



Published in final edited form as:

J Immunol. 2014 June 15; 192(12): 5679–5686. doi:10.4049/jimmunol.1302755.

Deletion of CD24 impairs development of heat shock protein gp96 -driven autoimmune disease through expansion of myeloid derived suppressor cells

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Abstract

CD24 binds to and suppresses inflammation triggered by danger associated molecular patterns (DAMPs) such as heat-shock proteins (HSPs) and HMGB1. Paradoxically, CD24 has been shown to enhance autoimmune disease. Here we attempt to reconcile this paradox by deletion of CD24 (24KO) in a lupus-like disease model driven by forced expression of HSP gp96 at the cell surface (tm). As expected, tm24KO mice showed increased CD11c⁺ DC activation coupled to a significant increase in DC-specific IL-12 production compared to tm mice. However, tm24KO mice showed less CD4 T cell activation and peripheral inflammatory cytokine production in comparison to tm mice. We characterized an enhanced immune suppressive milieu in tm24KO mice distinguished by increased TGF- β and greater Treg suppressive capacity. We found greater absolute numbers of MDSCs in tm24KO mice and showed that the Ly6C⁺ MDSC subset had greater suppressive capacity from tm24KO mice. Deletion of CD24 in tm mice led to diminished lupus-like pathology as evidenced by anti-nuclear antibody deposition and glomerulonephritis. Finally, we show that expanded MDSC populations were mediated by increased free HMGB1 in tm24KO mice. Thus, the deletion of CD24 in an HSP-driven model of autoimmunity led to the unexpected development of Treg and MDSC populations that augmented immune tolerance. Further study of these populations as possible negative regulators of inflammation in the context of autoimmunity is warranted.

Keywords

autoimmunity; MDSC; CD24; gp96; HMGB1

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¹Jessica Thaxton is funded by Department of Defense BCRP fellowship W81X1H.

²This work was supported in part by NIAID, NIH grant 2R01AI077283.

Introduction

Autoimmune diseases occur in response to a disruption in immune tolerance triggered by aberrant innate and adaptive immune cell activation resultant in tissue degradation and organ failure (1). Endogenous danger-associated molecular patterns (DAMPs) are released during the course of disease and enhance inflammation (2). The classic DAMP HMGB1 has been shown to trigger inflammation in the context of autoimmunity and to exacerbate diseases such as systemic lupus erythematosus (SLE) and rheumatoid arthritis (RA) (3). DAMP heat shock protein 90 (HSP90) is increased in sera of SLE patients and associated with increased autoantibody production (4). Similarly, auto-antibodies to gp96 have been detected in patients with SLE and RA (5, 6).

The heat stable antigen CD24 is a receptor for HMGB1 as well as heat shock proteins 70 and 90. Ligation of CD24 with HMGB1 or HSPs leads to recruitment of the ITIM Siglec10/G and subsequent abrogation of inflammatory cytokine signals through blockade of NF- κ B activation (7). Loss of CD24 in the context of exogenously introduced insult resulted in HMGB1-driven DC inflammatory response. The CD24 inflammatory axis is specific to signaling initiated by DAMPs as pathogen associated molecular pattern (PAMP) lipopolysaccharide (LPS), response did not differ between CD24KO and WT cells (8). It is not known how functional loss of CD24 will affect immune tolerance and subsequent disease progression in an autoimmune context.

Myeloid derived suppressor cells (MDSCs) have a well-defined role as pro-tumorigenic through suppression of host inflammatory mechanisms (9). While the role of MDSCs in autoimmune disease is less defined, reports demonstrate that suppression of host immunity ameliorates disease (3). MDSCs contribute to an immune-suppressive atmosphere through signaling of TGF- β or production of IL-10 to increase Treg cell activity and quell activation of naïve CD4 T cells (10). A mechanism of MDSC development and recruitment may be DAMP HMGB1 as tumor models show (11). HMGB1 may also lead to MDSC expansion in a tumor context to enhance tumor growth and in the context of autoimmune disease to suppress clinical manifestations.

Gp96, or grp94, is an abundant heat shock protein located in the endoplasmic reticulum with homology to HSP90 (12). We previously developed a murine model of forced gp96 expression at the cell surface and shown that the onset of lupus-like disease occurred in these animals and could be abrogated by depletion of T cells (13, 14). CD24 has been shown to be essential for the development of experimental autoimmune encephalomyelitis in the mice (15, 16). Moreover, polymorphism of CD24 is associated with a number of autoimmune diseases including multiple sclerosis, lupus, and rheumatoid arthritis (17–22). However, the role of CD24 in the context of endogenous DAMP driven autoimmune disease has not been investigated. Here we present the unexpected emergence of a population of MDSCs in tm24KO mice that correlated with decreased T cell activation and marks of inflammatory suppression. In the context of autoimmune disease we determined decreased lupus-like clinical manifestations due to the presence of MDSC and the consequent decreased inflammatory atmosphere. Finally, we show that elevated levels of free HMGB1 mediate MDSC infiltration in the absence of CD24 in an autoimmune disease context.

Materials and Methods

Mice

CD24^{-/-} mice, which were produced using ES cells from C57BL/6 mice, have been described (23). Membrane-bound gp96 transgenic mice (tm) (on pure C57BL/6 background) were developed as described previously (13). CD24KO and tm mice were backcrossed for 8 consecutive generations (tm24KO). Mice used for experiments were aged 6–12 months unless otherwise indicated. The Division of Laboratory Animal Resources (DLAR) of Medical University of South Carolina (MUSC) maintained mice according to established guidelines.

Ab and reagents

All flow cytometry and fluorescent microscopy antibodies were purchased from ebioscience unless otherwise indicated. CD11c, CD40, CD44, CD80, CD86, CD69, CD25, CD11b, Gr1, Ly6C, CD19, CD138, CD4 (BD Bioscience), and CD8 (BD Bioscience) were used for extracellular staining. BRDU (BD Biosciences) IFN γ , TNF α , IL-12, IL-10, and Foxp3 were used for intracellular staining and Foxp3 Buffer was used for permeabilization (eBioscience).

