ORIGINAL ARTICLE

Human and Mouse Mast Cells Express and Secrete the GPI-Anchored Isoform of CD160

Nicolas Ortonne[1,5], Caroline Ram-Wolff[1,5], Jérôme Giustiniani[2], Anne Marie-Cardine[2], Martine Bagot[2,3], Salaheddine Mecheri[4] and Armand Bensussan[2]

CD160 is expressed by human and mouse natural killer (NK) cells and other cytotoxic lymphocyte subpopulations. CD160 is mostly expressed as a trimeric 83 kDa glycosylphosphatidylinositol (GPI)-anchored activating NK receptor, cleaved upon IL-15 stimulation in a secreted trimeric soluble form (sCD160) that binds to major histocompatibility complex (MHC) class I molecules, while a transmembrane isoform appears. sCD160 exhibits immunoregulatory function as it inhibits CD8$^+$ T-lymphocyte cytotoxic activity. We show that human mast cells (MCs) express CD160. In human and mouse skin, resident MCs expressed CD160, whereas in C57BL/6-*Kit*$^{W\text{-}sh/W\text{-}sh}$ mice, CD160$^+$ cells were only identified at the site of reconstitution with syngeneic cultured MCs. In the human mast cell line, HMC-1, we only identified the transcripts of the GPI-anchored CD160 isoform. Furthermore, CD160 was identified in HMC-1 and mouse MC supernatants, suggesting that MCs release sCD160. Supporting this hypothesis, HMC-1 express the GPI-specific phospholipase D variant 2 involved in the NK lymphocyte membrane cleavage of CD160, and morphological studies highlighted a relative loss of CD160 expression in inflammatory skin sites, where MC degranulation is expected to occur. We also demonstrated an inhibition of T-cell cytotoxicity by HMC-1 supernatant that was partially reversed by anti-CD160 mAb. In conclusion, sCD160, produced by MCs, may have a role in T-cell–MC interactions *in vivo*.

Journal of Investigative Dermatology (2011) **131,** 916–924; doi:10.1038/jid.2010.412; published online 30 December 2010

INTRODUCTION

CD160 is a natural killer (NK) receptor initially identified as a 83-kDa molecule (Bensussan *et al*., 1994). CD160 appears to be unique as it is encoded by a gene located on human chromosome 1, it is expressed as a glycosylphosphatidylinositol (GPI)-anchored molecule at the cell surface, and its expression, which is induced upon lymphocyte activation, is highly regulated by circulating CD4$^+$CD25$^+$ T regulatory lymphocytes (Nikolova *et al*., 2009). CD160 was initially shown to be expressed by CD56^{dim} NK cells (Maiza *et al*., 1993; Anumanthan *et al*., 1998; Agrawal *et al*., 1999), where its engagement by major histocompatibility complex (MHC) class I molecules triggers cytotoxicity and elicits cytokines production (Le Bouteiller *et al*., 2002; Barakonyi *et al*., 2004). CD160 was also shown to be expressed by CD8 T cells (Nikolova *et al*., 2005; Rey *et al*., 2006), γδ lymphocytes, cutaneous dermal CD4$^+$ T cells (Abecassis *et al*., 2007), intestinal intraepithelial CD8$^+$ T cells (Nikolova *et al*., 2005; Rey *et al*., 2006), and, more recently, activated endothelial cells (Fons *et al*., 2006). Recent data have shown that the minor subset of circulating CD4$^+$ T cells that express CD160 are activated lymphocytes. In these lymphocytes, crosslinking of CD160 via an interaction with herpes virus entry mediator (HVEM) inhibits the CD3- and CD28-mediated activation (Cai *et al*., 2008). However, the function of CD160 in T cells remains to be clarified, as it can act as a co-activating receptor in CD28^{nul} T lymphocytes (Nikolova *et al*., 2002). Interestingly, CD160 was found to be expressed in mice, both in NK cells (Maeda *et al*., 2005) and CD8$^+$-activated and memory T cells (Tsujimura *et al*., 2006). In murine NK cells, CD160 can interact with both classical and nonclassical MHC class I molecules, as in humans, and CD1d (Maeda *et al*., 2005). More recently, we have reported in NK cells a transmembrane isoform of CD160, produced through alternative splicing of CD160 mRNA and obtained only upon activation by IL-2, IL-12, or IL-15 (Giustiniani *et al*., 2009). Finally, in human NK cells, we further identified a soluble form of CD160, produced by phospholipase D (PLD)-specific cleavage of the GPI anchor (Giustiniani *et al*., 2007), which impairs *ex vivo* the cytotoxic CD8$^+$ T-cell and NK cell

[1]*AP-HP, Groupe Hospitalier Henri Mondor–Albert Chenevier, Department of Pathology, and Université Paris 12, Faculté de Médecine, Créteil, France;* [2]*Institut National de la Santé et de la Recherche Médicale (INSERM) U976, Hôpital Saint-Louis, Equerre Bazin, Paris, France;* [3]*Department of Dermatology, AP-HP, Hôpital Saint-Louis, Paris, France and* [4]*Unité de Biologie des Interactions Hôte Parasite (BIHP), Institut Pasteur de Paris, Paris, France*

[5]*Nicolas Ortonne and Caroline Ram-Wolff contributed equally as first authors.*

Correspondence: Nicolas Ortonne, Département de Pathologie, Hôpital Henri Mondor, 51 Avenue du Maréchal de Lattre de Tassigny, F-94010 Créteil Cedex, France. E-mail: nicolas.ortonne@hmn.aphp.fr

Abbreviations: BMMC, bone marrow mast cell; FSMC, fetal skin mast cell; GPI, glycosylphosphatidylinositol; HVEM, herpes virus entry mediator; MC, mast cell; MHC, major histocompatibility complex; NK, natural killer; PLD, phospholipase D; PMC, peritoneal mast cell

Received 11 February 2010; revised 29 July 2010; accepted 24 September 2010; published online 30 December 2010

© 2011 The Society for Investigative Dermatology

functions, possibly by a competitive binding to MHC class I molecules.

Mast cells (MCs) are key players of innate and adaptative immunity and are involved in many allergic and inflammatory diseases, especially in the skin. In the past 10 years, MCs have been described for their regulatory functions of the adaptative immunity (Secor *et al.*, 2000; Lee *et al.*, 2002; Bryce *et al.*, 2004; Grimbaldeston *et al.*, 2006; Lu *et al.*, 2006), although the mechanisms by which they exert such immunoregulatory effects remain unknown. To date, MCs were not shown to produce regulatory mediators that may directly interact with effector cytotoxic lymphocytes. In this study, we show that human and mouse MCs produce soluble CD160, a known ligand for classical and nonclassical MHC class I molecules, previously shown to inhibit $CD8^+$ cytotoxic T lymphocyte activities.

