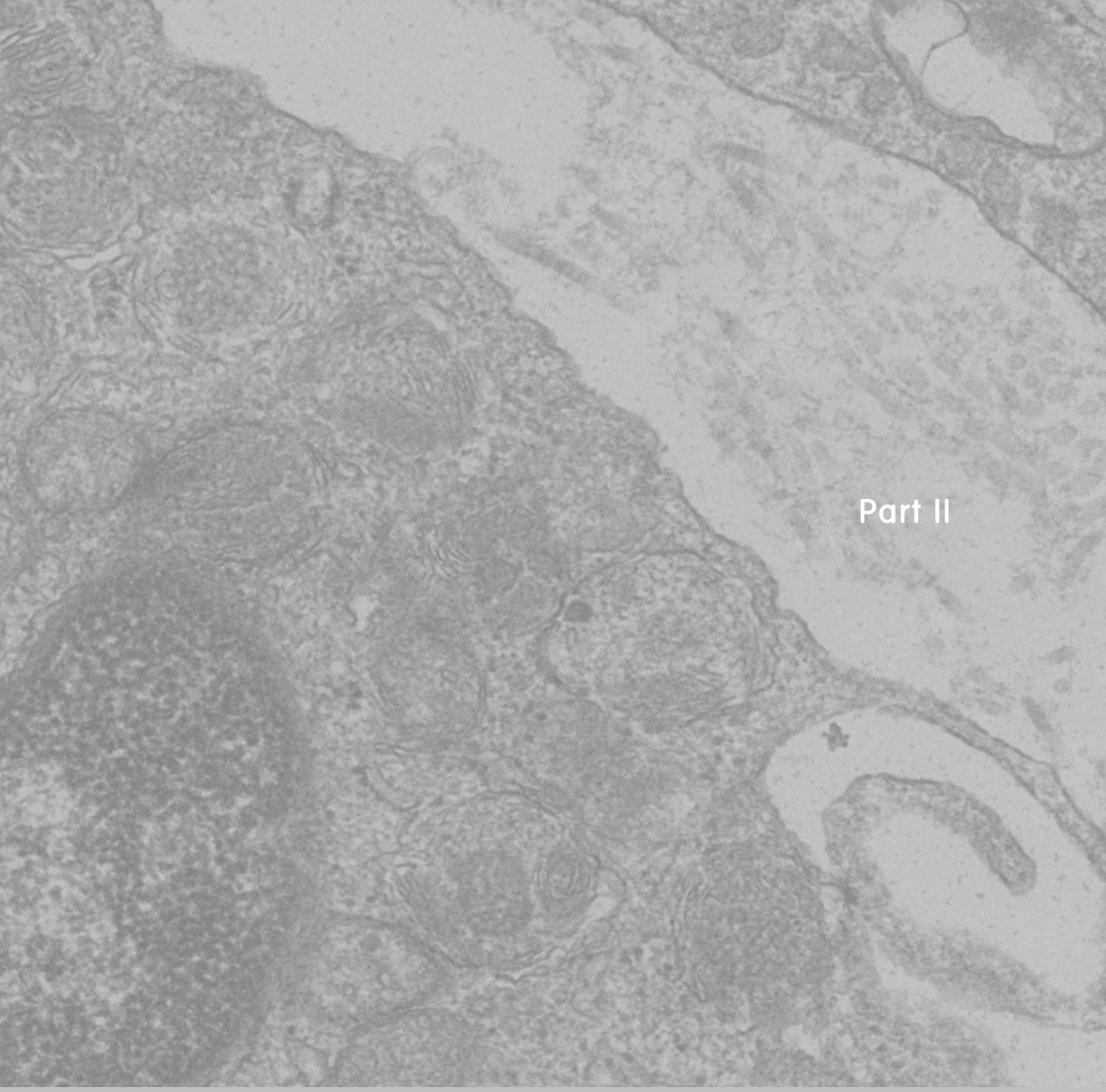

Part II

Interactions between mast cells and $CD4^+$ T cells

COMMUNICATION BETWEEN HUMAN MAST CELLS AND CD4$^+$ T CELLS THROUGH ANTIGEN-DEPENDENT INTERACTIONS

Chapter 7

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ABSTRACT

Mast cells (MCs) are immune cells residing in tissues where pathogens are first encountered. It has been indicated that MCs might also be involved in setting the outcome of T-cell responses. However, little is known about the capacity of human MCs to express MHC class II and/or to capture and present antigens to $CD4^+$ T cells.

To study the T-cell stimulatory potential of human MCs, $CD34^+$ stem cell derived MCs were generated. These cells expressed HLA-DR when stimulated with IFN-γ, and, importantly, presented peptide and protein for activation of antigen-specific $CD4^+$ T cells. The interplay between MC and T cell led to increased HLA-DR expression on MCs. MCs were present in close proximity to T cells in tonsil and expressed HLA-DR and CD80, indicating their ability to present antigens to $CD4^+$ T cells in T-cell areas of human LNs.

Our data show that MCs can present native antigens to human $CD4^+$ T cells and that HLA-DR expressing MCs are present in tonsil tissue, indicating that human MCs can directly activate T cells and provide a rationale to study the potential of MCs to prime and/or skew human T-cell responses.

INTRODUCTION

Mast cells (MCs) are most well known because of their role in IgE-mediated immune responses as they express the high-affinity FcεRI. MCs are present at strategic locations of the environment/host interface where they can encounter pathogens. Therefore, they have been implicated in the regulation of adaptive immune responses as well as in the regulation of T-cell immunity. Secretion of TNF by skin MCs was shown to be essential for LN hypertrophy and T-cell recruitment during bacterial infection (*1*).

Furthermore, MCs were shown to be capable of migrating from the skin to the draining LNs in murine models of contact hypersensitivity and UV radiation (*2, 3*). In the draining LNs, the secretion of chemokines by MCs has been implicated in the recruitment of T cells (*2, 4*). These studies, therefore, indicate that MCs can be involved in the regulation of T-cell trafficking toward lymphoid organs, suggesting that MCs may have a role in the induction of primary and/or local memory T-cell responses. Indeed, anti-CD3-stimulated T cells displayed enhanced proliferation and cytokine secretion in the presence of MCs, indicating that MCs are capable of enhancing T-cell responses (*5-7*).

