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Original Article

Myeloid-derived suppressor cells promote immune responses after induction by alum

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Abstract: *Objective*: The present study is to study the role of myeloid-derived suppressor cells (MDSCs) in immune responses in mice and human induced by alum. *Methods:* $CD11b^+Gr1^+Ly6C^+Ly6G^+(Ly6G^-)$ cells were obtained by alum-induced murine model. Phenotype, endocytosis, antigen presenting ability, and T cell suppression assays were performed by flow cytometry analysis. *Results:* After intraperitoneal injection of alum into C57BL/6 mice, the percentage of $CD11b^+Gr1^+$ cells was increased in mice spleen and bone marrow. Compared with control mice, Ly6C, Ly6G, F4/80 and CD86 expression were higher in alum-treated mice. Alum-MDSCs showed strong antigen uptake and antigen-presentation ability, which were accompanied by increase in F4/80 molecule. In addition, proliferation of $CD4^+$ T cells was promoted. *Conclusion:* The present study demonstrates that alum-induced MDSCs function as APC alone, directly prime to adoptive immune cells and trigger subsequent immune responses.

Keywords: MDSC, CD11b, ALUM

Introduction

Alum is the most widely used adjuvant in human vaccines and it is experimentally used to promote immune responses [1]. However, the precise mechanism by which alum promotes immune responses is still unclear [2]. Alum-induced immune response processes include aggregation and adsorption of antigens on the surface of aluminum particles, which facilitate interactions between antigens and immune cells. Uptake of antigens by immune cells (dentritic cells, macrophages, and monocytes) is a pivotal process for antigen-presenting cells-triggered humoral immune response (B cells) and cellular immune response (T cells) [3, 4]. Therefore, an ideal adjuvant should elicit enough immune response to help antigens achieve protective immunity with minimal toxicity and individual tolerance [5].

Myeloid-derived suppressor cells (MDSCs) are a heterogeneous population of cells that expand in cancer, inflammation, infection and transplantation. MDSCs have a remarkable ability to regulate adaptive and innate immune responses [6-10]. MDSCs consist of myeloid progenitor cells and immature myeloid cells, which can propagate continuously in pathological conditions, resulting in the up-regulated expression of arginase 1 (ARG1), inducible nitric oxide synthase (iNOS), nitric oxide (NO) and reactive oxygen species (ROS) [11, 12]. Due to the lack of the expression of cell-surface markers that are specifically expressed by monocytes, macrophages or dendritic cells (DCs), MDSCs are characterized by co-expression of myeloid-cell lineage differentiation antigen GR1 and CD11b in mice [13, 14]. $CD11b^+Gr1^+$ MDSCs can be classified into three subsets [15-17] depending on differential expression of Ly6C and Ly6G: $Ly6G^{high}Ly6C^{med}$ (granulocytic), $Ly6G^{med}Ly6C^{high}$ (monocytic), and $Ly6G^{med}Ly6C^{med}$ (non-monocytic and non-granulocytic). We speculate that MDSCs may participate in adjuvant-induced immune responses before or at the same time with antigen-presenting cells (APCs)-started immune cascades. Therefore, MDSCs are the main source of APCs. Although a number of studies have set light on the mechanism of MDSCs and how to eliminate their role on immunosuppression, reports on the effect of adjuvant-MDSCs on normal individual immune systems are rare [18]. In the present study,

we construct alum-induced MDSCs *in vivo*, and test their potential interaction with $CD4^+$ T cells *in vitro*. This study will provide a comprehensive understanding of alum-induced immune response and a valuable reference for the safe use of adjuvants in clinic.

Material and methods

Animals

Male BALB/c (H-2d) and C57BL/6 (H-2b) mice (6-8 weeks) were purchased from Institute of Zoology, Chinese Academy of Sciences. Potassium alum (Sigma-Aldrich, St. Louis, MO, USA) was well vortexed before used. Mice without treatment were used as blank control. For mice in experimental group, 0.5 ml alum turbid liquid was given intraperitoneally to each mouse. The same dose of PBS was given to mice in control group. We extracted cell suspension from spleen and bone marrow of all mice on day 7. The dose and time were chosen in accordance with preliminary tests. Three mice were included in each group, and each test was repeated for three times. All animal care and procedures were approved by the Institutional Animal Care and Use Committee of Jilin University, and mice were used in accordance with the Association for research in Vision and Ophthalmology Statement for the Use of Animals in Ophthalmic and Vision Research.

Cells

MDSCs and $CD4^+$ T cells were isolated by magnetic separation using $CD11b^+$ positive isolation kit and $CD4^+$ T negative isolation kit (Miltenyi Biotec, Bergisch Gladbach, Germany) according to the manufacturer's manual. The sorted cells were of great purity as detected by flow cytometry (> 90%).