RESULTS

$CD160^+$ MCs are detected in human and murine tissues

Immunohistochemical studies revealed the presence of $CD160^+$ MCs in normal human skin (resident MCs), inflammatory dermatoses (lichen, psoriasis (Figure 1a and b),

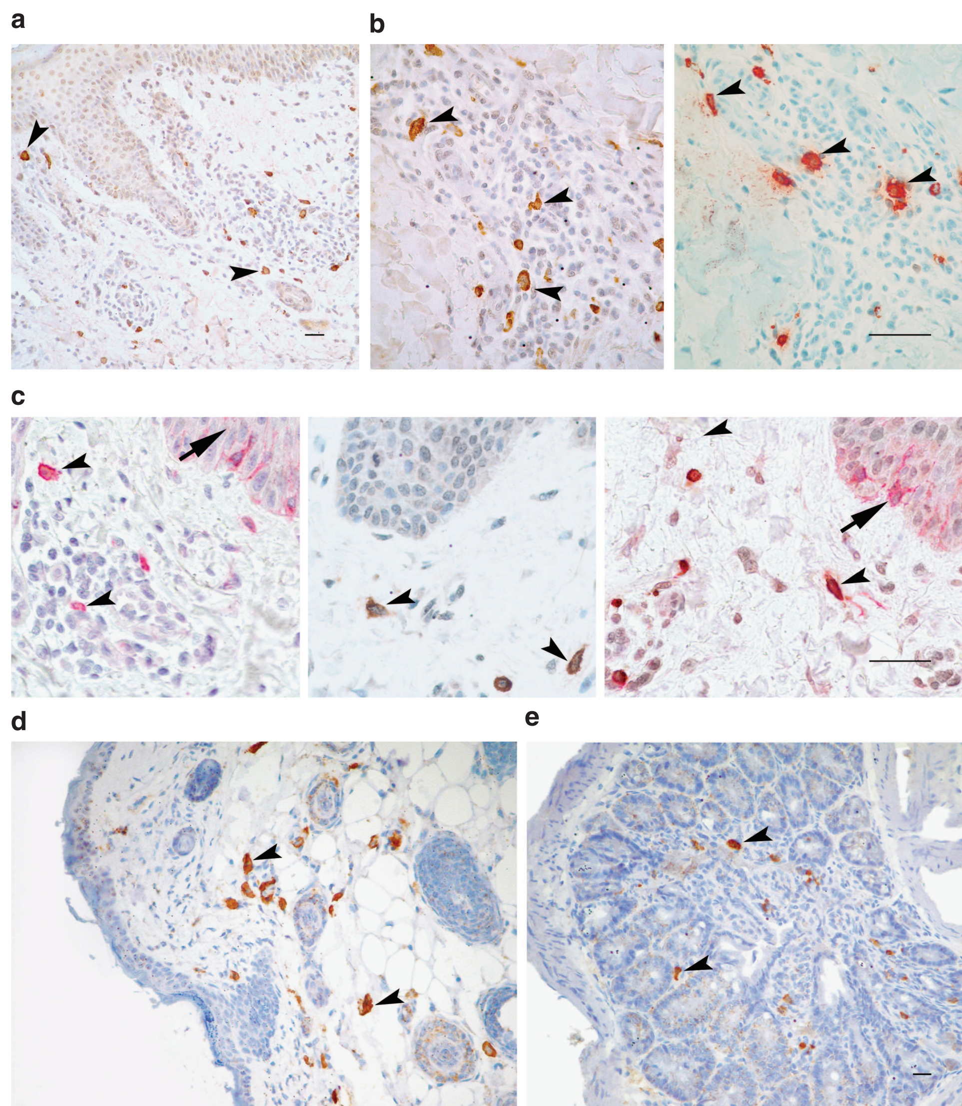

Figure 1. ***In situ* immunostaining of human and murine tissues allows detection of $CD160^+$ mast cells (MCs).** (**a**, **b**) Single immunostaining of human inflammatory skin from a patient with psoriasis showing MCs displaying cytoplasmic CD160 expression (**a** and left panel of **b**, arrowheads). MCs were recognized morphologically and according to mast-cell tryptase expression (right panels of **b**, arrowheads). (**c**) In this skin sample from inflammatory skin (contact hypersensitivity reaction), CD117/c-Kit (left panel) and CD160 (middle panel) expression both delineated MCs (arrowheads), whereas melanocytes, used as internal controls, only express CD117, as expected (left panel, arrow), highlighting their dendritic morphology within basal keratinocytes. With double stainings (right panel), the dermal MCs coexpressed CD117/c-Kit (red) and CD160 (brown) (arrowheads), whereas melanocytes remained only positive for CD117/c-Kit (arrow). (**d**) Dermal resident MCs displayed a cytoplasmic granular expression of CD160 in skin biopsy samples from a nude mouse (arrowheads). (**e**) A similar staining was seen in resident MCs of the intestine mucosa. Slides are shown at $\times 100$ original magnification in **a**, **d**, and **e** and $\times 400$ for **b** and **c**. Bar = 0.05 mm.

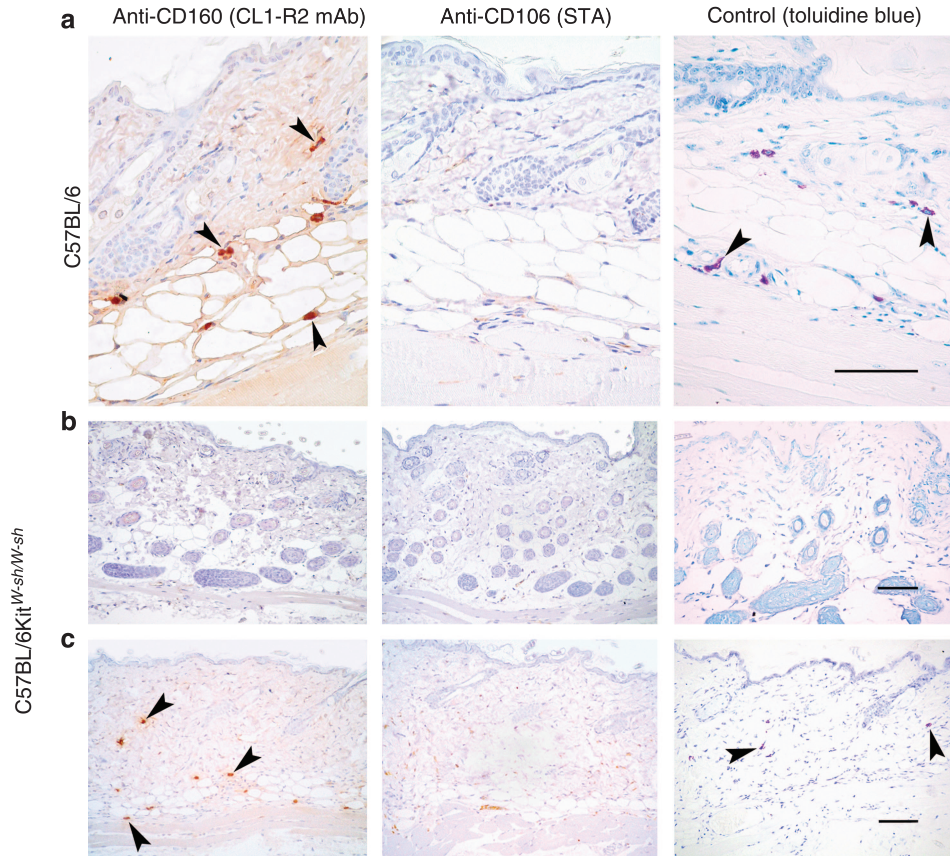

Figure 2. CD160^{+} cells are detected in C57BL/6-*Kit*$^{W-sh/W-sh}$ mice only after reconstitution with syngeneic cultured mast cells (MCs). (**a**) CD160^{+} MCs were detected in the dermis of the wild-type C57BL/6 mouse (left panel), otherwise characterized by toluidine blue staining (right panel), whereas no staining was evidenced with the isotypic-matched antibody directed to CD106 (middle panel). (**b**) In the C57BL/6-Kit$^{W-sh/W-sh}$ MC-deficient mouse, immunostaining with anti-CD160 antibody was negative, as well as negative (CD106) and positive (toluidine blue) controls. (**c**) In the dorsal skin taken after reconstitution by intradermal injection of syngeneic fetal skin-derived cultured MCs (+), CD160^{+} cells were seen in the dermis, with a similar staining with toluidine blue (right panel). Slides are shown at ×400 original magnification in **a** and ×200 for **b** and **c**. Bar = 0.1 mm.

