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Inhibition of TGF-β signaling in combination with TLR7 ligation re-programs a tumoricidal phenotype in Tumor-Associated Macrophages

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Abstract

Inadequate immunity that occurs in a tumor environment is in part due to the presence of M2-type tumor-associated macrophages (TAMs). TGF-β has a multi-functional role in tumor development including modulating the biological activity of both the tumor and TAMs. In this study, using an in vitro TAM/tumor cell co-culture system ligation of TLR7, which is expressed on TAMs but not the tumor cells, in the presence of TGF-β receptor I inhibitor re-programmed the phenotype of the TAMs. In part they adopted the phenotype characteristic of M1-type macrophages, namely they had increased tumoricidal activity and elevated expression of iNOS, CD80 and MHC class II, while TGF-β secretion was reduced. The reprogrammed phenotype was accompanied by enhanced NF-κB nuclear translocation. The pro-angiogenesis factor VEGF was down-regulated and in vivo the number of CD31-positive tumor capillaries was also reduced. Furthermore, in vivo we observed that TLR7 ligation/TGF-β receptor I inhibition increased tumor apoptosis and elevated the number of CD4+, CD8+, and CD19+ cells as well as neutrophils infiltrating the tumor. Our data demonstrate that selective TLR stimulation with TGF-β inhibition can reprogram TAMs towards an M1-like phenotype and thereby provides new perspectives in cancer therapy

1. Introduction

Innate and adaptive immunity co-operate to protect the host from microbial invasion and tumor formation. However, recent data indicate that tumor associated immune cells including dendritic cells, regulatory T cells, macrophages and neutrophils may function both to protect and promote tumor progression [1, 7, 21, 22]. The characterization of tumor associated macrophages (TAMs) has generated considerable interest. In lung, prostate and breast cancers and recently, Hodgkin's lymphoma clinical studies have established a positive correlation between TAMs present in the tumor and poor prognosis [18, 30]. More recent reports have indicated that TAMs can protect tumor cells from chemotherapy [34]. In animal studies, the depletion of TAMs by Clodronate encapsulated in liposomes (clodrolip) [8, 33] or genetically modified IκB kinase β expression can reprogram the functional phenotype of TAMs [6, 11] leading to reduced tumor progression and prolonged survival. These observations highlight the potential importance of targeting TAMs in tumor therapy.

TAMs express the phenotypic characteristics of M2 macrophages [27] which differ from those of M1 cells (classical activated macrophages) [21]. They produce low levels of pro-inflammatory cytokines such as TNF-α, IL-6, and large amounts of the anti-inflammatory cytokines IL-10 and TGF-β. TAMs also express VEGF and promote both matrix formation and the activation of tumor related regulatory T cells, all of which promote tumor progression [29]. Previous studies have indicated that the polarization of TAMs can be modified by their environment. The combination of CpG and anti-IL-10 receptor antibody treatment of tumor bearing mice reprogrammed the phenotype of the tumor infiltrating macrophages to that of M1 cells inducing TNF-α and IL-12 production and promoting tumoricidal adaptive immunity [9]. By altering the expression of intracellular signaling molecules such as STAT6, STAT3 and SHIP it has been demonstrated that the polarity of M2 cells can be changed [19]. However, it is not clear whether the changes are due to direct effects on TAMs or through changes in the microenvironment that alter the interaction between tumor cells and TAMs. More recent studies focused on the contribution of intracellular signaling pathways, such as c-MYC, in alternative activation of TAM activity [24]. TGF-β is an important growth factor in both the development of normal cells and tumor formation. Although it has been suggested that TGF-β functions in the prevention of tumor development during the early stages [28], in advanced tumors and in metastasis high expression of TGF-β is observed which correlates with the degree of malignancy. Consequently many anti-TGF-β treatments have been developed as potential tumor therapies [16]. The ability of TAMs to produce TGF-β [21] is well established for example in vitro altered TGF-β signaling mediated by expression of dominant-negative TβRII enhanced the tumor cytolytic activity of the macrophage cell line RAW [17]. Some studies illustrated the application of shRNA of

TGFβRII in reducing tumor metastasis [32]. However the contribution of TAM derived TGF-β in the tumor progression or in maintaining the M2 phenotype is ill defined.

Angiogenesis is a vital process in tumor development and is the target of many anti-tumor therapies. TAMs are thought to help in angiogenesis through the production of VEGF and the subsequent increase in CD31 positive tumor capillaries [33]. Two trans-membrane proteins TIE-2 and NRP-1 appear to be essential for angiogenesis, both of which are expressed in tissue macrophages and play important roles in vessel formation through the downstream gene VEGF [5]. The expression of TIE-2 and NRP-1 in TAMs is low [25] but whether TGF-β has any effect on the expression of these two molecules is unknown. Determining if the reprogramming of the phenotype of TAMs also inhibits their ability to promote angiogenesis will provide information that may be useful in developing novel therapies.

In brief, in this study, we have characterized the phenotype of TAMs isolated from fibrosarcoma cells stimulated with TLR ligands in the presence of specific TGF-β receptor I (TβRI) inhibitors. We report that TLR7 ligation in the presence of TGF-β inhibition in vitro re-programs TAMs to M1 cells and reduced their ability to produce angiogenesis promoting factors in macrophage/tumor cell co-cultures. In vivo treatment with a T□RI inhibitor and a TLR7 agonist increased the tumoricidal activity of the TAMs and reduced tumor progression.

2. Materials and methods

2. 1 Mice and reagents

Inbred male C57BL/6 mice (6-8 weeks old) were used. The experimental protocol was approved by the Committee on the Use of Live Animals in Teaching and Research, the University of Hong Kong. SB432542 was purchased from Tocris Bioscience, Bristol, UK; Rat anti-mouse CD11b, Rat anti-mouse CD11c, TGF-β Elisa kit was from BD Bioscience, San Jose, CA; Dispase I was from Roche Applied Science, Penzbeg Germany; DNase I was from Invitrogen, Carlsbad CA; Gardiquimod™, ODN1585, were from Invivogen, San Diego, CA; LPS (Salmonella), N-Acetylmuramyl-L-alanyl-D-isoglutamine hydrate (MDP) and SB505124 were from Sigma-Aldrich St. Louis, MO; Antibodies: rabbit anti-mouse F4/80 biotin-conjugated, CD4, CD8, CD19 antibodies, Elisa kit for TNF-α, IL-10 and IL-12 P40 were from BioLegend, San Diego, USA. Rat anti-mouse neutrophils antibody was purchased from ABCAM, MA, USA. Phoenix-Eco packaging cells were obtained from GENTAUR Europe, Kampenhout, Belgium. Rabbit anti-mouse NF-κB (P65) was from Santa Cruz, CA, USA

2. 2 Mouse fibrosarcoma tumor model and TAMs isolation and culture

Mouse fibrosarcoma model was established by intramuscular injection of $1x10^5$ MN/MCA1 cells into caudal thigh muscle as previously described [26]. Tumor mass was removed three weeks later. The tumor tissue was digested with Dispase I (2.4U/ml) with DNase I (25μg/ml) for 10 minutes with constant agitation at 37°C. The mixture was filtered through nylon mesh and further purified with F4/80 biotin-conjugated antibody together with anti-biotin microbeads. The purity of TAMs was >95% as determined by CD11b+CD11c- staining and measured by flow cytometry. Then isolated TAMs were cultured in RPMI medium with 10% FBS at 37ºC in 5% CO2 before treatment.

2. 3 Histology and Immunohistochemical analysis

4μm frozen or paraffin sections were cut and stained with Haematoxylin & Eosin. Immunohistochemical analysis was performed by cold acetone fixation, endogenous peroxidase blocking solution (Dako Corp. Carpinteria, CA) and 10% goat serum to prevent non-specific binding. The sections were incubated with primary antibodies for 16 hours at 4°C and the signals were detected with a DAKO EnVisionTM+ System. Rat anti-mouse antibodies specific for CD4(1:100), CD8(1:100), F4/80(1:200), CD31(1:200) and neutrophils(1:100) were used in the experiments.

2. 4 Transfection of luciferase into MN/MCA1

MN/MCA-Luc cells were generated by retroviral transduction. To prepare pMIGR.Luc retroviral constructs, the full length luciferase gene was excised from pGL3 vector between BglII and BamHI sites and cloned into pMIGR retroviral vector at the BglII site. The pMIGR.Luc constructs were then transfected into Phoenix-Eco packaging cells with lipofectamine™ for synthesis of retroviral supernatants. Supernatants were collected on day 2 post-transfection. For retroviral transduction, MN/MCA cells were cultured with 50% (v/v) of the retroviral supernatant in the presence of polybrene (8μg/ml) at 37ºC for 24 hours. Culture supernatants were then removed and replaced with fresh medium. GFP-luciferase positive cells were isolated by cell sorting.

2. 5 Quantitative -PCR

The total mRNA was isolated with TRIZOL®. Q-PCR was performed according to manufacturer's instructions. Briefly, RNA was reversed transcribed random primer and SuperScript II reverse transcriptase (Invitrogen Corp). PCR was performed using Taq polymerase (Applied Biosystems) with the SYBR® Green-Based Detection system. Relative quantification of mRNA expression was normalized with control GAPDH and analyzed using the Delta Delta Ct (2-ΔΔCT) method. Primers sequences (Forward/Reverse): Ym-1: gcataccttt atcctgagtg acc/gacattgtca taaccaaccc actc; iNOS: gccgtggcca acatgctact/ ggtcttccrg ggctcgatct g; TLR4: tgacaccctcc atagacttc/ tgcttctgtt ccttgaccca c; TLR7: ggacctcagc cataaccagc/cccaccagac aaaccacaca gc; TLR9: atcgcatcag tgggccttca acg/ggcaggaact gagagccatt gac; NOD2: gtgctttttt gccgctttct acttgg/ttcttgactc tggagccctg gatac. VEGF: gctactgccg tccgattgag a/ ggtgaggttt gatccgcatg a; NRP1: gatgagtgtg acgacgacca ggcca/tcccagtggc agaatgtctt gtgag.

