HUMAN MAST CELLS CO-STIMULATE T CELLS THROUGH A CD28-INDEPENDENT INTERACTION

Chapter 8

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ABSTRACT

Mast cells are innate immune cells usually residing in peripheral tissues, where they are likely to activate T-cell responses. Similar to other myeloid immune cells, mast cells can function as antigen presenting cells. However, little is known about the capacity of human mast cells to co-stimulate CD4+ T cells.

Here, we studied the T cell-stimulatory potential of human mast cells. Peripheral blood-derived mast cells were generated and co-cultured with isolated CD4+ T cells. In the presence of T-cell receptor triggering using anti-CD3, mast cells promoted strong proliferation of T cells, which was 2-5 fold stronger than the "T-cell promoting capacity" of monocytes. The interplay between mast cells and T cells was dependent on cell-cell contact, suggesting that co-stimulatory molecules on the mast cell surface are responsible for the effect. However, in contrast to monocytes, the T cell co-stimulation by mast cells was independent of the classical co-stimulatory molecule CD28, or that of OX40L, ICOSL, or LIGHT.

Our data show that mast cells can co-stimulate human CD4+ T cells to induce strong T cell proliferation, but that therapies aiming at disrupting the interaction of CD28 and B7 molecules do not inhibit mast cell-mediated T-cell activation.

INTRODUCTION

Mast cells are innate immune cells derived from myeloid progenitors. They have been originally described as one of the effector cells in allergic IgE-mediated responses. More recently, their role in immune regulation, and specifically in regulation of adaptive immune responses, has been recognized.(*1*) For example, we and others have shown that mast cells can function as antigen presenting cells through cognate interactions with $CD4^+$ T cells both in human and mouse.(*2-4*) $CD4^+$ T cell activation in this context was dependent on recognition of specific antigens in the context of MHC class II. Activation and skewing of T cells generally requires the presence of 3 signals, consisting of antigen presentation through MHC, co-stimulation and specific cytokine signals.(*5*) Co-stimulation is the interaction of membrane-bound receptors on T cells and antigen presenting cells that enhance signals through the T cell receptor, and that is necessary to induce full T cell activation, proliferation and survival. Classical co-stimulation consists of CD28-mediated signaling by B7 family members on antigen presenting cells, although different molecules, such as members of the TNF receptor superfamily or signaling

lymphocyte activation molecule (SLAM) family are known to regulate the balance between T cell death and survival as well.(*6*)

We have previously shown the presence of mast cells in T cell areas of lymphoid organs,(*2*) as well as their colocalization in synovial tissue,(*7*) suggesting that mast cells could modulate T cell responses. Although mast cells have been shown to function as antigen presenting cells, their capacity to provide co-stimulatory signals to T-cells has been studied sparsely. In the mouse, TCR-stimulated T-cells displayed enhanced proliferation and cytokine secretion in the presence of mast cells, indicating that mast cells are capable of enhancing T-cell responses.(*8, 9*) This effect in the mouse was directly mediated through TNF production by the mast cells. However, several important differences have been shown between mouse and human mast cells, one of which being the low production of TNF by human mast cells in contrast to their mouse counterparts.(*10*)

Co-stimulation pathways are therapeutic targets, for example in autoimmune diseases. CTLA4-Ig, which targets the CD28-dependent interaction between T cells and antigen-presenting cells through binding to B7 molecules on antigen presenting cells, is used as treatment for rheumatoid arthritis.(*11*) Recently, mast cells were shown to play an important role in T cell-dependent inflammation in a mouse model of arthritis, suggesting that mast cells may directly regulate T cell responses in this disease.(*12*) Therefore, it is important to understand if mast cells can regulate T cell activation through co-stimulation, and if CTLA4-Ig can regulate this interaction.

Our data show that human mast cells can induce robust proliferation responses of $CD4^+$ T-cells in the presence of T cell receptor stimulation. This interaction depends on cell-cell contact, but is independent of CD28. Together, these data indicate that mast cell-T cell interactions contribute to the outcome of adaptive immune responses in humans, and that these interactions are not targeted by "conventional" co-stimulation blockade.

RESULTS

$CD4^+$ T CELL ACTIVATION AND PROLIFERATION IS GREATLY ENHANCED BY MAST CELLS

To evaluate the co-stimulatory function of mast cells, isolated $CD4^+$ T cells were activated with anti-CD3 in the presence or absence of human peripheral blood-derived mast cells. T cell activation was measured by evaluating the expression of activation markers CD69 and CD25 (Figure 1A-C). To gate out mast cells ($CD117^+$) from the co-culture, T cells were gated as $CD3^+CD117^-$ cells (Figure 1A).

