

OUTSTANDING OBSERVATION

A role for the MHC class I-like Mill molecules in nutrient metabolism and wound healing

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MHC class I family members serve multiple functions beyond antigen presentation. We provide insight into the structure, expression and function of the Mill subfamily. This family includes two surface glycoproteins, Mill1 and Mill2. Protein sequences for Mill1 and Mill2 are most highly related to the NKG2D ligands, MICA and MICB, but neither of them bound to NKG2D. Computer-based protein modelling indicated that hereditary haemochromatosis protein (HFE), a molecule involved in iron uptake, was most similar. Mill1 and Mill2 were observed on cycling thymocytes, proliferating smooth muscle cells and fibroblasts. Using soluble Mill proteins, we found evidence for a soluble ligand in serum. Like HFE, the Mill family may be involved in nutrient metabolism. Skin was one of the only three organs found to express transcripts for both Mill1 and Mill2. Addition of antibodies specific for Mill2 to wounded skin enhanced healing. Our results suggest a role for the Mill proteins in cellular metabolism, with possible therapeutic significance.

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The family of major histocompatibility complex class I-related proteins (MHC-I) can be broadly divided into two groups, classical MHC-I antigens that present intracellular peptides to CD8+ T cells¹ and non-classical MHC-I homologues.² Classical MHC-I molecules are type I integral membrane proteins with three extracellular domains ($\alpha 1$ – $\alpha 3$) that associate non-covalently with $\beta 2$ -microglobulin ($\beta 2M$). The $\alpha 1$ and $\alpha 2$ domains together provide a β -sheet scaffold topped by two antiparallel α -helices. Between these two α -helices lies a peptide-binding groove that associates with intracellular peptides.² Although the genes encoding the classical MHC-I antigens are clustered within the MHC, many of the genes encoding MHC-I homologues are unlinked,³ and sequence homology between family members is generally low (under 30%) creating difficulty when attempting to assign functional attributes to linked molecules based on this criterion.⁴ All MHC-I family members encode a 'minimum class I folding unit' composed of $\alpha 1$ and $\alpha 2$ domains, although many do not bind to peptides. Some of the MHC-I homologues, such as CD1⁵ present non-peptide antigens. Many MHC-I homologues have functions completely distinct from antigen presentation (for review of MHC-I homologues see Braud *et al.*⁶). Several of these molecules, including MICA/B, the ULBPs, Rae1, H-60 and MULT-1 are ligands for the natural killer (NK)-cell activation receptor, NKG2D^{7–11} and have been implicated in cancer and autoimmunity.^{12,13} Others such as hereditary haemochromatosis protein (HFE)¹⁴ and Zinc-alpha(2)-glycoprotein (ZAG)^{15,16} play fundamental physiological roles in

nutrient metabolism via their activity in iron transport and lipid mobilization, respectively.

MHC class I-like molecules located near the leukocyte receptor complex, Mill1 and Mill2, were originally identified as mouse MHC-I homologues using a bioinformatic approach.^{3,17} Recent work has elucidated their structure as glycosylphosphatidylinositol (GPI)-linked membrane proteins¹⁸ with typical MHC-I structure, containing $\alpha 1$, $\alpha 2$ and $\alpha 3$ domains. Mill2 encodes two isoforms generated by alternative splicing of an exon located between the signal peptide and the $\alpha 1$ domain, a feature not normally present in MHC-I proteins. Only nine of the 33 sites known to dock H2-K to $\beta 2M$ are conserved in Mill1 and Mill2. In a recent report, however, both Mill1 and Mill2 were reported to associate with $\beta 2M$. Further, only Mill1 appeared to require $\beta 2M$ for cell-surface expression.¹⁸

Mill1 and Mill2 are of potential immunological interest because they are encoded in the vicinity of the leukocyte receptor complex on mouse chromosome 7. This complex encodes many genes critical to immunobiology including the paired inhibitory receptors (PIR), NKP46, DAP10 and DAP12.^{19,20} Moreover, as judged by primary amino-acid sequence homology, the closest relative to Mill1 and Mill2 is the human NKG2D ligand, MICA (33 and 35% amino-acid homology, respectively), followed by HFE (31–32%), ZAG (26%) and FcRn (24–27%).³ Recently, the small groove formed between $\alpha 1$ and $\alpha 2$ of Mill1 and Mill2 has been shown incapable of binding peptides generated through TAP, a feature similar to MICA and

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MICB.¹⁸ Given these findings and the lack of any known murine orthologues for MICA/B or human orthologues for Mill1/2, it has been suggested that MICs and Mills might serve similar functions.^{3,21}

Here we provide a comprehensive characterization of the Mill proteins. We demonstrate that Mill1 and Mill2 are not ligands for NKG2D and that their tertiary structure most closely resembles that of HFE. We also report and further dissect the association between β 2M and Mill1, Mill2 and the latter's splice variants. Using specific monoclonal antibodies, we show that Mill1 is expressed on a subset of thymocytes and Mill2 on fibroblasts and smooth muscle cells (SMC). By generating soluble forms of Mill1 and Mill2, we show that ligand(s) for these molecules are present in serum and on developing activated, but not, resting haematopoietic cells. Finally, we provide evidence that Mill1 and Mill2 may play a role in wound healing and suggest that the Mills may function in nutrient metabolism.