2. 6 Western blot analysis

10μg protein were denatured and separated by electrophoresis. Proteins were electro-transferred onto PVDF-membranes and then blocked with TBST with 5% low-fat milk. This was followed by an overnight incubation at 4°C with anti-TRAF6 and anti-NF-κB antibodies (1:1000) and further incubated with a horseradish peroxidase (HRP)-conjugated secondary antibody. The signals were developed using ECL chemi-luminescence (Amersham). The membrane was then stripped and reprobed with anti-actin antibody.

2. 7 ELISA measurement cytokines expression

The secreted cytokines in the culture supernatants were measured by ELISA according to manufacturer's instructions. In order to measure the total concentration of TGF-β, supernatants were acidified. All the measurements were

made in triplicate and minimum 2 sets of independent experiments for each treatment were performed.

2. 8 Flow cytometry

The cell surface activation markers CD40, CD86, MHC class II and CD80 were analyzed using flow cytometry. After tissue digestion or cell culture, single cell suspensions were labeled with antibodies and analyzed by flow cytometry. Appropriate isotype-matched IgGs were used as negative controls.

2. 9 Luciferase activity measurement

For testing the tumoricidal activity of TAMs in response to the different treatment regimens, 1x105 cells (TAMs) were co-cultured with 2,500 MN/MCA1-Luc for 3 days. The luciferase activity was measured using Dual-Luciferase® Reporter Assay System (Promega) with MicroBeta® TriLux β-counter (Perkin Elmer, MA). Each sample was performed in triplicate and the averages of each point were calculated.

2. 10 *In vivo* analysis of biological effects of TLR7 ligation together with TGF-β inhibition on tumor progression

The tumor model used here has been described previously (Section 2.2). In brief, $1x10^5$ MN/MCA1 cells were injected in caudal thigh muscle. SB505124 was dissolved in DMSO then further in PBS and the Gardiquimod in PBS. Less than 30µl of DMSO was injected intraperitoneally in mice and the vehicles alone were used as control groups. The treatment was initiated 3 days after tumor cell inoculation and continued for 2 weeks with delivery on alternative days. At end of treatment, the tumor size was analyzed by an IVIS imaging system (PerkinElmer, MA, USA) and photographed. The mice were then sacrificed and the tumors were isolated for further analysis by immunostaining.

2. 11 Tunnel assay

The tissue apoptosis was evaluated by using In Situ Cell Death Detection Kit Fluorescein (Roche Diagnostics (Hong Kong) Ltd) following the manufacturer's instructions. The immuno-fluorescent signals (Annexin-V-FITC) were detected with Nikon 80i fluorescent microscope and photographed. DAPI was used for control nuclear staining.

2. 12 Statistical analysis

All the data here are presented as means±SEM. In order to determine statistical significance results were analyzed with the Student paired t test when two sets of

data were compared or one way ANOVA for the comparison of three set of data. Bonferroni's Multiple Comparison Test was used to compare the difference among the groups. $p<0.05$ was considered significant. The data were collected and analyzed using GraphPad Prism software (GraphPad Software, Inc., CA).

3. Results

3. 1 Effect of tumor supernatants on TGF-β1 and TNF-α biosynthesis by TAMs

In order to test the effects of microenvironmental changes on the function of the TAMs they were cultured with tumor derived supernatants (50% v/v) in the presence and absence of LPS and TGF-β1 and TNF-α production was measured (Fig.1A). In the untreated TAM cultures only basal levels of TGF-β1 were detected which remained unaltered by the addition of the tumor supernatants. Stimulation with LPS at 10 and 200ng/ml only minimally enhanced TGF-β1 production. However, the addition of tumor supernatants reduced TGF-β expression in LPS stimulated TAMs to below basal levels (from 344pg/ml to 37pg/ml, $p<0.05$). Regarding TNF-α, the basal levels recorded in the absence of LPS stimulation was slightly reduced by exposure to the tumor supernatants. LPS stimulation increased TNF-α production which was significantly reduced following the addition of the tumor derived supernatants (Fig 1A). Similarly, IL-10 and IL-12 P40 secretion was also reduced in response to TS treatment. These results suggest that the tumor microenvironment can modulate TAM activity as regards cytokine expression. In subsequent experiments, therefore, tumor supernatants were added to replicate some aspects of *in vivo* conditions.

3. 2 Effects of TGF-β receptor I inhibitors on TAM function

TGF-β signaling was blocked by the addition of the TGF-β receptor I inhibitor, SB431542 (SB4x) and the effects on tumoricidal activity were examined after 3 days. Exposure to LPS in the presence and absence of SB4x had no obvious effects on TAM morphology compared to the control cultures (Fig.1B). However, when TAMs were co-cultured with tumor cells (TAM:MN/MCA1=10:1) and stimulated with LPS there was a slight inhibition of tumor cell growth. The phenomenon was more marked when LPS and SB4x were both present in the co-cultures as most of the cells died (Fig.1B).

3. 3 Expression of pattern recognition receptors

Since the tumoricidal effect was minimal when the ratio of TAMs to MN/MCA1 was reduced to 3:1 we investigated whether it could be increased by enhanced activation of the TAMs. Macrophage activation can be achieved by ligation of pattern recognition receptors (PRR) such as Toll-like receptors (TLRs) or Nod-like receptors (NODs). The expression of TLR4 on tumor cells can aid escape from immune surveillance and increase resistance to apoptosis [12, 13]. Therefore, PRR expression was compared on MN/MCA1 and TAMs in order to identify a receptor that was present on TAMs but absent or reduced on MN/MCA1 cells thereby allowing activation of the TAMs but not the tumor cells when stimulated with the appropriate

ligand. The levels of specific transcripts for TLR4 and NOD2 were similar in both MN/MCA1 cells and TAMs. However, there were major differences in the expression of TLR7 and TLR9, which were present at very low levels in the MN/MCA1 cells compared to TAMs (Fig.2A). Following stimulation with appropriate ligands the expression of TLR4, 7, 9 and NOD2 was measured in the presence and absence of SB505124 (SB5x), a TGF-β receptor I inhibitor which is 5 fold more potent than its analogue SB4x [2]. TLR4 and NOD2 expression was slightly reduced by treatment with specific ligand or ligand plus SB5x compared to the untreated co-cultures. Transcripts for TLR9 were increased following stimulation with ODN1585 but remained unchanged when SB5x was added. The ligation with Gardiquimod had minimal effect on TLR7 expression but in the presence of SB5x treatment expression was markedly increased (Fig.2B).

3. 4 Effects of TLR7 stimulation together with TGF-β inhibition on the phenotype of TAMs

The effects of TLR7 ligation on TAM tumoricidal function were investigated. Tumor cells transfected with luciferase (MN/MCA1-Luc) were used as target cells to allow quantitation of the killing activity. Compared to the untreated control group of tumor cells alone the addition of Gardiquimod, SB5x or two together has no obvious effects on tumor cell proliferation since similar luciferase activity was observed in all these conditions (Fig.3A). However, when the tumor cells were co-cultured with TAMs in the presence of Gardiquimod alone tumor cell proliferation was reduced by 40% ($p<0.01$). Similarly the addition of SB5x alone also reduced tumor cell proliferation but it was less marked than that of Gardiquimod ($p>0.05$). The effect was further enhanced when SB5x and Gardiquimod were added together (70% inhibition, $p<0.001$; Fig.3A MN+TAM).

As regards cytokine production TGF-β was significantly reduced in co-cultures treated with Gardiquimod and SB5x compared to the controls ($p>0.05$) and in contrast, TNF-α was slightly increased but was not statistically significant (Fig.3B). Combined treatment with Gardiquimod and SB5x resulted in an up-regulation of iNOS mRNA ($p<0.01$) and down-regulation in the expression of Ym-1mRNA ($p<0.001$) in the co-cultures (Fig.3C). Furthermore, cell surface expression of MHC class II and CD80 was elevated in the combined treatment versus Gardiquimod treatment alone (Fig.3D).

3. 5 The effects of TLR ligand/TGF-β inhibitor treatment on intracellular signaling

A notable difference of M2 and M1 cells is their NF-κB activity, which is reflected in enhanced expression of the p65 intra-nuclear activity of the NF-κB subunit [10]. Therefore intracellular expression of TRAF6 and NF-κB were examined in order to determine if the level of intracellular signaling changes in TAMs in response to TLR

ligand/TGF-β inhibitor treatment. For TRAF6, there was a modest increase in expression induced by Gardiquimod which was further elevated by the addition of the TGF-β inhibitor. A similar outcome was observed for NF-κB expression (Fig.4A). NF-κB expression and the nuclear translocation were examined in the co-cultures and compared to the untreated control treatment with Gardiquimod and SB5x greatly increased the expression of NF-κB with enhancement of nuclear expression (Fig.4B).

3. 6 In vivo effects of TLR7 ligation and TGF-β inhibition on tumor progression

In this set of experiments we investigated if treatment with Gardiquimod and SB5x reduced tumor growth in vivo. Tumors were established by MN/MCA1 inoculation for 2 days and then the compounds were delivered by intra-tumoral injection. Compared to the control group Gardiquimod and SB5x treatment greatly increased tumor apoptosis as demonstrated by TUNEL (Fig.5a). Analysis of the cellular infiltrate by flow cytometry revealed an increase in CD4+, CD8+ and CD19+ cells in the treated mice (Fig.5b). In addition within the tumor mass there was an increase in CD11b+ cells which expressed higher levels of MHC class II and CD86, although the total number of CD11b+ cells was slightly reduced (data not showed). Immuno-histochemical studies confirmed the increase in CD4+ and CD8+ cells. We also detected increased numbers of neutrophils, but the number of TAMs was slightly reduced (Fig.5C). The inhibition of tumor progression was determined by *in vivo* luciferase activity imaging and when compared to the untreated group treatment with SB5x and Gardiquimod markedly reduced the tumor size (Fig.5D).

