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Functional Changes in Myeloid-Derived Suppressor Cells (MDSCs) during Tumor Growth: FKBP51 Contributes to the Regulation of the Immunosuppressive Function of MDSCs

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Myeloid-derived suppressor cells (MDSCs) are increased by tumor-derived factors and suppress anti-tumor immunity. MDSCs obtained at a late time point after tumor injection had stronger suppressive activity than MDSCs obtained at an early time point, as measured by T cell proliferation assays. To find factors in MDSCs that change during tumor growth, we analyzed gene expression profiles from MDSCs at different time points after tumor injection. We found that immune response-related genes were downregulated but protumor function-related genes were upregulated in both monocytic MDSCs (Mo-MDSCs) and polymorphonuclear granulocytic MDSCs (PMN-MDSCs) at the late time point. Among differentially expressed genes, FK506 binding protein 51 (FKBP51), which is a member of the immunophilin protein family and plays a role in immunoregulation, was increased in the Mo-MDSCs and PMN-MDSCs isolated from the late time points. Experiments using small interfering RNA and a chemical inhibitor of FKBP51 revealed that FKBP51 contributes to the regulation of the suppressive function of MDSCs by increasing inducible NO synthase, arginase-1, and reactive oxygen species levels and enhancing NF-κB activity. Collectively, our data suggest that FKBP51 is a novel molecule that can be targeted to regulate the immunosuppressive function of MDSCs.

In mice, CD11b and Gr-1 (Ly-6G/Ly-6C) are widely used surface markers of MDSCs (1). Although CD11b+Gr-1+ cells exist in normal conditions, they are significantly increased and become suppressive in particular conditions. Several mechanisms of MDSC-mediated immune suppression have been studied (2, 3). MDSCs inhibit T cell proliferation and function via the depletion of l-arginine by arginase-1 (ARG1) (10) and the production of NO and reactive oxygen species (ROS) by inducible NO synthase (iNOS) (2). In addition, the depletion of cystine and cystein (3), CD40-mediated suppression (11), and the production of immunosuppressive cytokines (12) are known to be involved in MDSC-mediated immune suppression. However, the factors that regulate these molecules are not well understood.

Because of the important role of MDSCs in tumor-associated immune suppression, intensive studies examining the elimination of MDSCs or the inhibition of the suppressive function of MDSCs are ongoing (13–21). We have demonstrated that with the help of activated NKT cells, MDSCs could be converted into immunogenic APCs that generate anti-tumor immunity (16, 17). However, MDSCs obtained at a late time point after tumor injection (late MDSCs) were not efficiently converted into immunogenic APCs in contrast to MDSCs obtained at an early time point (early MDSCs) (17).

In this study, we showed that MDSCs change in the process of tumor growth, and we examined the genes that change from early MDSCs to late MDSCs. Using microarray analysis, we found that many genes that are associated with an immune response and tumor-promoting function were differentially expressed in late MDSCs compared with early MDSCs. Among those genes, we found that FK506 binding protein 51 (FKBP51) was increased in late MDSCs. Knockdown of FKBP51 with small interfering RNA (siRNA) and use of an FKBP51 inhibitor reduced the MDSC-mediated suppression of T cell proliferation, indicating that FKBP51 contributes to MDSC-mediated immune suppression. Therefore, we suggest that FKBP51 is a novel molecule that can be targeted to regulate the immunosuppressive function of MDSCs.

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The microarray data presented in this article have been submitted to the Gene Expression Omnibus database (http://www.ncbi.nlm.nih.gov/geo/) under accession number GSE35398.

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The online version of this article contains supplemental material.

Abbreviations used in this article: ARG1, arginase-1; FKBP51, FK506 binding protein 51; iNOS, inducible NO synthase; MDSC, myeloid-derived suppressor cell; NSP, neutrophil serine protease; PPIase, peptidyl-prolyl isomerase; ROS, reactive oxygen species; sCtrl, a commercially available control siRNA; siRNA, small interfering RNA.
Materials and Methods

Mice

All experiments were approved by the Institutional Animal Care and Use Committee of Seoul National University. BALB/c mice and C57BL/6 mice (Charles River Laboratories, Seoul, Korea) were purchased at 6 wk of age and used in all of the experiments. The mice were kept under specific pathogen-free conditions in the Animal Center for Pharmaceutical Research at Seoul National University.