RESULTS AND DISCUSSION

To initiate our study, we transduced a variety of cell types (EL4, 293, Daudi, P815) with N-terminal flag-tagged versions of Mill1 and Mill2. For Mill2, alternative splicing generates long (Mill2-L) and short (Mill2-S) isoforms that differ in size by 15 amino acids near the N terminus.³ Unless otherwise noted, Mill2 will refer to both isoforms in this report.

Kajikawa *et al.*¹⁸ recently reported that Mill family members associate with β 2M, including the finding of an apparent absolute requirement of β 2M for cell-surface expression of Mill1. Both findings

were observed during the initial phase of our study, thus confirming these results (data not shown).

The specific properties of the association between Mill2-L and Mill2-S, and β 2M remain unknown. Thus, a cell system initially devoid of β 2M was designed to explore this issue. We chose Daudi cells as our reporter cell because they do not express β 2M. Transduction efficiency in Daudi cells using lentivirus is poor (<5%). Therefore, we sorted Daudi cells transduced with an ecotropic receptor-eGFP fusion protein (DE cells), which are very sensitive to transduction with ecotropic retrovirus. Next, we generated a β 2M-positive variant of DE cells (DEM cells) using a retrovirus encoding mouse β 2M. DE or DEM cells were then transduced with Mill1 or Mill2. One week after transduction of DEM cells, approximately 50% of each population expressed cell-surface Mill1 or Mill2. As expected, DE cells supported expression of both Mill2 isoforms but not of Mill1 (Supplementary Figure S1). However, Mill1 was expressed intracellularly in DE cells (data not shown). We were surprised that neither Mill2-L nor Mill2-S required β 2M for surface expression and speculated that bovine β 2M in our *in vitro* culture system might be stabilizing Mill2. Accordingly, we positively sorted DE and DEM cells expressing Mill2-L or Mill2-S and removed cell-surface Mill with trypsin. These cells were then cultured for 72 h in serum-free medium and the recovery of Mill2 was assessed. Nearly 100% of Mill2-S transductants recovered full cell-surface expression irrespective of β 2M expression. In contrast, only 20% of DE cells and 44% of DEM cells recovered cell-surface expression of Mill2-L, and the fluorescent