3. 7 The effects of TLR7 ligation and TGF-β inhibition on angiogenesis

A characteristic of TAMs is their ability to promote tumor angiogenesis. Therefore, we investigated the effects of TLR7 ligation/TGF-β receptor 1 inhibition on the expression of NRP-1 and VEGF mRNA. In TAMs plus MN/MCA1 co-cultures, Gardiquimod alone reduced NRP-1 expression however when both compounds were delivered there was no significant down-regulation (Fig6A). In contrast, Gardiquimod treatment alone had no effect on VEGF expression whereas SB5x alone and Grad plus SB5x significantly reduced its expression. In parallel in vivo we stained for CD31+ cells and observed that the number of CD31+ cells in the tumor capillaries in combination treated group was greatly reduced (44.6%, $p<0.001$).

4. Discussion

TGF-β1 can be detected in tumor tissue [4] isolated from both cancer patients and experimental animal tumor models [23]. Here we have examined the regulation of TGF-β1 production by TAMs that occurs following their interaction with fibrosarcoma (MN/MCA1) tumor cells in vitro. Our results demonstrated that TAMs alone expressed low levels of activated TGF-β1 which were unaffected by the addition tumor cell derived supernatants. Activated TGF-β was increased after LPS stimulation but this response was reduced in the presence of tumor supernatants. Thus the tumor microenvironment seems to be important in regulating TGF-β production even in the presence of TLR mediated activation. For the mouse MN/MCA1 cells TGF-β was not essential for their proliferation as the TGF-β inhibitor had no effect suggesting that TGF-β1 production may be primarily regulating TAMs activity. TNF-α is effective in tumor killing [3] but recent findings suggest that it may also promote tumor metastasis[14]. Here we observed that the basal production of TNF-α by TAMs was low but was greatly increased by LPS stimulation. However, in the presence of the tumor derived supernatants this LPS mediated induction of TNF-α was significantly reduced. The effect of the tumor supernatants which in part mimic the tumor microenvironment indicates that TAMs activity is modulated by the tumor cells. It has been reported that tumor supernatants promote myeloid-derived suppressor cell (mainly macrophages) production of VEGF and bFGF resulting in enhanced angiogenesis[15]. Our results are in agreement, in the context that some tumors can produce factors that are capable of altering macrophage function.

The effect of TGF-β signaling on the phenotype of TAMs was investigated in the presence of TGF-β receptor 1 inhibitors. Exposure to LPS or LPS together with SB4x had no obvious effects either on tumor cell proliferation or TAM morphology (data not shown). However, in the TAM/tumor cell co-cultures the addition of LPS and TGF-β inhibitor resulted in reduced tumor cell growth. It has been suggested that macrophages with characteristics of M1 cells display tumoricidal activity when activated with LPS [20]. Our data here suggest that it is possible to partially reverse the phenotype of the TAMs towards that of M1 type macrophages. However, in vitro the tumoricidal effect was limited at reduced ratios of TAMs to MN/MCA1 cells. It has been suggested that the expression of TLR4 in tumor cells reduces apoptosis [12], which prompted us to look for other ways of amplifying the response of the TAMs without activating the tumor cells. We observed that basal expression of TLR7 and 9 was much lower in MN/MCA1 cells than in the TAMs. In TAM/tumor cell co-cultures stimulation with TLR7 specific ligand in the presence of TGF-β inhibitor the expression of TLR7 was markedly elevated. The combination of TLR7 signaling and TGF-β blockade also inhibited tumor cell proliferation, reduced TGF-β expression, increased iNOS expression and lowered that of Ym-1 in the TAMs. Both MHC class II and CD80 were up-regulated suggesting that the treatment both activates and

reprograms TAMs towards the M1 phenotype which would favor the induction of anti-tumor adaptive immunity.

It has previously been reported that NF-κB signaling is reduced in TAMs but can occur in the presence of STAT1 implying a functional IFN-β/IFN-γ pathway. Furthermore, TGF-β1 is negative regulator of TLR4 and MyD88. The inhibition of TGF-β signaling together with TLR7 stimulation increased NF-κB activation through the up-regulation of TRAF6 and increased translocation of NF-κB. However, the failure to detect the early phase of MKK activation after stimulation with LPS and the TGF-β inhibitor suggests that the reprogramming towards to a M1 phenotype, induced by TLR7 ligand stimulation, is only partial.

In order to establish proof of principle that TAM reprogramming can occur in vivo and prevent and/or reduce tumor progression mice were treated with the combination of SB5x and TLR7 ligand. This resulted in the reprogramming of an M1 phenotype in the TAMs and marked killing of the tumor cells in vivo. The reduction in tumor cells may be partly due to an increase in the number of inflammatory cells infiltrating the tumor. This implies that the immunosuppressive effect of the TAMs is diminished perhaps as the result of reduced of TGF-β production and increased MHC class II expression. Furthermore, the combination treatment also reduced the ability of TAMs to promote angiogenesis. It has been reported that TGF-β1 stimulates VEGF expression via MKK3 and activation of p38α and p38δ MAPK-dependent pathway in murine mesangial cells [31]. The reduction in TGF-β1 expression will have a direct effect on VEGF synthesis. Here we observed no TIE-2 expression (data not shown) and the basal expression levels of NRP-1 were not affected by TGF-β inhibition suggesting in TAMs that VEGF expression may be more related to TGF-β signaling than TIE-2 and NRP-1.

The results also revealed that the balance of tumor cell proliferation and tumoricidal activity of reprogrammed TAMs was altered. At early time points treatment effectively controlled tumor growth however, if it commenced on day 7 after tumor cell inoculation the effect was minimal. This outcome may due to a large tumor burden in vivo since we observed in the in vitro studies that with higher numbers of tumor cells (i.e. TAM/tumor cell ratios of 1:3) the tumoricidal effect was limited. Similar observations were reported following with anti-IL-10 antibody and CpG treatment where the minor effect on tumor progression could be potentiated by adding CCL16 to recruit more DCs or macrophages to the tumor [9]. In agreement with that we have observed that treatment together with low dose of cisplatin (1mg/kg) enhanced tumor killing (data not showed) which confirms that reprogramming TAMs may facilitate tumor treatment.

In summary our results demonstrate that TLR mediation signaling in combination with TGF-β inhibition can partially reprogram TAMs to a M1 phenotype and reduce tumor progression in vitro and in vivo in this mouse model of fibrosarcoma.

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Conflict of Interest Statement:

No potential conflicts of interest were disclosed

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Figures legends

Figure 1: Effects of tumor supernatant on TAM cytokines expression and TGF-β inhibition on the modulation of TAMs phenotype

1A Effects of tumor cell derived supernatants on TGF-β, TNF-α, IL-10 and IL-12 production. TAMs ($1x10^6$/well) were cultured in 2ml RPMI medium with 10% FBS alone (RPMI full) or with medium supplemented with 50% (v/v) tumor cell culture supernatant (TS). Tumor culture supernatants were harvested from tumor cell cultures after 3 days. Levels of TGF-β, TNF-α, IL-10 and IL-12 P40 in TAMs cultures were measured by ELISA. LPS10, LPS200 indicated LPS at concentrations of 10ng/ml and 200ng/ml respectively. $p<0.05$ n=4 compared to no tumor supernatant added group. **1B** TAMs were isolated from mice inoculated with tumor cells (MN/MCA1) for three weeks. Cells ($1x10^6$/wells) were cultured in medium with tumor supernatants alone or with tumor cells ($1x10^5$/wells) in the presence of LPS (10ng/ml) or LPS+SB4x (10μM) for 3 days and the cells photographed. (n=4)

Figure 2: Expression of TLR4, 7, 9 and NOD2 in TAM and tumor cells and changes in their expression in response to the ligands

2A Untreated TAMs, tumor cells and **2B** Co-cultures of TAMs and tumor cells (MN/MCA1) in the presence of respective TLR4 ligand LPS (10μg/ml), TLR7 ligand Gardiquimod (5μg/ml), TLR9 ligand CpG (5μg/ml) or NOD2 ligand MDP, 1μg/ml) with and without TGF-β inhibition (SB5x, 5μM) were harvested after 24hrs and specific transcripts for TLR4, 7, 9 and NOD2 were measured by Q-PCR. Each experiment was performed in triplicate and at least two independent experiments were performed. The *p* value for the statistical significance is indicated in the graph. **2C** Western blot analysis of the expression of TLR4 and TLR7 in TAMs and MN/MCA1 cells (n=2, one representative experiment is presented).