Cellular Preparation, Stimulation, and Isolation

Spleens and lymph nodes were harvested in RPMI media supplemented with 10% FBS. Single cell suspensions were obtained by tissue dissociation with glass slides and subsequent lysis of RBCs. For direct flow cytometry cells were kept on ice and immediately placed in PBS 2% FBS for staining with fluorescent conjugated antibodies. For re-stimulation cells were treated with or without PMA/ionomycin (0.2 μ g/mL, 1 μ g/mL (Sigma) for 4–6 hours with BFA (BD Biosciences) and extracellular/intracellular flow cytometry antibody staining was performed. BrdU staining protocols were followed per manufacturer's instructions (BD Biosciences). PBMCs were obtained by harvesting blood into heparinized tubes. Blood was stained at RT for 20 minutes with fluorescent conjugated Abs then washed and lysed with ACK. Cells were fixed or permeabilized for intracellular staining. For Western blot cells were lysed with RIPA buffer on ice for 1 hr.

Cell Cultures & Greiss Reaction

Cells were cultured in RPMI supplemented with 10% FBS and antibiotics. For TGF- β ELISA supernatants were collected after 24 hours of culture. For Greiss reaction, cells were plated at 1×10^5 cells per well in 96 well plates and treated with or without 1mg/mL LPS (Sigma) for 24 hours. Supernatants were harvested and Greiss reaction was performed per manufactures' instructions (Life Technologies).

BrdU & CFSE Assays

Mice were given BrdU (0.8 mg/mL) in drinking water for 3 consecutive days. CFSE was loaded into CD8 T cells per manufactures' instructions (Invitrogen) and plated at ratios of 1:1 and 10:1 (T: Suppressor). CD8 T and suppressor cell subsets were isolated from single cell splenic suspensions via magnetic bead sort per manufactures' instructions (Miltenyi:

Treg Kit, MDSC Kit). The 1:1 ratios were plated at 1×10^5 cells in 96 well plates coated with anti-CD3 (ebioscience). On day 3 of co-culture cells were washed and CFSE dilution was read via flow cytometer.

ELISA and Western Blot

ELISA was used to detect TGF- β per manufacturer's protocol (R&D Systems), IL-12p40 (BD Bioscience) and IgM, IgA, IgG1, IgG3, IgG2b, and IgG2c (Southern Biotech). Serum western blot for HMGB1 was performed on diluted sera samples (1:10) and Comassie blue stain of gel was used to detect albumin band as loading control. Lysates and sera were measured by BSA assay (Thermo Fisher). Denatured proteins were run on 10% SDS gel and primary proteins were blotted for overnight at 4C. Primary antibody for HMGB1 was obtained from Cell Signaling, respectively. Anti-rabbit HRP secondary antibody was used for protein detection.

Chemotaxis Assay

Recombinant HMGB1 was obtained from HMGBiotech. HMGB1 was rigorously tested by HMGBiotech and determined to be LPS free. I.P. injection of 100 ng/mouse in sterile PBS was administered and PBMC were assayed 1 hr pre and 4 hrs post injection, respectively.

Immunofluorescence detection of glomerulonephritis and clinical evaluation of the severity of kidney disease and Detection of ANA

Kidney sectioning, immunofluorescent staining, H&E, ANA detection and clinical assessments were performed and assessed as previously described (24). For ANA and GN immunofluorescence we used a 5-point scale for assessment of clinical disease where fluorescent intensity was graded 1 (negative staining), 2 (dim staining), 3(dim-moderate staining), 4 (moderate-bright staining), 5 (bright staining).

Statistics

Students' t test two tailed paired statistics were performed. Paired tests were used where age matched controls were measured unless otherwise specified. Values of $p < 0.05$ were considered significant. Mice analyzed were $n = 3$ pairs or more for all experiments.

Results

Loss of CD24 in tm mice leads to increased DC activation

We previously engineered transgenic mice to express gp96 at the cell membrane (tm) and demonstrated that these mice developed lupus-like disease 4 months after birth (13). We further showed that IL-12 and CD11c⁺ DCs contributed to disease development (14). CD24 is a critical receptor for DAMP HMGB1 on DCs and in the absence of CD24 DCs show amplified inflammatory activity to DAMPs upon exogenous stimulation (8). In order to address the role of CD24 in response to DAMPs released due to endogenous inflammation we crossed CD24KO mice to tm mice (tm24KO). At 6 months after birth we assessed CD11c⁺ cell populations and noted that mice from tm24KO groups consistently showed greater CD11c⁺ populations as a percentage of peripheral blood mononuclear cells. The

increase in CD11c⁺ cells was not significant in splenocyte populations (Figure 1A). We next assessed DC activation as measured by CD80, CD86, and CD40. We found that PBMC-DC populations from tm24KO had significantly increased expression as measured by MFI of CD80 and CD86 as compared to tm levels (Figure 1B). We next asked whether tm24KO peripheral blood DCs showed enhanced IL-12 production compared to tm mice. Direct *ex vivo* data show that PB-DCs from tm24KO mice have higher MFI of IL-12 than PB-DCs from tm mice (Figure 1C). We further quantified levels of serum IL-12p40 and noted that enhanced DC activity in tm24KO mice correlated to significantly elevated levels of IL-12p40 in tm24KO mice as compared to tm mice (Figure 1D).

Decreased inflammatory CD4 T cells in tm24KO mice

IL-12 is an inducer of Th1 differentiation and leads to enhanced T cell proliferation and IFN- γ production (25). We assessed CD4/CD8 populations in tm and tm24KO mice and did not note a difference between these populations (data not shown). We further investigated CD4 T cells by measurement of early activation marker CD69. We found that splenic tm24KO CD4 T cells expressed less CD69 than tm CD4 T cells. To determine whether tm24KO CD4 T cells were truly “less active” than tm CD4 T cells we fed mice BrdU water and assessed BrdU incorporation after 3 days. We found that CD4/CD69⁺ populations of tm24KO mice showed decreased BrdU incorporation as compared to tm mice and this effect was significant in splenocytes. These results indicate low CD4 T cell proliferation in tm24KO mice (Figure 2A). To quantify inflammatory potential of T cells we isolated and stimulated (PMA/ionomycin) mixed lymphocytes from tm and tm24KO mice. We found increased IFN- γ (top panels) and TNF- α (data not shown) production from mesenteric lymph nodes (mIn) of tm mice as compared to tm24KO mice (Figure 2B). We further assessed CD4 T cell activation in spleens and mIn by analysis of CD44 expression. We determined that IFN- γ (bottom panels) and TNF- α production (data not shown) were produced by CD44^{high} CD4 T cell subsets in tm and tm24KO mice (Figure 2B). Though not significant, tm24KO mice consistently showed less inflammatory cytokine production from CD44^{high} CD4 T cell subsets. Due to enhanced TNF- α and IFN- γ in lymph nodes that approached significance, we focused on T cells in circulation. We performed stimulation of CD4 T cells from peripheral blood of tm and tm24KO mice. Production of TNF- α and IFN- γ were increased in tm CD4 T cells as compared to tm24KO CD4 T cells (Figure 2C). Therefore it is likely that enhanced activation of T cells led to increased peripheral migration and subsequent inflammatory surveillance in tm mice.