eczema, mycosis fungoides, mastocytosis), and skin tumors stroma (basal cell and squamous cell carcinomas, nevus, melanoma, and neurofibroma). CD160^{+} resident MCs were also identified in other organs (lung, colonic mucosae, and salivary gland). CD160 was mostly expressed in the form of a cytoplasmic granular staining. MCs were recognized morphologically and using consecutive sections stained with MC tryptase. Double-staining experiments revealed that CD160^{+} cells in all cases were MCs, as they coexpressed CD117 as shown in Figure 1c. In the nude mouse, CD160^{+} tissue-resident MCs were identified in all investigated organs, including the skin (Figure 1d) and intestine mucosae (Figure 1e). We next analyzed the dorsal skin of wild-type and MC-deficient Kit$^{W-sh/W-sh}$ mice. As expected, we identified CD160^{+} MCs in the dermis of the wild-type mouse skin (Figure 2a), whereas a negative staining was obtained in the deficient mouse (Figure 2b). Interestingly, in mice reconstituted by intradermal injection of syngeneic fetal skin MCs, CD160^{+} cells were readily detected in the deep dermis, near to subcutaneous muscle (Figure 2c).

Two distinct transcripts corresponding to the two isoforms of the GPI-anchored CD160 are expressed in the HMC-1 cell line

CD160 transcripts were detected in HMC-1 (human mast cell line) cells. Interestingly, as in NK cells, two distinct isoforms were evidenced, which are expected to correspond to the GPI-anchored form of CD160 (CD160-GPI) and to the CD160ΔIg-GPI variant (Figure 3a). The latter lacks the sequences encoding for the extracellular immunoglobulin domain. In contrast, the transcripts of the transmembrane isoform of CD160 were not detected. The direct sequencing of reverse transcriptase-PCR products clearly established that they corresponded to wild CD160 mRNA, according to the already described sequence in NK cells (Giustiniani *et al.*, 2009).

The HMC-1 cell line and cultured murine MCs display intracellular expression of CD160 protein

Immunoprecipitation and immunoblotting of HMC-1 cell lysates revealed a weak 83-kDa band that we interpreted as the known trimeric form of CD160 (Figure 3b). In addition, at

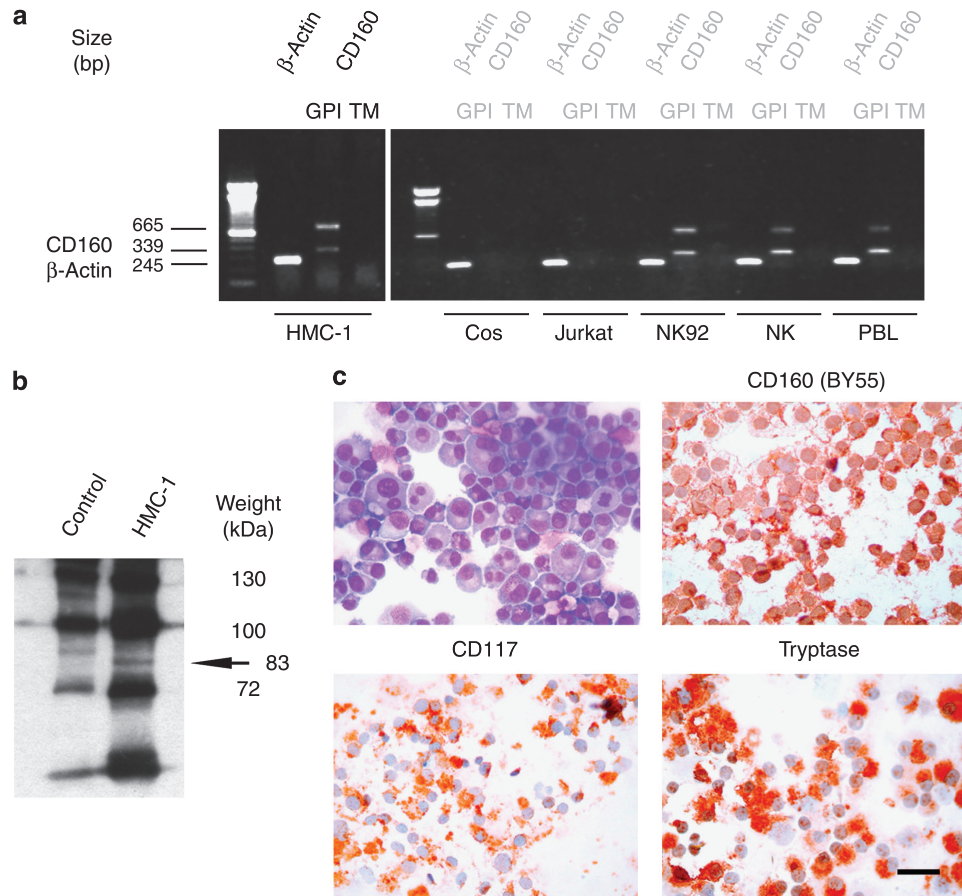

Figure 3. Detection of CD160 mRNA and protein in HMC-1 cell line. (**a**) Total mRNAs of HMC-1 were isolated and submitted to reverse transcription. Amplification of both the membrane-bound (glycosylphosphatidylinositol (GPI)) and the transmembrane (TM) isoforms of CD160 together with β-actin complementary DNA (cDNA) was performed using specific primers. The amplified products were separated on a 1% agarose gel. The CD160^{-} Cos and Jurkat cell lines were used as negative controls and the CD160^{+} NK92 cell line together with normal peripheral blood lymphocytes (PBLs) and natural killer (NK) cells from a healthy donor were used as positive controls. (**b**) Immunoprecipitations of HMC-1 cell lysates were performed with anti-CD160 mAb (CL1-R2) or with isotype-matched negative control antibody. The proteins were then transferred onto a nitrocellulose membrane and subjected to western blot analysis under nonreductive conditions using the anti-CD160 (CL1-R2, 5 μg ml^{-1}) and horseradish peroxidase (HRP)-conjugated goat anti-mouse antibodies. (**c**) Cytospin slides of HMC-1 were immunostained with anti-CD160 (BY55, upper right panel), anti-CD117 (lower left panel), and anti-tryptase (lower, right panel) mAbs followed by biotinylated secondary anti-mouse antibodies and avidin-biotin peroxidase complex using the Nexes device (Ventana). Slides were developed with 3′-3′-diaminobenzidine and counterstained with hematoxylin. Bar = 0.02 mm.

longer exposure times, a shorter, barely detectable, 50-kDa band was also seen (data not shown). Although it was difficult to distinguish from the mAb light chains, this short band was interpreted as a possible dimeric form of CD160. In addition, CD160 staining of HMC-1 demonstrated an intracytoplasmic granular staining pattern, consistent with expression of CD160 in MC granules (Figure 3c). Similar results were obtained with mouse MCs, including fetal skin MCs (FSMCs), bone marrow MCs (BMMCs), and peritoneal MCs (PMCs) (data not shown). Using flow cytometry on permeabilized cells without supplemental pretreatment, the 2C7 mAb detected CD160 only in BMMCs (Figure 4, left panels). Interestingly, when the same experiment was conducted after incubation of cells with monensine, before labeling, CD160 was observed in the three types of murine MCs analyzed (Figure 4, right panels). When performed on unpermeabilized HMC-1 cells, flow cytometry with anti-CD160 mAb showed negative results.