Fluorescence-activated cell sorting (FACS)

Anti-CD4-APC, anti-CD11b-APC, anti-Gr1-PerCP5.5, anti-Ly6C-PE, anti-Ly6G-PE, anti-CD40-PE, anti-CD80-PE, anti-CD86-PE, anti-TLR2-PE, anti-TLR4-PE, anti-MHC-II-PE, FITC-conjugated OVA (OVA-FITC) and isotype control antibodies were obtained for FACS analysis (eBioscience, San Diego, CA, USA).

Isolated $CD11b^+$ cells (1×10^5/well) were resuspended in complete medium and incubated at 37°C in the presence of 5% CO_2. OVA-FITC was added at a final concentration of 1 mg/mL or the same ratio of $CD11b^+$ and $CD4^+$ T cells was used instead. The cells were washed four times with cold phosphate-buffered saline (PBS) and 5% bovine AB serum, and then analyzed by FACScalibur (Becton Dickinson) using FlowJo 7.6 software (https://www.flowjo.com/).

MDSCs suppression assays

Suppression assays were performed in 96-well plates in triplicate. Fresh isolated naïve $CD4^+$ T cells (1×10^5/well) were incubated with MDSCs at a ratio of 1:1 for 5 days. T cell proliferation was determined by CFSE dilution profile. The suppression percentage was calculated using the following formula: suppression percentage (%) = (No. of T cells without MDSCs - No. of T cells with MDSCs)/(No. of T cells without MDSCs) × 100%.

Statistical analysis

The results were analyzed using SPSS13.0 statistical software (IBM, Armonk, NY, USA). Student's t test was performed for statistical analysis. Allograft survival data were generated as Kaplan-Meier survival curves, and log-rank analysis was conducted for comparisons between groups. Data are presented as means ± standard deviations. Differences with $P < 0.05$ were considered statistically significant.

Results

MDSCs are dramatically increased in spleen and marrow by alum induction

To investigate the expression of MDSCs in alum-induced mice, the percentage of MDSCs in alum-induced mice on day 7 was analyzed. The percentage of $CD11b^+Gr1^+$ cells was increased 5 times in spleen and 4 times in bone marrow compared with control group. Further analysis showed that, among $CD11b^+Gr1^+$ cells, granulocytic $Ly6G^{high}Ly6C^{med}$ subset accounted for 46% in spleen and 48% in bone marrow, while the percentage of monocytic $Ly6G^{med}$ $Ly6C^{high}$ subset was elevated to 30%, and the percentage of $Ly6G^{med}Ly6C^{med}$ subset was increased to 5%. The percentage of alum-induced Cd11b, Gr1, Ly6G, and Ly6C cells was significantly increased compared with control group ($P < 0.05$) (**Figure 1**). The results suggested that MDSCs are dramatically increased in spleen and marrow by alum induction.

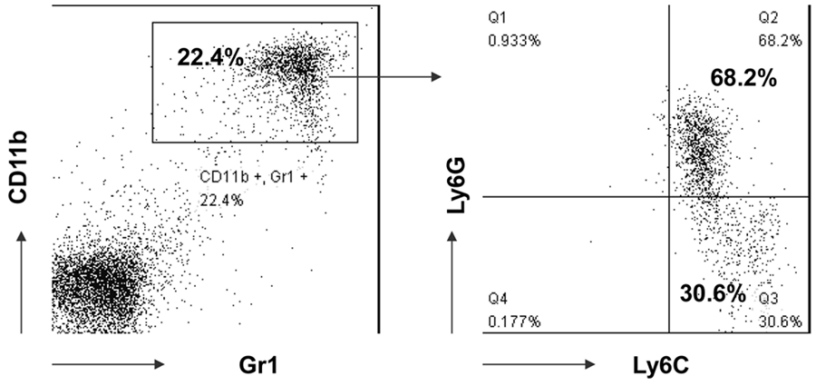

Figure 1. MDSCs expression on day 7 after alum injection. Percentage of $CD11b^+Gr1^+Ly6C^+Ly6G^+$ cells was analyzed by flow cytometry.