Hallmarks of anti-inflammatory immunity

To better understand the cause of decreased CD4 T cell activation in tm24KO mice we assessed parameters of anti-inflammatory immunity. The cytokine TGF- β is tied to activity of immune-suppressive populations such as Treg cells and MDSCs (26, 27). We measured active TGF- β secretion from PBMC cultured 24 hours or from serum. We found significantly increased active TGF- β in culture supernatants and sera from tm24KO mice compared to tm mice (Figure 3A). To further investigate these data we isolated and counted absolute numbers of Tregs from tm or tm24KO mouse spleens and found significantly increased numbers of Tregs in tm24KO mice (Figure 3B). We next directly evaluated the role of Tregs in tm24KO mice as compared to tm mice. IL-10 is a hallmark anti-

inflammatory cytokine associated with Treg activation and suppression of inflammatory immunity (28). CD25, the high affinity IL-2 receptor, is a mark of Treg cell activation indicative of active IL-2 signaling and subsequent active Treg cell suppression (29). Compared to tm mice, Tregs from tm24KO mice displayed significantly increased ratios of CD25⁺ when measured from total Foxp3⁺/CD4⁺ Tregs (top panel) (*=p<0.05, tm=0.56±0.03, tm24KO=0.77±0.04). Further, intracellular cytokine stain for IL-10 production showed increased IL-10 from splenic Tregs in tm24KO mice compared to tm mice (bottom panel) (*=p<0.05, tm=1.2±0.7, tm24KO=7.8±2.1). (Figure 3C). CFSE suppression assay at a ratio of 10:1 showed increased suppression from tm24KO Tregs compared to tm Tregs (*=p<0.05, tm=1261±271, tm24KO=1945±174) (Figure 3D). Taken together, increased TGF-β, IL-10, and active Tregs indicate an enhanced regulatory immune axis in tm24KO mice as compared to tm mice.

Tm24KO mice have increased MDSC populations

We wished to further understand the driving force behind increased suppressive immunity in tm24KO mice as compared to tm mice. Decreased naïve T cell activation, enhanced Treg cell activity and increased TGF-β signaling are hallmarks of MDSC activation (10, 30). Furthermore, reports demonstrate that HMGB1 has the potential to drive enrichment of granulocytic and myeloid derived suppressor cell populations (11, 31). Due to loss of HMGB1 receptor CD24 in tm24KO mice we wished to assess a potential contribution of MDSC populations in these mice. Absolute numbers of splenocytes indicated a significant increase in the CD11b⁺ population in tm24KO mice as compared to tm mice (Figure 4A). We used flow cytometry to carefully assess the lineage of increased CD11b⁺ cells. We gated myeloid and lymphoid cells and looked at CD11b⁺/Gr1⁺ status. Both CD11b⁺/Gr1^{high} and Gr1^{dim} populations were increased in spleens of tm24KO mice when compared to tm mice. Further gating of the CD11b/Gr1 populations distinguished a heterogeneous population of cells defined by Ly6C^{high}/Gr1^{low} and Ly6C^{low}/Gr1^{high} expression. Both populations were increased in tm24KO mice (Figure 4B). We next evaluated these populations in blood taken from tm or tm24KO mice. We found significantly increased percentages of MDSCs in circulation (PBMC) in tm24KO mice as compared to tm mice. Gating of these cells indicated both Ly6C^{high}/Gr1^{low} and Ly6C^{low}/Gr1^{high} were significantly increased in tm24KO mice as compared to tm mice (Figure 4C).

We next measured the suppressive capacity of MDSCs between tm and tm24KO mice. Ratios of 1:1 and 10:1 were plated for Ly6G⁺ and Ly6C⁺ MDSC subsets. CFSE dilution of effector cells was measured by MFI. The Ly6C⁺ subset from tm24KO mice showed greater suppressive capacity of effector cell proliferation than the subset from tm mice (*=p<0.05, tm=1075±109, tm24KO=1850±232) (Figure 4D). To further measure Ly6C⁺ subset suppression we used Greiss reaction to assess nitrite release in response to LPS stimulation (32). Ly6C⁺ MDSCs from tm24KO mice showed significantly increased nitrite production in response to LPS compared to subsets from tm mice (Figure 4E).

Presence of MDSC reduce clinical manifestations of lupus-like disease

Our previous investigation of tm mice highlighted the development of lupus-like disease marked by anti-nuclear antibody deposition (ANA), glomerulonephritis (GN) and

hypergammaglobulinemia (13, 14, 24). Due to the suppressive atmosphere in tm24KO mice evidenced by increased MDSCs and Tregs, enhanced TGF- β axis and decreased CD4 T cell activation we wished to assess the manifestation of lupus-like disease in these mice as compared to tm mice. We first categorized ANA with a clinical score between 1 and 5 where 5 is the strongest fluorescent intensity. Among n=10 pairs of mice ANA score was significantly increased in tm mice as compared to tm24KO mice. Representative panels for tm and tm24KO ANA results are shown (Figure 5A). We next assessed glomerulonephritis in kidneys of tm or tm24KO mice. As categorized by fluorescent intensity (score 1–5), tm24KO mice showed decreased GN as compared to tm mice (n=5 pairs) (Figure 5B). Further, we performed H&E staining on kidneys from tm or tm24KO mice and counted nuclei per equal area section via 20X microscopy (13). Tm mice showed significantly more nuclei stain per field of area than tm24KO (Figure 5C). These data indicate higher levels of endothelial and mesangial cells in glomeruli of tm mice. These data indicate significantly less autoimmune disease in tm24KO mice as compared to tm mice. Increased gammaglobulin levels are a hallmark of lupus-like disease. We investigated serum levels of IgM, IgA, IgG1, IgG3, IgG2c, and IgG2b between tm and tm24KO mice (n=5 pairs). Only IgM production was significantly less in tm24KO mice as compared to tm mice (Figure 5C). Further analysis of B cell activation and markers of plasma cell development suggested there were no detectable differences between B cell activity in tm and tm24KO mice (data not shown). These data suggest that B cells were not affected by the enhanced suppressive milieu or increased MDSC populations found in tm24KO mice. Taken together, these results shown a decreased progression of lupus-like disease in tm24KO mice in an immune suppressive atmosphere marked by increased MDSCs, TGF- β axis, activated Tregs and decreased naïve T cell activation.