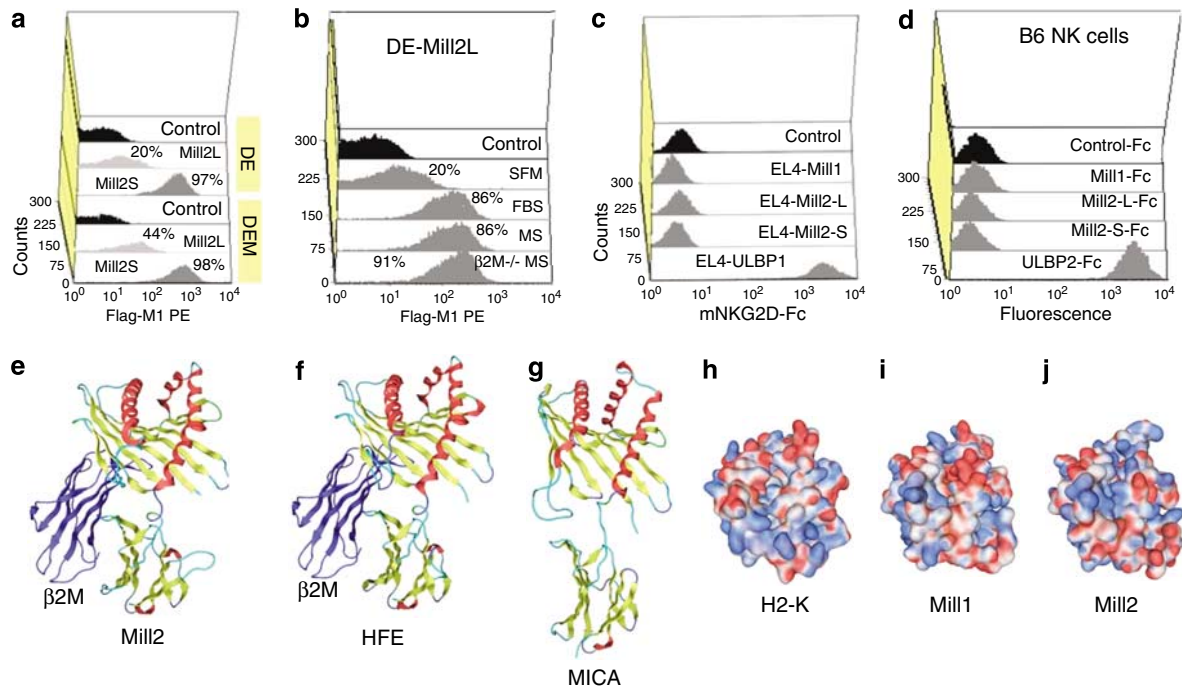


Figure 1 Mill1 and Mill2 are MHC-I homologues that more closely resemble HFE than MICA. (a) DE cells or DEM cells expressing Mill2L or Mill2S (as indicated) were treated with trypsin for 10 min at 37 °C to remove surface expression of the Flag-tagged Mill proteins. Cells were subsequently cultured for 72 h in SFM and stained with anti-Flag PE. (b) DE-Mill2L cells were trypsinized and cultured for 72 h without serum or 48 h without serum, followed by 24 h with 1% fetal bovine serum, 1% B6 serum or 1% B6 β 2M^{-/-} serum (as indicated). DE cells are shown in black. (c) EL4 cells stably expressing mouse IL4-R (control), Flag-tagged Mill1, Flag-tagged Mill2-L, Flag-tagged Mill2-S or ULBP1 were stained (as indicated) with mouse NKG2D-LZ and an anti-leucine zipper mAb. (d) B6 splenocytes were depleted of CD4⁺, CD8⁺ and IgM⁺ cells and cultured in IL-15 for 3 days. The resulting population, which was greater than 95% NK1.1+/TCR β ⁻, was stained (as indicated) with p3-Fc (negative control), Mill1-Fc, Mill2L-Fc, Mill2S-Fc or ULBP2-Fc (positive control). (e–i) Ribbon diagrams of predicted three-dimensional structure of Mill2 (e) and crystal structures of HFE (f) and MICA (g). β 2M, when present, is coloured blue. (h–j) Space-filling models of grooves generated between the α 1 and α 2 domains for H2Kd (h), Mill1 (i) and Mill2S (j). Surface electrostatic charge is highlighted such that blue is positive and red is negative. HFE, hereditary haemochromatosis protein; mAb, monoclonal antibody; MHC-I, major histocompatibility complex class I-related proteins; SFM, serum-free medium; β 2M, β 2-microglobulin.

intensity was more than 10-fold less compared to Mill2-S (Figure 1a). Thus, cell-surface expression of Mill2-L is partially dependent on β 2M and a serum factor, whereas excision of exon 2 from the Mill2 transcript renders the surface expression of Mill2S completely independent of both β 2M and a serum factor. Mill1 demonstrated similar dependence on a serum factor as Mill2-L but also required the expression of β 2M (data not shown). Importantly, endogenous classical MHC-I reached normal levels irrespective of the transduced gene as long as β 2M was co-expressed (data not shown). The presence of 1% fetal bovine serum or 1% mouse serum during the last 24 h of culture resulted in nearly full recovery of Mill2-L cell-surface expression, even when the serum was derived from β 2M-deficient mice (Figure 1b). TAPI, a broad-spectrum metalloprotease (MMP) inhibitor, could not mimic the effects of serum when added to this system (data not shown). This suggested that a serum factor(s) other than β 2M did not block cell-surface shedding of Mill2-L, but rather promoted the recovery or stabilized Mill2-L at the cell surface. The involvement of a serum factor in the cell-surface expression of Mill1 and Mill2-L was the first sign that these molecules play a role in nutrient metabolism.

On the basis of the association of the Mill family of proteins with β 2M, its possible interaction with a serum factor and the low amino-acid sequence homology with MICA ($\sim 30\%$), we hypothesized that these molecules were unlikely candidates as ligands for NKG2D. Thus, we stained EL4 cells, retrovirally transduced with Mill1 or Mill2, with a soluble mouse NKG2D-leucine zipper fusion protein (mNKG2D-LZ). EL4 cells were chosen because they do not express NKG2D ligands endogenously.²² mNKG2D-LZ bound with EL4-ULBP1 (positive control²²), but not with EL4-Mill1 or EL4-Mill2 (Figure 1c). Soluble Mill1-hFc and Mill2-hFc fusion proteins bound neither mouse NK cells (Figure 1d) nor human NK cell-like YT cells transduced with mNKG2D (data not shown). Cytotoxicity assays using interleukin (IL)-2 or IL-15 activated C57BL/6 NK effector cells, and Mill-transduced EL4 target cells resulted in the same level of killing as that observed with EL4 cells transduced with IL-4R (control). As previously reported, EL4-ULBP1 were more than threefold more sensitive to lysis than the control (data not shown).²² Our data indicate that Mill family members are not ligands for NKG2D nor other NK cell receptors on mature NK cells. We do not exclude the possibility that they are involved in other immunological processes.

To identify an MHC-I family member that more closely resembled the characteristics ascribed to Mill1 and Mill2, the tertiary structures of Mill1, Mill2-L and Mill2-S were predicted using computer-based protein homology using HFE (PDB ID 1A6Z) as a template.²³ A complete model for Mill2-L could not be generated because the 15 N-terminal amino acids found in Mill2-L but not Mill2-S fell outside the alignment to the structure template. These 15 amino acids, nevertheless, are of obvious interest because in their absence, expression of Mill2-S is completely independent of β 2M or a serum factor.

Our model revealed that Mill1 and Mill2-S contain 33 and 31 predicted contact sites with β 2M, respectively. Alignment of Mill1 and Mill2 demonstrated that 20 β 2M contact sites were identical and 27 conserved in position. Alignment of Mill1 and Mill2 with H2-K^d established that although only nine β 2M-binding residues were conserved; 26 of the 33 contact sites between H2-K^d and β 2M were located in the same position with those found for Mill1 or Mill2 (Supplementary Figure S2). Thus, our model predicts that orientation rather than conservation of residues serves to explain the unexpected association between Mill and β 2M. Although our models did predict greater surface contact between Mill1 and β 2M versus Mill2 and β 2M ($\sim 40 \text{ \AA}^2$), they could not immediately explain the absolute

dependence of Mill1 on β 2M. Solving the crystal structure for these complexes will ultimately resolve this issue.