A first direct demonstration that MCs can also present antigens to T cells came from mouse studies showing that OVA-specific TCR-transgenic $CD8^+$ T cells can be activated by MCs via

MHC class I (*8, 9*). Likewise, using OVA-specific TCR-transgenic $CD4^+$ T cells, it was demonstrated in vitro that mouse MCs can also activate MHC class II restricted T cells when loaded with OVA peptide, but were less efficient in presenting OVA protein (*10, 11*).

Thus far, little information is available on the antigen-presenting capacity of human MCs. Human MCs have also been shown to express MHC class II, especially after incubation with IFN-γ, which suggests their capacity to present antigen to $CD4^+$ T cells (*12-14*). Indeed, activation of T-cell hybridomas with superantigens by these cells has been reported (*14*). However, T-cell activation by superantigen does not require the presentation of antigenic peptides by MHC molecules or the processing of protein via endogenous pathways. Human MCs have been reported to be present in the interfollicular area in tonsil (*7*), indicating that they may be involved in the activation of T-cell responses in lymphoid organs. However, it is unknown whether these MCs possess the required molecular make-up, such as HLA-DR and costimulatory molecules, to present antigen and activate T cells in lymphoid organs.

As it is unclear whether human MCs are able to process and present native antigen to $CD4^+$ T cells, we aimed to study the interaction between these cells. Our data show that human MCs can present both peptide and entire protein antigens to $CD4^+$ T cells, using two different antigen-specific $CD4^+$ T-cell responses as read-out. Interaction of $CD4^+$ T cells with MCs led to the enhanced expression of HLA class II molecules on MCs via release of IFN-γ by T cells. In addition, we show that human MCs, analyzed ex vivo from tonsil, are present in T-cell areas where they express HLA-DR and CD80. Together, these data indicate that antigen-specific MC–T cell interactions could contribute to the outcome of immune responses in the human.

RESULTS

HUMAN PERIPHERAL BLOOD DERIVED MC EXPRESS HLA-DR WHEN STIMULATED WITH IFN-γ

Human MCs were generated from $CD34^+$ stem cells. Typical flow cytometry plots identifying MCs by combined expression of CD117, CD203c (Ectoenzyme E-NPP3), FcεRI, and intracellular tryptase are shown in Figure 1A, indicating that the 6-week culture results in mature ($CD117^+$ $tryptase^+$) MCs. No expression of the DC markers DC-SIGN and CD1c (BDCA-1) was found on our cultured MCs (data not shown). Only low, but detectable, levels of HLA-DR were detected on resting MCs (Fig. 1B and C).

Because it was previously shown for MC lines that IFN-γ could induce expression of HLA class II, we evaluated whether this could lead to upregulation of HLA-DR on our cultured

MCs as well. Incubation with 50 ng/mL IFN-γ for 24 h induced upregulation of HLA-DR (Fig. 1B and C). No induction of expression of HLA class II was found after stimulation with TLR ligands, anti-IgE, or recombinant IL-4 (data not shown). These results indicate that in the presence of IFN-γ, MCs can acquire the expression of HLA class II, and possibly the ability to present antigen to $CD4^+$ T cells.

Of the costimulatory molecules tested (CD80, CD86, ICOSL, OX40L, CD40L, and CD40), we found expression of CD80 but not CD86 or any of the other markers, as assessed by flow cytometry (Fig. 1D and E). Together, these data indicate that human MCs can express molecules required for stimulation of $CD4^+$ T cells.

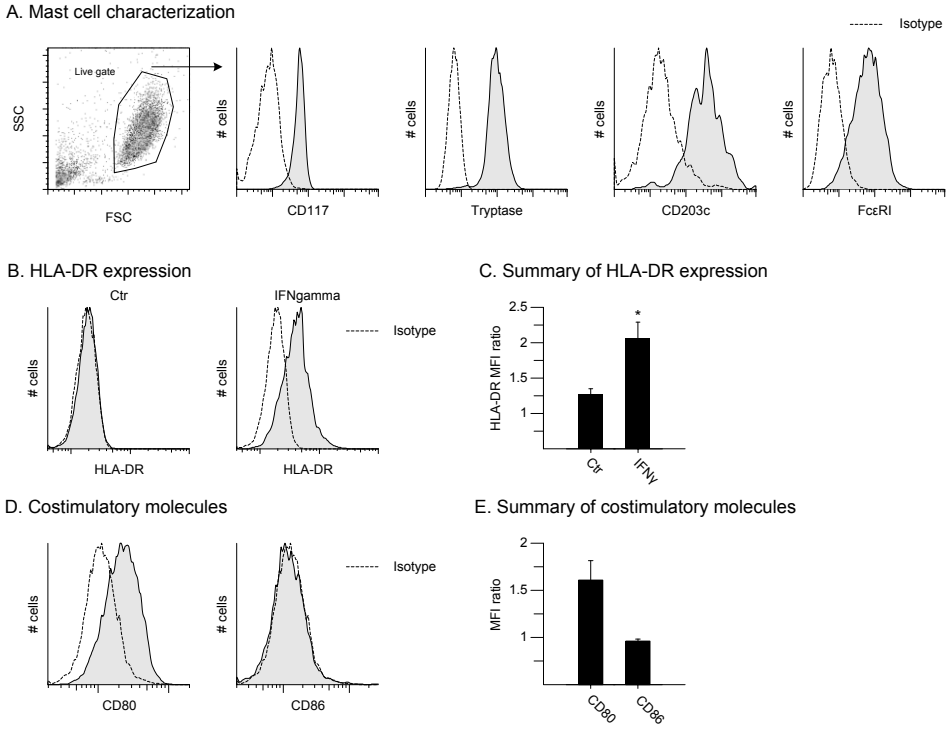


Figure 1. Expression of HLA-class II and costimulatory molecules by human peripheral blood derived mast cells (MCs). (A) Representative flow cytometric plots of MC characterization after 6–8 weeks differentiation showing expression of CD117, intracellular tryptase, FcεRI, and CD203c. Histograms show gated live cells based on forward scatter (FSC) and sidescatter (SSC) characteristics as shown in the first panel. (B, C) MCs were cultured in the presence of 50 ng/mL IFN-γ for 24 h, after which expression of HLA-DR was assessed. (D, E) Expression of CD80 and CD86 in resting MCs. (B, D) Representative examples of MCs gated as $CD117^+$ $CD203c^+$. (C, E) Summaries (mean +SEM) of independent MC cultures (n = 3). Expression (MFI ratio) is indicated as MFI of the marker of interest (e.g. HLA-DR) divided by the MFI of the matching isotype control. Asterisk in (C) indicates significant ($p < 0.05$) increase in IFN-γ-treated cells compared with ctr MCs, using Student's t-test.