In the absence of CD24 free HMGB1 recruits MDSCs to impede progression of lupus-like disease

CD24 is a DAMP ligand for HMGB1 and HSPs (8). The role of soluble HMGB1 is not well defined as studies show both a pro and anti-inflammatory role for the cytokine (33). We wished to determine whether loss of CD24 led to altered levels of HMGB1 in the periphery of tm24KO mice. We performed western blot on serum of tm and tm24KO mice and probed for HMGB1. We noted increased HMGB1 in WT and tm24KO sera as mice aged that correlated with progression of autoimmune disease. Along these lines, tm24KO mice showed greater levels of sera HMGB1 at 12 months when compared to WT mice (Figure 6A). Reports demonstrate that HMBG1 has the potential to recruit granulocytic and myeloid derived cells (31, 34). To determine whether HMGB1 acted as a chemo-attractant for myeloid and granulocytic populations in tm and tm24KO mice, we injected mice with low dose (100 ng/mouse) HMGB1 and assessed myeloid cells in circulation prior to and 4 hours post injection. We found that in both tm and tm24KO mice injection of HMGB1 caused significant increases in CD11b⁺/Gr1⁺ populations found in blood 4 hours post injection (Figure 6B). Unexpectedly, tm24KO mice showed significantly more recruitment of CD11b⁺/Gr1⁺ cells as compared to tm mice. Data are plotted as percentage increase from baseline (Figure 6C). Taken together, enhanced levels of HMGB1 in serum were likely due to loss of CD24 as levels were largely detectable in tm24KO, but not tm mice. In the context

of autoimmunity, free-HMGB1 may act as a chemo attractant for MDSCs that led to relative amelioration of lupus-like disease in tm24KO mice.

Discussion

CD24 has been shown to play important but apparently opposite roles in adaptive and innate immunity. On one hand, Chen et al (8) demonstrated CD24 expressed on dendritic cells binds to DAMPs and represses inflammatory responses by innate immune effectors. On the other hand, CD24 has been shown to be a costimulatory molecule on antigen-presenting cells for activation of CD4 and CD8 T cells to antigens (35, 36), especially in the context where the host lacks robust costimulation by CD28 (36, 37). In addition, CD24 on T cells is essential for T cell homeostatic proliferation (38). In the context of autoimmune diseases, CD24-deficiency abrogates experimental autoimmune encephalomyelitis by preventing T cell expansion in CNS (15) and by promoting clonal deletion of autoreactive T cells (39, 40). The apparently opposite function of CD24 in inflammation and autoimmune diseases remain to be reconciled. Here we investigated the role of CD24 in an autoimmune context where endogenous DAMPs are consistently released.

Surprisingly, CD24 deficiency ameliorated clinical manifestations of autoimmune disease in a model of DAMPs-driven autoimmune disease. While CD24 deletion does not affect B cell activation and antibody production (Figure 6), it reduces T-cell activation, proliferation and cytokine production (Figure 2). We have previously shown that depletion of T cells leads to amelioration of the tm driven autoimmune phenotype (14). The T cell proliferation defect that occurs in the absence of CD24 may play a role in decreased inflammatory T cell proliferation and activity in tm24KO mice ultimately leading to decreased Th1 abetted autoimmunity.

We have reported enhanced suppressive capacity of Tregs in tm mice (24). Here we showed that Treg suppressive capacity is further enhanced by CD24 deletion. Our data show an increase in absolute numbers of Tregs and greater IL-10 production in tm24KO mice. Correspondingly, we noted an increase in splenic IL-10 and TGF- β activity in tm24KO mice (Figure 3). Moreover, perhaps because these cytokines are also associated with enhanced MDSC activation (10) we noted a significant increase in the MDSC population found in tm24KO mice. This population was heterogeneous in nature consisting of Ly6C^{high}/Gr1^{low} and Ly6C^{low}/Gr1^{high} populations. Increased numbers of MDSC in tm24KO may account for enhanced overall suppressive of SLE-like symptoms (Figure 5). MDSC expansion was consistent with increased TGF- β and diminished T cell activity in tm24KO mice, respectively (Figure 2–4). Further, Ly6C^{high}/Gr1^{low} MDSCs from tm24KO mice have enhanced suppressive capacity as compared to the matched tm subset. Upon ligation with TLR4 ligand LPS, we saw increased nitrite release from Ly6C^{high}/Gr1^{low} MDSCs in tm24KO mice. These data point to a role for CD24 in regulation of NO₂ release and subsequent MDSC regulation. Taken together, the increased Treg and MDSC suppressive activity in tm24KO mice are causative factors in diminished effector T cell activation and diminished SLE-like symptoms.

Nuclear protein HMGB1 is released from both necrotic and apoptotic cells and promotes inflammatory responses in both cancer and autoimmune diseases (41). The mechanisms through which HMGB1 promotes inflammation are not well defined, and it is important to note that cellular receptors for HMGB1 range from CD24, TLRs, HSPs and CXCL12 (42). In autoimmune contexts HMGB1 has been shown to exacerbate disease through several mechanisms. Nucleosomes containing HMGB1, detected in the serum of SLE patients, led to production of inflammatory cytokines IL-6, IL1 β , and TNF- α coupled to DC activation in a TLR-2 dependent manner (43). SLE patients with inflammatory skin lesions release HMGB1 at the lesion site upon exposure to UVB exposure (44). As a whole, reports show that HMGB1 is considered a potent inflammatory mediator in acute settings when associated with autoimmune disease. It is not understood how persistent low-level HMGB1 exposure will effect development of pro and anti-inflammatory immunity in autoimmune diseases. The data presented here show that increased levels of HMGB1 appeared in aged mice and correlated with the appearance of MDSC populations in tm24KO mice (Figures 4 & 6). Loss of CD24 as a potent HMGB1 receptor may explain increased free serum HMGB1 in tm24KO mice. We further show that indeed, HMGB1 acts as a powerful chemo-attractant for myeloid populations in with both tm and tm24KO mice, and tm24KO mice recruited significantly more myeloid cells than tm mice in response to acute HMGB1 (Figure 6). Thus, while CD24 also suppress host response to HMGB1 in DAMPs-driven autoimmune diseases, recruitment of MDSC provided a new mechanism to explain the apparently opposite function of CD24 in adaptive and innate immunity.