The MHC family is very closely related structurally. Figures 1e–g illustrate the similarity between the predicted structure for Mill2 and the crystal structures of HFE (1A6Z)²³ and MICA (1HYR).²⁴ In a previous report, MICA was predicted to be the closest relative of the Mill family.¹⁷ Using protein-threading analysis (in house method) against 6692 known structures (ASTRAL SCOP^{25–28}), both Mill1 and Mill2 were most highly related to HFE. ZAG, an MHC class I homologue involved in lipid metabolism,^{15,16} was fifth closest to both Mill1 and Mill2, whereas MICA was the 149th and the 13th thirteenth closest to Mill1 and Mill2, respectively. Analysis using other threading engines (3D-PSSM)²⁹ generated slightly different hierarchies but reiterated the conclusion that Mill family members most closely resemble HFE.

Hereditary haemochromatosis protein is an MHC-IB family member that associates with β 2M^{14–16,23,30} and the transferrin receptor (CD71). The dimeric complex formed between HFE and CD71 binds to and internalizes transferrin. Binding of HFE to iron is pH-dependent and release occurs in the acidic environment of early endosomes.^{14,31,32} We therefore examined the electrostatics of the groove formed between α 1 and α 2 of Mill1 and Mill2. Unlike H2-K, both Mill1 and Mill2 were heavily charged. Mill1 was acidic, whereas Mill2 was basic (Figures 1h–j). Mill1 demonstrated similar dependence on a serum factor as Mill2-L but also required the expression of β 2M (data not shown). The generation of soluble Fc-fusion proteins of Mill1 and Mill2 allowed us to search for cellular counter structures and to test the effect of pH on any observed binding. Supporting the specificity of binding with these fusion proteins, versions of Mill1 and Mill2 fused to mouse IgG1 Fc inhibited staining when added in excess (data not shown). Similar binding patterns were observed for both molecules, although the magnitude of binding was always greater with Mill2-Fc. A series of mouse cells and cell lines was stained with Mill1-Fc and Mill2-Fc. EL4 (thymoma) and 70Z/3 (pre-B lymphoma) were highly positive whereas P815 (mastocytoma) and WEHI (plasmacytoma) were negative. RMA (T-cell lymphoma) and RAW-264 (macrophage-like) were marginally positive (data not shown). In mouse bone marrow (BM), 10–15% and 40–50% of cells bound to Mill1-Fc and Mill2-Fc, respectively (Figure 2a). We identified the positive populations in the BM as B cells and erythrocyte precursors. In the B-cell lineage, staining with Mill-Fc decreased as the cells differentiated from early (CD43⁻B220⁺IgM+IgD⁻) to late pre-B cells (CD43⁻B220⁺IgM/D⁻) and finally to immature B cells (Figures 2b–c; Mill2-Fc shown); CD43⁺ pro-B cells did not stain (data not shown). An analogous pattern was observed in the erythroid lineage. Developing erythrocytes can be ordered from least (Figure 2d, gate 1) to most differentiated (Figure 2d, gate 4) via loss of CD71 expression on the Ter119⁺ population (Figure 2d). Figure 2e shows that staining with Mill2-Fc decreased as the erythrocyte population matured. Thus, for both lineages, soluble Mill proteins bound preferentially to less-differentiated/highly proliferative cells with greater nutrient demands. Similarly, for splenic T and B cells, we found that mitogen-activated and proliferating, but not resting, cells bound Mill2-Fc (Figures 2f–g; resting cells not shown). In this case, very little staining was observed with Mill1-Fc. For Flt3-L-mobilized plasmacytoid dendritic cells (PDC), we found that stimulation of PDC with CpG induced expression of a counter structure for Mill2-Fc. In contrast, stimulation of granulocyte-macrophage colony-stimulating factor-mobilized myeloid dendritic cells (MDC) resulted in a complete loss of Mill2-Fc staining (Figure 2g). This opposite result obtained with stimulated MDCs may be because, unlike T cells, B cells and PDC that proliferate