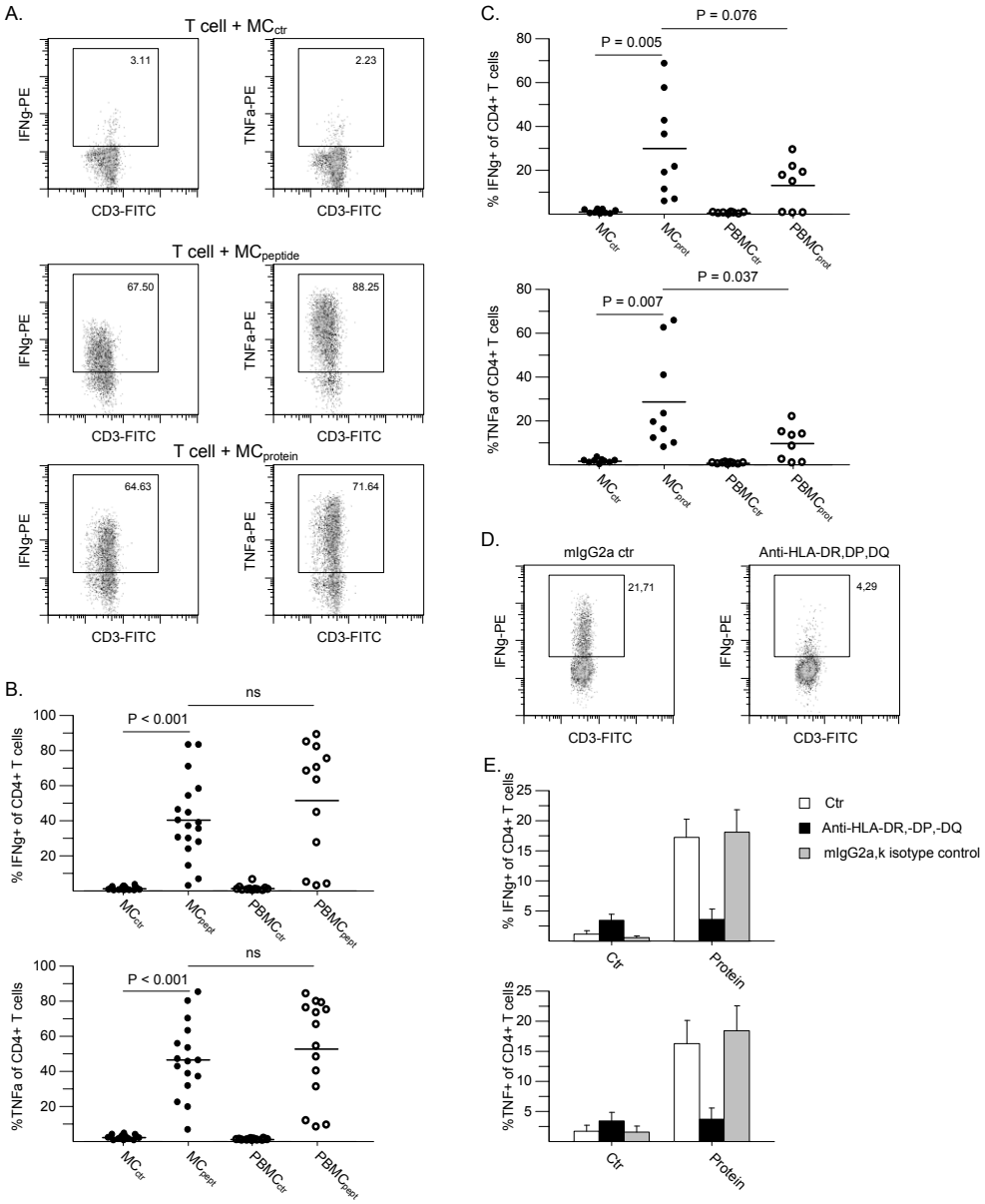


Figure 2. Antigen presentation by human mast cells (MCs) leads to activation of adenovirus-specific T cells. (A) Representative flow cytometric plots of TNF-α and IFN-γ production by gated $CD3^{+}CD4^{+}$ T cells after overnight coculture with MCs preincubated with medium (ctr), adenovirus-specific peptide or protein, in the presence of brefeldin A. (B, C) Summary of T-cell activation after preincubation of MCs with peptide and protein. Each dot shows an independent MC culture or PBMCs donor (n = 18 and n = 9 for peptide and protein, respectively). p values comparing the indicated conditions were calculated using Student's t-test. (D, E) Cytokine production by T cells after preincubation of MCs with blocking HLA class II antibodies (n = 3). (E) Data are shown as mean +SEM pooled from (n = 3) independent MC cultures.

HUMAN MC CAN PRESENT PEPTIDE AND PROTEIN TO CD4$^+$ T CELLS

To evaluate the potential of human MCs to present antigen to CD4$^+$ T cells, MCs were preincubated with an adenovirus-derived peptide or hexon IID protein. Subsequently, MCs were cocultured with an adenovirus-specific T-cell clone recognizing the particular peptide. As shown by intracellular cytokine staining, MCs preincubated with peptide, in contrast to control MCs, were capable of inducing robust cytokine production by the T cells (Fig. 2A and B). The capacity of MCs to induce T-cell responses with the peptide was comparable to that of PBMCs. Importantly, MCs preincubated with the hexon IID protein were also capable of activating T cells, indicating the capacity of MCs to take up, process, and present native protein antigen to CD4$^+$ T cells (Fig. 2A and C).