Although the role for HMGB1 in MDSC development has not been studied, HMGB1 has been shown to promote regulatory cell activation that enables suppression of host anti-tumor immunity. For example, neutralization of tumor-derived HMGB1 blocks Treg derived IL-10 and enables CD8 T cells and IFN- γ production resultant in tumor rejection (45). Further, in head and neck cancers it has been demonstrated that HMGB1 is a chemo-attractant for Tregs and promotes their suppressive capabilities (46). Promotion of Treg cell suppressive functions is associated with increased MDSC utility at the tumor site (47). In the context of cancer it is well understood that suppressor cells are detrimental to host anti-tumor immunity. However, in inflammatory-mediated autoimmune diseases Treg and MDSC populations may benefit the host. In keeping, cellular therapeutic strategies focus on restoration of immune tolerance through enhancement of these suppressor populations (48–50). The data presented here strongly support a potential role for Tregs and MDSCs in diminishing inflammation mediated autoimmune clinical manifestations. Further, the potential for low-level HMGB1 to drive these populations deserves further study.

Acknowledgments

We would like to thank members of the Li lab and members of Medical University of South Carolina Department of Immunology & Microbiology for continued support.

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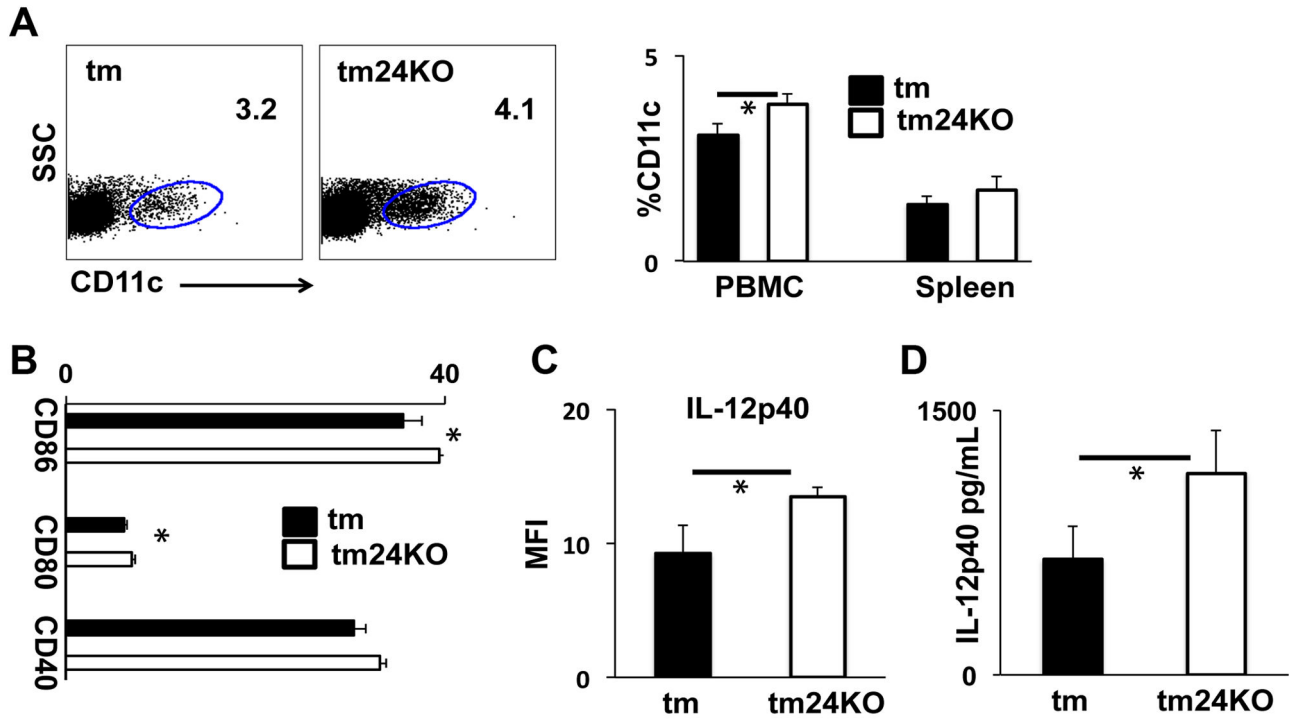


Figure 1. DC activation and IL-12 production in tm and tm24KO mice

Tm and tm24KO mice were subjected to flow cytometric analysis. (A) Surface staining of peripheral blood CD11c⁺ single-positive populations gated against SSC. Data of one representative mouse from 3 pairs of mice is shown. Averages of 3 mice per group from PBMC (*=p<0.05) or splenic CD11c⁺ populations are shown. (B) Single-positive CD11c⁺ population is gated and surface staining for CD80, CD86, and CD40 populations are gated and represented as average of MFI for 3 pairs of mice, (*=p<0.05). (C) PBMC from tm or tm24KO mice CD11c⁺ single-positive populations were gated on and intracellular IL-12 production was assessed. Data are graphed as average MFI of n=3 mice per group, (*=p<0.05). (D) Serum level of IL-12p40 at 6 months averaged for 6 pairs of mice, (*=p<0.05).

