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Characterisation of murine MICL (CLEC12A) and evidence for an endogenous ligand

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Abstract

Inhibitory receptors are required for the control of cellular activation and they play essential roles in regulating homeostasis and immunity. We previously identified a human inhibitory C-type lectin-like receptor, MICL (CLEC12A), a heavily glycosylated monomer predominantly expressed on myeloid cells. Here we characterise the murine homolog of MICL (mMICL), and demonstrate that the receptor is structurally and functionally similar to the human orthologue (hMICL), although there are some notable differences. mMICL is expressed as a dimer and is not heavily glycosylated; however, like hMICL, the receptor can recruit inhibitory phosphatases upon activation, and is down-regulated on leukocytes following stimulation with selected TLR agonists. Using novel monoclonal antibodies, we demonstrate that, like the human receptor, mMICL is predominantly expressed by myeloid cells. However, mMICL is also expressed by B cells and $CD8^+$ T cells in peripheral blood, and NK cells in the bone marrow. Finally, we show that mMICL recognises an endogenous ligand in a variety of murine tissues, suggesting that the receptor plays a role in homeostasis.

Keywords

C-type lectin; Endogenous ligand; Inhibitory receptors; Myeloid cell; Myeloid inhibitory C-type lectin

Introduction

C-type lectin-like receptors (CTLR) are type II transmembrane receptors and belong to the C-type lectin superfamily of proteins, which are classified into a number of groups based on the arrangement of their C-type lectin domains [1]. The CTLR can be broadly categorized as activation or inhibitory receptors. Most CTLR that trigger cellular activation lack cytoplasmic signalling motifs, but contain charged residues in their transmembrane domains that allow association with signalling partners, such as DAP12 or DAP10. Other activation

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CTLR, such as Dectin-1, are able to directly induce intracellular activation signals through their cytoplasmic tails [2]. The cytoplasmic tails of inhibitory receptors, on the other hand, possess one or more immunoreceptor tyrosine-based inhibitory motifs (ITIM), which become tyrosine phosphorylated following receptor ligation. This leads to the recruitment of phosphatases, such as SHP-1 or SHP-2, which mediate the downstream inhibitory effects.

Although a number of activation and inhibitory myeloid-expressed CTLR have been identified, the ligands and functions of many of these "orphan" receptors are unknown [3]. We identified and characterised one such receptor, human myeloid inhibitory C-type lectin-like receptor (hMICL) [4, 5] that has also been described by three other groups [6-8]. This receptor is found in the "Dectin-1 cluster" of related CTLR [9], which is conserved across species [10]. MICL is variably spliced and highly *N*-glycosylated, and is predominantly expressed on myeloid cells, particularly granulocytes and monocytes, and has been identified as a cellular marker in acute myeloid leukaemia [4-6]. Furthermore, expression of this receptor is down-regulated following inflammatory conditions *in vitro* and *in vivo* [4, 5]. MICL contains a single ITIM in its cytoplasmic tail that can associate with the signalling phosphatases SHP-1 and SHP-2 [4, 8], and has been shown to modulate cellular activity [4, 7, 8]. Here we have identified and characterised the murine orthologue of MICL (mMICL) and present evidence that mMICL recognises an endogenous ligand(s) in various tissues.

Results and discussion

mMICL is an inhibitory group V C-type lectin

mMICL was identified based on its genomic location within the "Dectin-1 cluster" [9] and homology to the human orthologue [4]. Analysis of the amino acid sequence indicates that the receptor has a structure typical for an inhibitory group V C-type lectin [1] (Fig. 1A and B). However, compared to the other receptors in this cluster [10], mMICL shows only 49% overall amino acid sequence identity (65% similarity) to its human homologue, implying that some evolutionary divergence has occurred between species (Fig. 1A and data not shown).

We then compared the characteristics of HA-tagged hMICL and mMICL (MICL-HA), which was expressed on the surface of transduced NIH3T3 fibroblasts (Fig. 1C). For this analysis, cell lysates were analysed by Western blotting under reducing and non-reducing conditions (Fig. 1D). As we have previously described, hMICL was detected predominantly as a glycosylated monomer [4, 5]. In contrast, both high (~90 kDa) and low molecular mass (~45 kDa) forms of murine MICL were observed under non-reducing conditions, which could be resolved to a single band (~45 kDa) under reducing conditions, indicating dimerisation of the receptor. Furthermore, there was a reduction in molecular mass upon treatment of the cell lysates with PNGase F, suggesting that mMICL was *N*-glycosylated. However, the glycosylation of this receptor was less extensive than that observed for the human homologue, a difference which is likely to be due to the loss of one of the *N*-linked glycosylation sites in the stalk region (Fig. 1A and B). Given that both hMICL and mMICL possess cysteines in the stalk region, it is possible that the extensive glycosylation of the human receptor prevents dimerisation [4].