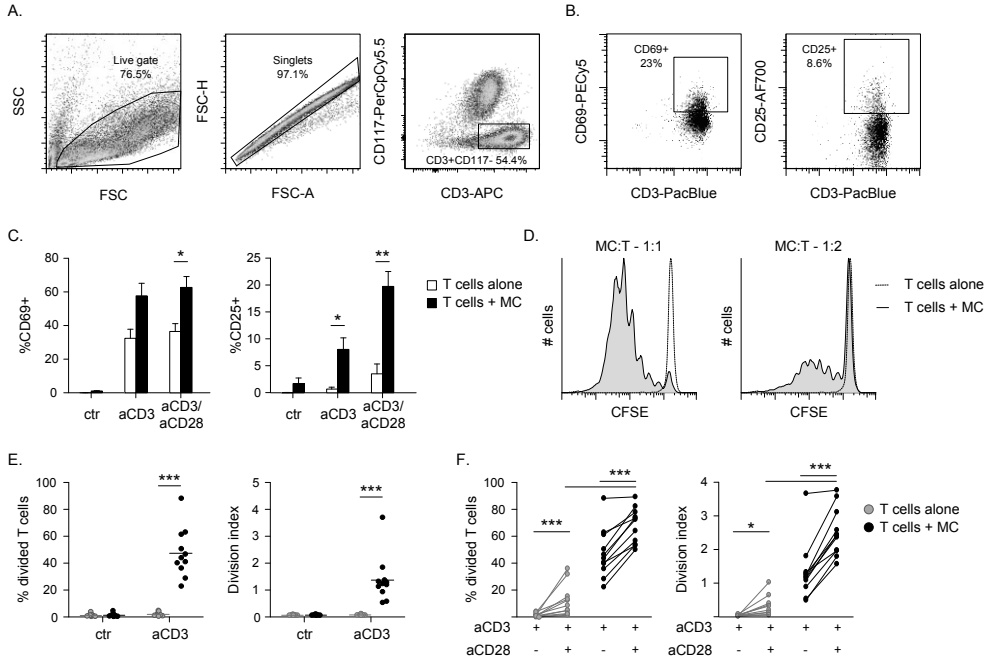


Figure 1 *(left)*: CD4$^+$ T cell co-stimulation by mast cells. A-C) Isolated human CD4$^+$ T cells were co-cultured with autologous peripheral blood-derived mast cells for 24 hours, in the presence or absence of anti-CD3 and anti-CD28, after which they were stained for activation markers CD69 and CD25. T cells were gated as CD3$^+$CD117$^-$ cells as shown in A. Representative flow cytometry plots of T cells in co-culture with mast cells and in the presence of anti-CD3 (B) and summary (C) of 3 independent experiments are shown. D-F) Isolated CD4$^+$ T cells were CFSE-labeled and co-cultured with autologous mast cells for 5 days, in the presence or absence of anti-CD3 and anti-CD28, after which they were stained for flow cytometry. T cells were gated as CD3$^+$CD117$^-$ cells as shown in panel A. Representative flow cytometry plots for CFSE (D) and summary of 11 independent experiments performed in duplicate with anti-CD3 (E) or anti-CD3 and anti-CD28 (F) are shown. G) Isolated total and naïve CD4$^+$ T cells were CFSE-labeled and co-cultured with autologous mast cells for 24 hours, in the presence or absence of anti-CD3 and anti-CD28, after which they were stained for flow cytometry. T cells were gated as CD3$^+$CD117$^-$ cells as shown in A. Representative example of 2 experiments performed in duplicate is shown. The percentage of divided T cells is depicted as % of the input frequency, indicating how many cells from the original cell population have divided. Division index represents the average number of cell divisions that a cell in the original population has undergone. Results are expressed as mean±SEM (C,G), or as individual dots where each dot/line indicates an independent experiment (E,F). Asterisks indicate statistically significant difference (*p<0.05; **p<0.01; ***p<0.005) using Students T-test.

Anti-CD3-stimulation led to an upregulation of these markers on T-cells. However, the upregulation, in particular that of CD25, was much more pronounced when T cells were co-cultured with mast cells and anti-CD3, suggesting that mast cells can enhance T-cell activation in the presence of a TCR stimulus.

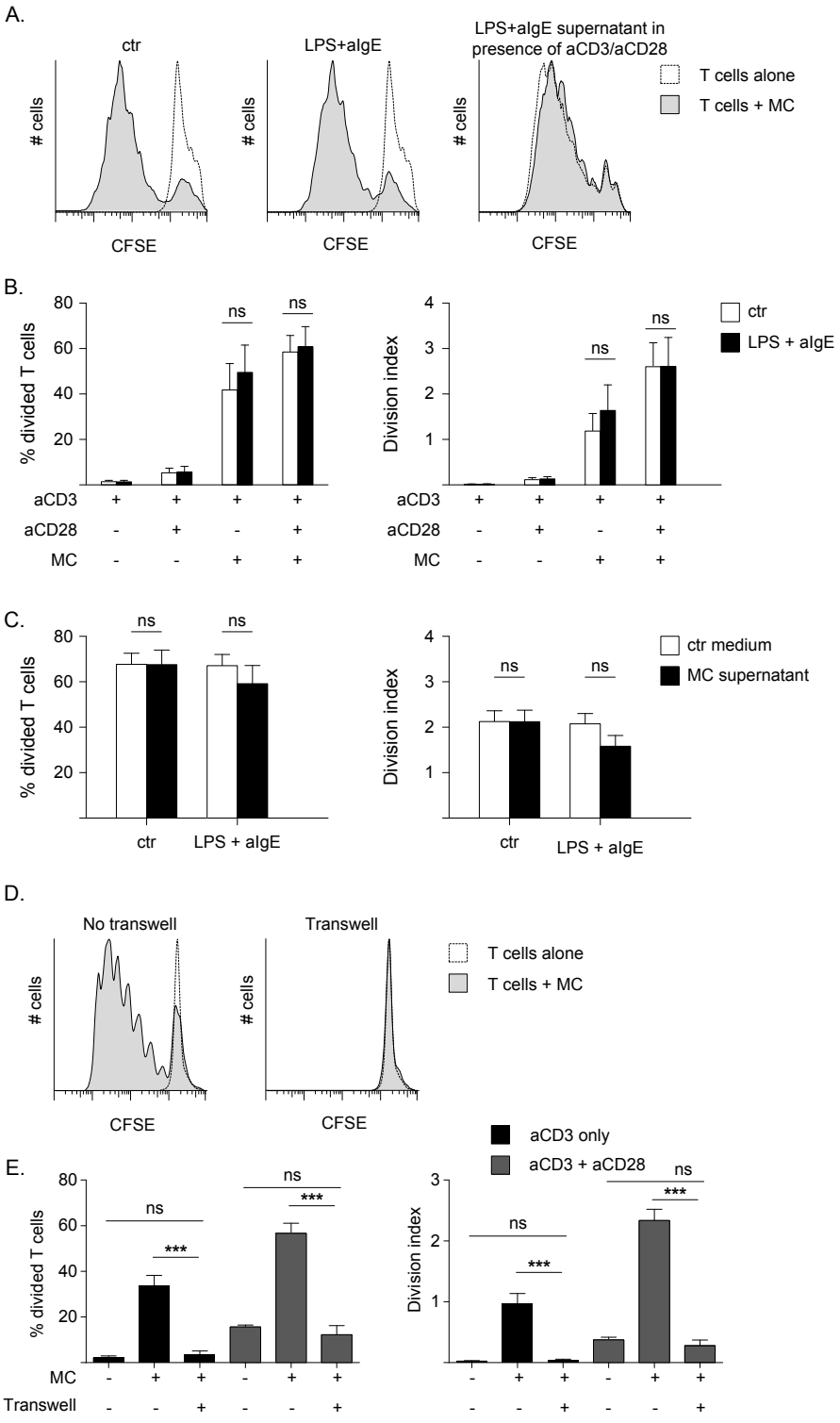
To confirm the enhanced activation of T cells after co-culture with mast cells, we next analysed T cell proliferation by a CSFE-dilution assay (Figure 1D,E). As expected, in the absence of mast cells or other forms of co-stimulation, virtually no T cell proliferation was observed in response to anti-CD3 alone. In line with the requirement of co-stimulation to trigger full T cell activation, anti-CD28 signalling significantly enhanced T cell proliferation (Figure 1F). Intriguingly, an even more pronounced T-cell activation was observed in the presence of mast cells.