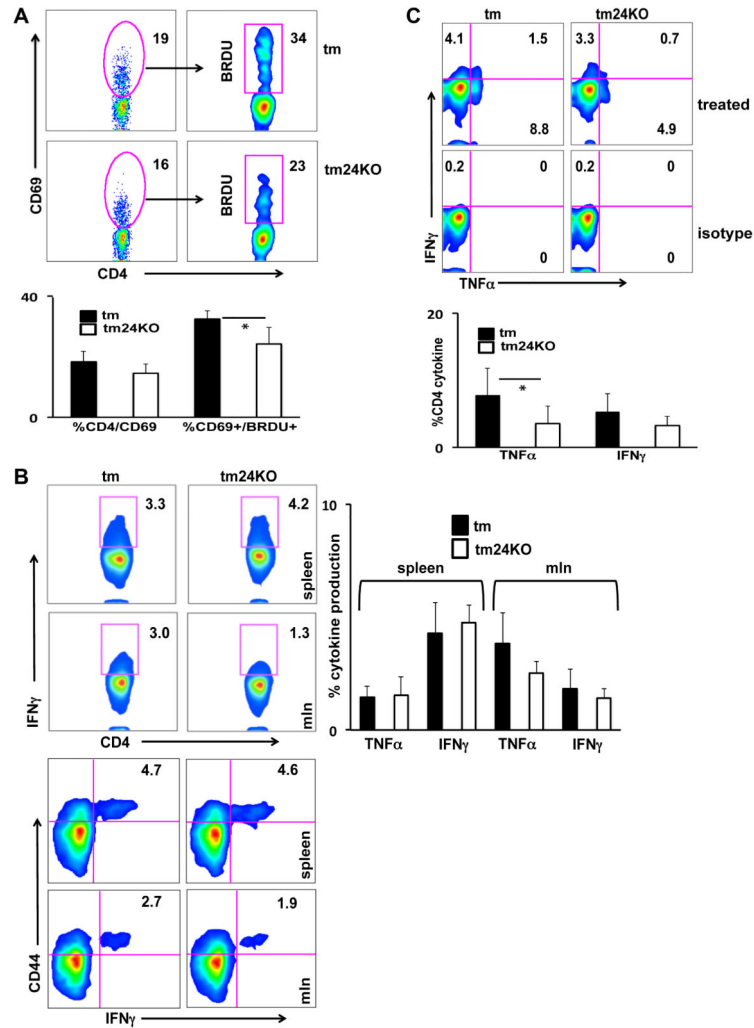


Figure 2. Decreased T cell activation, proliferation, and cytokine production in tm24KO mice
 Tm and tm24KO mice were subjected to flow cytometric analysis. CD4⁺ single-positive populations were gated on. (A) Splenocytes from 6 month tm or tm24KO mice were surface stained for CD69⁺ and intracellularly stained for BrdU after 3 days of BrdU water treatment. Data of one representative pair of mice from 4 pairs of mice is shown. Graph depicts statistical significance for CD4/CD69⁺ and CD4/CD69/BrdU⁺ cells, (*=p<0.05) (B) Splenocytes or mesenteric lymph node cells were incubated with PMA/Ionomycin in the presence of brefeldin A for 4–6 hours. Intracellular stain of IFN-γ (shown) and TNF-α were performed. Top flow panels show CD4-specific IFN-γ production. Bottom panels are gated on CD4⁺ single-positive cells and assessed for CD44/IFN-γ double positive populations. Data shown are one pair of mice, representative of 4 pairs of mice. Graph depicts statistical significance for CD4/TNF-α⁺ or CD4/IFN-γ⁺ splenocytes and mesenteric lymph node cells. (C) Peripheral blood mononuclear cells were incubated with PMA/Ionomycin in the presence of brefeldin A for 4–6 hours. Intracellular stain of IFN-γ (shown) and TNF-α were performed. Data of one representative pair of mice from 5 pairs of mice is shown. Graph depicts statistical significance for CD4/TNF-α⁺ or CD4/IFN-γ⁺ PBMCs, (*=p<0.05).