The soluble form of CD160 (sCD160) is found in HMC-1 and murine MC supernatants, and HMC-1 expresses the transcripts for the GPI-PLD1 variant 2

After immunoprecipitation and immunoblotting of supernatants of HMC-1 cells with anti-CD160 mAb (CL1-R2), we observed, in addition to the expected 83 kDa band, a 50 kDa molecule that might correspond to a dimeric form of CD160 (Figure 5a, right panel), whereas in mouse (FSMCs), only the 83 kDa band could be detected (Figure 5a, left panel). Interestingly, HMC-1 cells, like the YT NK cell line used as positive control, were found to express the GPI-PLD1 variant 2 transcript (Figure 5b), whereas, similar to NK cells, GPI-PLD1 variant 1 was not detected.

Diminished expression of CD160 by dermal MCs in inflammatory skin

In normal skin, all CD117 MCs expressed CD160. In skin biopsies from inflammatory dermatoses, double-staining

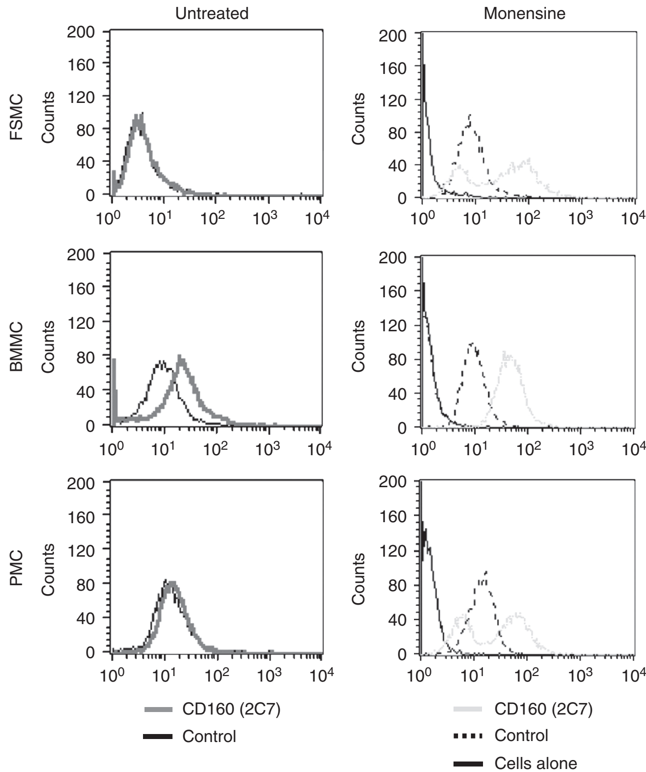

Figure 4. Detection of CD160 protein in murine mast cells (MCs). Fetal skin MCs (FSMCs), bone marrow MCs (BMMCs), and peritoneal mouse MCs (PMCs) were fixed, permeabilized, and incubated with biotinylated anti-CD160 mAb (2C7) for flow cytometry studies. Streptavidin-peroxidase was used as negative control. Cells were permeabilized and analyzed with (right panels) or without (left panel) previous incubation with monensine. All experiments were done in duplicate.

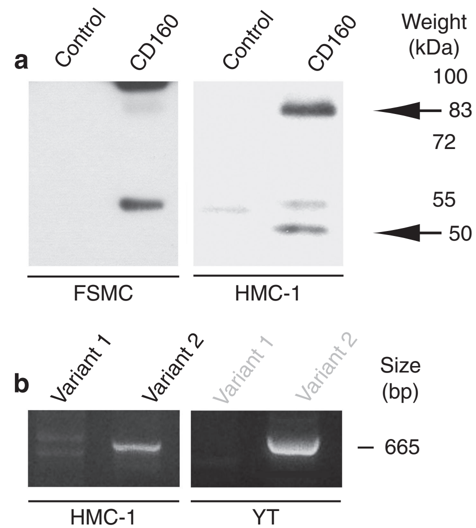

Figure 5. Presence of the soluble form of CD160 in culture cell supernatants of human and murine mast cells (MCs) and detection of the transcript of glycosylphosphatidylinositol-phospholipase D1 (GPI-PLD1) variant 2 protease in human MCs. (**a**) Immunoprecipitation of HMC-1 cells (right figure) and fetal skin MC (FSMC; left figure) supernatants were performed with anti-CD160 mAb (CL1-R2) or with isotype-matched control antibody. The proteins were then transferred onto a nitrocellulose membrane and subjected to western blot analysis using the anti-CD160 (CL1-R2, 5 $\mu g\,ml^{-1}$) and horseradish peroxidase (HRP)-conjugated goat anti-mouse antibodies. The migration was done under reductive condition for FSMCs and nonreductive condition for HMC-1. Thus, the 55 kDa band observed in the FSMC supernatant is expected to correspond to mAb light chains. (**b**) Total mRNAs of HMC-1 cells were isolated and submitted to reverse transcription. Amplifications of complementary DNA (cDNA) of GPI-PLD variants 1 and 2 were performed with specific primers. The amplified products were separated on a 1% agarose gel. The natural killer (NK) cell line YT was used as a positive control.

experiments revealed that a proportion of CD117$^+$ MCs showed a low or almost negative staining for CD160. We found a statistically significant reduction of CD160 expression by MCs in inflammatory areas in comparison with resident MCs found in the neighboring, spared, areas as illustrated in Figure 6a. Indeed, by direct counting at high-power fields, we found a mean of 28% (range 6–48%) of MCs with a strong CD160 expression (CD160$^+$CD117$^+$) in inflammatory areas versus 83% (range 75–96%) in noninflammatory areas (Figure 6b, $P<0.0001$). Altogether, these findings support the hypothesis that dermal MCs release CD160 during inflammatory processes *in vivo*.

In vitro inhibition of T-cell cytotoxicity by HMC-1 supernatant is reversed by anti-CD160 mAb

As shown in Figure 6c, MHC-I-restricted CD8$^+$ T-cell cytotoxicity was partially reduced by incubation of the target cells with HMC-1 supernatant, with an inhibition of 25% of specific lysis, at both 15:1 and 7:1 effector-to-target ratios. Interestingly, the inhibition was partially reversed when anti-CD160 mAbs were added to medium.

