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HUMAN T CELLS UPREGULATE CD69 AFTER COCULTURE WITH XENOGENEIC GENETICALLY-MODIFIED PIG MESENCHYMAL STROMAL CELLS

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Abstract

Mesenchymal stromal cells (MSC) obtained from $\alpha 1,3$ -galactosyltransferase gene knock-out pigs transgenic for the human complement-regulatory protein CD46 (GTKO/CD46 pMSC) suppress in vitro human anti-pig cellular responses as efficiently as allogeneic human MSC. We investigated the immunoregulatory effects of GTKO/CD46 pMSC on human CD4⁺ and CD8⁺ T cell proliferation in response to pig aortic endothelial cells (pAEC). pMSC efficiently suppressed T cell proliferation, which was associated with downregulation of granzyme B expression. No induction of CD4⁺CD25⁺Foxp3^{hi} regulatory T cells or T cell apoptosis was documented. In correlation with T cell proliferation, CD25 expression was upregulated on T cells in response to pAEC but not to pMSC. In contrast, CD69 expression was upregulated on T cells in response to both pMSC and pAEC, which was associated with a significant increase in the phosphorylation of STAT5. GTKO/CD46 pMSC possibly regulate human T cell responses through modulation of CD69 expression and STAT5 signaling.

Keywords

CD69; GTKO/CD46; Mesenchymal stem cells; Pig; STAT5; T cells; Xenotransplantation

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CONFLICT OF INTEREST

DA is an employee of Revivacor, Inc. No other author has a conflict of interest.

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1. INTRODUCTION

Mesenchymal stromal cell (MSC), with self-renewal, multi-differentiation, anti-inflammatory, and immunomodulatory properties, have been widely studied in preclinical and clinical trials. Although relatively little is known about their *in vivo* biological activity, allogeneic MSC have been used in the clinic to treat severe acute graft-versus-host disease [1, 2]. The biological properties of MSC have extensively studied in experimental organ transplantation [3–5].

Our previous studies demonstrated that MSC obtained from genetically-engineered pigs were very weakly immunogenic, and had similar immunosuppressive properties to human-derived MSC [6, 7]. MSC obtained from $\alpha 1, 3$ -galactosyltransferase gene knock-out pigs additionally transgenic for the human complement-regulatory protein CD46 (GTKO/hCD46 pigs) inhibited human T cell proliferation, suggesting that GTKO/hCD46 pMSC might be beneficial in therapeutic cell Xenotransplantation.

The mechanisms by which MSC modulate the immune response are still under investigation; evidence from previous *in vitro* studies has demonstrated that both cell-cell contact and soluble factors contribute to their immunomodulatory function [8–11]. MSC can directly suppress T cell immune responses in a cell contact manner [12], as well as modulate the immune response indirectly through an effect on antigen-presenting cells [12]. Several reports have implicated interleukin 10, hepatocyte growth factor, transforming growth factor- β , and/or prostaglandin E₂ as the soluble factors responsible for the suppressive effects exerted by MSC [13–15].

In the present study we investigated the underlying mechanisms of the immunomodulatory effect of GTKO/CD46 pig adipose-derived MSC on human CD4⁺ and CD8⁺ T cells.