Figure 3: Effect of the TLR7 ligand Gardiquimod on modulating TAM gene expression and activation

3A MN/MCA1-Luc (2,500/well, MN, left) or MN/MCA1+TAMs ($2,500+1x10^5$, respectively, MN+TAM, right) were cultured in 96 wells culture plate in the presence of Gardiquimod (Gard, 5μg/ml), SB5x (5μM) and Gard+SB5x for three days and the luciferase activity were measured. Each treatment was triplicate and the average was shown. **3B** Levels of TGF-β and TNF-α biosynthesis in TAMs ($3.8x10^5$/well) alone or co-cultured with MN/MCA1 ($3.8x10^4$/well) in the presence of Gardiquimod, SB5x or together (same concentration as mentioned above). Supernatants were collected after 24hrs and cytokine levels measured by ELISA. **3C** Expression of iNOS and Ym-1 mRNA in the presence of Gardiquimod +/- SB5x was measured after 4 hr of treatment by Q-PCR. **3D** Cell surface expression of CD40, CD80, CD86 and MHC class II was measured by flow cytometry after 24hrs treatment with Gardiquimod

together with TGF-β inhibition and the mean fluorescent intensity (MFI) was shown. n=3 in each data sets and the *p* value for the statistical significance is indicated in the graph

Figure 4 Modulation of intracellular signaling in response to LPS or Gardiquimod in presence of TGF-β inhibition

4A The TAMs were treated with LPS (10μg/ml), LPS with SB5x (5μM), LPS+SB5x, Gardiquimod (Gard, 5μg/ml) or Gardiquimod with SB5x (Gard+SB5x) for 24hrs. The protein was extracted for western blotting. n=3 and one representative was shown. **4B** For the NF-κB translocation assay cells were treated with Gardiquimod with SB5x (same concentration as above) for 30min and fixed in 4% paraformaldehyde for 10 min, followed by immunohistochemical staining of NF-κB p65 and compared to untreated group. Nuclear position was determined with DAPI staining. Green Arrow showed the nuclear expression of NF-κB in TAMs

Figure 5: In vivo analysis of Gardiquimod and TGF-β blockade on tumor progression

5A TUNEL assay. H-E staining; FITC-labelled annexinV and DAPI staining for nuclear location in tissue sections. **5B** Analysis of tumor infiltrate. The tumor mass was removed and single cell suspension prepared. Cells were stained for CD4, CD8, CD19 and TAMs were stained with CD11b, CD86 and MHC Class II then analyzed by flow cytometry. **5C** Immunohistochemical analysis of immune cells infiltrate compared with untreated controls and those mice receiving Gardiquimod + SB5x treatment; the sections were stained with anti-CD4, anti-CD8, anti-F4/80 for macrophages and anti-neutrophils and the nuclear were counterstained with methyl green. **5D** Effect of SB5x+Gard in tumor formation at early time points. The drugs (Gardiquimod 1mg/kg and SB5x 10mg/kg) were injected at day 2 after tumor cell (Luc-MN/MCA1) inoculation. The tumor formation was monitored twice daily and at day 14, the luciferase activity was measured and photographed with IVIS® Imaging System (n=6 in each treatment, 3 in each group were showed)

Figure 6: analysis of Gardiquimod and TGF-β blockade on tumor angiogenesis

6A Expression of NRP-1 and VEGF mRNA in vitro in response to Gard, SB5x and combine treatment. TAMs ($3.8x10^5$) and MN/MCA1 ($3.8x10^4$) were co-cultured in the presence of reagents for 24hrs and the expression of NRP-1 and VEGF was measured by Q-PCR. The drug concentration used was same as previously described for SB5x (5μM) and Gardiquimod (Gard, 5μg/ml). n=3 and the *p* value for the statistical significance is indicated in the graph. **6B** CD31 Immunohistochemical staining in frozen tumor tissue sections for the control untreated and Gard+SB5x treated groups. The brown color showed the positive signals and the number was counted as shown in **6C**

Figure 1A

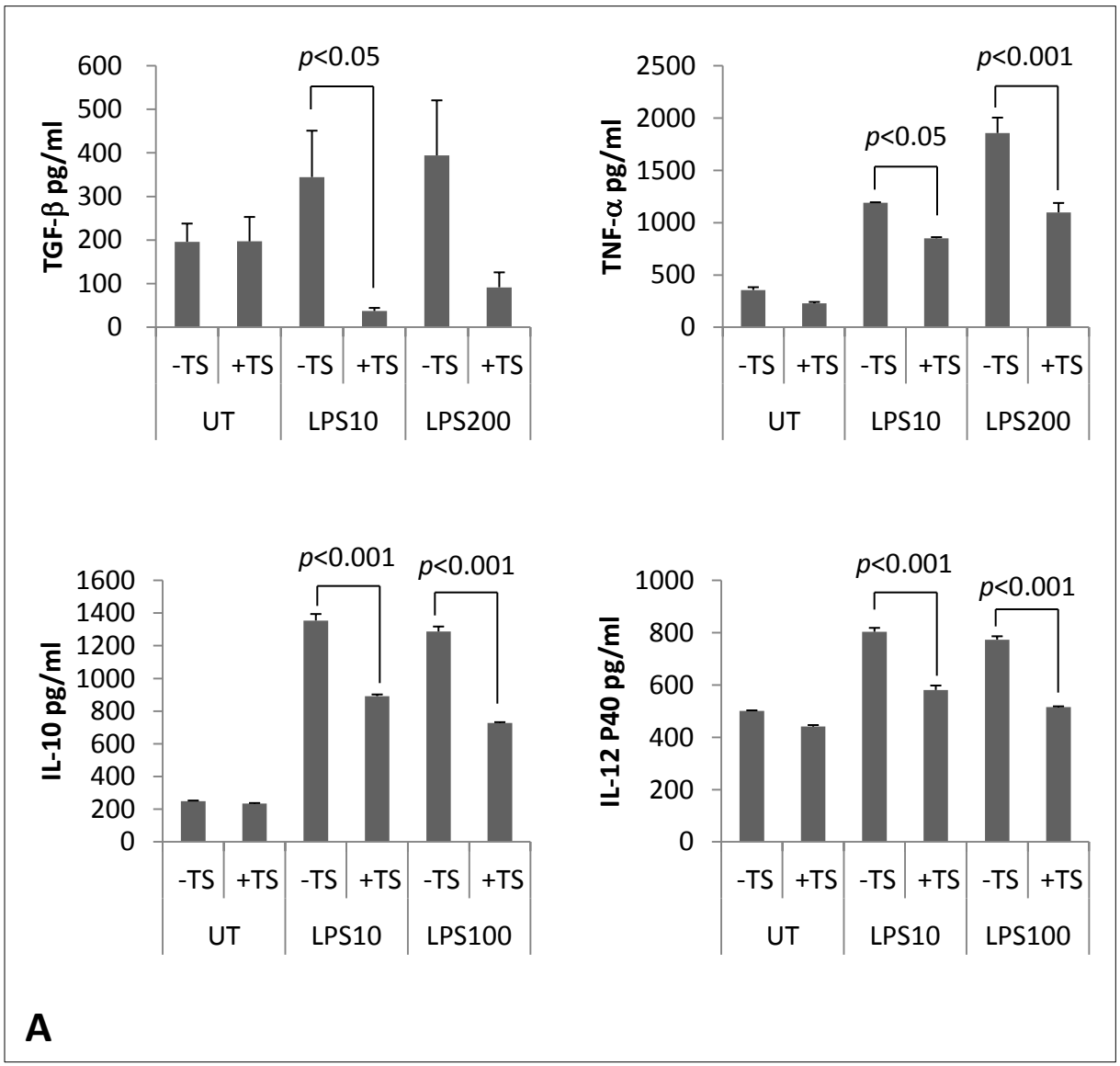

TGF-β pg/ml
600
500
400
300
200
100
0
p<0.05
-TS +TS -TS +TS -TS +TS
UT LPS10 LPS200
TNF-α pg/ml
2500
2000
1500
1000
500
0
p<0.05
p<0.001
-TS +TS -TS +TS -TS +TS
UT LPS10 LPS200
IL-10 pg/ml
1600
1400
1200
1000
800
600
400
200
0
p<0.001
p<0.001
-TS +TS -TS +TS -TS +TS
UT LPS10 LPS100
IL-12 P40 pg/ml
1000
800
600
400
200
0
p<0.001
p<0.001
-TS +TS -TS +TS -TS +TS
UT LPS10 LPS100
A