Although CD28-signalling further enhanced T-cell proliferation in the presence of mast cells, the contribution of mast cells did not require the concomitant presence of a CD28-dependent signal. However, the effect of mast cells was dependent on T cell receptor triggering, as no effect of mast cells was observed in the absence of anti-CD3 (Figure 1E).

CO-STIMULATION BY MAST CELLS IS CELL-CONTACT DEPENDENT

As co-stimulatory molecules on professional antigen presenting cells, such as dendritic cells, can be upregulated after their activation, the effect of stimulation of mast cells was evaluated. Mast cells were activated using anti-IgE and LPS, two stimuli that induce a robust activation of mast cells and result in degranulation and cytokine production.(*13*) As shown in Figure 2A-B, the activation of mast cells had no effect on T cell proliferation, indicating that resting mast cells already have the capacity to induce T cell proliferation.

Importantly, the co-stimulatory capacity of mast cells was not dependent on crosslinking via FcγR (Supplementary Figure 1). Whereas IL-8 production by mast cells was significantly reduced in the presence of blocking FcγR antibodies (anti-CD32A), no effect on T cell proliferation was observed. Together these results indicate that co-stimulation by mast cells is independent of activation, by TLR ligands or Fc receptor crosslinking.


A.
ctr
LPS+aIgE
LPS+aIgE supernatant in presence of aCD3/aCD28
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C.
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MC supernatant
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No transwell
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E.
aCD3 only
aCD3 + aCD28
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Transwell

Figure 2 *(left)*: $CD4^+$ T cell co-stimulation by mast cells is cell-contact dependent. A-C) Isolated human $CD4^+$ T cells were CFSE-labeled and co-cultured with autologous mast cells or mast cell supernatant for 5 days, in the presence of anti-CD3 and/or anti-CD28, after which they were stained for flow cytometry. T cells were gated as $CD3^+CD117^-$ cells as shown in Figure 1A. Mast cells were left unstimulated (ctr) or were stimulated with LPS + aIgE. Summary of 3 independent experiments is shown. A) Representative flow cytometry plots of T cell proliferation in the presence of unstimulated mast cells and anti-CD3 (left), mast cells stimulated with LPS + anti-IgE in the presence of anti-CD3 (middle), or mast cell supernatant in presence of anti-CD3 and anti-CD28 (right). B) T cell/mast cell coculture. Summary of 3 independent experiments is shown. C) T cell culture in the presence of mast cell supernatant. Summary of 6 independent experiments is shown. D,E) Isolated $CD4^+$ T cells were CFSE-labeled and co-cultured in transwell plates with autologous mast cells for 5 days, in the presence of anti-CD3 and/or anti-CD28, after which they were stained for flow cytometry. Mast cells were cultured in the same well as the T cells, or were separated by a transwell membrane (+). D) Representative flow cytometry plots of T cells cultured in presence of anti-CD3 and in the presence of mast cells either together in the same well (left) or separated by a transwell (right). E) Summary of 3 independent experiments is shown. The percentage of divided T cells is depicted as % of the input frequency, indicating how many cells from the original cell population have divided. Division index represents the average number of cell divisions that a cell in the original population has undergone. Results are expressed as mean±SEM. Ns indicates no significant difference was found using students T-test (B,C). Asterisks indicate statistically significant difference (***$p<0.005$) using ANOVA with Bonferroni's post-hoc test (E).