The cytoplasmic tail of mMICL contains an archetypal ITIM, a motif known to recruit inhibitory phosphatases [11]. To verify the ability of this receptor to engage these enzymes, we examined by Western blotting immunoprecipitated signalling complexes from cell lysates of pervanadate-stimulated RAW264.7 macrophages, transduced with mMICL-HA (Fig. 1C and E). Using a polyclonal antibody that recognises both SHP-1 and SHP-2, we could demonstrate that MICL associated with these inhibitory phosphatases following cellular activation. By contrast, MICL did not recruit SHIP (data not shown). Equivalent

loading was demonstrated by probing the immunoprecipitates with an anti-HA antibody. A similar association with inhibitory phosphatases was also obtained upon immunoprecipitation of MICL in primary cells (data not shown). Overall, these data indicate that mMICL is expressed as a glycosylated dimer and functions as an inhibitory receptor.

Expression and regulation of mMICL

To investigate the expression of mMICL, we generated novel rat mAb specific to this receptor, using a soluble Fc-MICL fusion protein as the immunogen. Hybridomas were generated and screened, as described in the *Material and methods*, and two mAb (206 and 309), were chosen for further analysis. These mAb specifically recognised murine MICL, as demonstrated by flow cytometry, and were not cross-reactive with hMICL (Fig. 2A and data not shown).

Using these mAb, we next analysed the distribution of MICL on cells isolated from a number of tissues by multicolour flow cytometry (Fig. 2B). In peripheral blood, mMICL was detected on monocytes ($CD11b^+$, $F4/80^+$), neutrophils ($7/4^{high}$, $Gr\text{-}1^{high}$), eosinophils ($7/4^{low}$, $F4/80^+$, SSC^{high}), and basophils (SSC^{high}, IgE^+). However, unlike the hMICL [5], the receptor was also detected on B cells ($CD19^+$, $MHCII^+$) and at low levels on $CD8^+$ T lymphocytes ($DX5^-$, $CD3^+$, $CD8^+$). Other cell subsets, including $CD4^+$ T lymphocytes, NK and NKT cells were largely negative for MICL expression (data not shown). A similar expression pattern was also seen in the bone marrow, with the exception of NK cells, which clearly expressed the receptor (Fig. 2C). High levels of MICL expression were also detected on thioglycolate-elicited neutrophils and macrophages, as well as on bone marrow-derived dendritic cells (BMDC) (Fig. 2D). Thus, the expression of mMICL is broader than that of the human orthologue.

As expression of hMICL can be down-regulated upon cellular activation [5], we wondered if the murine receptor could be similarly modulated. For this analysis, we stimulated thioglycolate-elicitied macrophages and neutrophils, as well as BMDC, with a variety of TLR agonists *in vitro*, and monitored changes in MICL surface expression by flow cytometry (Fig. 2E-G). In neutrophils (Fig. 2E), and to a lesser extent macrophages (Fig. 2F), we observed that MICL expression was down-regulated to a variable extent by many TLR ligands. In contrast, only zymosan affected the expression of MICL in BMDC (Fig. 2G and data not shown). Overall, these data indicate that the expression of mMICL can be down-regulated upon exposure to selected microbial components *in vitro*.

Antibody-mediated receptor-cross-linking experiments have recently demonstrated that hMICL can modulate DC function *in vitro* [7]. Following a similar approach, we next attempted to modulate the function of primary murine leukocytes using our novel mAb. However, despite considerable effort, we were unable to demonstrate any effect of antibody cross-linking on a variety of leukocyte responses (data not shown).

Evidence for an endogenous MICL ligand

The ligands for myeloid-expressed inhibitory CTLR are largely unknown, but it is possible that they recognise endogenous molecules [3]. To explore this, we probed cells isolated from numerous murine tissues by flow cytometry, using the Fc-mMICL fusion protein described above, as a probe. Using this approach, we detected prominent binding of Fc-MICL to cells isolated from heart, lung, liver, spleen and kidney (Fig. 3A). This interaction appeared to be specific, as similar binding was not obtained with Fc-Dectin-1, and was binding not dependent on divalent metal cations (Fig. 3A and data not shown).