Figure 1B

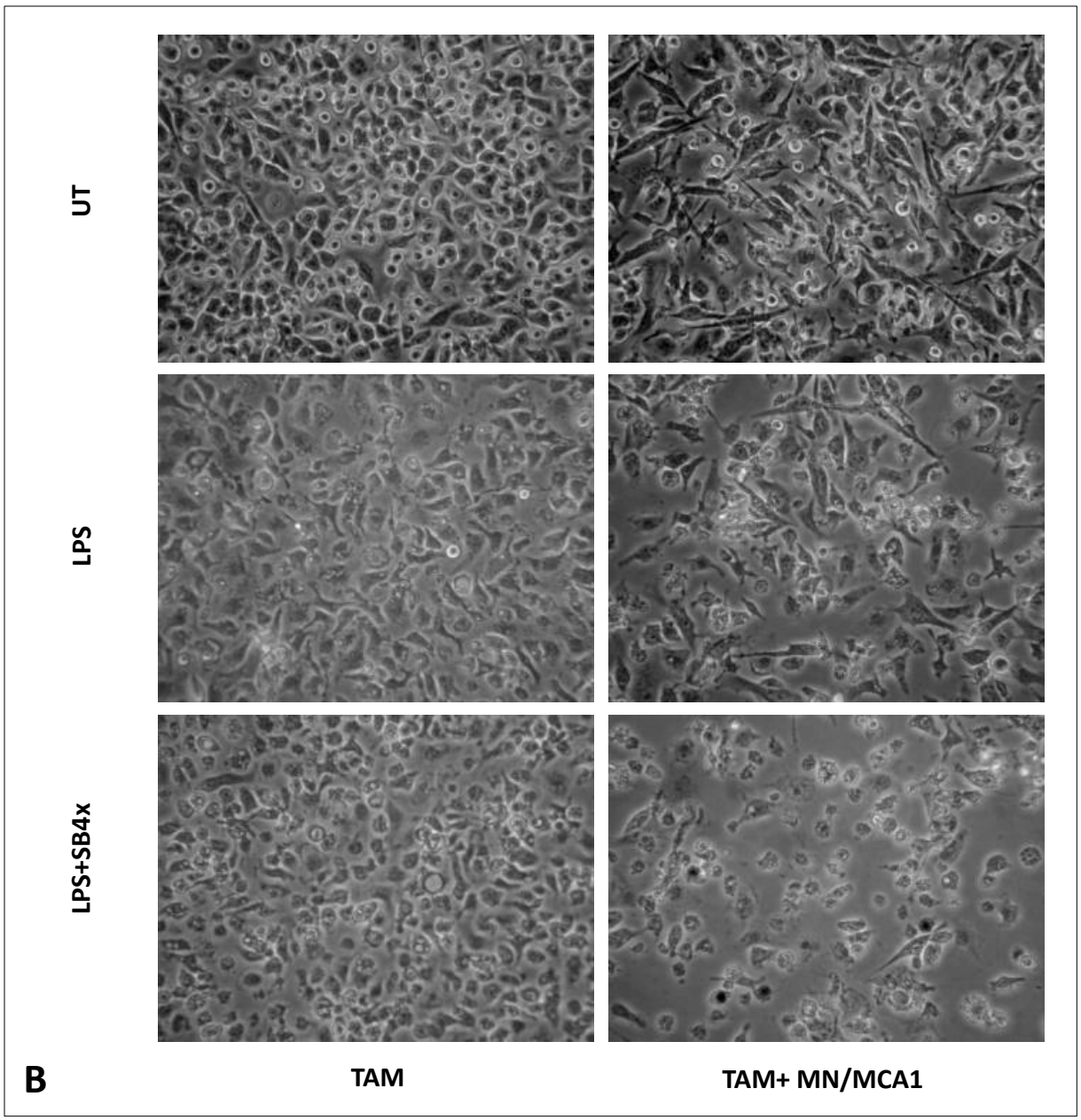
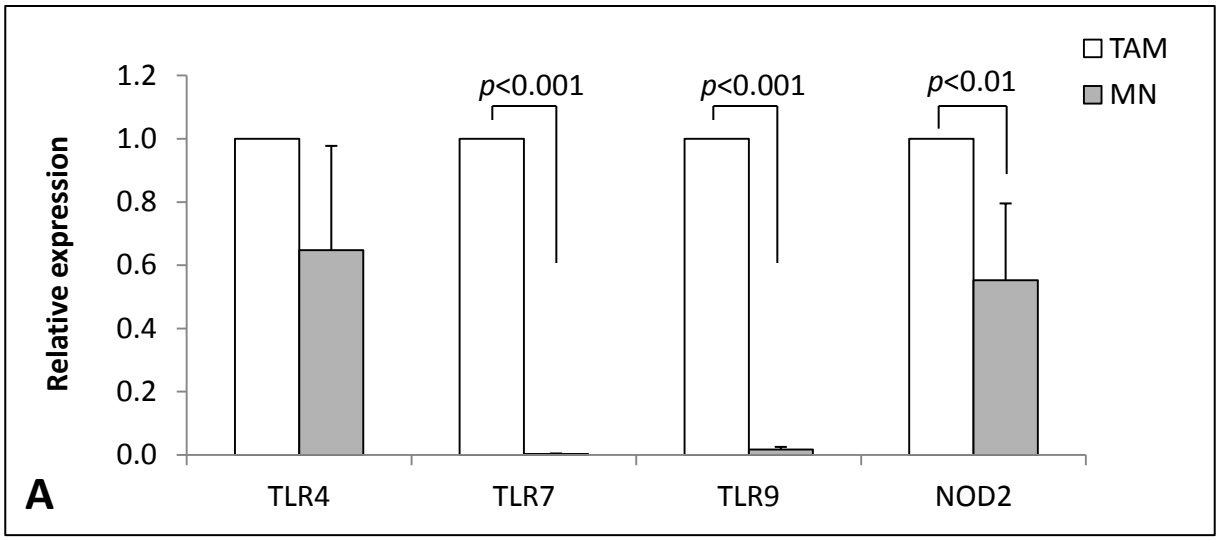
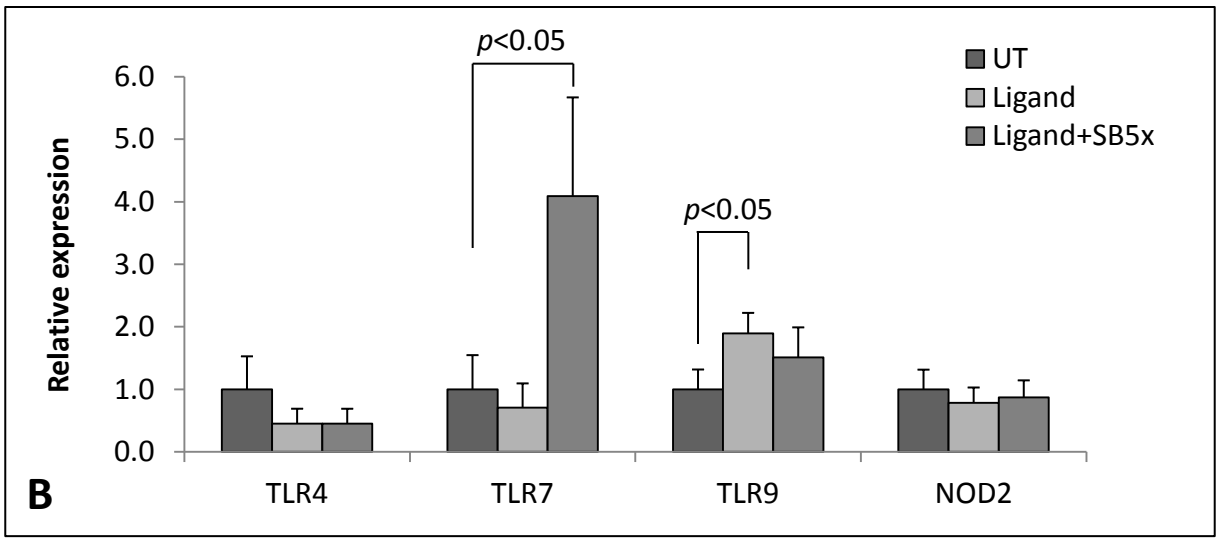


Figure 2A - 2C


TAM
MN
Relative expression
1.2
1.0
0.8
0.6
0.4
0.2
0.0
p<0.001
p<0.001
p<0.01
TLR4
TLR7
TLR9
NOD2
A


UT
Ligand
Ligand+SB5x
Relative expression
6.0
5.0
4.0
3.0
2.0
1.0
0.0
p<0.05
p<0.05
TLR4
TLR7
TLR9
NOD2
B