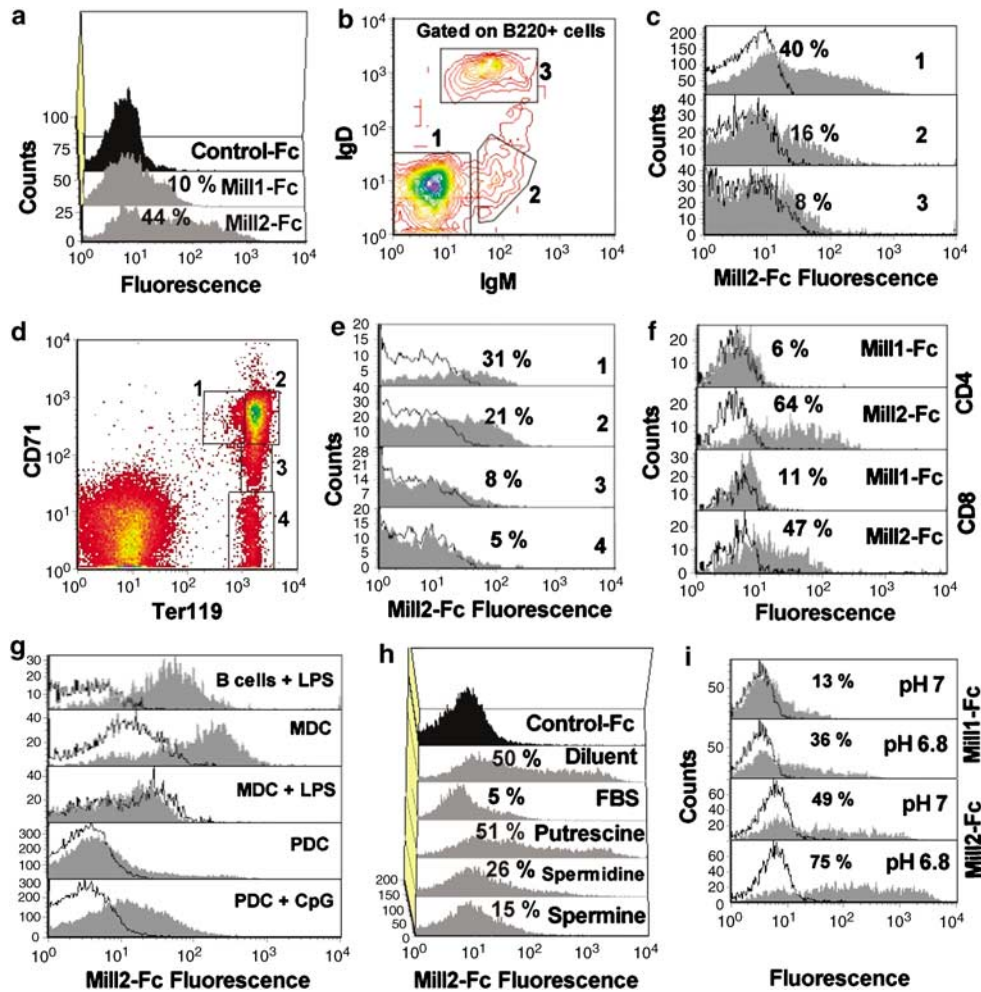


Figure 2 Characterization of cellular and soluble ligand(s) for Mill1 and Mill2. (a) BM cells from B6 mice were stained with either ULBP3-Fc (control), Mill1-Fc or Mill2-Fc (as indicated). (b) B6 BM gated on live (FCS/SSC) B220⁺ cells and subfractionated into (1) early pre-B cells, (2) late pre-B cells and (3) immature B cells. Staining of fractions 1–3 from (b) with Mill2-Fc (filled histograms) is depicted in (c). (d) B6 BM subfractionated into four subsets based on CD71 and Ter119 as follows: (1) pro-erythroblasts, (2) basophilic erythroblasts, (3) chromatophilic erythroblasts and (4) ortho-chromatophilic erythroblasts and erythrocytes. Staining of fractions 1–4 from (d) with Mill2-Fc (filled histograms) is shown in (e). (f) B6 splenocytes were activated with anti-CD3 for 48 h, gated on CD4⁺ and CD8⁺ cells (as indicated) and assessed for labelling with Mill1-Fc and Mill2-Fc (as marked). In the upper panel of (g), B6 splenocytes were activated for 72 h with LPS, gated on CD19⁺ cells and assessed for labelling with Mill2-Fc. In the bottom four panels, BM cells were cultured in GM-CSF (MDC) or Flt-3L (PDC) for 7 or 9 days, respectively and stained with Mill2-Fc prior to or after activation for 24 h (as indicated). For MDC, cells were gated as CD11b⁺/CD11c⁺. For PDC, cells were gated as CD11c⁺/CD11b⁻. (h) B6 BM was stained with Mill2-Fc in the presence of the indicated molecules. Staining with ULBP3-Fc (control) is shown as the black histogram. (i) B6 BM was stained with Mill1-Fc or Mill2-Fc at pH 7 or pH 6.8 (as marked). For (c), (e–g) and (i), staining with ULBP3-Fc (control) is shown as open histograms. BM, bone marrow; GM-CSF, granulocyte-macrophage colony-stimulating factor; MDC, myeloid dendritic cells; PDC, plasmacytoid dendritic cells.

upon stimulation, MDC do not. The highly charged grooves between $\alpha 1$ and $\alpha 2$ of Mill1 and Mill2 suggested that pH might change Mill-Fc staining patterns. Interestingly, a small decrease in pH from 7 to 6.8 resulted in a significant increase in fluorescence intensity for all cells that bound Mill-Fc (data not shown) including BM (Figure 2i). Our model predicted that a drop in pH from 7 to 6.8 would have no effect on groove charge for Mill1 or Mill2. This suggests that the pH-dependent increase in binding to Mill-Fc could be a function of the putative ligand or a minor structural change in the Mills. Thus, Mill1 and Mill2 bind to a ligand on proliferating cells in a pH-dependent manner, a finding similar to the binding properties of HFE for transferrin (that is, pH-dependent) and the expression pattern of CD71 on proliferating cells (that is, upregulated). Moreover, there was nearly a complete overlap of staining with an anti-CD71 monoclonal antibody (mAb) and Mill2-Fc on BM cells (data not shown). These

data support the hypothesis that like HFE, Mill proteins may also be involved in nutrient metabolism.