2. MATERIALS AND METHODS

2.1. Antibodies and reagents

The following antibodies were used for flow cytometry:- PerCP-CyTM 5.5 mouse anti-human CD3 (Cat #552852); FITC-mouse anti-human CD4 (Cat #556615); PE mouse anti-human CD69 (Cat #557050); PE anti-human CD3 (Cat #552127); Alexa Fluor® 700 mouse anti-human granzyme B (Cat #560213); PE mouse IgG1 κ isotype control (Cat #556650); PE-Cy7 mouse anti-human CD4 (Cat #557852); APC mouse IgG2b κ isotype control (Cat #555745); PE Annexin-V (Cat #556421); and FITC Annexin-V Apoptosis Detection Kit I (Cat #556547) (all from BD Biosciences, Franklin Lakes, NJ). PE-Cy7 anti-human CD25 (Cat #E07538-1631) and anti-human FoxP3 (Cat #17-4776-42) (eBioscience, San Diego, CA). APC mouse anti-human CD4 (Cat #551980) and goat IgG control PE (Cat #1C108P) (BioLegend, San Diego, CA). Alexa Fluor® 700 mouse anti-human CD8 (Cat #557945, Alexa Fluor® Dyes, Grand Island, NY). Anti-human LAG-3 PE goat IgG (Cat #FAB2319P, R&D Systems, Minneapolis, MN). For pSTAT5 expression by flow cytometry, anti-pSTAT5 (#9351; Cell Signaling Technology, Danvers, MA) was used as 1st antibody and Alexa Fluor® 647 anti-rabbit IgG (Cat #4414, Cell Signaling Technology, Danvers, MA) was used as 2nd antibody. For western blot, the following antibodies were used:- anti-STAT5 (#9363) and anti-pSTAT5 (#9351) (Cell Signaling Technology, Danvers, MA).

2.2. Pigs

Adipose tissue was harvested from GTKO/hCD46 pigs of Large White/Landrace/Duroc cross-breed and blood type non-A (O) (Revivicor, Blacksburg, VA). Porcine aortic endothelial cells (pAEC) were harvested from GTKO pigs.

2.3. Isolation of pMSC and pAEC

Adipose tissue was harvested from the anterior abdominal wall of the pigs and stored temporarily in cold PBS solution with antibiotics. Aliquots of 10g fat tissue were minced with sterile scissors in 50ml falcon tubes, then digested with 15ml 0.15% collagenase type (Worthington, Lakewood, NJ) for 1h at 37°C. The fat suspension was then centrifuged at 1200rpm at 4°C for 5min. The pellet was suspended and washed with PBS, then successively filtered with a metal filter and a 100µl filter. The cell pellet was collected and seeded in 25/75cm² collagen-coated flasks (Becton Dickinson, Franklin Lakes, NJ). The cultures were then incubated at 37°C in a humidified atmosphere containing 5% CO₂. MSC culture medium consisted of low-glucose-DMEM (Gibco, Grand Island, NY), 10% fetal bovine serum (Sigma-Aldrich, St Louis, MO), 1% HEPES buffer, penicillin G (10,000µg/ml), and streptomycin (10,000µg/ml). Non-adherent cells were removed and the medium was changed after 24h. Adherent cells were harvested using trypsin-EDTA (Invitrogen, Grand Island, NY) when MSC reached 90–95% confluence. pAECs were obtained from freshly-harvested GTKO pigs aortas, and cultured as previously described [16]. Both pMSC and pAEC were used from passages 2–6.

2.4. Isolation of human PBMC, CD4⁺ T and CD8⁺ T cells

Human (h) peripheral blood mononuclear cells (hPBMC) used for the MLR were isolated from fresh human blood type-O buffy coats (Institute for Transfusion Medicine, Pittsburgh, PA, USA) using Ficoll-paque density gradient centrifugation. CD4⁺ T cells were isolated using Kit II #130-091-155 and CD8⁺ T cells were isolated using MicroBeads (#130-045-201) (both from Miltenyi Biotec, Auburn, CA).

2.5. Mixed lymphocyte cocultures

Human PBMC, CD4⁺ or CD8⁺ T cells were used as responders. pMSC and pAEC were used as stimulators. The stimulators were harvested and resuspended in AIM-V medium (Invitrogen) and plated in round-bottom 96-well plates (Corning, Lowell, MA). Human T cells (0.2×10⁶/well) were added and cultured in an incubator at 37°C and 5% CO₂. The proliferation was measured after 5 days of culture, and 16h after addition of ³H-thymidine (1µCi/well), using a beta-scintillation counter (PerkinElmer, Waltham, MA). All of the proliferation assays were performed in triplicate. The results are represented as counts per minute (CPM).