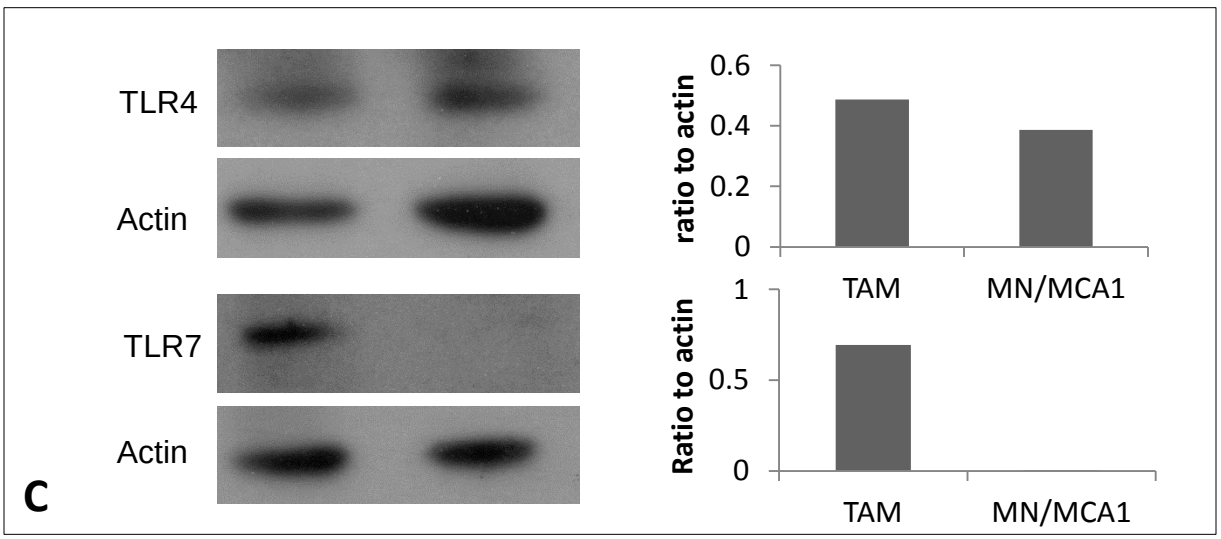
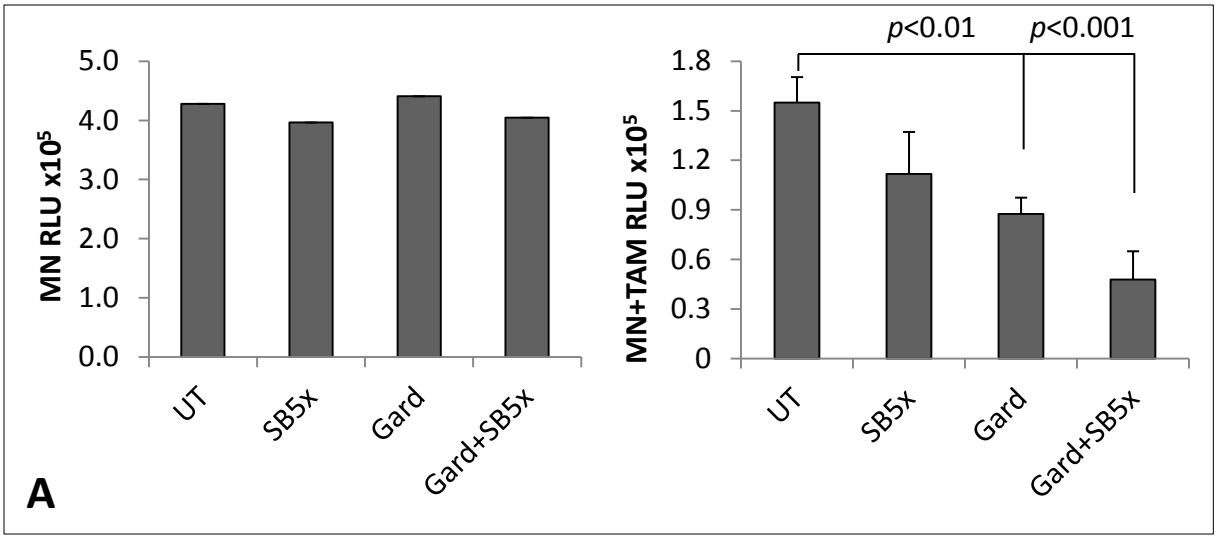
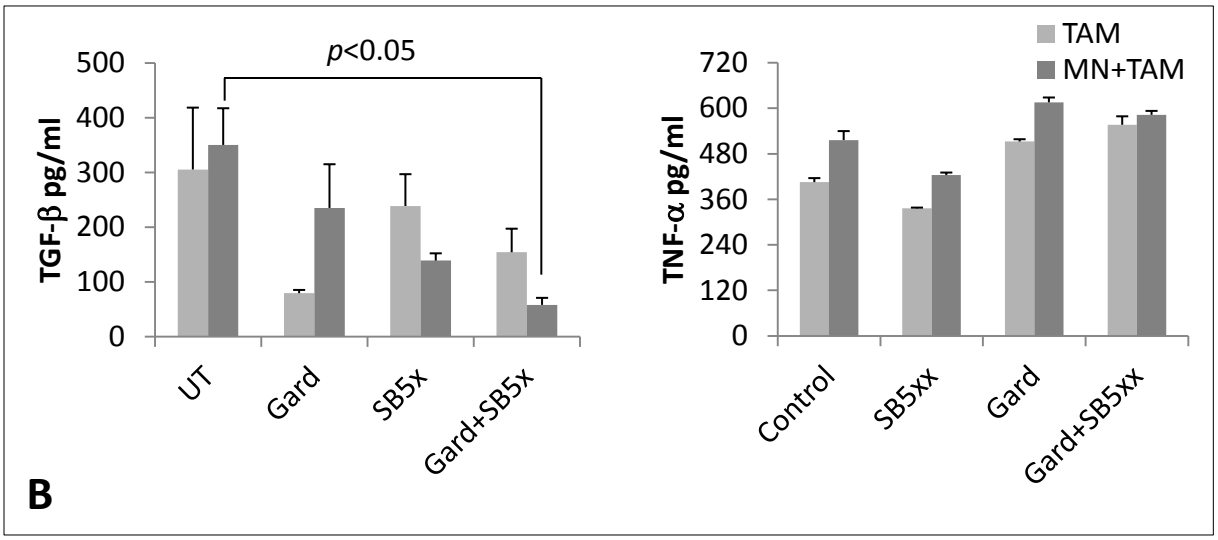

TLR4
Actin
TLR7
Actin
C
ratio to actin
0.6
0.4
0.2
0
TAM
MN/MCA1
Ratio to actin
1
0.5
0
TAM
MN/MCA1

Figure 3A-3D


MN RLU x10⁵
5.0
4.0
3.0
2.0
1.0
0.0
UT
SB5x
Gard
Gard+SB5x
MN+TAM RLU x10⁵
p<0.01
p<0.001
1.8
1.5
1.2
0.9
0.6
0.3
0
UT
SB5x
Gard
Gard+SB5x
A


TGF-β pg/ml
p<0.05
500
400
300
200
100
0
UT
Gard
SB5x
Gard+SB5x
TNF-α pg/ml
TAM
MN+TAM
720
600
480
360
240
120
0
Control
SB5xx
Gard
Gard+SB5xx
B

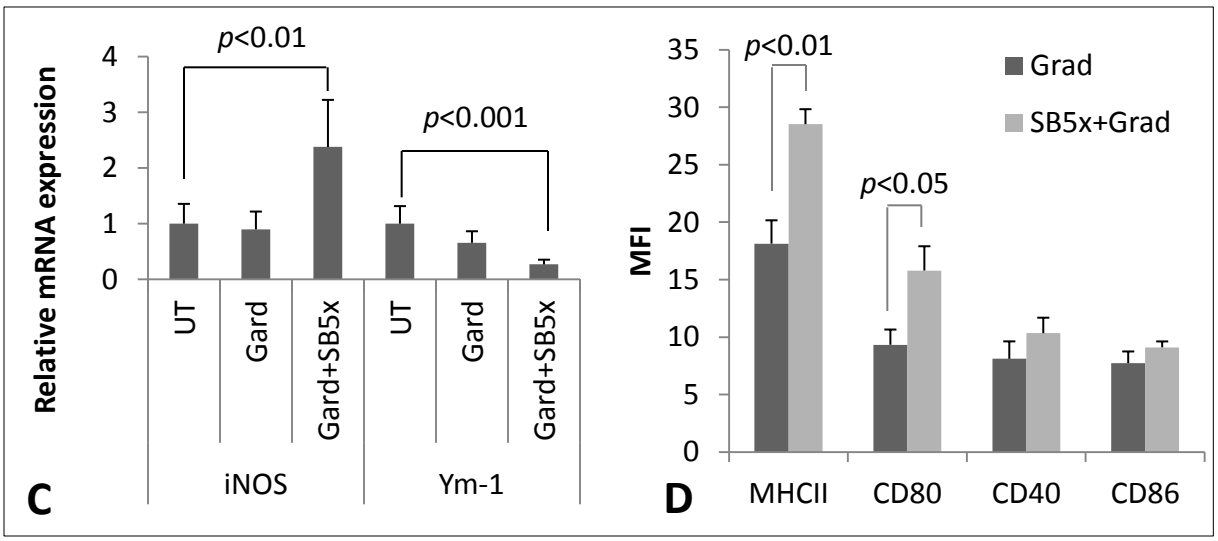
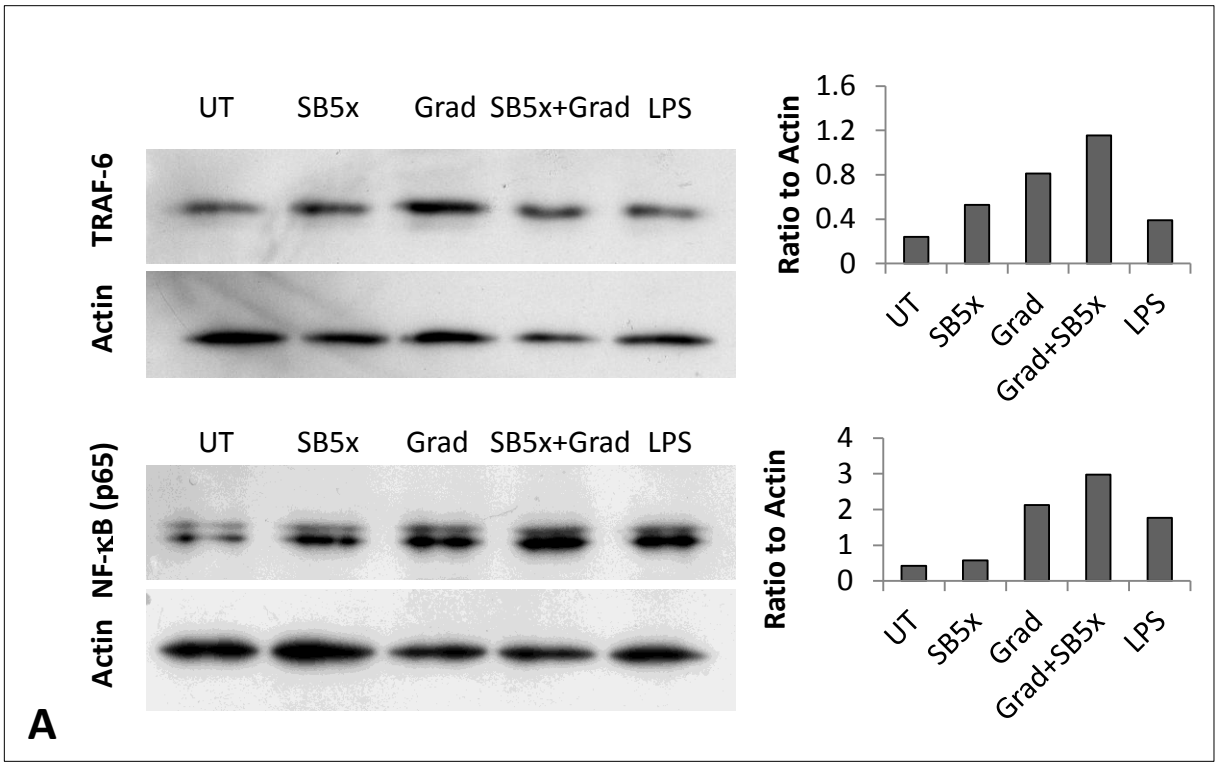