Presentation of protein antigens by MCs appeared rather efficient, as 5 μg/mL protein, equivalent to 225 nM, was still able to activate adenovirus-specific T cells. Furthermore, although there was no difference between the T-cell responses induced by MCs compared with PBMCs when they were incubated with peptide, MCs were more efficient than PBMCs in inducing T-cell responses with the protein antigen (Fig. 2C). Similar results were obtained when T-cell-derived cytokine levels in supernatant were measured by ELISA (data not shown).

To confirm that these T-cell responses were HLA class II dependent, MCs were next incubated with blocking HLA class II or isotype control antibodies. CD4$^+$ T-cell responses induced by MCs preincubated with hexon IID protein were almost completely inhibited by HLA class II blocking antibodies, compared with the respective isotype control (Fig. 2D and E), further confirming that the MCs stimulated the T cells via HLA class II.

Although the data presented above indicate that human MCs can present protein antigens to T cells, they do not show whether polyclonal T cells can be activated by MCs. To determine whether polyclonal T cells, rather than cloned T cells potentially harboring a high-avidity TCR to the peptide–MHC complex, are also activated by MCs, we next generated CD4$^+$ bulk T cells expanded for 1 week with pooled tetanus toxoid, tuberculin, and Candida albicans. Subsequently, autologous MCs were incubated with these recall antigens and used as APCs for polyclonal bulk T cells. MCs were capable of activating autologous polyclonal CD4$^+$ T cells, confirming their T-cell-activating capacity (Fig. 3). The response by CD4$^+$ T cells that was induced by MCs was comparable to or better than our positive control, autologous adherent PBMCs.

Together, these results indicate that human MCs are capable of taking up and processing native proteins for presentation to CD4$^+$ T cells in the context of HLA class II.

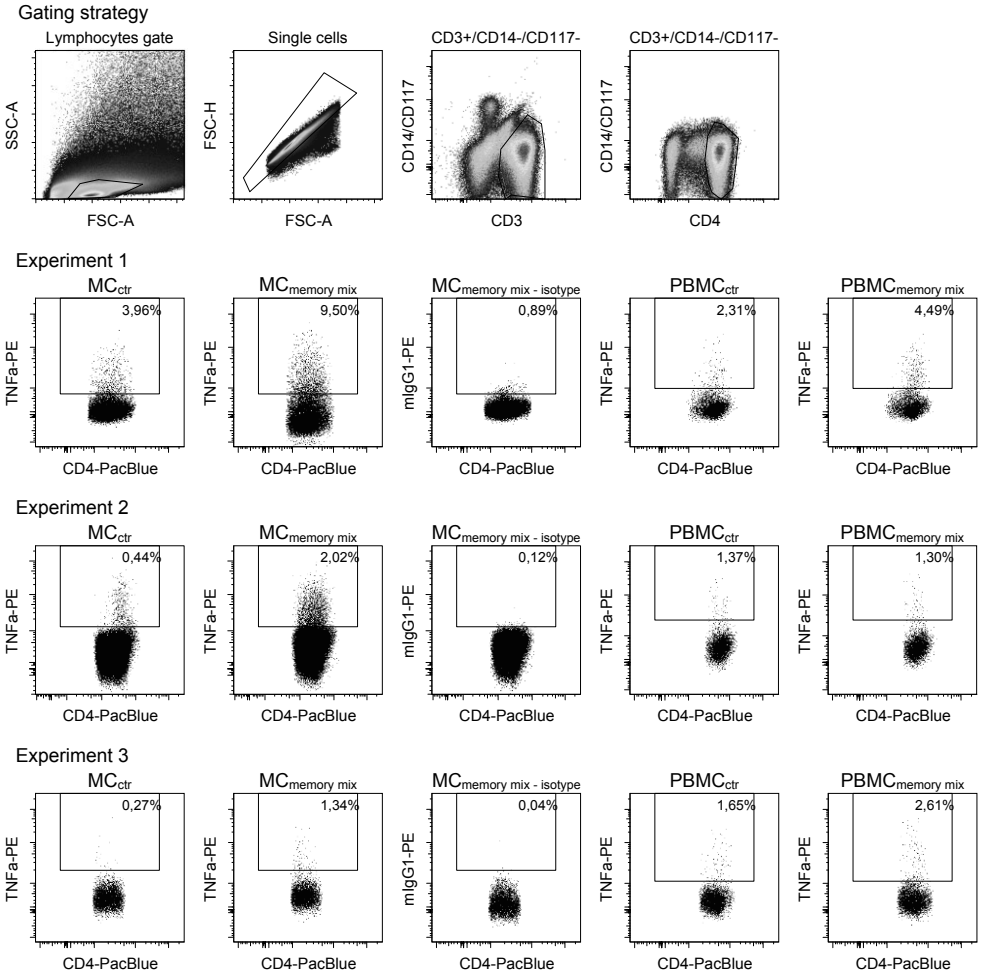


Figure 3. Antigen presentation by human mast cells (MCs) to autologous bulk $CD4^+$ T cells. Polyclonal $CD4^+$ T-cell bulks were generated by activation of PBMCs with the recall antigens tetanus toxoid, tuberculin purified protein derivative, and Candida albicans for 7 days. Autologous MCs or adherent PBMCs preincubated with medium (ctr) or these recall antigens (memory mix) were cultured overnight with the bulk T cells in the presence of brefeldin A. Flow cytometric plots of TNF-α production by T cells in three independent experiments are shown. T cells were gated based on FSC/SSC characteristics, CD14/CD117 negativity, $CD4^+$, and $CD3^+$ as shown in top panel.

CD4$^+$ T CELLS CAN MODULATE MC VIA IFN-γ

As the interaction between APCs and T cells can be bidirectional, the potential of CD4$^+$ T cells to modulate MC phenotype was analyzed. To this end, MCs preincubated with peptide were cocultured with adenovirus-specific CD4$^+$ T cells to evaluate potential effects of T-cell activation on the MCs. As shown in Figure 4A and B, the expression of HLA-DR by MCs was enhanced when T cells were activated by MCs preincubated with peptide. MCs cultured without T cells or MCs not preincubated with peptide did not show an increase in HLA-DR expression. Upregulation of HLA-DR was inhibited by blocking IFN-γ, ranging from 14 to 100% inhibition as compared with the isotype control (Fig. 4C and D).