2.6. T cell apoptosis assay

Apoptosis of cells was measured by an Annexin-V binding and propidium iodide uptake assay (FITC Annexin-V Apoptosis Detection Kit I, Cat #556547, BD Bioscience). Human PBMC were cultured alone or co-cultured with GTKO/hCD46 MSC in round-bottom 96-well plates for 5 days. The PBMC were then recollected, stained with FITC-conjugated Annexin-V and propidium iodide, and analyzed by flow cytometry. As a positive control, PBMC were treated with camptothecin (Sigma Aldrich) at a concentration of 5µM for 1×10⁶ cells for 4h.

2.7. T cell phenotype analysis

Human PBMC, or purified CD4⁺ and CD8⁺ T cells were suspended in AIM-V medium and cultured in round-bottom 96-well plates. T cells were either cultured alone, or cocultured with GTKO pAEC and/or GTKO/hCD46 MSC, both at a 1:10 ratio for 3 or 5 days as needed, and then harvested and examined by flow cytometry to determine MFI (mean fluorescence intensity). For analysis, cells were gated on CD3⁺CD4⁺ or CD3⁺CD8⁺ T cells.

2.8. Western blot analysis

Purified human CD4⁺ and CD8⁺ T cells were collected after coculture with pMSC. Protein lysates were separated by 10% SDS–polyacrylamide gel electrophoresis and then electrophoretically transferred to polyvinylidene fluoride membranes. After blocking, membranes were incubated with primary antibodies overnight at 4°C and then with secondary antibodies for 1h at room temperature. Band signals were visualized using an enhanced chemiluminescence kit (Pierce, Minneapolis, MN) and analyzed by image analysis software (Image J V1.34 for Mac OSX, National Institutes of Health, Bethesda, MD).

2.9. Statistical analyses

Statistical significance was obtained from data obtained within each experiment. Statistical analyses were carried out using the Student's t-test. P values <0.05 were considered significant.

3. RESULTS

3.1. GTKO/CD46 pMSC suppress human CD4⁺ and CD8⁺ T cell proliferation

Purified human CD4⁺ or CD8⁺ T cells were cocultured with GTKO/CD46 pMSC and GTKO pAEC for 5 days in MLR in the presence or absence of GTKO/CD46 pMSC. When GTKO pAEC were used as stimulators (in the absence of pMSC), the proliferation of both CD4⁺ and CD8⁺ T cells was significantly higher than to GTKO/CD46 pMSC. In addition, when GTKO pAEC and GTKO/CD46 pMSC were cocultured together with T cells, GTKO/CD46 pMSC suppressed the T cell proliferation to GTKO pAEC in a dose-dependent manner (Figure 1). These data suggest an immunoregulatory effect for GTKO/CD46 pMSC on CD4⁺ and CD8⁺ T cells proliferation in response to GTKO pAEC.

3.2. Reduced CD4⁺ and CD8⁺ granzyme B⁺ effector T cells after coculture with pMSC

It has been shown that induction of granzyme B-containing cytotoxic granules in T cells requires T cell proliferation [17]. Since GTKO/CD46 pMSC inhibit the proliferation of CD4⁺ and CD8⁺ T cells, we investigated whether pMSC could affect the expression of granzyme B in CD4⁺ and CD8⁺ T cells. Human CD4⁺ and CD8⁺ T cells were cultured with pMSC and pAEC in the presence or absence of pMSC for 5 days. Reduced expression of granzyme B was observed when T cells were cocultured with pMSC, in comparison to when cocultured with pAEC (Figure 2). Additionally, a reduction of intracellular granzyme B was observed when pAEC were cocultured with pMSC compared to pAEC alone.