Tumor models

To obtain MDSCs, 1 × 10^5 CT26 cells expressing Her-2/neu (Her-2/CT26) (22) were s.c. injected into the left flank of BALB/c mice, and 1 × 10^5 CT26 and EL4 cells (both from American Type Culture Collection) were i.p. injected into BALB/c mice and C57BL/6 mice, respectively. In the s.c. tumor model, we defined the early time point as 21–27 d after tumor injection, when the tumor size was less than 10 mm in diameter and the proportion of CD11b+Gr-1+ cells increased to ∼10% in the spleen. The late time point was defined as 42–48 d after injection, when the tumor size was ∼30 mm in diameter and more than 30% of the splenocytes were CD11b+Gr-1+ cells. Some mice had wounds in the tumor mass at the late time point; however, we excluded these mice from the experiment. In the i.p. tumor model, we defined the early and late time points as 7–9 d after tumor injection and 16–18 d after tumor injection, respectively.

Abs and flow cytometry

To detect CD11b+Gr-1+ MDSC populations, allopurinol-injected anti-CD11b and FITC-labeled anti-Gr-1 Abs (both from BioLegend, San Diego, CA) were used. To classify the subpopulations in the CD11b+ cells, FITC-labeled CD11b and FITC-labeled anti–Gr-1 Abs (both from BioLegend, San Diego, CA) and PE-labeled Ly-6G Ab (BioLegend) were used. To detect FKBP51, CD11b+Gr-1+ cells were intracellularly stained serially with rabbit anti-FKBP51 (Abcam, U.K.) and anti-rabbit IgG–PE Abs (BD Biosciences). Stained cells were analyzed by gating on viable cells using a FACSCaliber (BD, Franklin Lakes, NJ).

MDSC isolation

To obtain CD11b+Gr-1+ MDSCs, splenocytes were prepared from naive or tumor-bearing mice and stained with anti-CD11b microbeads (Miltenyi Biotec, Germany). The CD11b+Gr-1+ cells were isolated by MACS Cell Separation, and the purity of CD11b+Gr-1+ cells was greater than 99% (Supplemental Fig. 1A). To obtain MDSC subpopulations, CD11b+ cells, enriched by the MACS system were stained with an allopurinol-conjugated anti-CD11b Ab, an FITC-conjugated anti-Ly-6C Ab, and a PE-conjugated anti–Ly-6G Ab. The CD11b+Ly6C^-Ly6G^- monocytic MDSCs (PMN-MDSCs) and the CD11b+Ly6C^+Ly6G^- monocytic MDSCs (Mo-MDSCs) were isolated by a FACSaria II cell sorter (BD Biosciences). The purity of these populations was greater than 97% (Supplemental Fig. 1B, 1C).

Microarray and data preprocessing

Total RNA was purified from CD11b+Ly-6ChighLy-6Glow and CD11b+Ly-6C^-Ly-6G^- cells using Qiagen RNaseasy kits (Qiagen, Valencia, CA) and reverse-transcribed into cDNA. To analyze gene expression profiles, the GeneChip Mouse Gene 1.0 ST Array was performed. The RMA algorithm was used for expression summaries and signal calculation. Sketch outline normalization was performed for the signal normalization. Fold changes were applied to select the differentially expressed genes. Microarray data are available at the Gene Expression Omnibus database (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE35398).

siRNA nucleofection

We used a commercially available siRNA comprising three target-specific 19–25 nt siRNAs that was recommended for the inhibition of FKBP51 expression (siFKBP51) (23). A commercially available control siRNA (siCtrl) consisting of a scrambled sequence that should not lead to the specific degradation of any known cellular mrNA was also used (both from Santa Cruz Biotechnology, Santa Cruz, CA). To knock down target gene, MDSCs were nucleofected with 500 nM siFKBP51 or siCtrl using the Amaxa nucleofection system (Lonza, Germany).