These data indicate that the effect of activated T cells responsible for HLA class II upregulation on MCs was mainly mediated via IFN-γ. Together, these results show that MCs not only activate T cells, but also CD4$^+$ T cells that can modulate MCs by upregulation of HLA class II, indicating a bidirectional interplay between MC and T cell.

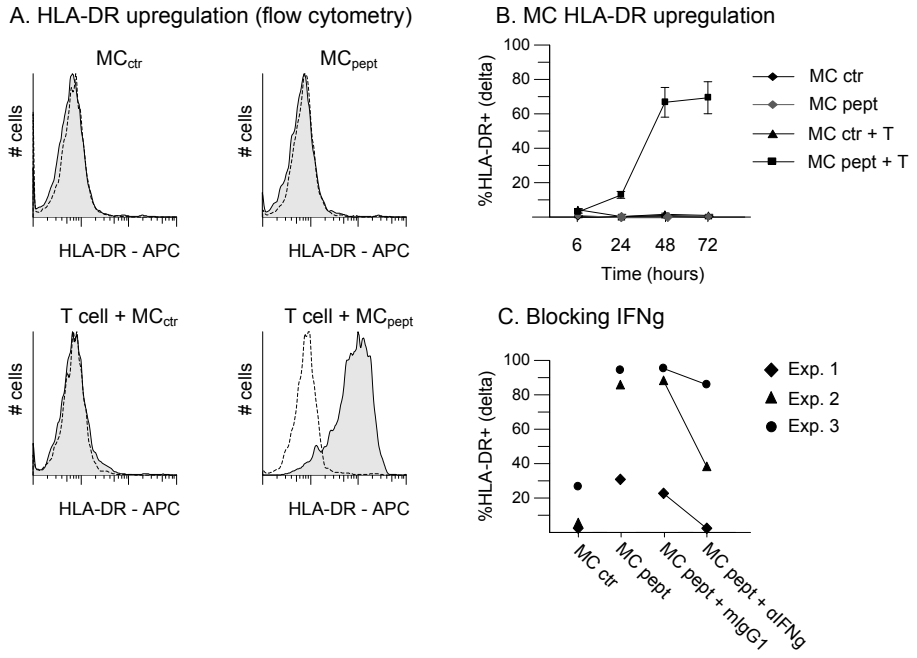
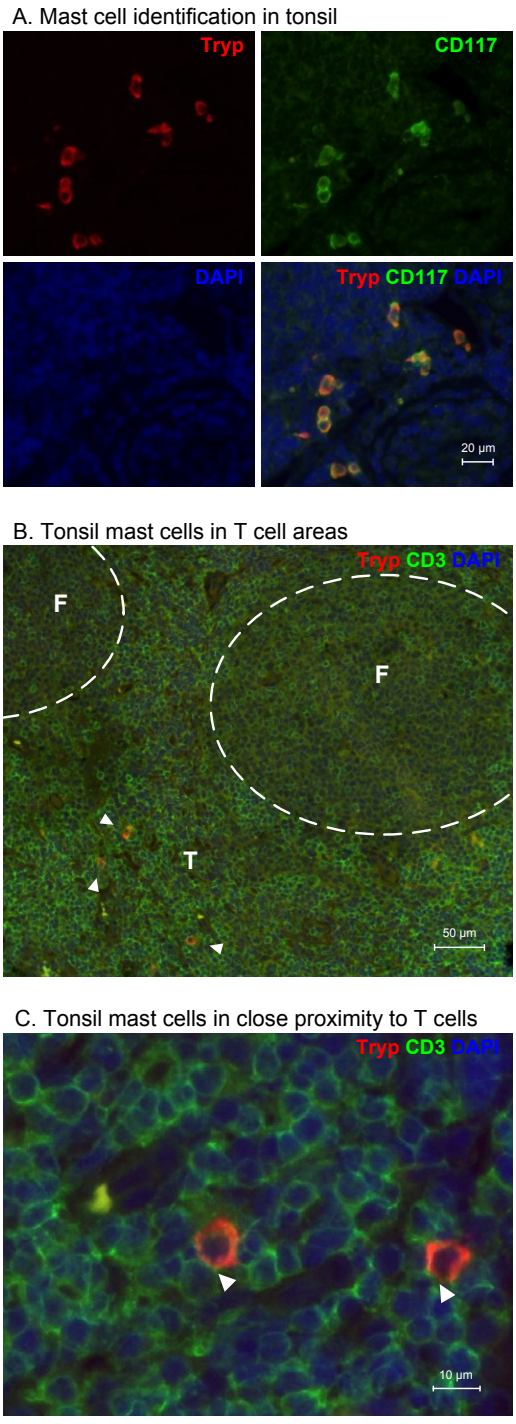


Figure 4. Induction of HLA class II on mast cells (MCs) through IFN-γ production by CD4$^+$ T cells. (A, B) Representative flow cytometric plots and summary of HLA-DR expression by MCs preincubated with medium (ctr) or adenovirus-specific peptide (pept), after coculture with or without adenovirus-specific CD4$^+$ T cells for the indicated time periods (n = 2). (C, D) Representative flow cytometric plots and summary of HLA-DR expression by MCs, after coculture for 48 h with adenovirus-specific CD4$^+$ T cells in the presence of isotype control antibodies (mIgG1) or blocking antibodies to IFN-γ (n = 3). MCs are gated as CD203c$^+$ cells as shown in Figure 1. Data are shown as mean ± SEM pooled from (n = 3) independent MC cultures.



HUMAN TONSILS CONTAIN HLA-DR$^+$ MC

To evaluate whether HLA-DR$^+$ MCs can also be found in vivo in T-cell areas of lymphoid organs, tonsil sections were stained for MCs. MCs were identified as CD117$^+$ tryptase$^+$ double-positive cells and were found in all (n = 6) tissues analyzed (Fig. 5A). All tryptase$^+$ cells expressed CD117, whereas also some CD117$^+$ tryptase- cells could be identified, indicating that CD117 alone cannot uniquely define tonsillar MCs. Tryptase$^+$ MCs were most often found in T-cell areas characterized by abundant expression of CD3, and were commonly observed in close proximity to CD3$^+$ T cells (Fig. 5B and C). These results indicate that MCs are present in T-cell areas of lymphoid organs and at locations allowing T cell–MC interactions.