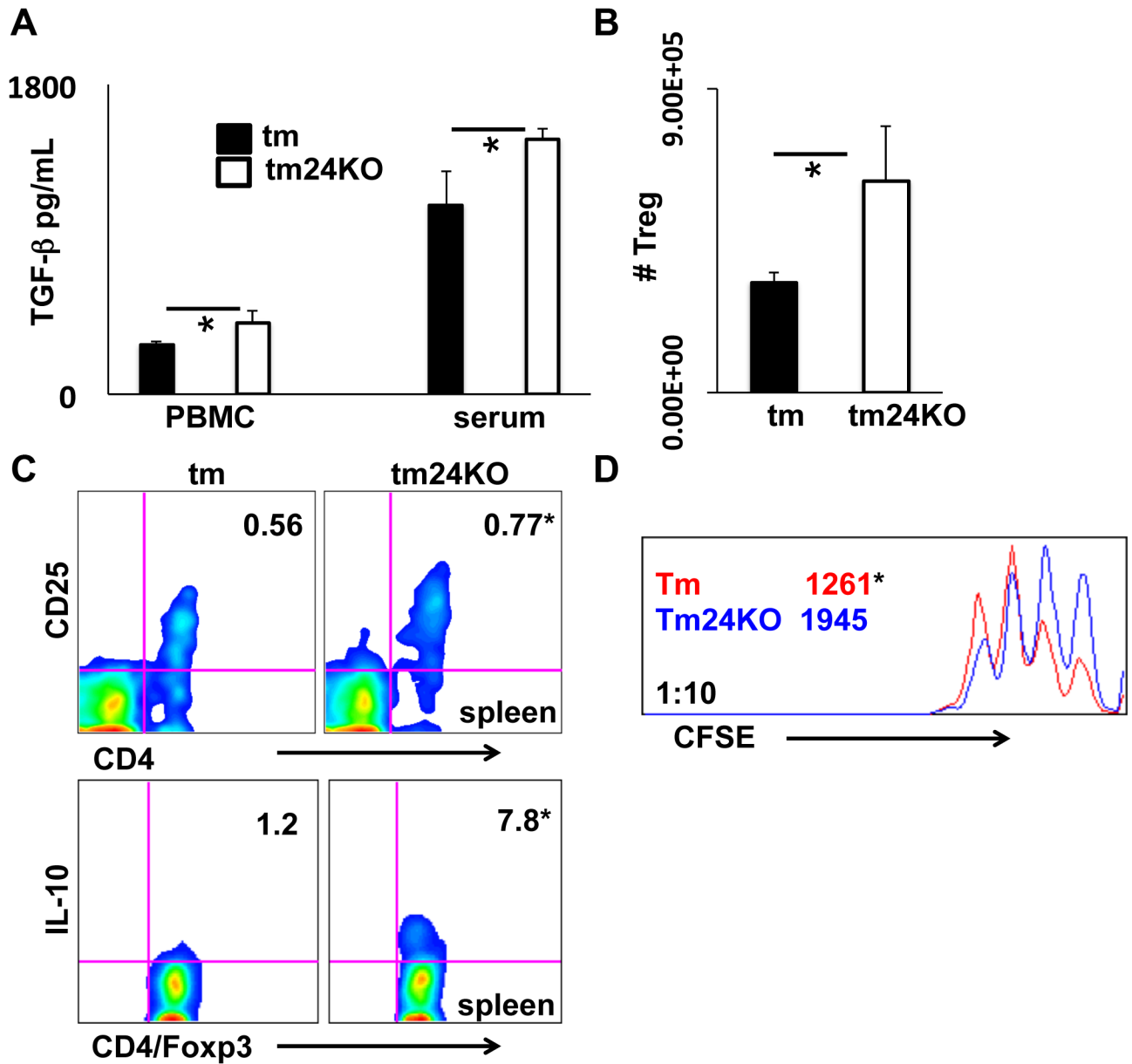


Figure 3. Measurement of Treg associated suppressive parameters in tm and tm24KO mice
 (A) Averages of active TGF-β measured by ELISA from 24 hour PBMC cultured supernatants (3 pairs of mice) or serum (6 pairs of mice), (*=p<0.05). (B) Tregs from spleens of tm or tm24KO mice were isolated and counted. Graphs are the averages for 3 pairs of mice, (*=p<0.05). (C) Splenocyte or mesenteric lymph node single-cell suspensions were extracellularly stained for CD4/CD25 (top panel). Splenocyte single-cell suspensions were incubated with PMA/Ionomycin in the presence of brefeldin A for 4–6 hours. Cells were extracellularly stained for CD4/CD25 and intracellular staining for Foxp3/IL-10 was performed. Bottom panels are gated on Foxp3+/CD4+ cells (*=p<0.05). Splenic data of one representative pair of mice from 3 pairs of mice is shown D) CD8 T cells and Tregs were isolated from tm or tm24KO spleens. CD8 T cells were labeled with CFSE and plated at

ratio of 10:1 with strain specific Tregs on CD3/28 coated plates. CFSE dilution was analyzed by flow cytometry 72 hours later. Representative histograms from 3 separate experiments are shown. Data are presented as average MFI of CFSE (*= $p < 0.05$).

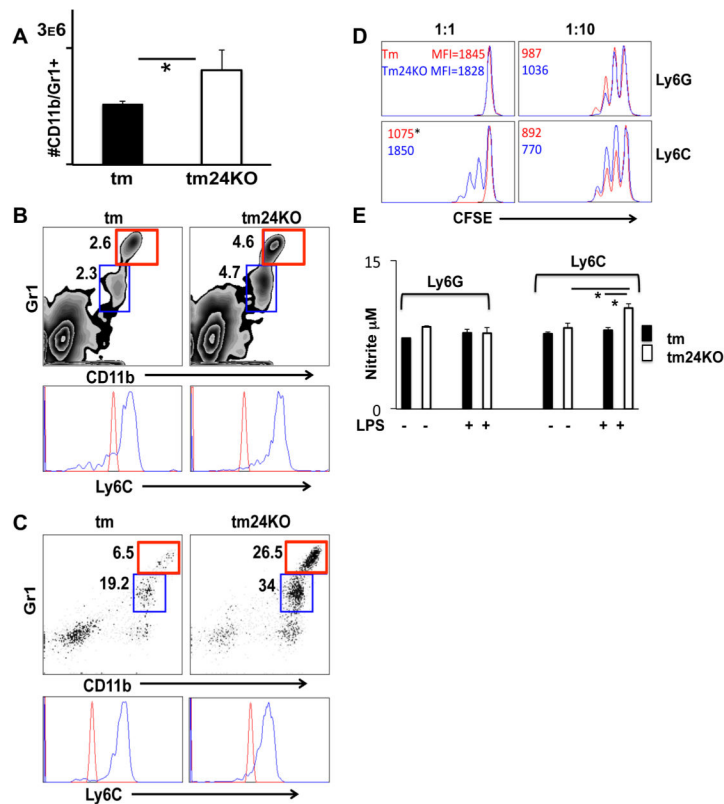


Figure 4. Characterization of MDSC populations in tm and tm24KO mice

Tm and tm24KO mice were subjected to flow cytometric analysis. Extracellular CD11b+/Gr1+ single-positive populations were gated, except for (D). (A) Spleen cells were counted and total number of Cd11b+/Gr1+ cells was calculated. Graph depicts averages from 3 pairs of mice, (*= $p < 0.05$). (B) Splenic or (C) peripheral blood CD11b+/Gr1^{high} (red) CD11b+/Gr1^{dim} (blue) cells are gated and histogram represents Ly6C⁺ dim (red) or high (blue) staining of these populations. (D) CD8 T cells, Ly6G⁺ and Ly6C⁺ cell fractions were isolated from tm or tm24KO spleens. Effector cells were labeled with CFSE and plated at ratios of 1:1 and 10:1 with strain specific MDSC subsets. CFSE dilution was analyzed by flow cytometry 72 hours later. Representative histograms from 3 separate experiments are shown. Data are presented as average MFI of CFSE (*= $p < 0.05$). (E) Ly6G⁺ and Ly6C⁺ cell fractions were isolated from tm or tm24KO spleens and 1×10^5 cells were plated with or without $1 \mu\text{g/mL}$ LPS for 24 hours. Greiss reaction was performed on cell supernatants (*= $p < 0.05$).