In vitro T cell suppression

OT-1 or DO11.10 cells (3 × 10^5/well) were stimulated with 250 μg/ml OVA protein (grade V; Sigma-Aldrich) and cocultured with or without MDSCs for 3 d. For the final 24 h, 1 μCi/well [3H]thymidine was added. [3H]Thymidine incorporation into the dividing cells was detected with a liquid scintillation counter (Wallac, Turku, Finland).

Quantitative real-time PCR

Total RNA was isolated from 3 × 10^6 MDSCs using the RNasey Mini Kit (Qiagen, Germany). Reverse transcription was performed using M-MLV reverse transcriptase (Invitrogen, Carlsbad, CA), and quantitative real-time PCR was conducted using SYBR Premix Ex Taq (Takara, Japan). The following primers were used: iNOS, forward 5’-AGG AAG TGG GCC GAA GGA GAA T-3’, reverse 5’-GAA ACT ATG GAG CAC AGC CAC AF-3’; ARG1, forward 5’-AAC AGC GGA GTG CCT TTA ACC T-3’, reverse 5’-GTC GTG ATG CCC CAG ATG GTT TTC-3’; NOX2, forward 5’-GAC CCA GAT GCA GGA AAG GAA-3’, reverse 5’-TCA TGG TGC ACA GGA AAG TGA T-3’; and GAPDH, forward 5’-CCT GGA GAA ACC TTC CCA GTG TA-3’, reverse 5’-GGA AGA AGA GTG GGA GGT GCT GGT G-3’.

Western blot analysis

MDSCs were cultured in media containing LPS and/or rapamycin prior to lysis. Western blot analysis was performed using mAbs to iKo or GAPDH (Abcam).

Statistical analysis

The Student t test was used to compare the differences between two groups. The p values <0.05 were considered significant at a 95% confidence interval.

Results

Suppressive activity of CD11b+Gr1+ MDSCs increases with tumor growth

We previously reported that MDSCs could be converted into immunogenic APCs and induce tumor Ag-specific immune responses in a Her-2/neu–expressing tumor model (16, 17). During these experiments, we found that MDSCs obtained at different time points after tumor injection had different characteristics. The percentage of MDSCs in splenocytes and tumor-infiltrated cells was dramatically increased as tumors grew (Fig. 1A). At an early time point, 21–27 d after tumor inoculation, the percentage of the CD11b+Gr-1+ cells increased to ∼10% in the spleen, but at the late time point, 42–48 d after tumor injection, more than 30% of the splenocytes were CD11b+Gr-1+ cells. Furthermore, the expression levels of MHC-related molecules, such as H-2Kd, I-A, and CD1d, and costimulatory molecules, such as CD86 and CD40, on CD11b+Gr-1+ cells were dramatically decreased (data not shown) (17), suggesting that the Ag presentation capacity of CD11b+Gr1+ cells might be decreased at late time points.

Next, we compared the suppressive activity of CD11b+Gr-1+ MDSCs in a T cell proliferation assay. Whereas CD11b+Gr-1+ cells from naive mice had no suppressive effect on T cell proliferation, CD11b+Gr-1+ MDSCs from tumor-bearing mice inhibited CD4+ T cell proliferation (Fig. 1B) in a MDSC dose-dependent manner. MDSCs also inhibited alloreactive CD8+ T cell proliferation (Fig. 1C), as described in other studies (24). Although early MDSCs had acquired suppressive activity against T cell proliferation, the suppressive activity of late MDSCs was much stronger than that of early MDSCs.