Table 1. Percentage of positive MDSCs in alum and PBS mice (control)

MDSCs	Alum		PBS	
	Spleen	Bone marrow	Spleen	Bone marrow
$CD11b^+Gr1^+$	5.29 ± 0.65***	20.09 ± 5.05***	0.93 ± 0.05	5.35 ± 0.25
$Ly6G^{high}Ly6C^{med}$	46.34 ± 2.70***	47.66 ± 2.34***	0.65 ± 0.20	2.82 ± 0.56
$Ly6G^{med}Ly6C^{high}$	32.65 ± 2.69***	28.02 ± 2.32***	0.37 ± 0.13	1.50 ± 0.25
$Ly6G^{med}Ly6C^{med}$	5.70 ± 0.12*	5.01 ± 0.84*	1.55 ± 0.13	1.31 ± 0.48
CD40	2.2 ± 0.09	1.03 ± 0.88	1.70 ± 0.18	0.79 ± 0.22
F4/80	10.2 ± 2.09***	8.03 ± 0.88***	0.87 ± 0.08	1.25 ± 0.43
CD86	3.70 ± 0.12*	5.01 ± 0.84*	0.47 ± 0.10	0.26 ± 0.03
MHC-II	60.13 ± 1.54	58.66 ± 3.34	61.0 ± 1.21	61.0 ± 2.82

Note: Data are presented as means ± standard deviations of triple independent experiments. Significant differences between the alum and PBS mice: $^*P < 0.05$; $^{**}P < 0.02$; $^{***}P < 0.01$.

MDSC cells tend to elicit immune responses by adjuvant induction

To examine MDSCs phenotype alteration of alum-induced mice, expression of various cell surface markers was detected by flow cytometry. The percentages of F4/80, CD86, Ly6C and Ly6G cells were significantly increased to that in sham group ($P < 0.05$). However, the percentages of CD40 and MHC-II were similar to that of PBS group ($P < 0.05$) (**Table 1**). These results indicate that MDSC cells tend to elicit immune responses by adjuvant induction.

MDSCs tend to be functioning as APCs

To determine the role of MDSC cells on antigen phagocyte and antigen presenting ability, OVA-FITC was used. Coculture of isolated $CD11b^+$ cells with OVA for 4 h showed that 55% $CD11b^+$ cells were conjugated to OVA. By contrast, only 30% $CD11b^+$ cells were conjugated to OVA in PBS mice. Co-stimulation with F4/80 increased the percentage to 33% as to that of 8% in PBS group ($P < 0.05$). Coculture of isolated $CD11b^+$ cells with OVA and $CD4^+$ T cells showed that 68% $CD11b^+$ cells are able to present OVA to $CD4^+$ T cells, whereas this percentage was 32% in PBS group ($P < 0.05$) (**Figure 2A** and **2B**). The results suggested that MDSCs tend to be functioning as antigen presenting cells.

Alum-MDSCs promote $CD4^+$ T cells proliferation

To determine the suppressive ability of MDSCs, $CD4^+$ T cells were co-cultured with $CD11b^+$ cells. The data showed that about 40% $CD4^+$ T cells in blank control could spontaneously split after 5 days of *in vitro* culture. Over 50% of $CD4^+$ T cells proliferation was achieved by alum-MDSCs, which has shown higher proportion of cells than PBS and blank groups ($P < 0.05$). However, the promotive effect in sham mice was not significantly different from that in PBS group or blank group ($P > 0.05$) (**Figure 3**). These findings indicated that alum-MDSCs promote $CD4^+$ T cells proliferation.

Discussion

Our results have shown that $CD11b^+Gr1^+$ cells are markedly increased in spleen and bone marrow at the site of injection. Moreover, alum-MDSCs participate in systemic immune responses. Before immune responses initiated by APCs, the MDSCs have independently started immunoreaction processes. MDSCs population mainly include granulocytic and monocytic cells (75%). Similar results are reported in malignant lymphoma model, chronic inflammatory dis-