DISCUSSION

MCs can regulate T-cell-mediated immune responses via several mechanisms, including (1) MHC class I, II, and costimulatory molecule-dependent antigen presentation (Malaviya *et al.*, 1996; Frandji *et al.*, 1998), (2) elicitation of T-cell migration to inflammatory sites (Rumsaeng *et al.*, 1997), (3) Th2 polarization of helper T cells (Jutel *et al.*, 2001), and (4) secretion of exosomes (Skokos *et al.*, 2001, 2003; Valadi *et al.*, 2007). On the other hand, MC functions are in return regulated by T cells, as the regulatory CD4$^+$CD25$^+$FoxP3$^+$ T-cell subsets can suppress MC degranulation through OX40–OX40L interaction (Gri *et al.*, 2008). Besides their proinflammatory role, MCs can also exert antiinflammatory or immunosuppressive roles (Brain and Williams, 1988; Hart *et al.*, 1998), which may be increased by UVB exposure in the skin (Byrne *et al.*, 2008), including the promotion of tissue allograft tolerance (Lu *et al.*, 2006).

MCs were previously shown to express various surface activating and inhibitory immune receptors, some of them being known as NK receptors (Li and Yao, 2004). Besides FcγRIIB, they express various inhibitory receptors bearing ITIMs (immunoreceptor tyrosine-based inhibition motifs), including Siglecs (sialic acid binding Ig-like lectins; Yokoi *et al.*, 2006; Bochner, 2009), the MC function-associated antigen (Butcher *et al.*, 1998), and LILRB4 (leukocyte immunoglobulin-like receptor subfamily B member 4; Katz, 2007). Activating receptors consist of 2B4 (CD244; Romero *et al.*, 2004; Bachelet *et al.*, 2006) and CD200R3 (Kojima

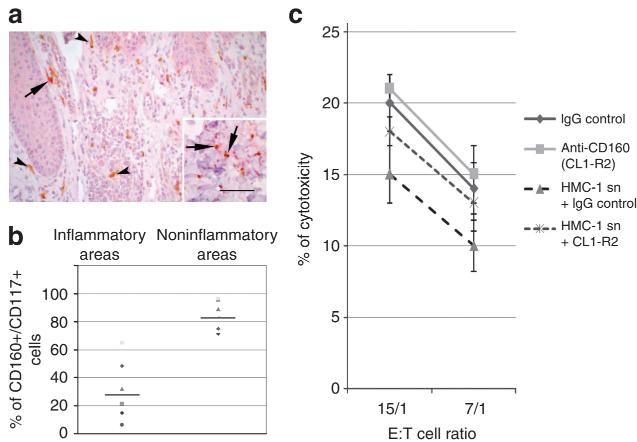

Figure 6. Diminished expression of CD160 by mast cells (MCs) in inflammatory skin areas and *in vitro* inhibition of major histocompatibility complex (MHC)-I cytotoxicity by HMC-1 supernatant. (**a**) Human MCs doubly stained with anti-CD160 (CL1-R2, red) and CD117 (brown), showing a diminished expression of CD160 in inflammatory areas (arrowheads). MCs with a strong CD160 expression (arrows) are more numerous within noninflammatory areas (inset). Slides are shown at $\times 400$ original magnification. Bar = 0.05 mm. (**b**) Double-staining experiments were done in nine skin biopsies. In each skin sample, the proportion of CD117 dermal MCs showing a strong coexpression of CD160 ($CD160^+/CD117^+$ cells) on doubly stained slides was evaluated on 10 high-power fields ($\times 400$ magnification) from one area showing cellular inflammation and from another with no inflammation. (**c**) 51Cr-release assay performed with irradiated Epstein–Barr virus (EBV)-transformed B cells as target cells, labeled with Na51CrO4, plated in 96-well V-bottom plates. The target cells were preincubated with medium from HMC-1 cells alone or completed with IgG1 control or CL1-R2 antibodies. The effector cells were then added at various effector-to-target (E:T) cell ratios, in triplicate. After 4 hours of culture, the determination of 51Cr release was done using a γ-counter and the percentage of specific lysis was determined ($\pm$SD). All experiments were done in triplicate.

et al., 2007), in addition to the high-affinity receptor for IgE, FcεRI. Among these receptors, some are involved in the regulation of the IgE-dependent activation of MCs (Yokoi *et al.*, 2008), whereas other are not, such as CD200R3 (Kojima *et al.*, 2007).

Regarding MC/T-cell interactions, direct interactions between MCs and MHC class I-restricted cytotoxic $CD8^+$ T cells are now well documented (Stelekati *et al.*, 2009). The finding of sCD160 in MC supernatants suggests that similar to NK cells, sCD160 may prevent or impair the MHC class I-restricted cytotoxic functions *in vivo* (Giustiniani *et al.*, 2007). The inhibitory effect of HMC-1 supernatant on MHC-I-restricted cytotoxicity of $CD8^+$ T cells, partially reversed by anti-CD160 mAb that we evidenced *in vitro*, supports this hypothesis. Interestingly, Cai *et al.* (2008) have shown that CD160 can be expressed by activated $CD4^+$ helper T cells, where it may have inhibitory functions by interacting with HVEM. In this study, we show that human and mouse MCs produce and secrete sCD160. It is therefore conceivable that MCs may also regulate helper T-cell function, by preventing the interaction of membrane-bound CD160 with antigen-presenting cell HVEM. Although structural traits regarding soluble CD160 are lacking, sCD160 might be expressed in HMC-1 as both 50 kDa dimeric and 83 kDa trimeric forms. Of course, we cannot exclude that the 50 kD band observed in immunoprecipitation experiments with the CL1-R2 mAb rather represents a partially reduced form of the trimeric CD160. It is noticeable that Cai *et al.* (2008) identified HVEM as a ligand for CD160 using a construct in which the extracellular domain of CD160 was fused to human IgG4 Fc (CD160-Ig). Such construct can be expected to represent a dimeric analog rather than the trimeric form of CD160. We therefore wonder if the putative dimeric form of sCD160 may act as a positive regulator of helper T-cell activation, by a specific binding to HVEM expressed at the cell surface of antigen-presenting cells, which may lead to conformational changes or constitute a steric hindrance. The dominant soluble form of CD160 produced by MCs may also be regarded as a possible mechanism by which these cells could contribute to control herpes virus infections, by preventing virus entry into surface HVEM-expressing cells.

In NK cells, previous studies have shown that the release of sCD160 relies on the proteolytic cleavage of the GPI motif by PLD1. In these cells, the release of membrane-bound CD160 correlated with the *de novo* synthesis of PLD1 variant 2, upon IL-15 treatment (Giustiniani *et al.*, 2007). We show that both humans (HMC-1) and murine MCs can express sCD160, without the need of a cytokine pretreatment, and that CD160 seems to be released by MCs in the inflammatory areas of pathologic skin. The mechanisms of CD160 release by MCs remain to be further studied, but the assumption can be made that CD160 may be associated within secretory granules, and might be released during the degranulation process. In support of this hypothesis, it is already known that human MCs express PLDs, including those involved in the specific cleavage of GPI structures (Metz *et al.*, 1992). We also found that untreated HMC-1 cells produce the PLD1 variant 2, shown to be involved in the release of sCD160 in NK cells (Giustiniani *et al.*, 2007). Interestingly, PLD was actually shown to have an important role in the degranulation process (Choi *et al.*, 2002).