As described previously, CD11b+Gr-1+ MDSCs consisted of two major subsets, a CD11b+Ly-6C^-Ly-6G^- subset and a CD11b+Ly-6C^-Ly-6G^+ subset. To investigate the changes in the MDSC subpopulations at early and late time points, the percentage and number of both Mo-MDSCs and PMN-MDSCs in total splenocytes were increased at the late time point (Fig. 1D). In particular, PMN-MDSCs exponentially increased and became the predominant MDSC population at the late time point both in the spleen and the tumor site (Fig. 1E). We sorted each population to evaluate the suppressive activity of each of the MDSC subpopulations. The sorted Mo-MDSCs were mononuclear cells, which had a light blue cytoplasm, and the
PMN-MDSCs were neutrophils, which had lobulated nuclei and faint cytoplasm (Fig. 1F). The sorted Mo-MDSCs and PMN-MDSCs were cocultured with OVA-stimulated OT-1 cells. Both Mo-MDSCs and PMN-MDSCs suppressed OVA-stimulated OT-1 T cell proliferation. However, Mo-MDSCs had a stronger suppressive capacity than PMN-MDSCs at each T cell/MDSC ratio (Fig. 1G). PMN-MDSCs suppressed T cell proliferation when they were present in high numbers (Fig. 1H). Although Mo-MDSCs constituted a smaller population, their high suppressive capacity can overcome their limited numbers. In contrast, PMN-MDSCs have weaker suppressive function but constituted a larger population. Taken together, these factors might contribute to the total suppressive function of MDSCs.

Mo-MDSCs and PMN-MDSCs express tumor-promoting genes at late time points

To identify the factors that license MDSCs to be more suppressive as tumors grow, we analyzed gene expression profiles in the two subsets of MDSCs at different time points after tumor injection. At the late time point, most of the increased genes were related to the cell cycle and cellular division (data not shown). We focused on the analysis of mRNA levels of genes related to cell-lineage markers, apoptosis, tissue remodeling, tumor metastasis, cell recruitment, and immune suppression. Immune response-related genes, including cell surface molecules, cytokines, chemokines, and cell signaling molecules, were downregulated in both Mo-MDSCs and PMN-MDSCs at the late time point (Fig. 2A, 2B), suggesting that the APC function of MDSCs might decrease with tumor growth. In contrast, some genes related to MDSC immunosuppressive function, such as S100a8 and S100a9 (26) in Mo-MDSCs and urokinase (Plau) (27) in PMN-MDSCs, were increased with tumor growth (Fig. 2C, 2D). In addition, lipocalin 2 (Lcn2), which is an iron-binding protein that promotes tumor metastasis (28, 29), and leukotriene A4 hydrolase (Lta4h), which is involved in leukotriene B4-mediated MDSC chemotraction (30), were also increased in Mo-MDSCs and PMN-MDSCs, respectively, at the late time point. These data suggest that MDSCs are genetically regulated to possess protumor functions as tumors grow.

Moreover, apoptosis-related factors, such as NLR family apoptosis inhibitory protein 2 and the proliferation-related factor Ki67, were also overexpressed in PMN-MDSCs at the late time point (Fig. 2E), suggesting that PMN-MDSCs might be dividing rapidly and be resistant to cell death. This finding may explain why PMN-MDSCs become such a large population at the late time point during tumor growth.

FKBP51 is associated with the suppressive function of MDSCs

We next investigated the role of the other differentially expressed genes in MDSCs. Among the upregulated genes, we focused on FKBP51 (Fkbp5)—which was overexpressed in both Mo-MDSCs and PMN-MDSCs at the late time point—because it is a member of the immunophilin protein family, which plays a role in immune
regulation (31, 32). The late Mo-MDSCs expressed an 8-fold higher level of Fkbp5 than that of the early Mo-MDSCs, and the late PMN-MDSCs expressed a 3.5-fold higher level of Fkbp5 than that of the early PMN-MDSCs (Fig. 2C, 2D). We confirmed the increase of FKBP51 expression in CD11b+Gr-1+ MDSCs by RT-PCR. Consistent with the gene chip data, gene expression level of FKBP51 was enhanced in late CD11b+Gr-1+ MDSCs (Fig. 3A). We also measured the levels of FKBP51 protein at different time points and confirmed that the expression level of FKBP51 was higher in the late CD11b+Gr-1+ MDSCs than that in the early CD11b+Gr-1+ MDSCs (Fig. 3B). In addition, in i.p. injected tumor models, FKBP51 was also increased in both splenic and tumor-infiltrated CD11b+Gr-1+ MDSCs at a later time point (Fig. 3C).