To analyse whether cell-cell contact or mast cell-derived soluble mediators were responsible for the co-stimulatory effects, we next cultured T cells in the presence of mast cell supernatant. However, the presence supernatant from mast cell cultures did not result in enhanced proliferation, but if anything, the opposite (Figure 2A,C). To directly address the requirement for cell contact between mast cells and T cells, transwell experiments were performed. The effect of mast cells on T cell proliferation was absent when mast cells were separated from T cells by the transwell membrane (Figure 2D-E). These results indicate that the co-stimulatory effect mediated by mast cells requires cell-cell-contact, and is not dependent on soluble mediators secreted by mast cells.

$CD4^+$ T CELL PROLIFERATION INDUCED BY MC COMPARED TO MONOCYTES

To compare the co-stimulatory capacity of mast cells to other cells known to effectively co-stimulate T-cells, we compared the T cell proliferation induced by mast cells to that induced by total PBMCs (Figure 3A). At a similar ratio of PBMCs/mast cells to T cells, mast cells displayed a considerable higher capacity to induce T cell proliferation in the presence of anti-CD3 as compared to PBMCs.

The superior co-stimulatory ability of mast cells compared to PBMCs was also observed in titration experiments as lower number of mast cells were required to achieve a similar T-cell proliferation (Figure 3B). Next, we also wished to compare the co-stimulatory capacity of mast cells to isolated $CD14^+$ monocytes. As shown in Figure 3C-D, mast cells were also better in supporting T cell proliferation than monocytes, further confirming the potent co-stimulatory ability of mast cells.

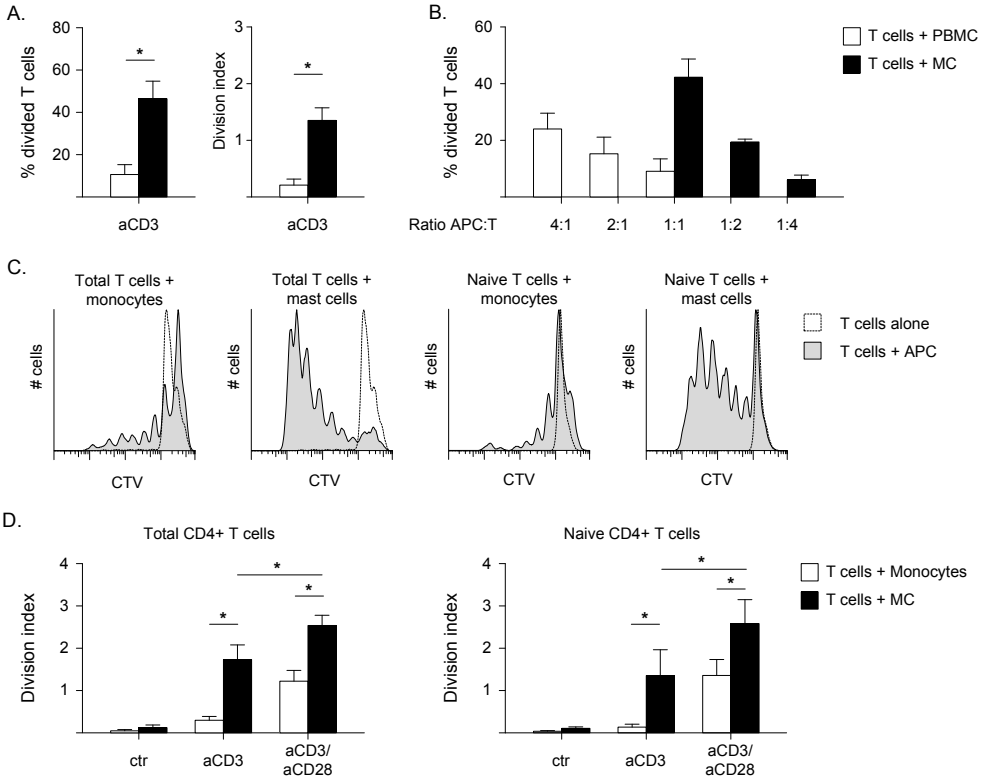


Figure 3: Mast cell co-stimulation compared to monocytes. A,B) Isolated human $CD4^+$ T cells were CFSE-labeled and co-cultured with autologous mast cells or PBMCs for 5 days, in the presence of anti-CD3, after which they were stained for flow cytometry. T cells were gated as $CD3^+CD117^-CD14^-$ cells as shown in Figure 1A. Ratios of co-stimulatory cells (APC) to T cells was 1:1 in 3 independent experiments (A) or titrated ranging from 4:1 – 1:1 for PBMCs and from 1:1 – 1:4 for mast cells in 2 independent experiments (B). C,D) Isolated total and naïve $CD4^+$ T cells were Cell Trace Violet (CTV) labeled and co-cultured with monocytes or mast cells for 5 days, in the presence or absence of anti-CD3 and CD28, after which they were stained for flow cytometry. C) Representative flow cytometry plots of CTV staining of T cells cultured in the presence of anti-CD3, gated as in Figure 1A. D) Summary of 3 independent experiments is shown. Asterisks indicate statistically significant ($p<0.05$) differences obtained using paired-samples T test (A), or Two-way ANOVA with Bonferroni's posthoc test (D).

As the co-stimulatory requirements of T cells differ between memory and naïve $CD4^+$ T cells, with naïve T cells being more dependent on a co-stimulatory signal,(*14*) we also evaluated the contribution of mast cells to proliferation of naïve $CD4^+$ T cells. As depicted in Ffigure 3C-D, mast cells were fully capable of inducing robust proliferation of naïve T cells in the presence of only anti-CD3, in contrast to monocytes which only induced minimal T cell proliferation. These results therefore indicate that mast cells are potent inducers of-stimulation of $CD4^+$ T cells in the presence of a TCR stimulus, also when the T-cells have a naïve phenotype.