Figure 5. Mast cells (MCs) are present in T-cell areas of human tonsil. (A) MCs in tonsils are characterized as tryptase (tryp)$^+$ CD117$^+$ double-positive cells. (B, C) Double staining of tryptase and CD3. (B) MCs are mainly found in T-cell areas of the tonsil. "F" indicates the B-cell follicles and "T" indicates the T-cell area, defined by expression of CD3. Arrowheads show Tryp$^+$ MCs present in the T-cell area. (C) MCs are often found in close proximity to T cells. Arrowheads show examples of Tryp$^+$ MCs in close contact with CD3$^+$ T cells. Original magnifications: 20× (A, B) and 40× (C). (A–C) Images are representative examples of fluorescent stainings of tonsils from six independent donors.

Nonetheless, as evaluated by immunofluorescent staining of tonsil sections, we could not detect HLA-DR on these tryptase$^+$ MCs (data not shown). As HLA-DR expression might be relatively low on tonsillar MCs, the detection of its expression might be obscured by the high HLA expression of other APCs that are abundantly present in tonsil. Therefore, we also analyzed MCs in tonsil cell suspensions using a more sensitive flow cytometric technique with antibodies recognizing CD117, CD203c, FcεRI, HLA-DR, and costimulatory molecules (Fig. 6). MCs were identified by their expression of high levels of CD117 and were further characterized by expression of the MC markers CD203c and FcεRI (Fig. 6A and B), and absence of the DC markers DC-SIGN and CD1c (data not shown). HLA-DR expression was found on MCs of all six donors analyzed, both when analyzed as percentage or mean fluorescence (Fig. 6C and D). The relative immunofluorescence was approximately tenfold lower than on other HLA-DR$^+$ cells in the tonsil, which is in line with the difficulty to detect HLA-DR on MCs in a convincing manner by staining of tonsil sections.

The expression of costimulatory molecules by tonsillar MCs was also evaluated (Fig. 6E and F). Although only low levels of CD86 were found, all four donors analyzed showed significant expression of CD80 compared with the isotype control, confirming the data obtained from MCs cultured from blood (Fig. 1C).

Together, these results show that MCs expressing HLA-DR and costimulatory molecules are present in human tonsil, and that the majority of these MCs are located in close proximity to T cells, arguing for MC–T cell interaction in human LNs.

DISCUSSION

In this article, we show for the first time that cultured human MCs are capable of presenting native protein antigens to CD4$^+$ T cells. The interaction between MCs and CD4$^+$ T cells modulated the MC phenotype, indicating that both cell types can influence each other in an antigen-dependent manner. Furthermore, the interaction between these cell types may be physiologically relevant as the presence of MCs in proximity to T cells was also shown in lymphoid organs.

A clear inherent limitation of our study is that we are unable to show the ability of MCs to present antigen in vivo. Likewise, there were some differences between the cultured MCs and their in vivo counterparts. Whereas the cultured MCs readily expressed HLA-DR after coculture with the T cells, the tonsil-resident MCs already expressed HLA-DR when evaluated directly ex vivo. This difference might be explained by the exposure of MCs to IFN-γ either in the tonsil or in the periphery by either T cells or innate IFN-γ-producing cells such as NK cells.