We next sought to clarify the role of FKBP51, particularly whether FKBP51 is involved in the suppressive function of MDSCs. We knocked down FKBP51 using siRNA. CD11b+Gr-1+ MDSCs were isolated and then transfected with either an irrelevant control siRNA (siCtrl) or an siRNA specific for FKBP51 (siFKBP51). When FKBP51 expression was measured by FACS staining after siRNA nucleofection, the expression of FKBP51 was reduced in the majority of cells (Fig. 4A). Nucleofection itself influenced cell viability; however, there was no difference in the viability of the cells receiving the siFKBP51 and the cells receiving the siCtrl (Supplemental Fig. 2). We then tested the role of FKBP51 in mediating immunosuppression by coculturing Ag-stimulated DO11.10 or OT-1 cells with siRNA-transfected MDSCs. We found that
proliferation of both CD4+ and CD8+ T cells was inhibited by MDSCs, but siFKBP51 significantly reduced the suppression compared with that by siCtrl (Fig. 4B, 4C). Collectively, these data suggest that FKBP51 contributes to the suppressive function of MDSCs on T cell proliferation.

A chemical inhibitor of FKBP51, rapamycin, reduces the suppressive effect of MDSCs

We sought to find a chemical inhibitor that regulates FKBP51 function for more in-depth analysis of the role of FKBP51 in MDSCs, and several reports demonstrated that FK506 and rapamycin inhibit the peptidyl-prolyl isomerase (PPIase) function of FKBP51 (33, 34) and regulate inflammation (35, 36). Thus, we tested whether FK506 and rapamycin could inhibit the suppressive activity of MDSCs. Purified MDSCs were preincubated with rapamycin and FK506 in vitro and then evaluated for their suppressive activity on T cell proliferation. When we compared the suppressive activities of inhibitor-pretreated MDSCs with untreated MDSCs, rapamycin, which has a higher affinity for FKBP51 than FK506 (33), reduced the suppressive activity (Fig. 5A). Also, we found that rapamycin reduced the suppressive function of MDSCs on T cell proliferation. When we compared the suppressive activities of inhibitor-pretreated MDSCs with untreated MDSCs, rapamycin, which has a higher affinity for FKBP51 than FK506 (33), reduced the suppressive activity (Fig. 5A). Also, we found that rapamycin reduced the suppressive function of MDSCs on T cell proliferation. When we compared the suppressive activities of inhibitor-pretreated MDSCs with untreated MDSCs, rapamycin, which has a higher affinity for FKBP51 than FK506 (33), reduced the suppressive activity (Fig. 5A). Also, we found that rapamycin reduced the suppressive function of MDSCs on T cell proliferation. When we compared the suppressive activities of inhibitor-pretreated MDSCs with untreated MDSCs, rapamycin, which has a higher affinity for FKBP51 than FK506 (33), reduced the suppressive activity (Fig. 5A). Also, we found that rapamycin reduced the suppressive function of MDSCs on T cell proliferation. When we compared the suppressive activities of inhibitor-pretreated MDSCs with untreated MDSCs, rapamycin, which has a higher affinity for FKBP51 than FK506 (33), reduced the suppressive activity (Fig. 5A). Also, we found that rapamycin reduced the suppressive function of MDSCs on T cell proliferation. When we compared the suppressive activities of inhibitor-pretreated MDSCs with untreated MDSCs, rapamycin, which has a higher affinity for FKBP51 than FK506 (33), reduced the suppressive activity (Fig. 5A). Also, we found that rapamycin reduced the suppressive function of MDSCs on T cell proliferation. When we compared the suppressive activities of inhibitor-pretreated MDSCs with untreated MDSCs, rapamycin, which has a higher affinity for FKBP51 than FK506 (33), reduced the suppressive activity (Fig. 5A). Also, we found that rapamycin reduced the suppressive function of MDSCs on T cell proliferation. When we compared the suppressive activities of inhibitor-pretreated MDSCs with untreated MDSCs, rapamycin, which has a higher affinity for FKBP51 than FK506 (33), reduced the suppressive activity (Fig. 5A). Also, we found that rapamycin reduced the suppressive function of MDSCs on T cell proliferation. When we compared the suppressive activities of inhibitor-pretreated MDSCs with untreated MDSCs, rapamycin, which has a higher affinity for FKBP51 than FK506 (33), reduced the suppressive activity (Fig. 5A). Also, we found that rapamycin reduced the suppressive function of MDSCs on T cell proliferation. When we compared the suppressive activities of inhibitor-pretreated MDSCs with untreated MDSCs, rapamycin, which has a higher affinity for FKBP51 than FK506 (33), reduced the suppressive activity (Fig. 5A). Also, we found that rapamycin reduced the suppressive function of MDSCs on T cell proliferation. When we compared the suppressive activities of inhibitor-pretreated MDSCs with untreated MDSCs, rapamycin, which has a higher affinity for FKBP51 than FK506 (33), reduced the suppressive activity (Fig. 5A). Also, we found that rapamycin reduced the suppressive function of MDSCs on T cell prol...
MDSCs (Fig. 1). The numbers and percentage of PMN-MDSCs became predominant among the MDSC subpopulations at late time point (Fig. 1) (24, 37). During tumor growth, increases of inflammation and tumor-derived factors, such as IL-1β (40), G-CSF (41, 42), and IL-6 (39), might be responsible for these changes. Indeed, we observed that IL-6 levels in the sera were also increased with tumor growth (C.-Y. Kang, unpublished observations).