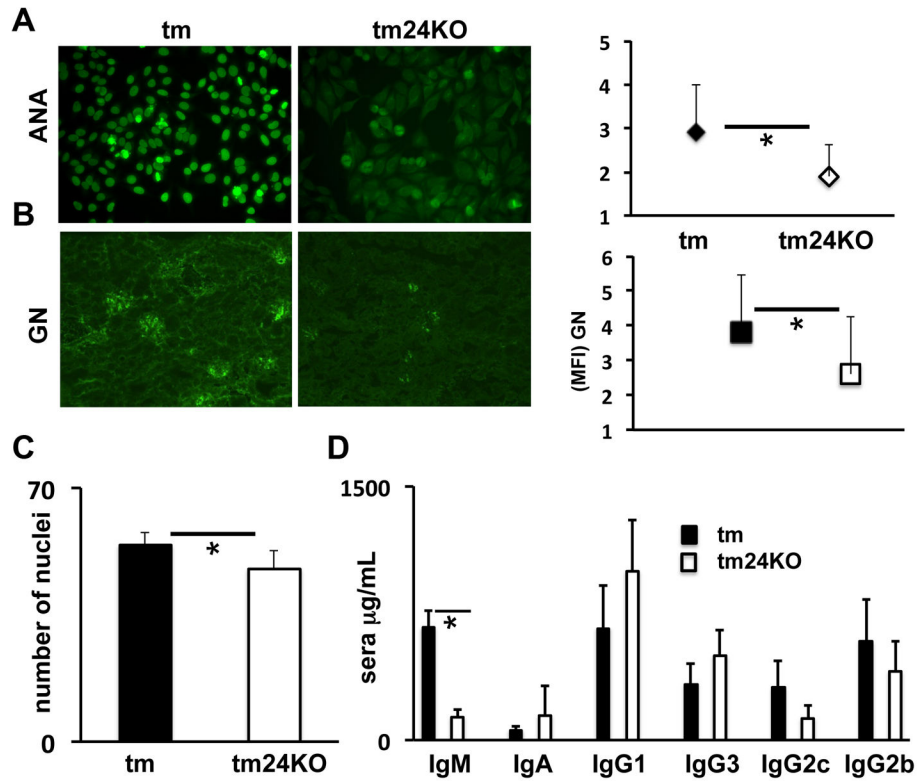


Figure 5. Decreased autoimmune disease in tm24KO mice as compared to tm mice
 Mean fluorescent staining intensity (MFI) of (A) ANA (B) GN in 6 month old tm or tm24KO mice categorized as clinical score 1–5 for disease severity (5 is severe disease). (C) Quantification of H&E number of nuclei per glomeruli (20X). Data are representative of one pair of 5 or more pairs of mice, (*=p<.05). (D) Serum level of IgM, IgA, IgG1, IgG3, IgG2b, IgG2c are averages of 5 pairs of mice, (*=p<.05).

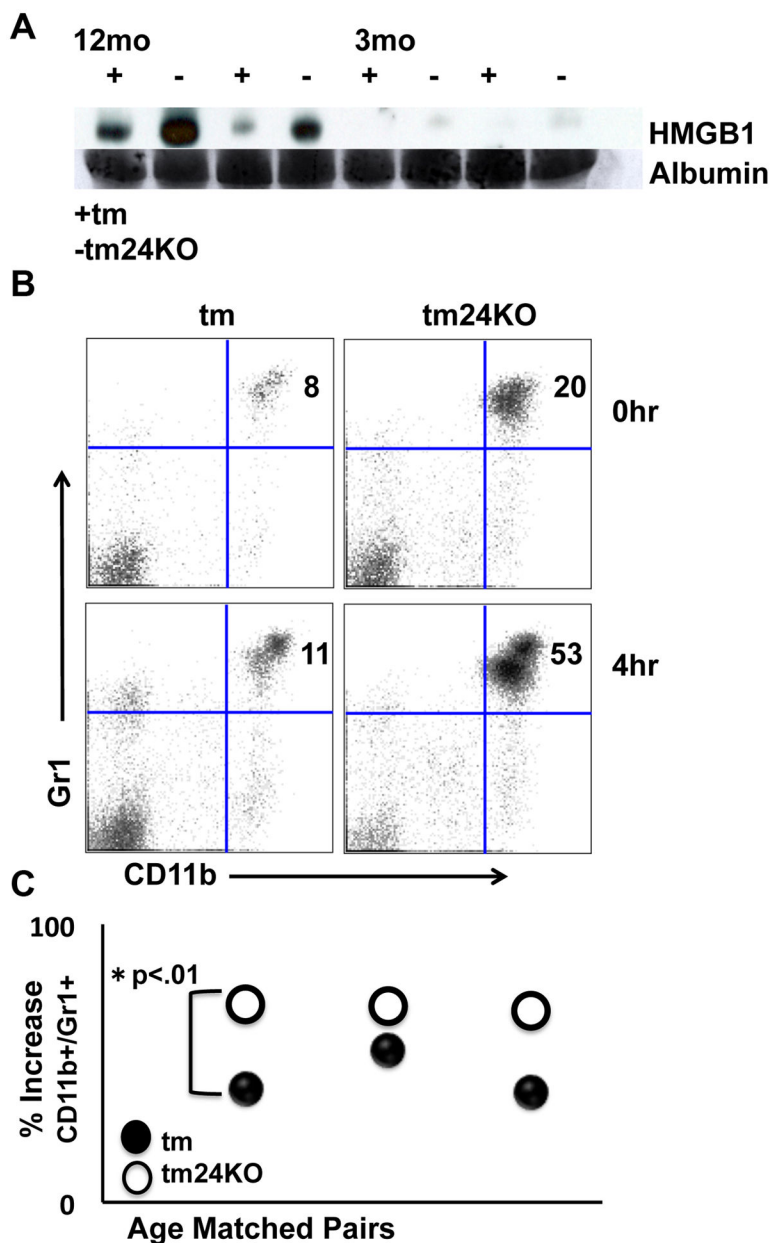


Figure 6. HMGB1 recruits increased numbers of MDSC in tm24KO mice
 (A) Western blot for HMGB1 on serum from tm (+) or tm24KO (-) mice aged 12 or 3 months. Data are representative of results from 4 pairs of mice. Albumin is used as a loading control from Coomassie blue de-stained gel. Tm or tm24KO mice were treated with 100 ng/mouse of rHMGB1 and flow cytometric analysis was performed on single-suspension PBMC pre (0 hr) and post (4 hr) injection for extracellular stain CD11b/Gr1⁺. (B) Data of one representative pair of mice from 3 pairs of mice is shown (C) Data are graphed as percentage increase of CD11b/Gr1⁺ cells from 0 hr to 4 hr time point (*=p<.01).