In conclusion, we have shown that human and murine MCs constitutively express the GPI anchored isoform of CD160. These cells are able to secrete CD160 in a soluble form (sCD160), but unlike NK cells, without the need of IL-15 exposure. In addition, our results suggest that in MCs, CD160 might be produced as both dimeric and trimeric forms. The function of CD160 produced by MCs *in vivo* remains to be studied, especially to determine whether it might impair $CD8^+$ T-cell cytotoxicity, as observed *in vitro*, and/or may be involved in the $CD4^+$ helper T-cell activation, by preventing HVEM-mediated inhibitory signals.

MATERIALS AND METHODS

Cells

The human MC line HMC-1, and Cos, Jurkat, YT, and NK-92 cell lines were cultured at 37 °C in RPMI-1640 medium (Gibco, Invitrogen, Brebières, France), supplemented with penicillin ($100\,IU\,ml^{-1}$), streptomycin ($100\,\mu g\,ml^{-1}$), L-glutamine (2 mM), sodium pyruvate (1%), and 10% of 56 °C-decomplemented fetal calf serum (Hyclone, Perbio Sciences, Brebières, France). The medium was supplemented with IL-3 and stem cell factor for the cultures of murine FSMCs, BMMCs, and murine PMCs.

Peripheral blood mononuclear cells and NK cells were isolated from the peripheral blood of healthy donors. Briefly, peripheral blood mononuclear cells were isolated from heparinized venous blood by density gradient centrifugation over lymphoprep (PAA Laboratories, Linz, Austria). NK cells were purified using the MACS NK cell isolation kit (Miltenyi Biotec, Paris, France). The selection of *in vitro* allogeneic MHC-I-restricted effector T lymphocytes directed toward Epstein–Barr virus-transformed B cells was performed as previously reported (Giustiniani *et al.*, 2007).

Antibodies

The anti-CD160 mAbs were the following: CL1-R2 (mouse IgG1) and BY55 (mouse IgM), produced by us and which recognize both human and murine forms of CD160, and 2C7, a biotinylated rat IgG2a anti-mCD160 antibody kindly provided by Dr K Tsujimura (Aichi Cancer Center Research Institute, Nagoya, Japan) (Tsujimura *et al.*, 2006). For immunohistochemical studies, we used a rabbit anti-CD117/c-Kit antibody (Dako, Glostrup, Denmark) and the anti-MC tryptase IgG1 mAb (AA1 clone, Abcam, Cambridge, MA). Isotype-matched antibodies were used as negative controls: STA (anti-CD106, IgG1), 5T-147 (IgM), and a biotinylated IGg2aκ (BD Biosciences, San Jose, CA).

Immunoprecipitation and immunoblotting

Immunoprecipitation and immunoblotting assays were done as previously described (Giustiniani *et al.*, 2007). Briefly, for supernatants analyses, cells were washed in RPMI-1640 medium, incubated for 4 hours, and supernatants were filtered and concentrated. For lysate analyses, cells were washed in phosphate-buffered saline and resuspended in a lysis buffer. Lysates were centrifugated for 15 minutes at 4 °C and 13,000 r.p.m. to eliminate cellular debris and nuclei. Immunoprecipitations were performed with CL1-R2 mAb or the negative control STA antibody. The proteins precipitated with G-Sepharose beads were separated by SDS-6% PAGE in nonreduced conditions for HMC-1 and reduced conditions for FSMCs.

RNA extraction, reverse transcription-PCR, complementary DNA amplification, and sequence analysis

Total RNA from 5×10^6 HMC-1 was isolated using the Trizol reagent (Invitrogen, Cergy-Pontoise, France). Reverse transcription was performed using an oligo-dT primer (Invitrogen) and the Prime Script reverse transcriptase (Takara, Otsu, Japan). Specific primers (Invitrogen) for the amplification of GPI-PLD1 variants 1 and 2 were published sequences (Schofield and Rademacher, 2000). The CD160 primers were either 5′-ATGCTGTTGGAACCCGGCAGA GGC-3′ (forward) and 5′-TTACAAAGCTTGAAGGGCCAC-3′ (reverse) or 5′-TGCAGGATGCTGTTGGAACCC-3′ (forward) and 5′-CCTGTGCCCTGTTGCATTCTTC-3′ (reverse) flanking 219 and 546 or 339 and 665 base pair segments from the two alternatively spliced short and long transcripts of the GPI-anchored CD160 isoforms. In addition, we searched for the transmembrane isoform of CD160 using the following reverse primer: 5′-TCAGTGA AACTGGTTTGAACTTTCCTG -3′, as previously described (Giustiniani *et al.*, 2009). β-Actin complementary DNA amplification was performed in parallel as internal control. After initial denaturation (94 °C, 2 minutes), each complementary DNA sample was subjected to denaturation (94 °C, 30 seconds), annealing (55 °C, 40 seconds), and extension (72 °C, 60 seconds) steps for 35 cycles. The amplified products were separated on a 1% agarose gel and sequenced with the same CD160 oligonucleotide primers and two others (5′-CAGCT GAGACTTAAAAGGGATC-3′ and 5′-CACCAACACCATCTATCCGA G-3′), and analyzed with an automated fluorescent DNA sequencer (ABI Prism/Applied Biosystems, Foster City, CA).

Animals

Female C57BL/6 and nude 6–8-week-old mice were purchased from R Janvier (Laval, France). C57BL/6-$Kit^{W\text{-}sh/W\text{-}sh}$ ($\mathrm{Kit}^{W\text{-}sh/W\text{-}sh}$) MC-deficient mice (Wolters *et al.*, 2005) were used as a model to study MC function *in vivo*. These mice were kindly provided from Peter Besmer (Sloan-Kettering Institute, New York) and were bred in our animal facility. All animal care and experimentation were conducted in accord with the Pasteur Institute animal care and use committee guidelines.

Preparation of murine MCs for MC-deficient mice reconstitution

Mouse FSMCs are often used as a model of connective tissue-type MCs that are distributed in the dermis. FSMCs were prepared according to the method described by Yamada *et al*. (2003). After 4 weeks of culture in the presence of IL-3 and stem cell factor ($3\,\mathrm{ng\,ml^{-1}}$), the cells express FcεRI, can be sensitized with IgE, and release histamine upon IgE aggregation, altogether showing a high degree of maturity. For these reasons, a short-term 7-day reconstitution period was carried out. $\mathrm{Kit}^{W\text{-}sh/W\text{-}sh}$ mice (8 weeks old) were reconstituted locally with MCs by intradermal injection in the dorsal skin of 50 μl phosphate-buffered saline containing 2×10^6 FSMCs. Mice were killed 7 days after MC reconstitution.