3.3. No induction of CD4⁺CD25⁺Foxp3^{hi} regulatory T cells after coculture with pMSC

To investigate the role of pMSC in the induction of regulatory T cells, human purified CD4⁺ T cells were cultured with pMSC in the presence or absence of pAEC for 5 days. On day 5 of culture, we evaluated the expression of Foxp3 on CD4⁺ T cells. No significant difference was observed when T cells were cocultured with pMSC, pAEC, or both (Figure 3).

3.4. pMSC does not induce human T cell apoptosis

MSC are known to prevent lymphocyte activation and proliferation through induction of apoptosis. We cultured human PBMC with or without GTKO/CD46 pMSC for 5 days. Using flow cytometry, apoptosis and cell death were analyzed by Annexin-V staining and propidium iodide incorporation. Similar to PBMC cultured alone, there was no increase in apoptosis and cell death when PBMC were cocultured with pMSC. Also, no evidence of apoptosis was observed when pMSC were cocultured with pAEC (Figure 4). In contrast, when PBMC were treated with camptothecin (5μM) for 4h, a high percentage of cell apoptosis was observed.

3.5. Upregulation of CD69 on CD4⁺ and CD8⁺ T cells after coculture with pMSC

We then evaluated the effect of pMSC on the activation of T cells. Purified human CD4⁺ or CD8⁺ T cells were cultured alone or cocultured with pMSC and pAEC in the presence or absence of MSC for 5 days. Afterwards, T cells were evaluated for their activation markers CD25, CD69, and LAG-3 (lymphocyte activation gene-3) using flow cytometry. There was no significant difference in LAG-3 expression when T cells were cultured either alone, or cocultured with pMSC, pAEC, or both (Figure 5). In contrast, the expression of CD25 was upregulated on CD4⁺ T cells following coculture with pAEC with or without pMSC, but not after coculture with pMSC.

Interestingly, CD69 expression was upregulated on both CD4⁺ (Figure 5A) and CD8⁺ (Figure 5B) T cells following coculture with pMSC (23.5% and 12.9%, respectively) which was higher than after coculture with pAEC (7.3% and 10.8%, respectively). Moreover, CD69 expression on CD4⁺ and CD8⁺ T cells was not downregulated when these cells were cocultured with both pAEC and pMSC (32% and 8.9%, respectively).

We evaluated CD69 expression on human T cells 3 and 5 days after coculture with pMSC. In comparison to cells cultured alone, there was a significant increase in CD69⁺T cells at day 3 and day 5 after coculture in CD4⁺T and CD8⁺T cells ($p<0.05$), with a significantly higher percentage on day 5 than on day 3 ($p<0.05$). CD69 MFI values were significantly higher after 5 days of coculture in comparison to cells cultured alone ($p<0.05$).

3.6. Phosphorylation of STAT5 in CD4⁺ and CD8⁺ T cells after coculture with pMSC

It is known that CD69 promotes activation of signal transducer and activator of transcription 5 (STAT5) [18]. Purified human CD4⁺ and CD8⁺ T cells were cocultured with GTKO/CD46 pMSC. Following coculture, human T cells were analysed for phosphorylation of STAT5 (pSTAT5) by western blot. T cells cocultured with pMSC showed significantly increased phosphorylation of STAT5 (Figure 6A), in comparison to T cells cultured alone ($p<0.05$). Also, we evaluated pSTAT5 expression on human T cells by flow cytometry after coculture with pMSC. To exclude pSTAT5 expression by pMSC after coculture, human T cells were gated on CD3⁺CD4⁺ or CD3⁺CD8⁺ T cells. As seen in Figure 6C, pSTAT5 was upregulated on T cells after coculture with pMSC in comparison to cells cultured alone, with significantly higher MFI ($p<0.05$). This suggests that upregulation of CD69 on CD4⁺ and CD8⁺ T cells after coculture with GTKO/CD46 pMSC is associated with upregulation of pSTAT5.