Repeated attempts to identify a cell surface ligand for Mill1 and Mill2 via immunoprecipitation and expression cloning failed. On the basis of our finding that a serum factor is involved in Mill expression, we hypothesized that a low molecular weight soluble serum factor (too small to be identified via gel electrophoresis) may bind non-covalently to proliferating cells. We therefore utilized an indirect approach to identify potential soluble ligands for Mill1 and Mill2 by screening an array of small charged molecules for their ability to block staining of BM with Mill1-Fc and Mill2-Fc. We observed that fetal bovine serum (Figure 2h) and MS (data not shown) completely blocked Mill-Fc staining (Mill2-Fc, shown). Our protein modelling showed that although the groove between $\alpha 1$ and $\alpha 2$ was too small to accommodate TAP-derived peptides,¹⁸ the charged grooves of Mill1

and Mill2 could accommodate small peptides of two or three amino acids, vitamins, sugars and nucleosides. The results of our screen indicated that the polyamines, spermidine and spermine (but not putrescine), highly charged oligomers of arginine, could completely inhibit binding of Mill-Fc to BM cells (Figure 2h; Mill2-Fc, shown). It was surprising that spermidine and spermine may bind both Mill family members as Mill1 and Mill2 possess oppositely charged grooves. On the basis of our models, however, these polyamines could bind in alternate modes to Mill1 and Mill2. Surface electrostatic calculations on these ligands while present in the binding groove indicate that the ligands possess a surface charge distribution that could allow their binding to these differentially charged grooves. Because highly charged molecules may associate non-specifically to Mill, we can only speculate that small charged molecules like polyamines resemble the true ligands. These likely bind within the grooves of Mill1 and Mill2, using different binding modes.

Using RT-PCR Kasahara³ reported that normal expression of Mill1 is scarce (mainly skin, thymus, ovary and kidney) whereas that of Mill2 is widespread. A similar pattern was observed using Taqman qRT-PCR (data not shown). Transcripts for Mill1 and Mill2 were nearly absent in whole fetus and embryonic stem cells, suggesting that their expression is limited to postnatal life. To examine Mill1 and Mill2 at the protein level, we generated mAbs specific for Mill1 (M575), Mill2-L (M247) and Mill2-S (M248; binds both Mill2-L and Mill2-S), which were used in flow cytometry and immunohistochemistry experiments. Immunohistochemical analysis of a panel of frozen tissue sections from BALB/c mice did not detect Mill1 protein expression on any of the tissues (data not shown). As detection of low-density surface antigens is often difficult using IHC but possible using flow cytometry, cell suspensions of thymocytes, 10–15% of which are dividing cells, were probed for expression of Mill1. Cycling but not resting thymocytes expressed low but appreciable levels of Mill1

(Figures 3a–b). Most resting thymocytes, however, did express intracellular Mill1 (Figure 3c), implying that Mill1 is transported to the cell surface when thymocytes undergo cell division. Within the proliferating thymocyte pool, Mill1 was only expressed on developing CD4⁺CD8⁺ and CD4⁻CD8⁻ thymocytes (data not shown). Consistent with the expression of Mill1 on developing thymocytes, we also observed low but appreciable expression of Mill1 on EL4 thymoma cells (data not shown). The fibroblast cell line, 3T3, was identified as positive for Mill1 (Supplementary Figure S3c). For Mill2, every tumour cell line tested expressed low but significant levels of the protein. The highest levels were observed on 3T3 and the squamous cell carcinoma, SCCVII (data not shown). Standard IHC staining of normal BALB/c tissue detected Mill2 expression in the skin, bladder, uterus and colonic muscularis (Figures 3e–h; isotype controls are shown in Supplementary Figure S4). In these tissues, staining was confined to SMC and fibroblasts. Flow cytometric analysis of primary SMC from carotid artery explants confirmed our results for Mill2 while producing negative results for Mill1 and an isotype control (Supplementary Figure S4a). The expression of Mill2 on stromal cells serves to explain its widespread expression via RT-PCR and qRT-PCR. Interestingly, both 3T3 and SMC bound strongly to Mill1-Fc and Mill2-Fc, suggesting a possible role for autocrine-like activity in these cell types (Supplementary Figure S3b and d).

Several aspects of our data suggested a role for Mill2 in nutrient metabolism during times of increased energy demands, such as cellular activation and proliferation, which occur during tissue injury and repair. To examine whether enhanced energy demands of SMC undergoing hyperplasia correlate with expression of Mill2, we stained cryopreserved colitic colon sections from two models of inflammatory bowel disease. *Mdr1a*^{-/-} mice suffer from chronic inflammation and hyperplasia of the Mill2⁺ muscularis externa,³³ whereas treatment of mice with dextran sulphate sodium results in acute destruction of