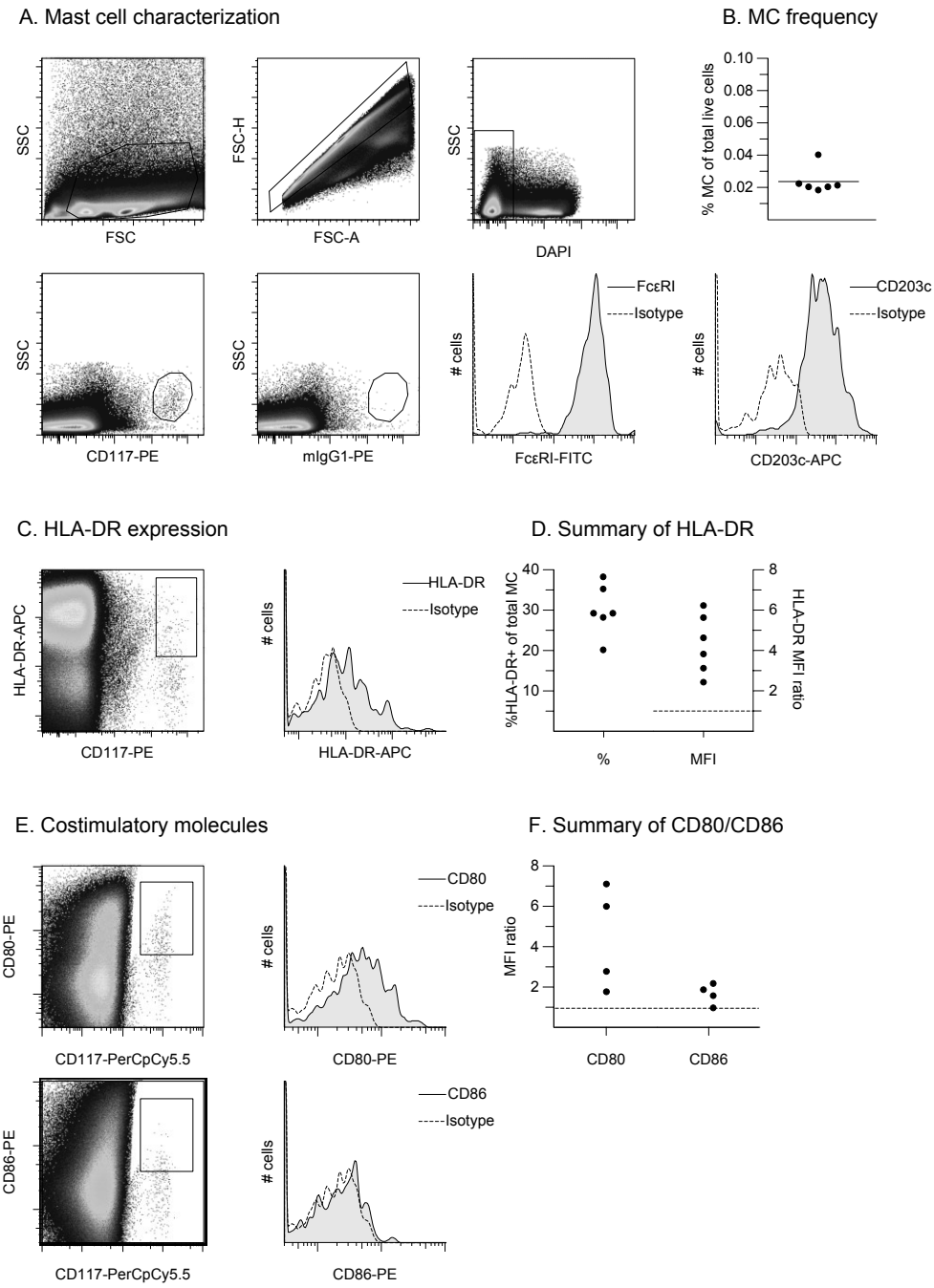

A. Mast cell characterization
B. MC frequency
SSC
FSC
FSC-H
FSC-A
SSC
DAPI
% MC of total live cells
0.10
0.08
0.06
0.04
0.02
SSC
CD117-PE
SSC
mIgG1-PE
cells
FcεRI
Isotype
FcεRI-FITC
cells
CD203c
Isotype
CD203c-APC
C. HLA-DR expression
D. Summary of HLA-DR
HLA-DR-APC
CD117-PE
cells
HLA-DR
Isotype
HLA-DR-APC
%HLA-DR+ of total MC
40
30
20
10
HLA-DR MFI ratio
8
6
4
2
%
MFI
E. Costimulatory molecules
F. Summary of CD80/CD86
CD80-PE
CD117-PerCpCy5.5
cells
CD80
Isotype
CD80-PE
MFI ratio
8
6
4
2
CD80
CD86
CD86-PE
CD117-PerCpCy5.5
cells
CD86
Isotype
CD86-PE

Figure 6 *(left)*. Mast cells (MCs) in tonsil express HLA-DR and CD80. (A, B) MC characterization in total tonsil cell suspensions by CD117, FcεRI, and CD203c (n = 6). Live cells were gated based on FSC/SSC characteristics and DAPI negativity and their expression of CD117 or isotype control are shown in the dotplots. Histograms show expression of FcεRI and CD203c by gated CD117hi cells as shown in the dotplots. (C, D) Expression of HLA-DR by MCs characterized as in (A) (n = 6). (E, F) Expression of CD80 and CD86 by MCs characterized as in (A) (n = 4). Expression (MFI ratio) in (D) and (F) is indicated as MFI of the marker of interest (e.g. HLA-DR and CD80) divided by the MFI of the matching isotype control. Dotted lines in (D) and (F) indicate absence of expression of the indicated marker compared with the isotype control (MFI ratio = 1). Data shown are representative of tonsils from four to six independent donors.

Nonetheless, as the phenotype of MCs for the other markers evaluated (CD117, tryptase and CD80) was comparable, we believe that the cultured MCs are a good representation of their counterparts in vivo, and that the biological effects observed in vitro are conceivably present in vivo as well.

The presentation of antigen to $CD4^+$ T cells by human MCs may serve to stimulate T cells in diverse locations, such as lymphoid organs and peripheral tissues. As MCs can produce a different set of cytokines than other APCs (*15-17*), our findings could potentially have important implications for the skewing of T helper cells and maybe even for the outcome of naïve T-cell priming in LNs. Since both cultured and tonsil-resident MCs express HLA class II and CD80, their potential in stimulating naïve $CD4^+$ T cells cannot be excluded. Skin MCs have been shown to migrate from infected/allergic areas to draining LNs in the mouse (*2, 3*). Nonetheless, mouse MCs, although able to activate memory T cells, were not able to prime naïve $CD4^+$ T cells, probably due to the lack of costimulatory molecules (*10, 11*).

As MCs only comprised approximately 0.02–0.04% of total tonsil cells as shown in Fig. 6, and as their expression of HLA class II molecules was much lower than that of other professional APCs such as DCs, we do not consider it likely that MCs play a crucial role in priming of T cells in primary immune responses. In contrast, we consider it more likely that they might be more important in activation of secondary T cells in either LN or peripheral tissue. Especially, the latter is an intriguing possibility as MCs are present at several strategic locations where pathogens are first encountered. It could be very beneficial to the host to swiftly activate effector memory T cells that are known to reside in tissues (*18*), by MCs that are well known for their immediate and strong actions upon activation.

Furthermore, MCs could produce several cytokines upon activation through activation via IgE and TLR, which may have an impact on T-cell activation or skewing. In addition, $CD4^+$ T cells could also influence MCs, e.g. by inhibiting or modulating their reactivity. As murine

MCs were shown to specifically enhance activation of $CD4^+$ Treg cells through MHC class II (*10*), such T cell–MC interactions could also function to inhibit MC responses, such as observed in a murine model of anaphylaxis (*19*). The expression of MHC class II by MCs depended on the production of IFN-γ by T cells in this study, showing the ability of T cells to influence MC phenotype. Therefore, interaction between MCs and T cells might direct the responsiveness of both cell types, allowing for optimal control of both cell populations.

We have not shown how the MCs have taken up antigen for presentation to T cells in our study. However, we observed no enhancement of antigen presentation when protein antigens were targeted to the FcεRI on the MCs via hapten-specific IgE and hapten-coupled protein (data not shown). The lack of enhancement of presentation by Fc-receptor-targeted antigens by human MCs contrasts findings obtained in mice, as two studies reported enhanced antigen presentation by Fc-receptor-mediated uptake (*11, 20*). Nonetheless, another study using murine MCs indicated decreased antigen presentation when antigen was routed to IgE/FcεRI, possibly because antigens were protected from proteolytic degradation and subsequent presentation as peptide in MHC (*10*). Further research into this aspect is required, but our data indicate that Fc-receptor-mediated uptake and presentation of Ig(E)-bound antigen by human MCs, if present, is likely to be less efficient as compared with the ability of DCs to take up and present immune-complexed antigens (*21*).

