1% yeast extract, 5% defibrinated sheep blood, 5  $\mu$ g/ml hemin, and 1  $\mu$ g/ml menadione at 37°C in an anaerobic atmosphere of 10% H<sub>2</sub>, 5% CO<sub>2</sub>, and 85% N<sub>2</sub> (76, 77). For the preparation of *P. gingivalis* for *in vitro* and *in vivo* infection studies, bacteria were harvested, centrifuged, and washed in PBS. The number of bacteria (CFU per milliliter) was determined by measuring the optical density at 600 nm and extrapolating using a standard curve (7, 78).

**Infection model.** Female C57BL/6 mice (8 to 10 weeks of age) were anesthetized with a solution of ketamine (100 mg/kg of body weight) and xylazine (50 mg/kg). The back of each mouse was shaved and prepped with 10% betadine. Microosmotic pumps (model 1002; 1.5 cm in length, 0.6 cm in diameter, 0.4 g in weight; Alzet Osmotic Pumps, Cupertino, CA) were loaded with 100  $\mu$ l of freshly harvested bacteria and then surgically implanted into the dorsolumbar region according to the manufacturer's instructions.

Bacteria were delivered daily for 14 days at a constant pumping rate of 0.25  $\mu$ l/h and then sacrificed (Fig. 1).

**Cell preparation.** Murine bone marrow (BM) cells were collected from the femurs and tibiae as previously described (79–82). Single-cell suspensions were prepared by mechanically dispersing the BM through a 40- $\mu$ m cell strainer. Erythrocytes were lysed using M-lysis buffer (R&D Systems, Minneapolis, MN), and BM cells were then washed twice with PBS and suspended in fluorescence-activated cell sorter (FACS) buffer (PBS containing 5% bovine serum albumin without sodium azide) at a concentration of  $1 \times 10^7$  cells/ml. For the preparation of spleen cell suspensions, spleens were removed from mice, processed through a cell strainer, and washed twice with PBS. Cells were then suspended in FACS buffer at a concentration of  $1 \times 10^7$  cells/ml.

**Cell sorting.** Suspensions of BM and spleen cells derived from *P. gingivalis*-infected and noninfected controls were stained with anti-mouse CD11b-fluorescein isothiocyanate (FITC), Ly6C-phycoerythrin (PE), and Ly6G-allophycocyanin (APC) antibodies (eBioscience, San Diego, CA) for 30 min on ice in the dark. After washing the cells twice with FACS buffer, cells were sorted for the isolation of MDSC subpopulations using a BD FACS Aria IIIu cell sorter (BD Bioscience, San Jose, CA) (Fig. 1). The data were analyzed with BD FACSDiva software (BD Bioscience) and FlowJo software (TreeStar, San Carlos, CA). The sorted MDSC subpopulations were suspended in RPMI 1640 medium supplemented with 10% heat-inactivated fetal calf serum, penicillin (50 U/ml), streptomycin (50  $\mu$ g/ml), L-glutamine (2 mM),  $\beta$ -mercaptoethanol (50  $\mu$ M), sodium pyruvate (1 mM), sodium bicarbonate (1.5 mg/ml), and HEPES (25 mM), counted, and plated according to the appropriate experimental conditions.