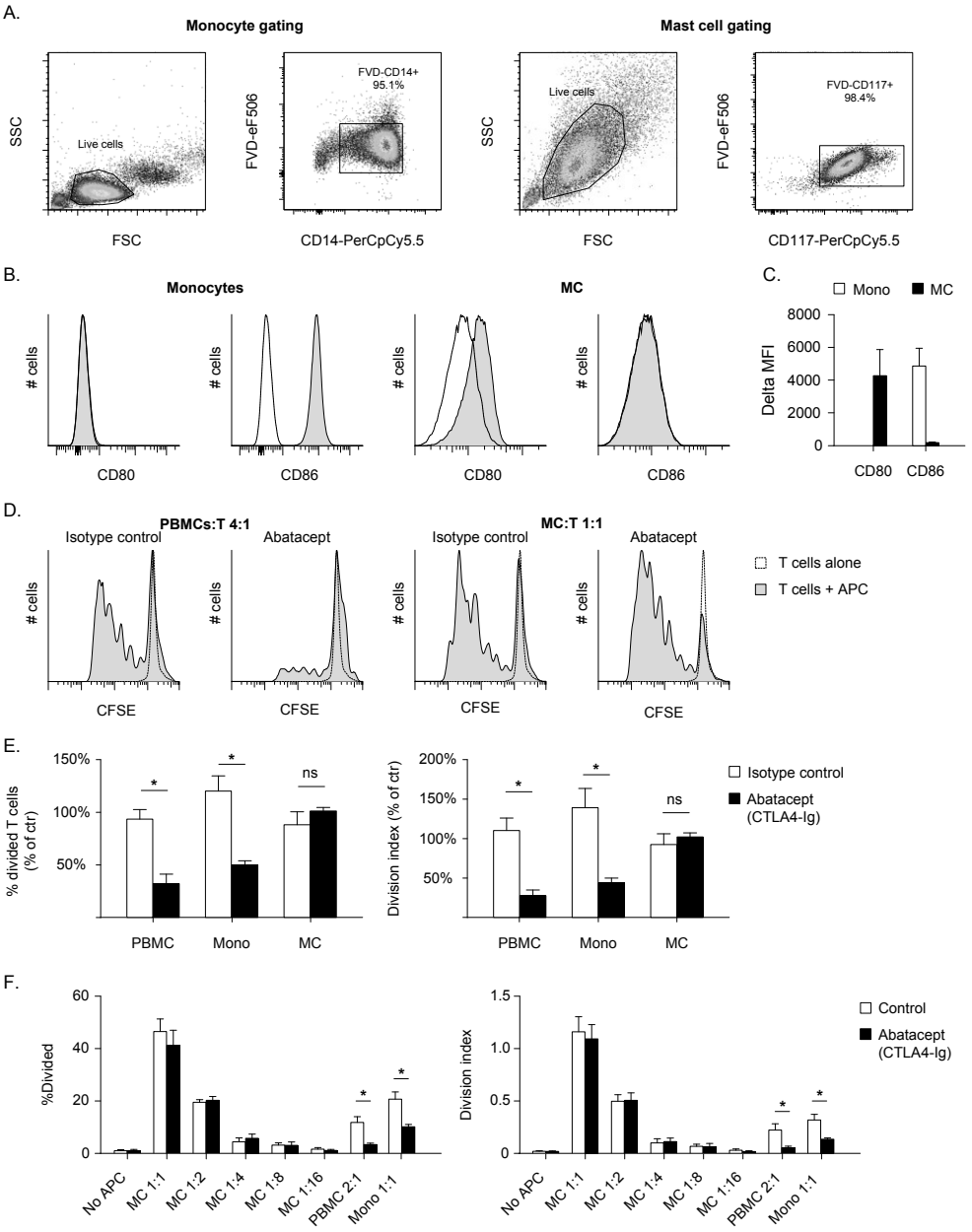
$CD4^+$ T CELL PROLIFERATION INDUCED BY MC DOES NOT DEPEND ON CLASSICAL CO-STIMULATION VIA CD28

We and others have shown previously that mast cells express CD80, which is known to interact with CD28 on T cells to induce co-stimulation.(*2, 4*) Therefore, it has been postulated that this molecule could be responsible for a co-stimulatory signal coming from mast cells. We first compared the expression levels of CD80 and CD86 on mast cells and monocytes (Fig 4A-C). Similar to our previous findings, human mast cells express CD80 but no CD86. In comparison, we observed that resting monocytes express CD86, but no CD80. However, we have to note that the high level of autofluorescence in mast cells (due to their granularity) makes it difficult to directly compare expression levels between the two cell types.

To analyse the contribution of this pathway to T cell co-stimulation by mast cells, we incubated mast cells, PBMCs or monocytes with CTLA4-Ig (Abatacept) or isotype control, prior to co-culture with the T cells. CTLA4-Ig remained present throughout the culture period. Whereas CTLA-4Ig significantly decreased T cell proliferation when T-cells were cultured with PBMCs or isolated monocytes, no effect of CTLA4-Ig was observed when mast cells were used as co-stimulatory cell (Fig 4D,E). Likewise, dilution of mast cells to achieve lower mast cell:T cell ratios to mimic the co-stimulatory capacity of PBMCs and monocytes did not lead to a detectable effect on T-cell proliferation supported by mast cells (Fig 4F).

Our results indicate that co-stimulation by mast cells is independent of CD28, in contrast to the ability of PBMCs and monocytes to co-stimulate T-cells.