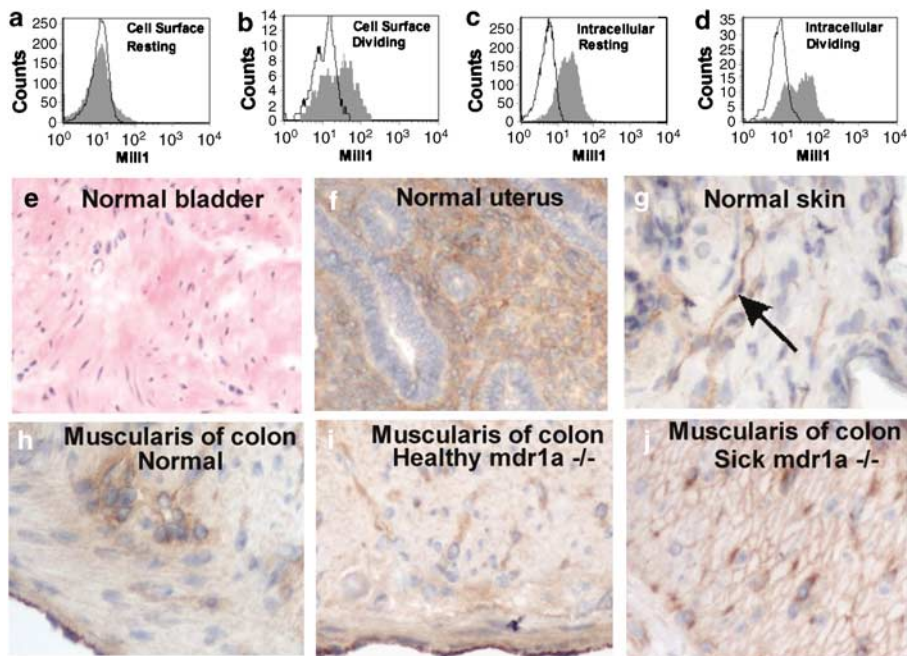


Figure 3 Mill1 and Mill2 expression on normal and inflamed tissues (a–d). Balb/c thymocytes were gated on resting and dividing populations (based on SSC/FSC) and stained for cell surface (a and b) and intracellular (c and d) Mill1 using M575. Staining was visualized with an anti-rat PE antibody. (e–h) Immunohistochemical staining of a panel of cryopreserved tissues (as shown) from BALB/c mice stained with M248. The arrow in (g) indicates a fibroblast. (i and j) Cryopreserved sections of the colonic muscularis from non-colitic *mdr1a*^{-/-} mice (i) or mildly colitic *mdr1a*^{-/-} mice (j) were stained with M248.

Mill2⁻ epithelial cells and no change to the muscularis.³⁴ As anticipated, Mill2 was upregulated only in the hyperplastic muscularis of colitic *mdr1a*^{-/-} mice (Figures 3i–j) but not in dextran sulphate sodium-treated mice (data not shown).

To test whether the expression of Mill was merely a correlate to increased energy demands or was of functional consequence, we asked what effect mAbs specific for Mill1 and Mill2 would have on another form of tissue injury, dermal wound healing. This model was chosen because the skin co-expressed Mill1 and Mill2, allowing us to test the antibodies against both molecules in a well-established assay. A skin organ culture system was chosen to diminish the number of variables potentially affecting our results such as infiltrating lymphocytes. Excised wounds were allowed to heal on surgical gelfoam for 7 days with or without the addition of antibodies. Wound closure was calculated as described in Materials and Methods. We found that compared to skin organ culture performed in media alone or with the addition of an isotype control mAb, the addition of M247 (binds Mill2-L) resulted in wounds about 50% smaller on day 7 (Figures 4a–k; $P < 0.0001$, Student's *t*-test). Addition of M575 (binds Mill1) and, to a greater extent, M248 (binds both Mill2-L and Mill2-S), appeared to augment wound closure to some degree but the values were not significant ($P > 0.01$). Importantly, heat denaturation of the M247 abolished its ability to augment wound closure (Figure 4k). It is possible that the difference between the effects of M247 and M248 are related to epitope location as (as assessed by flow cytometry) SMC and 3T3 stained more weakly with M248 than with M247. Affinity differences were not formally assessed although both antibodies behaved similarly using transduced EL4 cells and flow cytometry. To address whether M247 augmented wound healing by promoting keratinocyte proliferation, we pulsed these organ cultures with BrdU to enumerate proliferating cells. Treatment with M247 did not increase the number of BrdU+ cells (data not shown). Mill2 expression

during wound closure as assessed by IHC did not appear to change, but the fragility of the tissue after organ culture may have decreased the sensitivity of staining (data not shown). Nevertheless, we did observe specific staining for Mill1 in the hair follicles of wounded skin, an observation also made by Kajikawa *et al.*¹⁸ in the skin of 3-day-old mice but not older animals¹⁸ (Figure 4l).

Although we do not yet understand the mechanism of action of M247, it is interesting to note that other than transferrin, the only known iron transport vehicles are the same polyamines that blocked Mill-Fc staining of BM, that is, spermine and spermidine but not putrescine.³⁵ Like HFE, the Mill family may also be involved in iron metabolism. It is worth noting that haemochromatosis is exacerbated in $\beta 2M$ ^{-/-} mice compared to HFE^{-/-} mice, suggesting that a $\beta 2M$ -binding molecule other than HFE is involved in iron transport.^{36,37} M247 augmented wound healing without inducing the proliferation of cells surrounding the wound. As Mill2 is expressed on contractile SMC, M247 may have functioned by inducing the skin to 'pinch in'. M247 may act directly by sending a contractile signal to the cells or indirectly by inducing internalization of Mill2L and associated iron. In support of this explanation, we found that crosslinking Mill2 on EL4 cells resulted in altered motility/contractility as measured by enhanced adherence and spreading of these to plastic (without the induction of a calcium flux; data not shown).

In conclusion, we have demonstrated that the Mill family of MHC-I homologues resemble the structure and function of HFE, rather than the previously presumed relationship with MICA. We provided evidence for this similarity not only by demonstrating that neither of the Mill isoforms binds to the receptor for MICA (NKG2D), but also by presenting data indicating that Mill1 and Mill2 are linked to nutrient metabolism and can influence wound healing. Our data provide a template for future experimental investigation.