4. DISCUSSION

MSC have been extensively investigated as a promising therapy for various pathological conditions. Encouraging results have been achieved regarding MSC application in several experimental and clinical models. However, the mechanisms underlying the immunomodulatory effects of MSC on T cells are not fully elucidated. Many studies have demonstrated that MSC can inhibit different effector functions of immune cells, including T, B, dendritic, and natural killer cells [19]. Of relevance to transplantation, T cells are the primary effectors in the adaptive immune response. MSC can regulate effector T cells directly by cell-cell contact or indirectly through effects on antigen-presenting cells [20, 21], or by secreting cytokines such as transforming growth factor-beta, prostaglandin E₂, hepatocyte growth factor, and interleukin-10 [11, 22, 23].

Following xenotransplantation, intensive immunosuppressive therapy is frequently required to maintain xenograft survival, and so additional strategies have been proposed to minimize the intensity of immunosuppression needed. For example, pigs expressing the immunoregulatory molecule CTLA4-Ig have been produced [24] [25], and anti-

inflammatory agents have been shown to reduce the human T cell response to pig cells in vitro [16]. Our previous studies indicated that a large number of MSC can be effectively harvested from GTKO/CD46 pig fat tissue [26]. We have also shown that Gal expression was associated with increased human T cell responses to pig cells [27]. GTKO/CD46 pMSC do not express the Gal antigen, and express the human complement-regulatory protein CD46. Also, GTKO/CD46 pMSC express low levels of costimulatory molecules, e.g., CD80, induce minimal human T cell proliferation, and suppress pig antigen-induced human T cell proliferation as effectively as hMSC [7]. Recently, GTKO adipose-derived pMSC have been shown to induce less antibody production and cellular infiltration than wild-type pMSC in vivo [28].

Najar et al found that the suppressive effect of MSC on both CD4⁺ and CD8⁺ T cells was achieved in a dose-dependent manner [29]. In our study, we found a similar immunosuppressive effect of GTKO/CD46 pMSC on the proliferation of CD4⁺ and CD8⁺ T cells in response to GTKO pAEC. Induction of granzyme B-containing cytotoxic granules in (naïve and memory) T cells requires several rounds of cell division and cell cycle progression [17]. Benvenuto et al found MSC could decrease intracytoplasmic release of granzyme B by activated CD8⁺T cells [30]. Additionally, Rasmusson et al found that MSC were resistant to cytotoxic T lymphocyte lysis despite the expression of MHC class on their surface [31], which can promote prolonged survival of MSC in vivo. Our data show that, after coculture, intracellular granzyme B expression on both CD4⁺ and CD8⁺ T cells was increased in response to pAEC, while no increase was detected in response to pMSC. This observation correlates with the extent of T cell proliferation in MLR.

It has already been demonstrated that MSC can suppress the proliferation of CD4⁺ and CD8⁺ T cells in vitro, with or without a concomitant increase in regulatory T cells. We found no increase in CD4⁺CD25⁺ FoxP3^{high} regulatory T cells following coculture of CD4⁺T cells with GTKO/CD46 pMSC. MSC have been shown to promote T cell apoptosis [32]. Additionally, allogeneic MSC can rescue human T cells from activation-induced cell death [30], and can inhibit T cell proliferation through arrest of cell division, but not apoptosis [33]. In our studies, we did not observe any T cell apoptosis following coculture with pMSC.