We found that the immune response-related genes were decreased and the protumor function-related genes were increased in MDSCs at the late time point (Fig. 2). These genetic changes may license the functional changes of MDSC observed during the tumor growth. Although we and others have identified the function of several genes important in the immunosuppressive function of MDSCs (26–30), many genes still remain unknown. For instance, it is interesting that all neutrophil serine proteases (NSPs), including neutrophil elastase, cathepsin G, and proteinase-3, are increased in Mo-MDSCs, and serpinb1, which is an NSP inhibitor, was increased in both Mo-MDSCs and PMN-MDSCs (Fig. 2). It is well known that excess NSPs induce cytokine and neutrophil chemokine production in alveolar macrophages (43, 44). In addition, Serpinb1−/− mice showed an increase of TNF-α, KC, and IL-1β in the bronchoalveolar lavage fluid and G-CSF in the serum compared with wild-type mice after Pseudomonas aeruginosa infection (45). In addition, increased neutrophil apoptosis was observed in Serpinb1−/− mice compared with wild-type mice (45). Therefore, we suspect that NSPs and serpinb1 might be related to the accumulation and function of MDSCs. However, that hypothesis requires further investigation.

MDSCs provide an immunosuppressive tumor microenvironment and limit the effect of anti-cancer immunotherapy. Therefore, the effects of anti-cancer immunotherapy could be enhanced by the removal of MDSCs or the inhibition of the suppressive function of MDSCs (13). Several approaches have been used to overcome the suppressive effect of MDSCs in various experimental settings. Gemcitabine (13, 14) and 5-fluorouracil (15) eliminate MDSCs. α-Galactosylceramide (16, 17) and retinoic acid (18, 19) induce the differentiation of MDSCs into immunogenic APCs. In addition, sildenafil, an inhibitor of phosphodiesterase-5 (20), and sunitinib, a tyrosine kinase inhibitor (21), reduce the suppressive activity of MDSCs. Thus, blocking of FKBP51 with siRNA or rapamycin could be an alternative approach to overcome the effect of MDSCs.