We next evaluated whether other known co-stimulatory molecules were responsible for $CD4^+$ T cell activation by mast cells. Based on published microarray databases and RNA sequencing results, we identified the following potential T cell co-stimulatory molecules expressed by human mast cells that can be blocked by neutralizing antibodies: OX40L (TNFSF4), ICOSL (B7H2), and LIGHT (TNFSF14).(*15, 16*)



As shown in Figure 5, co-stimulation by mast cells was not inhibited using blocking antibodies against OX40L, ICOSL, or LIGHT, suggesting that the activation of $CD4^+$ T cells by mast cells was not mediated via these receptor/ligand systems and that other, possibly unknown, receptors are responsible for T cell co-stimulation by human mast cells.

Figure 4 *(left)*: Mast cell-mediated co-stimulation is independent of CD28A-C) Cultured mast cells and isolated monocytes were stained for CD80 and CD86 and analysed by flow cytometry. A) Debris and doublets were excluded based on forward and sideward scatter (FSC/SSC). Live monocytes were gated as $CD14^{+}FVD^{-}$ (fixable viability dye eFluor506). Live mast cells were gated as $CD117^{+}FVD^{-}$ cells. B) Representative examples of CD80 and CD86 expression by monocytes and mast cells. C) Summary expression of CD80 and CD86 by monocytes and mast cells. Delta-MFI was calculated by subtracting the MFI of isotype control from the MFI of CD80 or CD86 staining. D-F) Isolated $CD4^{+}$ T cells were CFSE-labeled and co-cultured autologous mast cells, monocytes or PBMCs for 5 days, after which they were stained for flow cytometry. Prior to co-culture, co-stimulatory cells were treated with control medium, isotype control IgG, or Abatacept (CTLA4-Ig), and these treatments remained present during the co-culture. D) Representative flow cytometry plots of CFSE staining of T cells cultured in the presence of anti-CD3, gated as in Figure 1A. T cells cultured with PBMCs as APC in a ratio 1:4 (left) and T cells cultured with mast cells as APC in a ratio 1:1 (right). E,F) Summary of 5 independent experiments using n=5 independent mast cell donors, n=3 independent PBMC donors and n=2 independent monocyte donors. E) T cell proliferation was normalized to control-medium treated PBMCs, monocytes or mast cells (T+APC ctr: 100%). F) T cell proliferation at different mast cell:T cell ratios as indicated in the axis titles. The percentage of divided T cells is the precursor frequency, indicating how many cells from the original cell population have divided. Division index is the average number of cell divisions that a cell in the original population has undergone. Results are expressed as mean±SEM. Asterisks indicate statistically significant difference (*$p<0.05$) using Students T-test. Ns indicates no significant difference was found using students T-test (E).

DISCUSSION

In this study we showed that mast cells can mediate T cell co-stimulation through a CD28-independent pathway. Although we analyzed a possible role for OX40L, ICOSL, and LIGHT, we have not identified the molecular mode of action explaining the ability of human mast cells to provide co-stimulation to T-cells. We also analyzed the contribution of FcγR crosslinking of mast cells by anti-CD3. Mast cells were activated by anti-CD3 antibodies in an FcγRIIA-dependent manner, but blocking of this receptor did not affect T cell proliferation induced by mast cells. Therefore, this activation of mast cells is unlikely to contribute to the T cell activation we observed.

We hypothesize that classical members of the B7 and TNF receptor families are unlikely to account for the observed co-stimulation by mast cells. For example, some of the molecular interactions that are described to be involved in priming of naïve $CD4^{+}$ T cells, HVEM-LIGHT, CD27-CD70 are either not expressed by mast cells, or were not inhibited by the blocking antibodies we used. However, there are many other members of the TNF receptor family and SLAM family, for which their role in co-stimulation of $CD4^{+}$ T cells is currently unknown.(*6*)