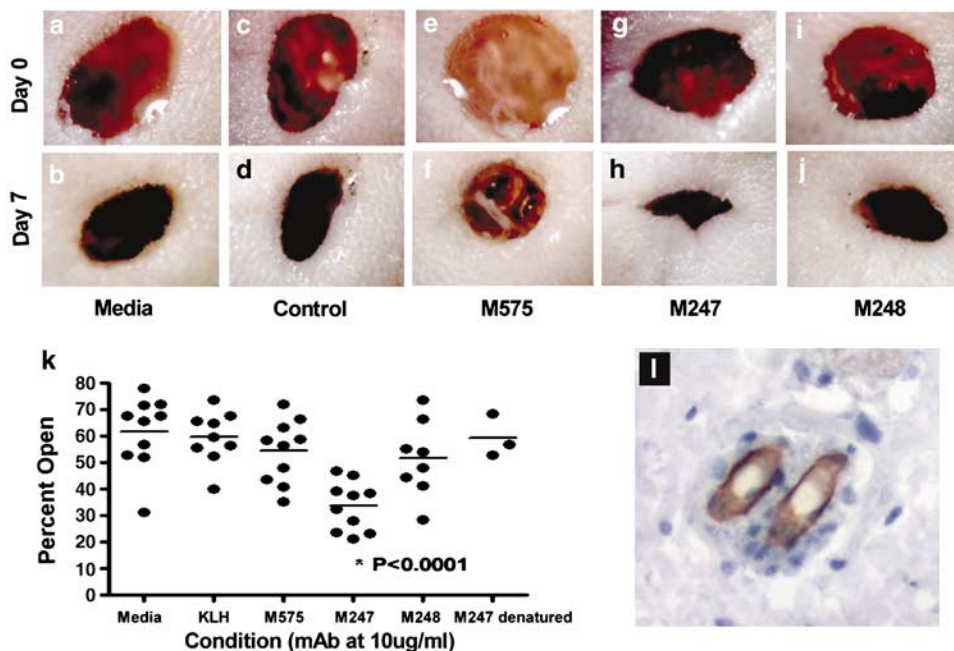


Figure 4 An antibody specific for Mill2-L augments dermal wound healing in SOC. (a–j) Two-millimeter wounds were introduced into dorsal skin sections of BALB/c mice and then cultured on surgical sponges for up to 7 days containing media alone, an isotype control mAb, M575, M247, M248 or heat-denatured M247 (as indicated). Antibody concentration was $10 \mu\text{g ml}^{-1}$ and media was changed on day 4. This experiment was repeated four times and the combined results are illustrated in (k). The y axis represents the percentage of the wound still open on day 7 compared to day 0. Each dot represents one piece of wounded skin. (l) Cryosections of wounded skin 5 days after wounding were stained with M575. mAb, monoclonal antibody; SOC, skin organ culture.

METHODS

Expression of Mill1 and Mill2

Mill1 and Mill2 were cloned from BALB/c uterus cDNA (Clontech, Mountain View, CA, USA) into pDONR222 (Invitrogen, Carlsbad, CA, USA). In some cases, the leader sequence was replaced with an IgK leader-Flag-M1 cassette. Mill1 and Mill2 were then recombined into either the transient expression vector pDC415G or the lentivector pLV410G (human EF1 α promoter) or LZRS-G retroviral vector via LR reaction (Invitrogen). Lentiviral and retroviral packaging with the VSV.G or ecotropic envelope protein and transduction of EL4 or P815 cells with Mill1 or Mill2 were performed as previously described.^{38,39} Daudi cells overexpressing the ecotropic receptor MCAT-1 fused to GFP (DE cells) were generated by transducing Daudi cells with pLV410-MCAT-1-eGFP (VSV.G) and sorting the eGFP+ cells (~1–2%). These cells were then transduced with LZRS encoding mouse- β 2M (ecotropic), generating DE cells that express β 2M and HLA-A/B/C (DEM cells).

Flow cytometry and Fc-fusion proteins

Fc fusion proteins of Mill1, Mill2-L and Mill2-S were generated as previously described.⁹ All primary and secondary antibodies for flow cytometry were purchased from Pharmingen (San Jose, CA, USA) and Jackson Labs (Bar Harbor, ME, USA), respectively, except PE-anti-Flag M1 (M1 conjugated with PE (Prozyme, San Leandro, CA, USA)).

Western blotting and immunoprecipitation

Western blotting and immunoprecipitation were performed using digitonin lysis buffer as previously described.³⁸

Computer-based protein modelling

The structures of H2-Kd, Mill1 and Mill2 were calculated using computer-based protein homology modelling based on the haemochromatosis protein HFE²³ (Protein Data Bank entry 1A6Z^{23,40}). The protein structure models were built using the Homology Modelling method within the Molecular Operating Environment software package (Chemical Computing Group, Montreal, Canada). To verify the Mill fold family, threading analysis was performed on a template library built using protein-level representative structures using ASTRAL SCOP version 1.69^{25–28} resulting in a fold library of 6692 structures. Threading is a method in which a protein sequence is matched onto a structure from a library of known protein structures and the resulting fold of the query sequence scored for suitability to the template fold.⁴¹

Monoclonal antibody generation and immunohistochemistry

Lewis rats were immunized with either Mill1-Fc or Mill2L-Fc protein as described.⁹ Hybridoma supernatants were screened via flow cytometry and immunohistochemistry on fibrin cell pellets using EL4 cells expressing Mill1 or Mill2. IHC studies were performed using standard techniques.

Skin organ culture and wound healing

Skin organ culture was performed as previously described⁴² using BALB/c mice between 8 and 10 weeks of age. Wound size was measured using Metavue Software (Metavue Corp., Madison, WI) on days 0, 4 and 7 of culture. Antibodies were added to the culture at 10 μ g ml⁻¹. Media was changed on day 4.

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