To verify these observations, we next utilized a cellular reporter system based on BWZ.36 cells [12], which were transduced to express a chimeric MICL/CD3ζ receptor. In these

reporter cells, ligand binding to the mMICL carbohydrate recognition domain (CRD) induces signalling through the CD3ζ cytoplasmic tail, and the induction of both β-galactosidase and IL-2 (Fig. 3B). Expression was confirmed by flow cytometry (data not shown). We then tested the functionality of this system by antibody cross-linking and were able to demonstrate specific induction of β-galactosidase and IL-2 under these conditions (Fig. 3C and data not shown). The mMICL/CD3ζ reporter cells did not respond to microbial ligands including gram-positive and gram-negative bacteria, or fungi, suggesting that the receptor does not recognise microbial ligands (data not shown).

We next co-cultured the reporter cells with single-cell suspensions isolated from various murine tissues, and looked for the induction of IL-2 (Fig. 3D). By comparison to the Dectin-1 reporter cells, significant induction of IL-2 was observed in the BM, thymus, heart, spleen, and kidney. In the BM, spleen and kidney, this response could be inhibited by the inclusion of soluble Fc-MICL, suggesting that this interaction was specific (Fig. 3E). These data therefore suggest that mMICL recognises an endogenous ligand(s), broadly expressed in many mouse tissues.

Concluding remarks

Here we have identified and characterised murine MICL and demonstrated that it is broadly expressed as a glycoslyated dimer and functions as an inhibitory receptor. We have also identified the presence of endogenous ligand(s) in multiple mouse tissues, suggesting that MICL plays a role in homeostasis.

Materials and methods

Generation of constructs

C-terminally HA-tagged mMICL was generated by PCR from mouse splenic cDNA, and cloning into pFB-neo (Stratagene, LaJolla, CA, USA) containing an HA-tag [4], using the following primers (forward 5′-GGGAGAATTCCACCATGTCTGAAGAAATTGTT-3′ and reverse 5′-CCGAGCTCGAGCCTGCTATCCTCTGGGAG-3′). Full-length HA-tagged human MICL was constructed as described [4].

To generate Fc-MICL recombinant protein, the mMICL ectodomain was fused with Fc fragment of human IgG. Briefly, the CRD of mMICL was amplified with following primers (forward 5′-ATACAGGTACCGCAACAGAAATGATAAAATCGAAT-3′ and reverse 5′-CCGAGGAATTCCCTGCTATCCTCTGGGAG-3′) and cloned into pSecTag2 vector (Invitrogen) containing the Fc fragment of human IgG [13]. Purification of Fc-MICL and FcDectin-1, from transduced HEK293T cells, was performed as previously described [13].

Receptor chimaeras consisting of the extracellular and transmembrane portions of Dectin-1 or MICL fused to the cytoplasmic tail of CD3ζ chain were generated by PCR and cloned in the pMX-IP retroviral vector (a gift from Toshio Kitamura, University of Tokyo, [14]) using the following primers: mDectin-1 (forward 5′-CCAACTAGTCCTTGGAGGCCCATTGCAGTGG-3′ and reverse 5′-TTTGCGGCCGCTTACAGTTCCTTCTCACAGAT-3′); mMICL (forward 5′-GATACTAGTCATTCACAGCAAAAAACAGTC-3′ and reverse 5′-GCGCGGCCGCGTAGCTACCTGCTATCCTCTGG-3′); and mCD3ζ (forward 5′-GTCTCGAGCCACCATGTTCAGCAGGAGTGCAG-3′ and reverse 5′-CGATTCTAGAGTAGGCTTCTGCCATCTTGTC-3′).

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All constructs were verified by sequencing, packaged into virions using HEK293T-based Phoenix ecotropic cells, as described previously [15], and used to transduce the various cell lines, as indicated.

Animals and cells

C57BL/6 mice and Wistar rats were obtained from the animal unit at the University of Cape Town. All animals were maintained under specific pathogen-free conditions and used at 6-10 weeks of age. All procedures were performed in accordance with ethical guidelines established by the University of Cape Town.