CD25, CD69, and LAG-3 are considered classical markers of activated T lymphocytes [34–36]. We evaluated the effects of GTKO/CD46 pMSC on the expression of CD69, CD25, and LAG-3 on activated T lymphocytes. MSC have been shown to regulate expression of CD25 and CD69 on lymphocytes [37–39]. In correlation with reduced T cell proliferation and granzyme B expression by pMSC, we found that the expression of LAG-3 and CD25 were not increased on T cells in response to pMSC (in contrast to the response to pAEC). However, we found that CD69 was upregulated on both CD4⁺ and CD8⁺ T cells after coculture with pMSC. Following pMSC coculture (with or without pAEC), the overall median increase in CD69 expression on CD8⁺ T cells was associated with reduced CD69^{high} cells, in contrast to CD8⁺ T cells cocultured with pAEC. This may be related to variation in T cell responses to pMSC versus pAEC, and the possible CD69-dependent regulation of pMSC-conditioned T cells. Moreover, we evaluated pSTAT5 expression on human T cells after coculture with pMSC. We found that, in association with upregulation of CD69, there was significant upregulation of pSTAT5 on T cells in comparison to T cells cultured alone. A potential ligand for human CD69 may exist on pMSC. Blocking the interaction of CD69 with such a ligand (which has not been identified) will be essential to verify whether upregulation of pSTAT5 in T cells is downstream of CD69.

CD69 has been recently considered as a marker of T cells with regulatory potential [40–42]. CD69-deficient mice have been shown to develop enhanced anti-tumor immunity [40].

However, CD69-deficient mice have also been shown to acquire augmented T and B cell responses against collagen in a model of collagen-induced arthritis [41]. Recently, CD69 has been associated with Jak3/STAT5 signaling, which in turn regulates Th17 T cell differentiation [18]. Also, regulation of differentiation of Th17 cells by MSC has been reported [43–45]. These observations suggest a regulatory role for CD69 on the balance between anti-inflammatory regulatory T cells and pro-inflammatory Th17 cells. Meanwhile, human MSC have been shown to induce sustained CD69 expression on activated allogeneic T cells [46]. In contrast, CD69⁺CD4⁺CD25[−] T cells have been recently recognized as regulatory T cells with immunosuppressive capacity through a cell-cell-dependent mechanism [47]. It is essential to further evaluate the role of CD69⁺CD4⁺CD25[−] T cells in regulating the human T cell responses by xenogeneic pMSC, as well as their suppressive capacity.

5. Conclusions

Following coculture with GTKO/CD46 pMSC, it is possible that upregulation of CD69 on human T cells initiates signaling events that would regulate CD4⁺ and CD8⁺ T cell activation and differentiation. The immunomodulatory effects of pMSC on human T cells through CD69 require further investigation, especially possible regulation of Th17 T cell differentiation. Through a better understanding of the mechanisms underlying the interplay between xenogeneic pMSC and human T cells, we may generate more promising immunotherapeutic strategies for the clinical application of pMSC.

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ABBREVIATIONS

AEC	pig aortic endothelial cells
Gal	galactose- α 1, 3-galactose
GTKO/hCD46	α 1, 3-galactosyltransferase gene-knockout/human CD46
LAG-3	lymphocyte activation gene-3
PBMC	peripheral blood mononuclear cells
MSC	pig mesenchymal stromal cells
pSTAT5	phosphorylated signal transducer and activator of transcription 5

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Highlights

- α 1,3-galactosyltransferase gene knock-out pigs expressing human CD46 (GTKO/CD46)
- GTKO/CD46 pig mesenchymal stromal cells (pMSC) regulate human T cell responses
- GTKO/CD46 pMSC do not induce human T cell apoptosis or regulatory T cells
- Human CD4⁺ and CD8⁺ T cells upregulate CD69 after coculture with GTKO/CD46 pMSC
- CD69 upregulation on human T cells is associated with phosphorylation of STAT5

Figure 1. Suppression of human CD4⁺ and CD8⁺ T cell proliferation

In MLR, human CD4⁺ (top) or CD8⁺ (bottom) T cell proliferation was measured when these cells were cultured alone (A), cocultured with GTKO/CD46 pMSC (B) or with GTKO pAEC (C) at a responder:stimulator ratio of 1:10. Additionally, GTKO/CD46 pMSC were mixed with GTKO pAEC at 2:1, 1:1, and 1:2 ratios (D, E, and F, respectively). GTKO/CD46 pMSC significantly downregulated the responses of CD4⁺ or CD8⁺ T cells in a dose-dependent manner. Data represent 3 different experiments, and are presented as mean $\pm$ SD of triplicates. * $p < 0.05$, ** $p < 0.01$.