**CD4<sup>+</sup> T cell proliferation assay.** Single-spleen cell suspensions from OT-II mice were cultured in 96-well plates at a concentration of  $2 \times 10^5$  cells/well in the presence or absence of the different sorted MDSC subpopulations at a 1:1 ratio. Cells were stimulated with the OVA<sub>323–339</sub> peptide (2.5  $\mu$ g/ml) for 72 h. Inhibition of NOS2 and of IFN- $\gamma$  was carried out by the addition of the NOS2 inhibitor 1400W [N-(3-aminomethyl)benzylacetamide, 2HCl; Millipore, Billerica, MA] at a concentration of 0.1 mM or by the addition of the IFN- $\gamma$  receptor 1 (eBioscience) blocking antibody at a concentration of 5  $\mu$ g/ml to the cocultures of MDSC and OT-II cells, respectively. The indicated concentration of the inhibitor and blocking antibody used was shown to be optimal in preliminary studies. The NOS2 inhibitor and the IFN- $\gamma$  blocking antibody had no detrimental effect on cell viability, as determined by trypan blue exclusion. Controls included cultures of OT-II spleen cells or sorted MDSC alone stimulated with the OVA<sub>323–339</sub> peptide or left unstimulated. Twenty-four hours prior to the termination of the assay, [<sup>3</sup>H]thymidine (0.5  $\mu$ Ci) (Perkin-Elmer, Waltham, MA) was added to each well. Cells were then harvested using a cell harvester (Brandel, Gaithersburg, MD), and the amount of [<sup>3</sup>H]thymidine taken up by OVA-specific CD4<sup>+</sup> proliferating T cells was determined in a scintillation counter (Beckman, Fullerton, CA).

**Cytokine production.** Spleen cells derived from OT-II mice were cultured in a 96-well plate at  $2 \times 10^5$  cells/well in the presence or absence of MDSC subpopulations at a 1:1 ratio. Cells were stimulated with OVA<sub>323–339</sub> peptide as described above, and the culture supernatants were harvested 72 h later and analyzed for the presence of IFN- $\gamma$  by ELISA (eBioscience) according to the manufacturer's instructions.

**Real-time quantitative PCR.** Total RNA was extracted from  $10^6$  cells of each sorted subpopulation of BM-derived MDSC to assess the expression of *Nos2* (nitric oxide synthase) and *Cat2* (cationic amino acid transporter 2) mRNA at 48 h using RNeasy minikits (Qiagen, Valencia, CA) according to the manufacturer's recommendations. cDNA was synthesized from 500 ng of total RNA by reverse transcription using QuantiTect RT kits (Qiagen). Real-time PCR was done using a LightCycler 480 (Roche Molecular Biochemicals) with a FastStart DNA Master SYBR green I reagent (Roche Applied Science). Relative

quantities of the tested gene were normalized to hypoxanthine phosphoribosyltransferase (HPRT) mRNA. The normalized data were expressed using the comparative  $2^{-\Delta\Delta CT}$  method.

**Transwell assay.** In order to determine if cell contact is required for MDSC-mediated inhibition of T cell-proliferative responses, spleen cells obtained from OT-II mice ( $2 \times 10^5$  cells/well) were cultured in plate wells, whereas sorted MDSC subpopulations were cultured in Transwell inserts (0.4- $\mu$ m pores; Corning, NY) at a 1:1 ratio, thus preventing direct CD4<sup>+</sup> T cell-MDSC contact. Cells were either left unstimulated or stimulated with the OVA<sub>323–339</sub> peptide for 72 h, and the proliferative responses of CD4<sup>+</sup> T cells were determined by [<sup>3</sup>H]thymidine uptake as described above.

**Differentiation of MDSC into OC.** Spleen and BM cells obtained from *P. gingivalis*-infected or noninfected mice were sorted into the different MDSC subpopulations as described above. Each sorted MDSC subpopulation was cultured in 24-well plates ( $5 \times 10^4$  cells/well) in  $\alpha$ -10 medium ( $\alpha$ -minimum essential medium, 10% fetal bovine serum,  $1 \times$  penicillin-streptomycin) in the presence of M-CSF (50 ng/ml) and incubated in a humidified 5% CO<sub>2</sub> atmosphere at 37°C. Cells were stimulated with RANKL (100 ng/ml), and the culture medium was replaced every 2 days. Cells were cultured for 4 to 5 days and stained for TRAP activity using a leukocyte acid phosphatase kit (Sigma-Aldrich, St. Louis, MO).

**Statistical analysis.** Statistical significance was evaluated by analysis of variance and the Tukey's multiple-comparison test using the InStat program (GraphPad Software, San Diego, CA). Differences between groups were considered significant at *P* values of <0.05 (\*), <0.01 (\*\*), and <0.001 (\*\*\*)

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