The isolation and preparation of peripheral blood and BM cells, elicited peritoneal cells and BMDC was performed essentially as described [16, 17]. Primary cells, NIH3T3 fibroblasts (ATCC no. TIB-71), RAW264.7 macrophages (ATCC no. TIB-71), BWZ.36 NFAT-lacZ cells (kindly provided by Wayne Yokoyama, Washington, USA), and HEK293T-based Phoenix ecotropic retroviral packaging cell line (a gift from Gary Nolan, Stanford University) were maintained in DMEM or RPMI medium, supplemented with 10% heat-inactivated FCS, 100 U/mL penicillin, 0.1 mg/mL streptomycin, and 2 mM L-glutamine, at 37°C with 5% CO_2. Transduced NIH3T3 and RAW263.7 cells were supplemented with 0.6 mg/mL G418 and transduced BWZ.36 NFAT-LacZ cells were supplemented with 100 μM 2-mercaptoethanol, 400 μg/mL hygromycin and 4 μg/mL puromycin.

For single-cell suspensions, mouse organs were removed aseptically and disaggregated by maceration through a 70-μm cell filter. Tissue debris was allowed to settle by sedimentation and the remaining cell suspension isolated. Following erythrocyte lysis, the cells were washed twice and resuspended in culture medium. All steps were performed at 4°C.

Generation of mAb against murine MICL

Wistar rats were immunized two to three times subcutaneously with purified Fc-MICL, in Titermax gold (Sigma, Aston Manor, South Africa). After a final intravenous boost, without adjuvant, rat splenocytes were harvested and fused with Y3 myeloma cells, as described [18]. Hybridoma supernatants were screened by flow cytometry of MICL transduced or control cell lines. Two mAb (206 and 309; both IgG1), which displayed similar characteristics by flow cytometry, were used interchangeably for further analysis. These antibodies were biotinylated as described [5].

Antibodies and flow cytometry

Live-cell three-colour flow cytometric analysis was performed as described previously [17]. The following antibodies were used in this study: CD11b-PerCPCy5, GR1-PE, MHCI-FITC, DX5-PE, CD3-PerCPCy5, B220-PerCPCy5 (all from BD Biosciences, Oxford, UK), CD19-PE, F4/80-PE, IgE-FITC (from Serotec, Oxford, UK), CD4-PE, CD8-FITC (from Caltag Laboratories, San Francisco, CA, USA), anti-HA (HA.11, Covance, Princeton, NJ, USA), 7/4-FITC, biotin-2A11 [18], biotin anti-human (HB3; [5]), anti-mouse (206 or 309, this study) MICL and rabbit anti-SHP-1/SHP-2 (Upstate, Biotechnologies, Lake Placid, NY, USA). In addition to the appropriately labelled isotype control antibodies, secondary reagents used included streptavidin-PE, streptavidin-APC (BD-Biosciences), donkey anti-rat IgG-PE (Jackson, West Grove, PA, USA), and anti-mouse HRP (Jackson). Flow cytometric analysis with Fc-MICL and Fc-Dectin-1 protein was performed using the Fc proteins at 10 μg/mL and detecting with PE-labelled donkey-anti human IgG (Jackson).

Cell stimulation and immunoprecipitation

To test the effect of TLR agonists on MICL surface expression, thioglycolate-elicited peritoneal neutrophils or macrophages, and BMDC were plated in 48-well plates and

stimulated for 40 h with agonists of TLR2/1 (Pam_3Csk_4, 100 ng/mL), TLR2/6 (Pam_2Csk_3, 100 ng/mL), TLR3 (poly I:C, 10 μg/mL), TLR4 (LPS, 100 ng/mL), TLR5 (flagellin, 100 ng/mL), TLR7 (loxoribine, 10 μg/mL), TLR9 (*E. coli K12* DNA, 10 μg/mL), all from (InvivoGen, San Diego, CA, USA), or zymosan (50 μg/mL, Sigma).

For ligand screening, reporter cells were co-cultured with isolated mouse primary cells, or 40 ng/mL PMA and 1.5 μg/mL ionomycin, for 20 h in 24-well plates. For antibody cross-linking, 24-well plates were pre-coated with sheep anti-mouse IgG (50 μg/mL), followed by isotype or anti-MICL mAb (10 μg/mL). Following stimulation, IL-2 released into the supernatants was quantified by ELISA (BD Pharmingen), and/ or the cells were stained for β-galactosidase expression. When required, FcMICL protein (10 μg/mL) was added to the reporter cells 1 h prior to the addition of primary mouse cells.

To test mMICL expression by biochemical approaches, normal and deglycosylated cellular extracts were prepared and analysed by SDS-PAGE and Western blotting, under reducing or non-reducing conditions, as previously described [5].