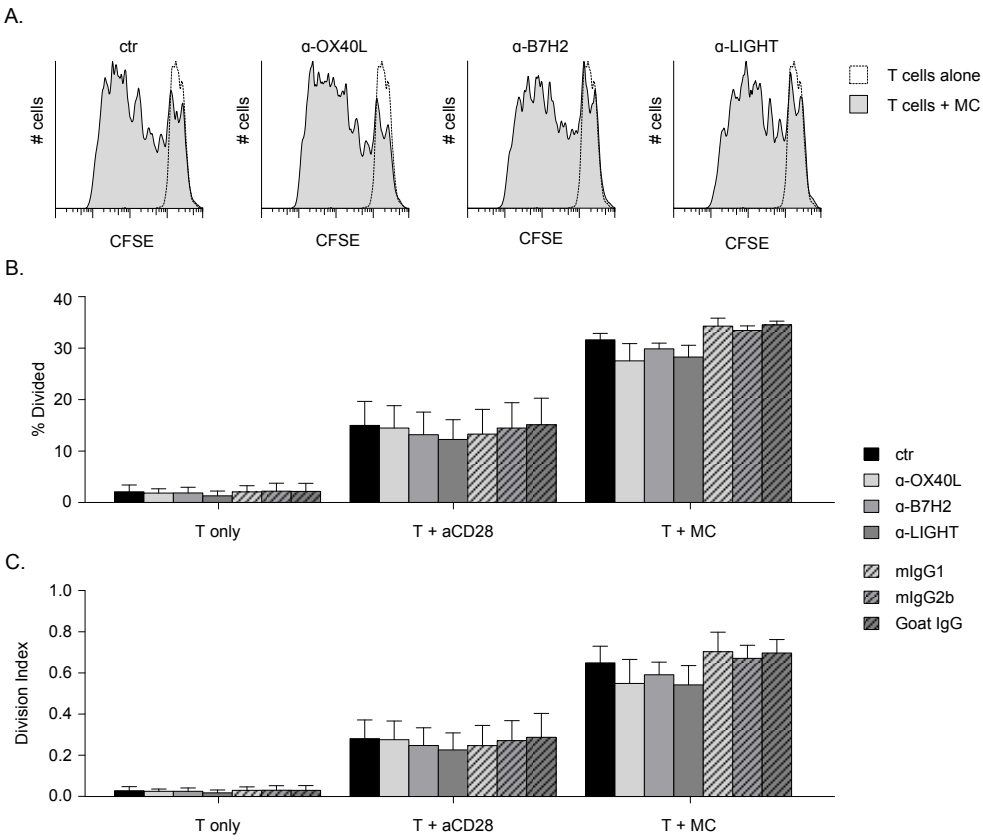


Figure 5: Blocking co-stimulatory molecules in the mast cell-T cell interaction. Isolated human $CD4^+$ T cells were labeled with cell trace violet (CTV) and co-cultured with mast cells for 4-5 days, in the presence of anti-CD3, after which they were stained for flow cytometry. Live T cells were gated as $CD3^+CD117^-CD14^-$ cells as shown in Figure 1A. As positive control, T cells were stimulated in the presence of anti-CD3 and anti-CD28 (T+aCD28). Prior to co-culture, mast cells were treated with control medium, isotype control mIgG1 (control for OX40L), mIgG2b (control for B7H2), goat IgG (control for LIGHT) or anti-OX40L, anti-B7H2 (ICOSL), anti-LIGHT, and these antibodies remained present during the co-culture. A) Representative flow cytometry plots of cell trace violet (CTV) staining. B,C) Summary of 3 independent experiments is shown. The percentage of divided T cells is the precursor frequency, indicating how many T cells from the original cell population have divided. Division index is the average number of cell divisions that a T cell in the original population has undergone. Results are expressed as mean±SEM. No statistically significant differences between blocking antibodies and their matching isotype control antibodies were found using students T-test.

Therefore, it remains to be shown which of these, or other unknown receptors, is responsible for co-stimulation by human mast cells. Other studies have shown T cell proliferation by activated mast cells was partially mediated by TNF (mouse mast cells) and OX40L/OX40 interactions (both mouse and human).(*8, 9, 17*) However, T cell activation

through TNF and OX40L/OX40 depend on mast cell activation, which is required for production or expression of these molecules.(*8, 9*) Furthermore, T cell activation by these molecules usually only occurs in pre-activated or effector T cells. (*18, 19*) As we observed strong T cell activation in the absence of mast cell stimulation, and activation of naïve T cells by mast cells was as strong as that of total $CD4^+$ T cells, we propose that these pathways are unlikely to account for the full co-stimulatory signal provided by mast cells, as also shown by transwell experiments and inhibition of OX40L.

Because a redundancy of co-stimulatory molecules might be present with respect to the ability of human mast cells to provide co-stimulatory signals to T-cells, it might be difficult to identify the pathway that is responsible for the observed effect.(*20*) However, know-how on the molecular details underlying the effects observed is likely of relevance as these effects may have important implications for therapies aiming to block co-stimulatory pathways such as employed in autoimmune diseases. Whereas CTLA4-Ig is being successfully used as treatment for a variety of autoimmune diseases, it does not completely inhibit T cell activation, suggesting that CD28-independent pathways may contribute to autoreactive T cell responses as well.(*11, 21-23*) Our results suggest that this treatment may not inhibit mast cell-mediated, and possibly other forms of T cell activation. Indeed, several studies point to an important contribution of CD28-independent T cell activation in autoimmune disease.(*24, 25*) Therefore, our results suggest that CD28-independent inhibitors of T cell activation may prove beneficial effects against CD28-independent autoimmune T cell responses such as those supported by mast cells and possibly other cells.

MATERIALS AND METHODS

MAST CELL CULTURE

Buffy coats from healthy volunteers were obtained from the blood bank (Sanquin, The Netherlands). PBMCs were isolated using a standard Ficoll procedure, after which $CD34^+$ hematopoietic stem cells were isolated with CD34 microbeads (Miltenyi Biotec). Isolated $CD34^+$ stem cells were differentiated into mast cells using serum-free medium (StemPro 34 + supplement, Gibco) with 30 ng/mL IL-3, 100 ng/mL IL-6 and 100 ng/mL Stem Cell Factor (SCF) at 50.000 cells/mL as described.(*26*) Half of the medium was replaced weekly with serum-free medium containing 100 ng/mL IL-6 and 100 ng/mL SCF. All recombinant cytokines were obtained from Peprotech. After 6-8 weeks, the purity of mast cells was determined by flow cytometric analysis of CD117 (c-kit), FcεRI and CD203c and intracellular tryptase. The purity of mast cells ranged from 90-99%.

T CELL ACTIVATION

For isolation of T cells, autologous CD45RO-negative (naïve) or total $CD4^+$ T cells were isolated from PBMCs by negative magnetic bead isolation (Miltenyi Biotec). The purity of isolated T cells was above 95% in each experiment. As positive controls, total PBMCs or isolated $CD14^+$ monocytes (Miltenyi Biotec) were used to stimulate T cells.