Figure 2. Downregulation of CD4⁺ granzyme B⁺ and CD8⁺ granzyme B⁺ effector T cells after coculture with pMSC

Purified human CD4⁺ (top) or CD8⁺ (bottom) T cells were separately cultured with (A) pMSC, (B) pAEC, or (C) pAEC + pMSC for 5 days, and then harvested for flow cytometry. Dot plots were boxed for granzyme B⁺ T cells. The percentages of granzyme B⁺ cells in the cocultures of CD4⁺ or CD8⁺ T cells with pAEC alone, pMSC alone, and pAEC + pMSC are indicated.

Figure 3. No induction of CD4⁺CD25⁺Foxp3^{hi} regulatory T cells after coculture with pMSC
Purified human CD4⁺ T cells were cocultured with pAEC in the presence or absence of pMSC for 5 days, and then harvested for flow cytometry. (A) in the dot plot, CD4⁺CD25⁺ T cells are gated. The percentages of cells expressing FoxP3 in the cocultures of CD4⁺ cells with (B) pAEC alone, (C) pMSC alone, and (D) pAEC + pMSC are indicated.

Figure 4. pMSC do not induce human T cell apoptosis

Human PBMC were cultured alone, with pMSC only, or with pMSC+pAEC for 5 days. PBMC were treated with camptothecin (5 μ M) for 4h (to induce T cell apoptosis, as a control), and then harvested for flow cytometry. Similar to cells cultured alone, minimal T cells were observed to be apoptotic/dead when either cultured with pMSC only or with pMSC+pAEC.

A

B **C**

Figure 5. Up-regulation of expression of CD69 on CD4⁺ and CD8⁺ T cells after coculture with pMSC

Purified human CD4⁺ (A) or CD8⁺ (B) T cells were cultured alone or cocultured with pMSC alone, pAEC alone, or pAEC and pMSC for 5 days, and then harvested for flow cytometry analysis after gating on CD3⁺CD4⁺ or CD3⁺CD8⁺ T cells, to determine the expression of activation markers CD25, CD69, and LAG-3. The percentages of cells positive for each activation marker are indicated. (C) Human PBMC were cocultured with pMSC for 3 and 5 days. CD69 expression was evaluated by flow cytometry as in (A) and

(B). Data represent average of four different human PBMC cocultures with pMSC. * $p < 0.05$ in comparison to cells cultured alone. # $p < 0.05$ in comparison to cell cocultured for 3 days.

A

B

Figure 6. Phosphorylation of STAT5 in CD4⁺ and CD8⁺ T cells after coculture with pMSC

(A) STAT5 and pSTAT5 were measured in cell lysates obtained from pMSC alone, human CD4⁺ (left) and CD8⁺ (right) T cells alone, or after coculture with pMSC. Arrows indicate pSTAT5. Blots shown are representative of 3 independent experiments with similar results. β -actin served as the loading control.

(B) Gel analysis was done using gray-scale image. The extent of T cell activation is indicated by the ratio of pSTAT5/STAT5. Compared with T cells cultured alone, after coculture with GTKO/CD46 pMSC, CD4⁺ and CD8⁺ T cells demonstrated significantly higher activity ratios. * $p < 0.05$.

(C) pSTAT5 expression was evaluated by flow cytometry. Histograms (left) represent the expression of pSTAT5 before (dashed line) and after (straight line) coculture with pMSC. Cells were gated on CD3⁺CD4⁺ or CD3⁺CD8⁺ T cells. Gray indicates isotype. The mean MFI (mean fluorescence intensity) of four different human PBMC cocultures with pMSC (right) demonstrating significantly higher pSTAT5 following pMSC coculture; * $p < 0.05$.