To identify MICL-associated phosphatases, murine MICL-HA expressing RAW264.7 macrophages were stimulated with pervanadate, for the times indicated. The cells were then were immediately lysed and proteins of interest were immunoprecipitated with anti-mouse MICL mAb (206; 10 μg /mL) and analysed under reducing conditions by Western blot, as previously described [4].

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Abbreviations

CRD	carbohydrate recognition domain
CTLR	C-type lectin-like receptor
hMICl	human MICL
MICL	myeloid inhibitory C-type lectin
mMICL	murine MICL

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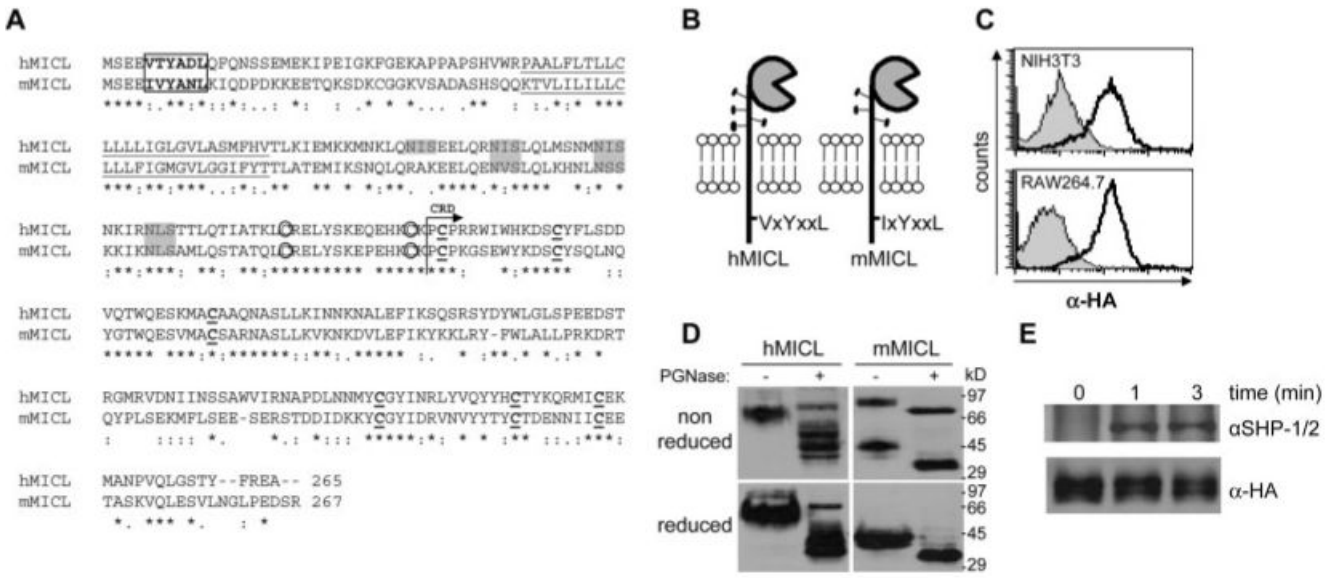

Figure 1.

Murine MICL is structurally and functionally similar to the human receptor. (A) ClustalW alignment of human and mouse amino acid sequences (GenBank accession numbers: NP_612210 and NP_808354). The ITIM sequences are in bold face and boxed, the transmembrane regions are underlined and the beginning of the CRD is indicated by arrow. The six conserved canonical cysteine residues of the CRD are in bold and underlined, while the two cysteines in stalk region, potentially involved in dimerisation, are ringed. Predicted *N*-glycosylation sites are highlighted in grey. "*", identity; ":", conservative substitutions; "." similar substitutions. (B) Cartoon representations of hMICL and mMICL. (C) Flow cytometric analysis demonstrating surface expression of mMICL-HA in transduced NIH-3T3 fibroblasts and RAW264.7 macrophages, as indicated. Anti-HA staining (bold line) and isotype control (grey filled histograms). (D) Anti-HA Western blot analysis of murine or human MICL-HA transduced NIH-3T3 cell extracts, under non-reducing (upper panels) or reducing (lower panels) conditions. Cell extracts were incubated with PNGase, as indicated. (E) Anti-phosphatase Western blotting analysis of immunoprecipitates prepared from mMICL-HA transduced RAW264.7 macrophages, stimulated with pervanadate for the times indicated. These data show representative results from at least three independent experiments.