In conclusion, our results show that cultured human MCs can function as APCs, by uptake and processing of protein, with subsequent presentation to $CD4^+$ T cells via HLA class II. Furthermore, expression of HLA class II and CD80 is found on MCs isolated from human tonsil, where they are found in close proximity to T cells. In addition, the interaction between MCs and $CD4^+$ T cells led to changes in the MC phenotype in an antigen-specific manner, indicating an intimate interaction between these two distinguished cell types.

MATERIALS AND METHODS

MAST CELLS

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 MCs 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 (*22, 23*). 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 MCs was determined by flow cytometric analysis of CD117 (c-kit), FcεRI and CD203c, and intracellular tryptase. The purity of MCs ranged from 90 to 99%.

CD4$^+$ T-CELL CLONE

An adenovirus-specific CD4$^+$ T-cell clone (M2.11) was used to determine antigen presentation by MCs (*24*). This clone recognizes a peptide derived from hexon protein II, in the context of HLA-DRB1*03:01. MCs from blood of HLA-DRB1*03:01 positive donors were generated as described above. MCs were incubated with 5 μg/mL peptide for 24 h, or 1–50 μg/mL protein (22.2 kDa polypeptide fragment D from hexon protein II (*24*)) for 6–72 h, before coculture with the CD4$^+$ T cells. Before coculture with the T-cell clone, MCs were thoroughly washed to remove all soluble protein or peptide. Subsequently, the MCs were added to the CD4$^+$ T cells in a 1:1 ratio in RPMI with 10% human serum and 100 ng/mL SCF at 200 000 cells/mL. After 1 h, 5 μg/mL brefeldin A was added and incubated overnight. To determine the effects of activation of the T-cell clone on the MCs, the cells were cultured without brefeldin A for the indicated timepoints.

Blocking antibodies and isotype controls for HLA class II (BD Pharmingen, clone Tü39, 10 μg/mL) were added to the MCs, and incubated for 1 h at 37°C before the MCs were added to the T cells. Blocking antibodies remained present during the coculture. Blocking antibodies and isotype controls for IFN-γ (eBioscience, clone NIB42, 20 μg/mL) were added to the cocultures directly.

CD4$^+$ T-CELL BULK RESPONSES AGAINST RECALL ANTIGENS

Polyclonal CD4$^+$ T-cell bulks were generated by activation of PBMCs with the recall antigens tetanus toxoid at 0.75 Lf/mL (Netherlands Vaccine Institute), tuberculin purified protein derivative (0.5 μg/mL) (Netherlands Vaccine Institute), and C. albicans 0.005% (HAL allergy) in IMDM/5% human serum for 7 days, after which antigen presentation by autologous MCs was evaluated. MCs or autologous PBMCs were incubated with the recall antigens mentioned above for 16 h, before coculture with autologous bulk CD4$^+$ T cells. After this incubation, the cells were thoroughly washed to remove all soluble protein. Autologous PBMCs were used as positive control and were allowed to adhere to the plate at 37°C for 2 h, after which nonadherent cells were removed. Subsequently, the CD4$^+$ T cells were added to the APCs in IMDM/5% human serum at 2 × 106 cells/mL. After 1 h, 10 μg/mL brefeldin A was added and incubated overnight.

TONSILS

Human tonsils were obtained after tonsillectomy. A portion of these tissues were fixed with 4% formaldehyde in PBS, stored in 70% ethanol, and embedded in paraffin for immunofluorescent staining of sections. The remaining part of the tonsils was washed with RPMI with 10% FCS and cut into small pieces. Subsequently, the tonsils were dispersed through a 70 µm filter to get single-cell suspensions. Cells were frozen until flow cytometric staining and acquisition. This study was performed in accordance with the declaration of Helsinki and local ethical guidelines.

IMMUNOFLUORESCENCE

Tonsil tissues were treated according to the method described by Schuerwegh et al. to deparaffinize and retrieve antigens with EDTA (*25*). Slides were incubated with 8 µg/mL goat anti-human tryptase, 26.5 µg/mL rabbit anti-human CD117, 60 µg/mL rabbit anti-human CD3, or matching isotype controls for 1 h at room temperature. After washing, slides were stained with 2 µg/mL donkey α-goat AF 568 and 2 µg/mL donkey α-rabbit AF 488 for 1 h at room temperature. After washing, slides were covered with Vectashield containing DAPI (Vector laboratories) to stain nuclei. Slides were analyzed on a Zeiss Axio ScopeA1 microscope.

FLOW CYTOMETRY

For detection of HLA-DR and costimulatory molecules expression by cultured MCs, cells were incubated with fluorochrome-conjugated antibodies recognizing CD117, CD203c, HLA-DR, CD80, and CD86 or matching isotype controls. Intracellular staining of tryptase was performed as described (*26*).

For detection of expression of HLA-DR and costimulatory molecules on tonsil MCs, cells were incubated with fluorochrome-conjugated antibodies recognizing CD117, CD203c, FcεRI, HLA-DR, CD86, and CD80 or matching isotype controls for each of these antibodies. Just prior to flow cytometric acquisition, 0.2 µM DAPI was added for exclusion of dead cells.

For intracellular cytokine staining by $CD4^+$ T cells, the cells were incubated with fluorochrome-conjugated antibodies recognizing CD3 and CD4, after which they were permeabilized using CytoFix CytoPerm Kit (BD Biosciences). To gate out any remaining monocytes in the $CD4^+$ T-cell bulks, antibodies to CD14 were added as well. After washing, cells were incubated with PE-labeled anti-IFN-γ, TNF-α, or matching isotype control. Cells were taken up in 1% paraformaldehyde until flow cytometric acquisition. Flow cytometry was performed on FACS Calibur (BD) or LSR II (BD). Analysis was performed using FACS Diva (BD) and FlowJo software.

STATISTICAL ANALYSIS

Results are expressed as mean ± SEM. Statistical analysis was performed using SPSS PASW 17.0 and GraphPad Prism 4. For differences between two groups, Student's t-tests were performed. p values of <0.05 were considered statistically significant.

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