FKBP51 encoded by the Fkbp5 gene possesses PPIase activity and participates in protein–protein interactions (31, 32). This PPIase activity is essential for IκBα degradation. Thus, FKBP51 overexpression results in NF-κB activation and relevant gene expression (31, 32). The NF-κB signaling pathway has been shown to regulate the protumor function of tumor-associated macrophages (46, 47). On the basis of these reports, we hypothesized that FKBP51 might be involved in the immunosuppressive function of MDSCs. Our data supported this hypothesis by showing that the late MDSCs, which expressed a higher level of FKBP51 than the early MDSCs, have stronger suppressive activity, and experiments using siRNA and a chemical inhibitor of FKBP51 reduced the suppressive activity of the late MDSCs. The reduction in the suppressive function of MDSCs was not complete (Figs. 4, 5), either because the inhibition of FKBP51 with the inhibitors was not complete or because other regulators were involved in the suppressive function. In addition, despite their low expression of FKBP51, the early MDSCs expressed a higher level of other immunosuppressive genes, such as S100a8 and S100a9. Therefore, we conclude that FKBP51 is one factor that contributes to the suppressive activity of the MDSCs. However, it remains unclear whether FKBP51 directly or indirectly affects the suppressive function.

Discussion

MDSCs are important immune suppressor cells in the tumor microenvironment, and they inhibit anti-tumor immunity (1–3). In the current study, we found that CD11b+Gr-1− MDSCs obtained at a late time point after tumor injection inhibited T cell proliferation more strongly than that by early MDSCs. In addition, our findings that FKBP51 is overexpressed in the late MDSCs and regulates the suppressive function of MDSCs suggest that FKBP51 is a new target for overcoming MDSC-mediated immune suppression.

The context of where MDSCs are present influences the function of MDSCs. For instance, inflammation and hypoxic conditions around the tumor site enhance the suppressive function of MDSCs (37, 39). MDSCs in the tumor microenvironment have upregulated iNOS and arginase and have more suppressive activity than splenic MDSCs (37). In this study, we highlighted the changes in MDSCs during tumor growth. The number and percentage of CD11b+Gr-1− MDSCs increased as tumors grew, and the late MDSCs had more potent suppressive activity on T cell proliferation than that of early MDSCs (Fig. 1). The numbers and percentage of PMN-MDSCs became predominant among the MDSC subpopulations at late time point (Fig. 1) (24, 37). During tumor growth, increases of inflammation and tumor-derived factors, such as IL-1β (40), G-CSF (41, 42), and IL-6 (39), might be responsible for these changes. Indeed, we observed that IL-6 levels in the sera were also increased with tumor growth (C.-Y. Kang, unpublished observations).

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and the relative contribution of FKBP51 to the suppressive function of MDSCs compared with that of other molecules should be studied.

Rapamycin inhibited the MDSC suppressive function in vitro and in vivo (Fig. 5). We hypothesize that these results were due to the blockade of FKBP51 PPIase activity by rapamycin (33, 34), and the results with siRNA supported this claim (Fig. 4). However, it cannot be excluded that a rapamycin–FKBP12 complex blocked the mTORC1 pathway and subsequently affected the MDSC suppressive activity. However, it was reported that mTORC1 inhibition by rapamycin treatment of granulocytes reduced c-Myc expression and resulted in rapid differentiation (48). Therefore,

![Figure 5](image1.png)

**FIGURE 5.** Rapamycin reduced the suppressive function of MDSCs. (A) Late MDSCs from Her-2/CT26 tumor-bearing mice were preincubated with 100 nM rapamycin or FK506 for 2 h, and then the chemicals were washed out. Rapamycin- or FK506-treated MDSCs were cocultured with Ag-stimulated OT-1 cells for 3 d. (B) Late MDSCs were preincubated with various concentrations of rapamycin for 2 h and then cocultured with Ag-stimulated OT-1 cells for 3 d. (C and D) Her-2/CT26 tumor cells were s.c. injected into the flanks of BALB/c mice. After 45 d, the tumor-bearing mice were i.p. injected with 30 µg rapamycin, and 2 d later, CD11b+ cells were sorted by MACS. Ag-stimulated DO11.10 (C) and OT-1 (D) cells were cocultured with MDSCs from rapamycin-treated or untreated tumor-bearing mice for 3 d. Representative data from three separate experiments are shown. *p < 0.05, **p < 0.005.