Relative mRNA expression
p<0.01
p<0.001
4
3
2
1
0
UT
Gard
Gard+SB5x
UT
Gard
Gard+SB5x
iNOS
Ym-1
C
MFI
p<0.01
p<0.05
Grad
SB5x+Grad
35
30
25
20
15
10
5
0
MHCII
CD80
CD40
CD86
D

Figure 4A, 4B


UT
SB5x
Grad
SB5x+Grad
LPS
TRAF-6
Actin
Ratio to Actin
1.6
1.2
0.8
0.4
0
UT
SB5x
Grad
Grad+SB5x
LPS
NF-κB (p65)
Actin
4
3
2
1
0
A

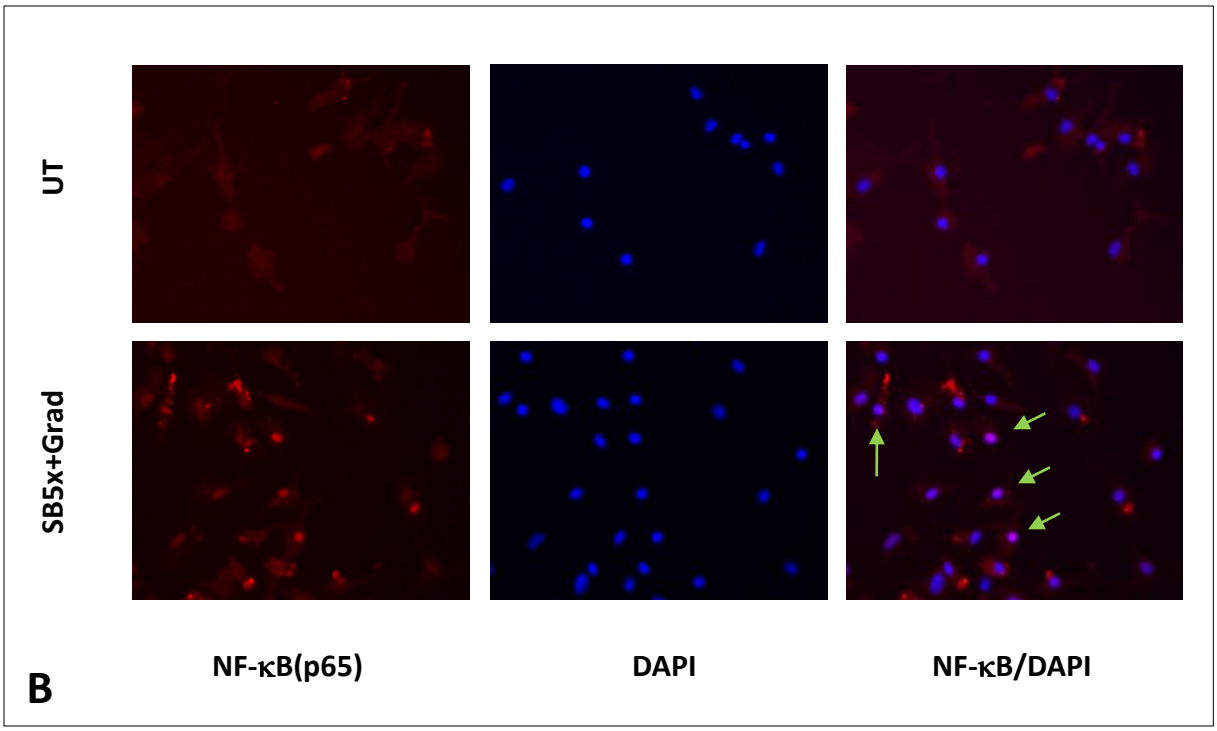
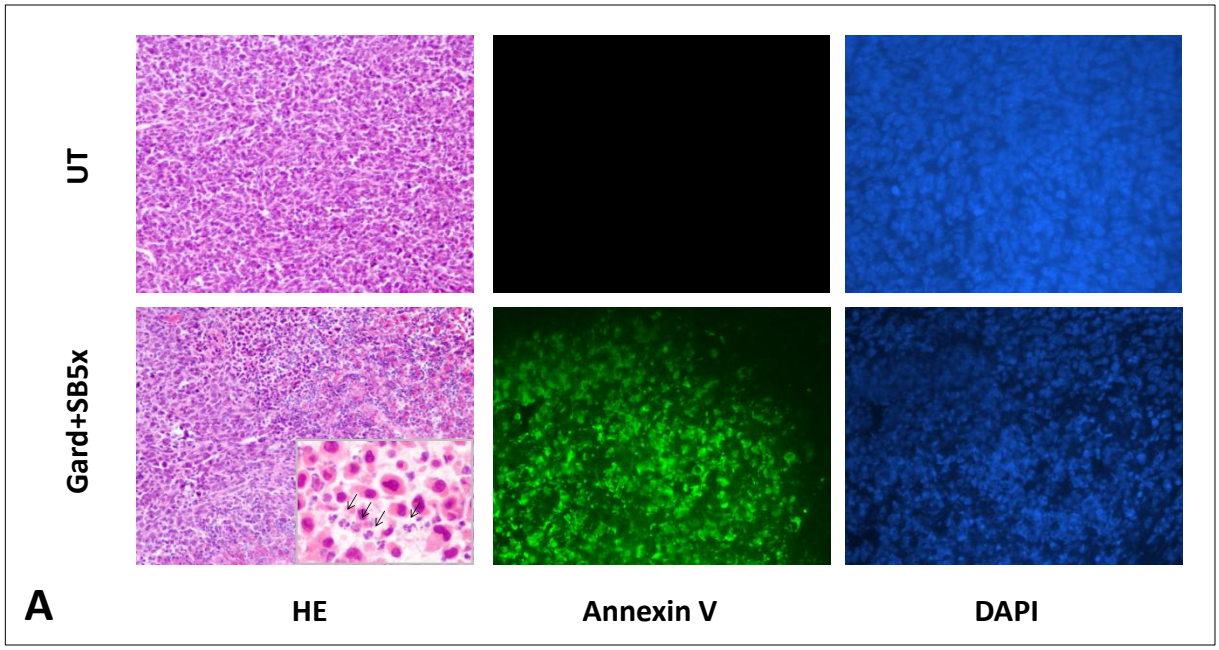

UT
SB5x+Grad
NF-κB(p65)
DAPI
NF-κB/DAPI
B

Figure 5A, 5B


UT
Gard+SB5x
A
HE
Annexin V
DAPI

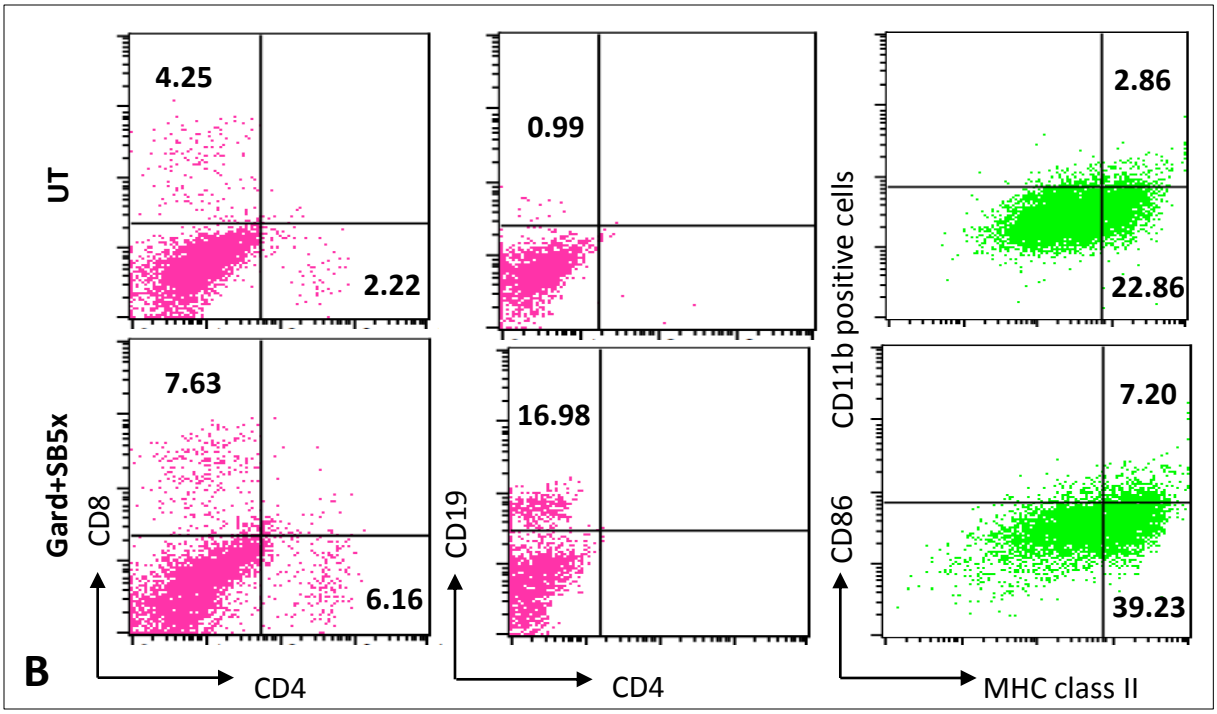

UT
Gard+SB5x
4.25
2.22
7.63
6.16
CD8
CD4
0.99
16.98
CD19
CD4
2.86
22.86
7.20
39.23
CD11b positive cells
CD86
MHC class II
B