Isolated T cells were labeled with Cell Trace CFSE or Cell Trace Violet (both from Invitrogen) to measure cell proliferation. T cells were activated in the presence or absence of 5 µg/mL platebound anti-CD3 (eBioscience; clone: OKT3) and/or 1 µg/mL soluble anti-CD28 (Sanquin; clone: CLB-CD28/1, 15E8) and were cultured in the presence or absence of mast cells in the indicated ratios. Activation of T cells was measured after 24h by flow cytometry for CD25 and CD69. Proliferation and cytokine production were measured after 4-5 days of incubation. To this end, T cells were harvested, washed, and restimulated using 50 ng/mL PMA (Sigma) and 500 ng/mL Ionomycin (Sigma). Cells were restimulated for 5 hours in the presence of 10 µg/mL brefeldin A (Sigma) for intracellular cytokine staining.

For mast cell activation, cells were sensitized with 0.1 µg/mL hybridoma IgE (Diatec) for a minimum of 18 hours, after which non-bound IgE was washed away. Mast cells were activated with 10 ug/mL goat anti-human IgE (Nordic) and 1 ug/mL E. Coli K12 lipopolysaccharide (LPS; InVivoGen).

For blocking experiments, mast cells, PBMCs or monocytes were incubated with 100 µg/mL CTLA4-Ig (Abatacept, Bristol-Myers Squibb), 100 µg/mL human IgG isotype control, 10 µg/mL anti-OX40L (MAB10541-100, R&D Systems), 10 µg/mL anti-B7-H2/ICOSL (MAB165-100, R&D Systems), 0.1 µg/mL anti-LIGHT/TNFSF14 (AF664-SP, R&D Systems), 10 µg/mL anti-CD32A (IV.3, StemCell), 10 µg/mL mouse IgG1 isotype control (16-4714, eBioscience), 10 µg/mL mouse IgG2b isotype control (16-4732, eBioscience), 0.1 µg/mL polyclonal goat IgG isotype control (AB-108-C, R&D systems), for 30 minutes at 37 ºC prior to co-culture with the T cells.

To analyse the requirement of cell-cell contact, transwell plates with 0.4 uM-pore membrane (Corning) were used.

FLOW CYTOMETRY

Antibodies to CD3, CD14, CD117, CD25 and CD69 were obtained from BD Biosciences. For surface staining, cells were incubated with fluorochrome-conjugated antibodies diluted in PBS 0,5% BSA at 4 ºC for 30 min. To exclude dead cells fixable viability dye eFluor506 (eBioscience) was added during incubation of surface antibodies. After washing, cells were suspended in 1% paraformaldehyde until flow cytometric aquisition on a FACS Calibur (BD)

or LSR-II (BD). Analysis was performed using FACS Diva (BD) and FlowJo software. Cell proliferation was analysed as described.(*27*) The % divided cells was defined as the probability that a cell has divided at least once from the original population. The division index was defined as the average number of cell divisions that a cell in the original population has undergone.

STATISTICAL ANALYSIS

Results are expressed as mean ± SEM. Statistical analysis was performed using GraphPad Prism 4. For differences between two groups, student's T tests were performed. For differences between more than two groups, One-way ANOVA with Bonferroni's posthoc test was performed. P values of <0.05 were considered statistically significant.

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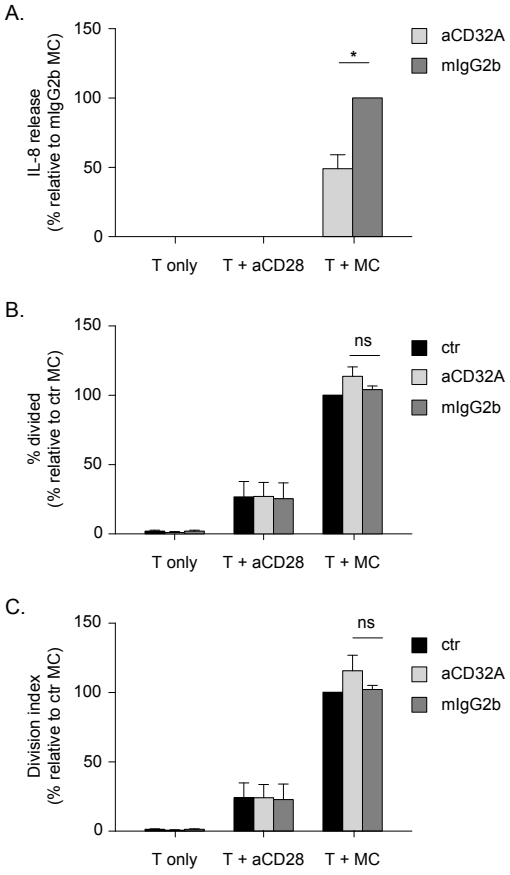
SUPPLEMENTARY FIGURE



Supplementary Figure 1: Blocking FcγR on mast cells. Isolated human $CD4^+$ T cells were labeled with cell trace violet (CTV) and cocultured with autologous mast cells for 5 days, in the presence of anti-CD3, after which they were stained for flow cytometry. T cells were gated as $CD3^+CD117^-CD14^-$ cells as shown in Figure 1A of the manuscript. Prior to coculture, mast cells were treated with control medium, isotype control mIgG2b, or anti-CD32A, and these antibodies remained present during the coculture. A) Release of IL-8 in presence of mIgG2b or anti-CD32A was measured by ELISA. No IL-8 was detected in the absence of mast cells. IL-8 production was normalized to mIgG2b control (100%). Asterisk indicates statistically significant difference calculated using Two-way ANOVA and Bonferroni's posthoc test. B,C) T cell proliferation in presence of mIgG2b or anti-CD32A. T cell proliferation was normalized to control-medium treated mast cells (T+MC ctr: 100%). Ns indicates no statistically significant differences were found using Two-way ANOVA and Bonferroni's posthoc test. Results are expressed as mean±SEM of 3 independent experiments.