![Figure 6](image2.png)

**FIGURE 6.** iNOS, ARG1, and ROS levels were decreased by FKBP51 inhibition. (A) CD11b+ cells were purified from Her-2/CT26 tumor-bearing mice at day 42 and nucleofected with 500 nM siFKBP51 or siCtrl. Four hours after siRNA nucleofection, 1 µg/ml LPS and 20 ng/ml GM-CSF were added into the cell cultures. After 48 h, the mRNA levels of iNOS, ARG1, and NOX2 were measured. (B and C) MDSCs were stimulated with 100 ng/ml LPS (B) or IFN-γ (C). To inhibit FKBP51 function, 100 ng/ml rapamycin or DMSO (control) was added. After 48 h, the mRNA levels of iNOS, ARG1, and NOX2 were measured. (D) MDSCs that were cultured with 100 ng/ml LPS and 100 nM rapamycin. Cell lysates were obtained at the indicated time points, and IκBα levels were measured by Western blotting. The data shown are representative of two separate experiments. *p < 0.05, **p < 0.005.
rapamycin seems to be advantageous for MDSC regulation through its FKBP51 and mTORC1 blocking activity. Notably, when the rapamycin-pretreated MDSCs were transferred to tumor-bearing mice, the tumor tended to grow more slowly than the tumors in the control group. We have previously reported that MDSCs can be converted into immunogenic APCs in certain conditions (16, 17). Therefore, we speculate that inhibiting of FKBP51 function with rapamycin might not only reduce the suppressive function of MDSCs but also facilitate the conversion of MDSCs into other effector cells to impair tumor growth. However, this hypothesis requires further investigation.

Although rapamycin was originally identified as an immunosuppressive drug and used to prevent rejection in organ transplantation, new biological functions of rapamycin have recently been discovered that would be beneficial for cancer therapy. Rapamycin inhibits tumor growth by not only suppressing angiogenesis (49) but also preventing the proliferation of cancer cells (50). Moreover, rapamycin enhanced the apoptosis of chemotherapy-resistant tumor cells (34) and reduced inflammation by regulating arginase, iNOS, and ROS (35, 36). In MDSCs, rapamycin enhanced gemcitabine-induced apoptosis in vitro (C.-Y. Kang, unpublished observations) and inhibited the immunosuppressive activity (Fig. 5).

In summary, according to the stage of tumor development, MDSCs have different characteristics of cell surface molecule expression, gene expression, and function. Therefore, this study provides new insight into MDSC regulation, demonstrating that different approaches are needed to overcome the different stages of MDSCs. In addition, this study suggests a new approach targeting FKBP51 to overcome the potent immunosuppressive activity of late MDSCs.

Disclosures
The authors have no financial conflicts of interest.

References


Supplemental figure 1. The purity of the isolated cells

A. Splenocytes from naive or tumor-bearing mice were stained with CD11b+ microbeads and then CD11b+ cells were isolated by two-round positive selection using the MACS system. The isolated cells were stained with a FITC-conjugated anti-CD11b Ab and a PE-conjugated anti-Gr-1 Ab, and the purity was measured by flow cytometry. (B and C) CD11b+ cells that were enriched by the MACS system were stained with an APC-conjugated anti-CD11b Ab, a FITC-conjugated anti-Ly-6C Ab and a PE-conjugated anti-Ly-6G Ab and sorted using a FACSARia II (BD Bioscience). After cell sorting, the purity of the Mo-MDSC (B) and PMN-MDSC(C) populations was measured by flow cytometry.
Supplemental figure 2. Cell viability after siRNA nucleofection

MDSCs were nucleofected with siFKBP51 or siCtrl using the Amaxa® nucleofection system. The cells were cultured in complete media with GM-CSF (20 ng/ml) for 24 hours, and the viable cells were evaluated by staining with PI and annexin V. The percentage of annexin V-PI live cells is shown.