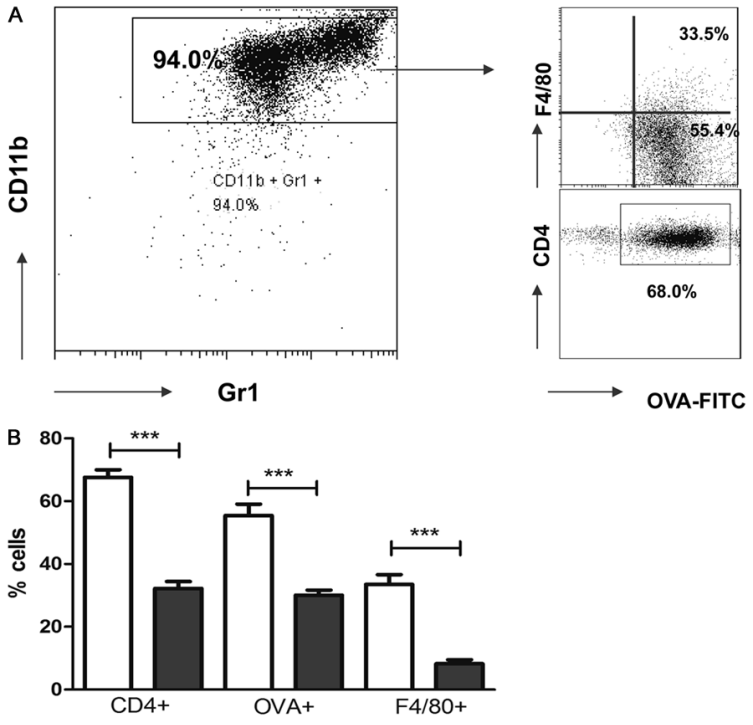

Figure 2. Phagocytic capacity and antigen presenting ability. A: MDSCs co-cultured with OVA-FITC or $CD4^+$ T cells after 4 h. B: Histogram presenting the percentages of CD4, OVA and F4/80-positive cells after being cultured with MDSCs induced by alum (white histogram) and PBS (black histogram). Data are means ± standard deviations of triple determinations. $*P < 0.05$, $**P < 0.02$, and $***P < 0.01$ indicate significant differences obtained by Wilcoxon rank sum test between alum and PBS mice.

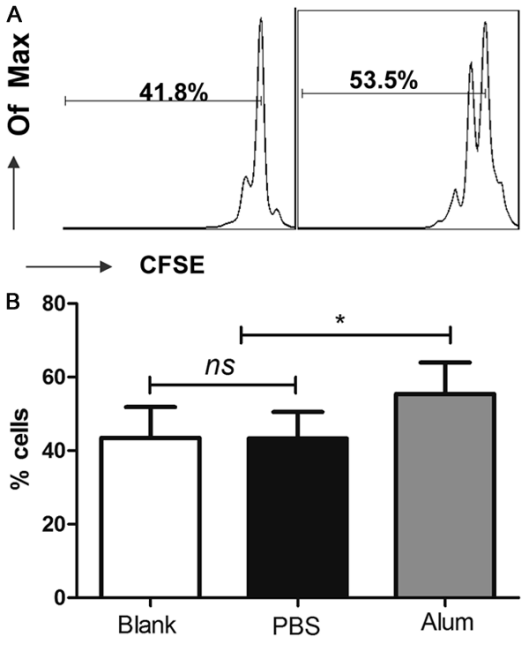

Figure 3. $CD4^+$ T cell proliferation promoted by alum-MDSCs. A: Percentage of CFSE dilution by culturing $CD4^+$ T cells or/and MDSCs for 5 days. B: Histogram presenting the percentage of $CD4^+$ T cells division of blank (white), PBS (black) and alum (gray). Data are means ± standard deviations of triple determinations. $*P = 0.034$ indicates significant difference obtained by *t* test between alum and PBS mice.

ease, virus or bacterial infection and acute graft-versus-host disease [19-23]. Most studies have confirmed that the function of MDSCs is to inhibit immune reactions. Although our results demonstrate that MDSCs promote immune responses *in vitro*, MDSCs can be acted as an independent factor to initiate cascades. Whether MDSCs play positive or negative roles in diseases depends on many factors such as cytokines, pathogenic factors and signal pathway [24-26].

MDSCs are a heterogeneous population of myeloid cells with immunoregulatory activity, and they include immature precursors of monocytes, macrophages, granulocytes and DCs. This population of myeloid progenitors are currently classified in two main subsets: monocytic and granulocytic, which differ in phenotypes and biological properties. In pathological conditions such as cancer, tumor-bearing hosts persistently produce MDSCs, which impair T cell and nature killer (NK) cell responses, resulting in tumor-induced immunosuppression and poor prognosis [27]. MDSCs have become a major therapeutic target in immunotherapy strategies. Consequently, an effective vaccine-adjuvant formulations are to be developed for cancer immunotherapy. The present study has demonstrated positive immune responses of MDSCs. Therefore, we provide possible ways to control immunoregulatory ability of MDSCs. Lee et al demonstrate that TLR7/8 against resiquimod differentiates tumor-induced MDSCs *in vitro* toward macrophages and DCs [28]. Adjuvant LPS is unable to differentiate tumor-induced MDSCs to mature APCs [29]. Alum may be a candidate for cancer vaccine formulations [30, 31]. Our data also

support the idea that alum-induced MDSCs could promote the proliferation of $CD4^+$ T cells, while LPS-induced MDSCs inhibit the proliferation of $CD4^+$ T cells.

The present study also shows that alum is the most commonly used adjuvant, and MDSCs function as APCs. In addition, alum has been demonstrated to elicit humoral immunity [18, 32]. In conclusion, the present study demonstrates that alum-MDSCs independently cause cellular immunity. Our research provides new ideas for the safe use of alum in clinic, and insights for the development of cancer vaccines.

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Disclosure of conflict of interest

None.

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