Figure 5C

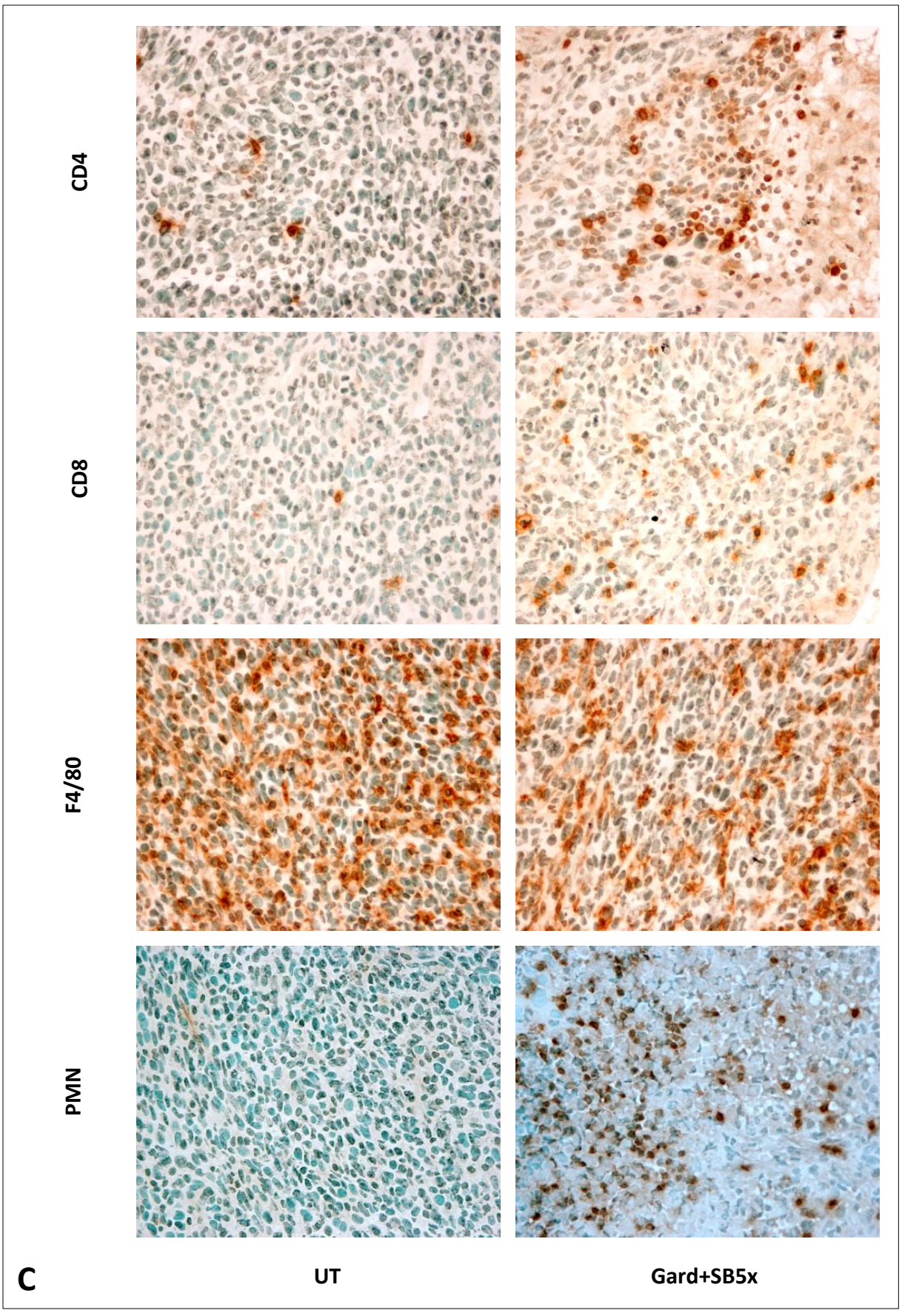


Figure 5D

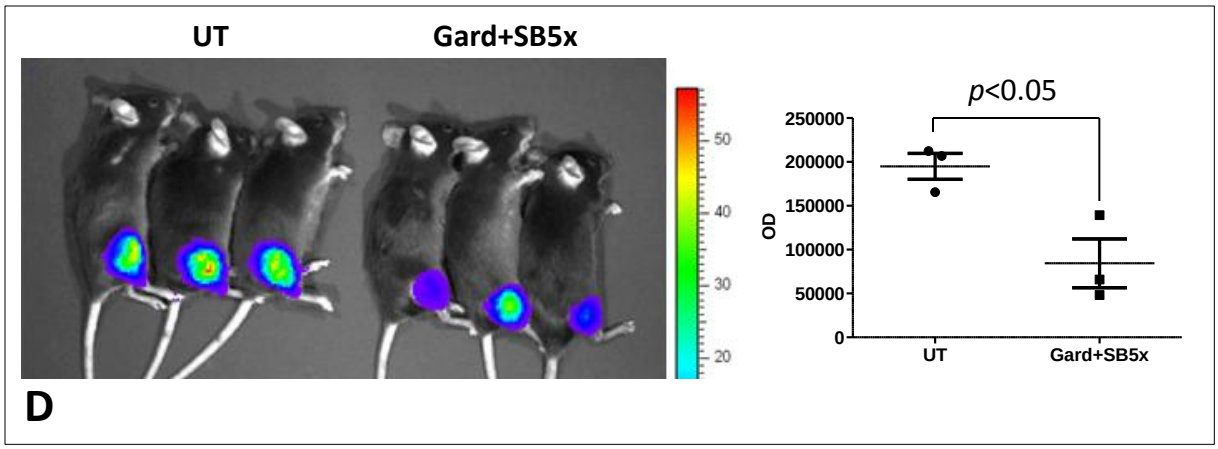
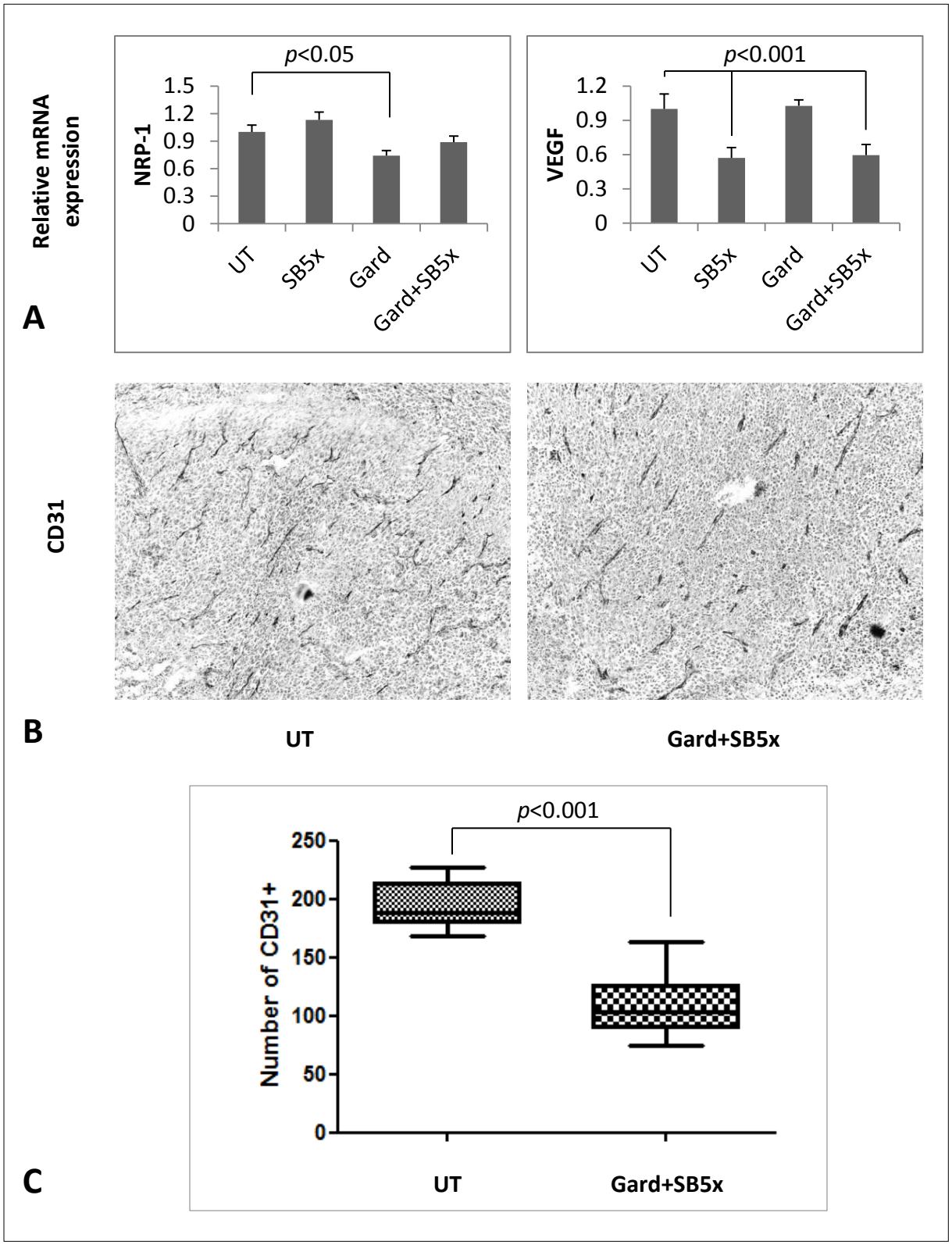

UT
Gard+SB5x
50
40
30
20
p<0.05
250000
200000
150000
100000
50000
0
OD
UT
Gard+SB5x
D

Figure 6


A
Relative mRNA expression
NRP-1
p<0.05
1.5
1.2
0.9
0.6
0.3
0
UT
SB5x
Gard
Gard+SB5x
VEGF
p<0.001
1.2
0.9
0.6
0.3
0
UT
SB5x
Gard
Gard+SB5x
B
CD31
UT
Gard+SB5x
C
p<0.001
Number of CD31+
250
200
150
100
50
0
UT
Gard+SB5x