Tissue samples, immunohistochemistry, and analysis of the density of CD160$^+$ MCs

A total of 19 formalin-fixed, paraffin-embedded human tissue samples were studied with normal (around a scar) and pathologic human skin (lichen, psoriasis, eczema, mycosis fungoides, mastocytosis, basal cell and squamous cell carcinomas, nevus, melanoma, and neurofibromas), and samples from human normal lung, colonic mucosae, and salivary gland were retrieved from archival material. Additionally, we analyzed formalin-fixed, paraffin-embedded tissue samples from a nude mouse (skin, digestive tract, lung, heart, kidney, spleen, and bone marrow) and dorsal skin sample from two wild mice, two MC-deficient mice ($\mathrm{Kit}^{W\text{-}sh/W\text{-}sh}$), and two MC-reconstituted ($\mathrm{Kit}^{W\text{-}sh/W\text{-}sh}$) mice. HMC-1, murine FSMCs, BMMCs, and PMCs were cytospinned using a Shandon cytospin 4 centrifuge (Thermo Electron Corporation, Waltham, MA), air dried, and fixed in aceton.

Immunohistochemical procedures were applied on 3 μm thick sections and cytospinned cells, either manually or using the Nexes device (Ventana, Tucson, AZ). After antigen retrieval in heat with citrate buffer, slides were incubated with the CL1-R2 mAb, followed by a secondary biotinylated anti-mouse antibody and streptavidin-alkaline phosphatase or streptavidin-peroxidase (Vectastain/Vector, Burlingame, CA), developed with naphtol-fast red or diaminobenzidine, respectively. For double-staining experiments, slides were then incubated in H_2O_2 for 15 minutes to block endogenous peroxidases and 10 minutes with blocking serum. Afterwards, slides were incubated with the anti-CD117 rabbit antibodies, followed by a

secondary anti-rabbit antibody conjugated with peroxidase for 35 minutes (Impress) and finally developed in 3′-3′-diaminobenzidine (Vectastain/Vector). All slides were counterstained with hematoxylin. Primary antibodies were used in a 1:50 dilution. Staining with toluidine blue and MC tryptase expression were used as positive controls for MCs.

For quantitative analyses of the $CD160^+$ MC population in human tissues, we performed direct counting of the $CD160^+$ and $CD160^-$ or $CD160^{low}$ MC subpopulations on sections doubly stained with CD117 on 10 high-power fields (× 400 magnification). We analyzed separately the inflammatory and noninflammatory areas within each skin sample. Statistical analysis was done using Student's *t*-test with the Statview software (Abacus concept, Piscataway, NJ).

Flow cytometry

Murine FSMCs, BMMCs, and PMCs were fixed and permeabilized (kit BD cytofix cytoperm with Golgi stop; BD Biosciences). Briefly, the cells were incubated overnight at 4 °C in the "fixation-permeabilization" solution from the kit. After washes, cells were incubated with the specific mAb for 30 minutes at 4 °C (CL1-R2, BY55 and respective isotype-matched negative control antibodies). Streptavidin-peroxidase (BD Pharmingen, San Jose, CA; streptavidin-phycoerythrin) was used as negative control for 2C7. After washes, cells were incubated with the appropriate FITC- or phycoerythrin-labeled secondary antibodies. After washing, cells were analyzed by flow cytometry on a FACScalibur (BD Biosciences). For golgi stop experiments, cells were incubated before permeabilization with monensine for 4 hours at 37 °C.

Lymphocyte-mediated cytotoxicity

Target cells were labeled with 100 μCi of Na51CrO4 for 90 minutes at 37 °C, washed in RPMI-1640 medium containing 10% fetal calf serum, and plated in 96-well V-bottom microtiter plates (Greiner, Courtaboeuf, France) for 1 hour at 37 °C. When necessary, the target cells were subjected to a preincubation step with 50 μl of complete medium or culture medium from HMC-1 cells completed with IgG1 control or CL1-R2 antibodies at 10 μg ml^{-1}, 30 minutes, at room temperature. The effector cells were added on 10^3 target cells in 15:1 and 7:1 effector-to-target cell ratios. After 4 hours of culture at 37 °C, the plates were spun down and supernatants were collected for the determination of 51Cr release with a gamma-counter (Packard Instrument, Rungis, France). The percentage of specific lysis was determined as previously reported (Giustiniani *et al.*, 2007).

CONFLICT OF INTEREST

The authors state no conflict of interest.

ACKNOWLEDGMENTS

This study was supported by a grant from the Institut National de la Santé et de la Recherche Médicale (INSERM), within the projet national de recherche en Dermatologie PNR Dermatologie, and by the Société Française de Dermatologie (SFD). We thank Pr Michel Arock, who initially provided the HMC-1 human mast cell line.

REFERENCES

Abecassis S, Giustiniani J, Meyer N *et al.* (2007) Identification of a novel CD160+ CD4+ T-lymphocyte subset in the skin: a possible role for CD160 in skin inflammation. *J Invest Dermatol* 127:1161–6

Agrawal S, Marquet J, Freeman GJ *et al.* (1999) Cutting edge: MHC class I triggering by a novel cell surface ligand costimulates proliferation of activated human T cells. *J Immunol* 162:1223–6

Anumanthan A, Bensussan A, Boumsell L *et al.* (1998) Cloning of BY55, a novel Ig superfamily member expressed on NK cells, CTL, and intestinal intraepithelial lymphocytes. *J Immunol* 161:2780–90

Bachelet I, Munitz A, Mankutad D *et al.* (2006) Mast cell costimulation by CD226/CD112 (DNAM-1/Nectin-2): a novel interface in the allergic process. *J Biol Chem* 281:27190–6

Barakonyi A, Rabot M, Marie-Cardine A *et al.* (2004) Cutting edge: engagement of CD160 by its HLA-C physiological ligand triggers a unique cytokine profile secretion in the cytotoxic peripheral blood NK cell subset. *J Immunol* 173:5349–54

Bensussan A, Gluckman E, el Marsafy S *et al.* (1994) BY55 monoclonal antibody delineates within human cord blood and bone marrow lymphocytes distinct cell subsets mediating cytotoxic activity. *Proc Natl Acad Sci USA* 91:9136–40

Bochner BS (2009) Siglec-8 on human eosinophils and mast cells, and Siglec-F on murine eosinophils, are functionally related inhibitory receptors. *Clin Exp Allergy* 39:317–24

Brain SD, Williams TJ (1988) Substance P regulates the vasodilator activity of calcitonin gene-related peptide. *Nature* 335:73–5

Bryce PJ, Miller ML, Miyajima I *et al.* (2004) Immune sensitization in the skin is enhanced by antigen-independent effects of IgE. *Immunity* 20:381–92

Butcher S, Arney KL, Cook GP (1998) MAFA-L, an ITIM-containing receptor encoded by the human NK cell gene complex and expressed by basophils and NK cells. *Eur J Immunol* 28:3755–62

Byrne SN, Limon-Flores AY, Ullrich SE (2008) Mast cell migration from the skin to the draining lymph nodes upon ultraviolet irradiation represents a key step in the induction of immune suppression. *J Immunol* 180:4648–55

Cai G, Anumanthan A, Brown JA *et al.* (2008) CD160 inhibits activation of human CD4+ T cells through interaction with herpesvirus entry mediator. *Nat Immunol* 9:176–85

Choi WS, Kim YM, Combs C *et al.* (2002) Phospholipases D1 and D2 regulate different phases of exocytosis in mast cells. *J Immunol* 168:5682–9

Fons P, Chabot S, Cartwright JE *et al.* (2006) Soluble HLA-G1 inhibits angiogenesis through an apoptotic pathway and by direct binding to CD160 receptor expressed by endothelial cells. *Blood* 108:2608–15

Frandji P, Mourad W, Tkaczyk C *et al.* (1998) IL-4 mRNA transcription is induced in mouse bone marrow-derived mast cells through an MHC class II-dependent signaling pathway. *Eur J Immunol* 28:844–54

Giustiniani J, Bensussan A, Marie-Cardine A (2009) Identification and characterization of a transmembrane isoform of CD160 (CD160-TM), a unique activating receptor selectively expressed upon human NK cell activation. *J Immunol* 182:63–71

Giustiniani J, Marie-Cardine A, Bensussan A (2007) A soluble form of the MHC class I-specific CD160 receptor is released from human activated NK lymphocytes and inhibits cell-mediated cytotoxicity. *J Immunol* 178:1293–300

Gri G, Piconese S, Frossi B *et al.* (2008) CD4+CD25+ regulatory T cells suppress mast cell degranulation and allergic responses through OX40-OX40L interaction. *Immunity* 29:771–81

Grimbaldeston MA, Metz M, Yu M *et al.* (2006) Effector and potential immunoregulatory roles of mast cells in IgE-associated acquired immune responses. *Curr Opin Immunol* 18:751–60

Hart PH, Grimbaldeston MA, Swift GJ *et al.* (1998) Dermal mast cells determine susceptibility to ultraviolet B-induced systemic suppression of contact hypersensitivity responses in mice. *J Exp Med* 187:2045–53

Jutel M, Watanabe T, Klunker S *et al.* (2001) Histamine regulates T-cell and antibody responses by differential expression of H1 and H2 receptors. *Nature* 413:420–5

Katz HR (2007) Inhibition of pathologic inflammation by leukocyte Ig-like receptor B4 and related inhibitory receptors. *Immunol Rev* 217:222–30

Kojima T, Obata K, Mukai K *et al.* (2007) Mast cells and basophils are selectively activated in vitro and in vivo through CD200R3 in an IgE-independent manner. *J Immunol* 179:7093–100

Le Bouteiller P, Barakonyi A, Giustiniani J *et al.* (2002) Engagement of CD160 receptor by HLA-C is a triggering mechanism used by circulating natural killer (NK) cells to mediate cytotoxicity. *Proc Natl Acad Sci USA* 99:16963–8

Lee DM, Friend DS, Gurish MF *et al.* (2002) Mast cells: a cellular link between autoantibodies and inflammatory arthritis. *Science* 297:1689–92

Li L, Yao Z (2004) Mast cell and immune inhibitory receptors. *Cell Mol Immunol* 1:408–15

Lu LF, Lind EF, Gondek DC *et al.* (2006) Mast cells are essential intermediaries in regulatory T-cell tolerance. *Nature* 442:997–1002

Maeda M, Carpenito C, Russell RC *et al.* (2005) Murine CD160, Ig-like receptor on NK cells and NKT cells, recognizes classical and nonclassical MHC class I and regulates NK cell activation. *J Immunol* 175:4426–32

Maiza H, Leca G, Mansur IG *et al.* (1993) A novel 80-kD cell surface structure identifies human circulating lymphocytes with natural killer activity. *J Exp Med* 178:1121–6

Malaviya R, Twesten NJ, Ross EA *et al.* (1996) Mast cells process bacterial Ags through a phagocytic route for class I MHC presentation to T cells. *J Immunol* 156:1490–6

Metz CN, Thomas P, Davitz MA (1992) Immunolocalization of a glycosylphosphatidylinositol-specific phospholipase D in mast cells found in normal tissue and neurofibromatosis lesions. *Am J Pathol* 140: 1275–81

Nikolova M, Lelievre JD, Carriere M *et al.* (2009) Regulatory T cells differentially modulate the maturation and apoptosis of human CD8+ T-cell subsets. *Blood* 113:4556–65

Nikolova M, Marie-Cardine A, Boumsell L *et al.* (2002) BY55/CD160 acts as a co-receptor in TCR signal transduction of a human circulating cytotoxic effector T lymphocyte subset lacking CD28 expression. *Int Immunol* 14:445–51

Nikolova MH, Muhtarova MN, Taskov HB *et al.* (2005) The CD160+ CD8high cytotoxic T cell subset correlates with response to HAART in HIV-1+ patients. *Cell Immunol* 237:96–105

Rey J, Giustiniani J, Mallet F *et al.* (2006) The co-expression of 2B4 (CD244) and CD160 delineates a subpopulation of human CD8+ T cells with a potent CD160-mediated cytolytic effector function. *Eur J Immunol* 36:2359–66

Romero X, Benitez D, March S *et al.* (2004) Differential expression of SAP and EAT-2-binding leukocyte cell-surface molecules CD84, CD150 (SLAM), CD229 (Ly9) and CD244 (2B4). *Tissue Antigens* 64:132–44

Rumsaeng V, Cruikshank WW, Foster B *et al.* (1997) Human mast cells produce the CD4+ T lymphocyte chemoattractant factor, IL-16. *J Immunol* 159:2904–10

Schofield JN, Rademacher TW (2000) Structure and expression of the human glycosylphosphatidylinositol phospholipase D1 (GPLD1) gene. *Biochim Biophys Acta* 1494:189–94

Secor VH, Secor WE, Gutekunst CA *et al.* (2000) Mast cells are essential for early onset and severe disease in a murine model of multiple sclerosis. *J Exp Med* 191:813–22

Skokos D, Botros HG, Demeure C *et al.* (2003) Mast cell-derived exosomes induce phenotypic and functional maturation of dendritic cells and elicit specific immune responses in vivo. *J Immunol* 170:3037–45

Skokos D, Le Panse S, Villa I *et al.* (2001) Mast cell-dependent B and T lymphocyte activation is mediated by the secretion of immunologically active exosomes. *J Immunol* 166:868–76

Stelekati E, Bahri R, D'Orlando O *et al.* (2009) Mast cell-mediated antigen presentation regulates CD8+ T cell effector functions. *Immunity* 31:665–76

Tsujimura K, Obata Y, Matsudaira Y *et al.* (2006) Characterization of murine CD160+ CD8+ T lymphocytes. *Immunol Lett* 106:48–56

Valadi H, Ekstrom K, Bossios A *et al.* (2007) Exosome-mediated transfer of mRNAs and microRNAs is a novel mechanism of genetic exchange between cells. *Nat Cell Biol* 9:654–9

Wolters PJ, Mallen-St Clair J, Lewis CC *et al.* (2005) Tissue-selective mast cell reconstitution and differential lung gene expression in mast cell-deficient Kit(W-sh)/Kit(W-sh) sash mice. *Clin Exp Allergy* 35:82–8

Yamada N, Matsushima H, Tagaya Y *et al.* (2003) Generation of a large number of connective tissue type mast cells by culture of murine fetal skin cells. *J Invest Dermatol* 121:1425–32

Yokoi H, Choi OH, Hubbard W *et al.* (2008) Inhibition of FcepsilonRI-dependent mediator release and calcium flux from human mast cells by sialic acid-binding immunoglobulin-like lectin 8 engagement. *J Allergy Clin Immunol* 121:499–505

Yokoi H, Myers A, Matsumoto K *et al.* (2006) Alteration and acquisition of Siglecs during in vitro maturation of CD34+ progenitors into human mast cells. *Allergy* 61:769–76