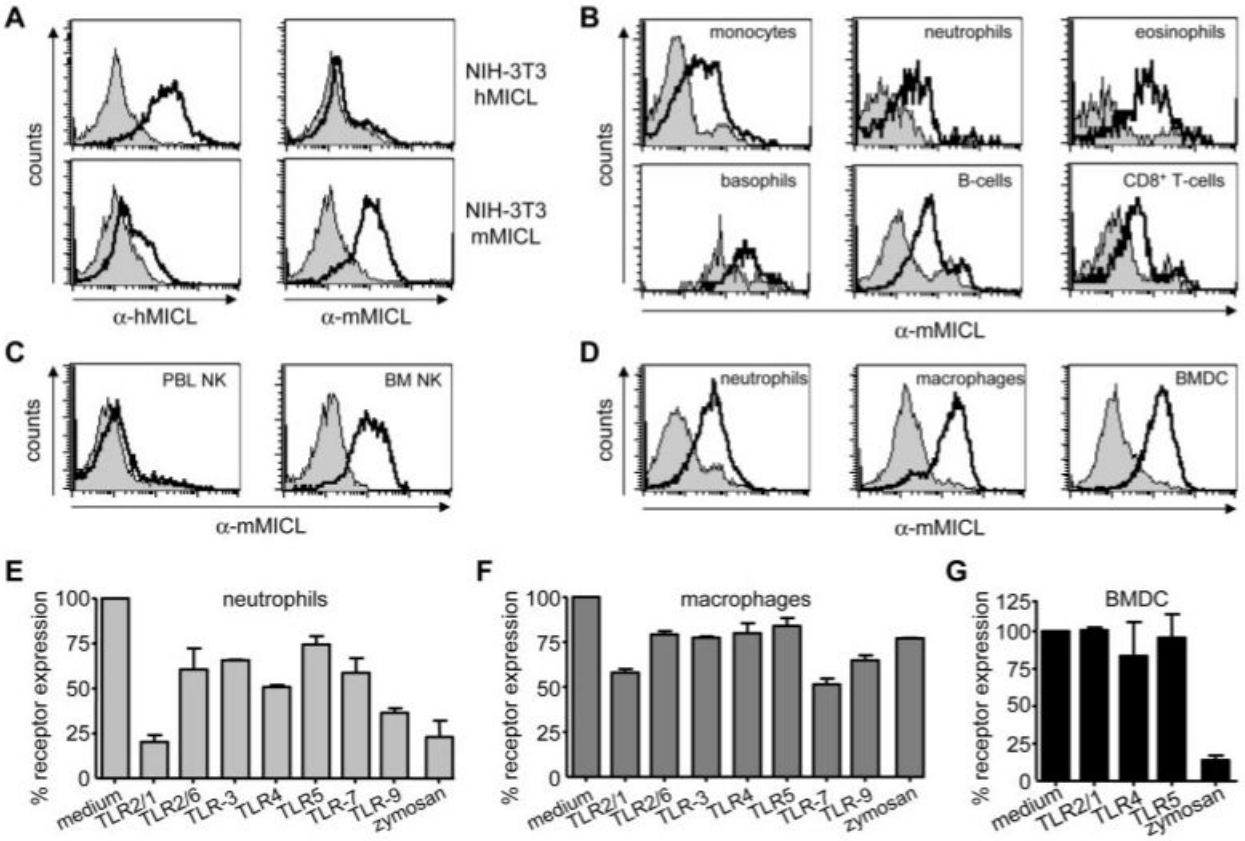

Figure 2.

Characterisation of mMICL expression and regulation on primary cells. (A) Flow cytometric analysis of transduced NIH3T3 fibroblasts stained with anti-hMICL or mMICL mAb, as indicated. Three-colour flow cytometric analysis of mMICL expression on (B) murine peripheral blood cells subsets, (C) peripheral blood (PBL) and BM NK cells, and (D) thioglycolate-elicited peritoneal neutrophils and macrophages, and BMDC. Dark lines indicate anti-mMICL staining and grey-filled histograms indicate isotype control staining. The histograms shown are representative of three independent experiments. Regulation of mMICL expression by various TLR agonists, as indicated, on (E) thioglycolate-elicited neutrophils and (F) macrophages, and on (G) BMDC. The results represent pooled data from two independent experiments and are presented as % receptor expression *versus* unstimulated controls ± SEM.

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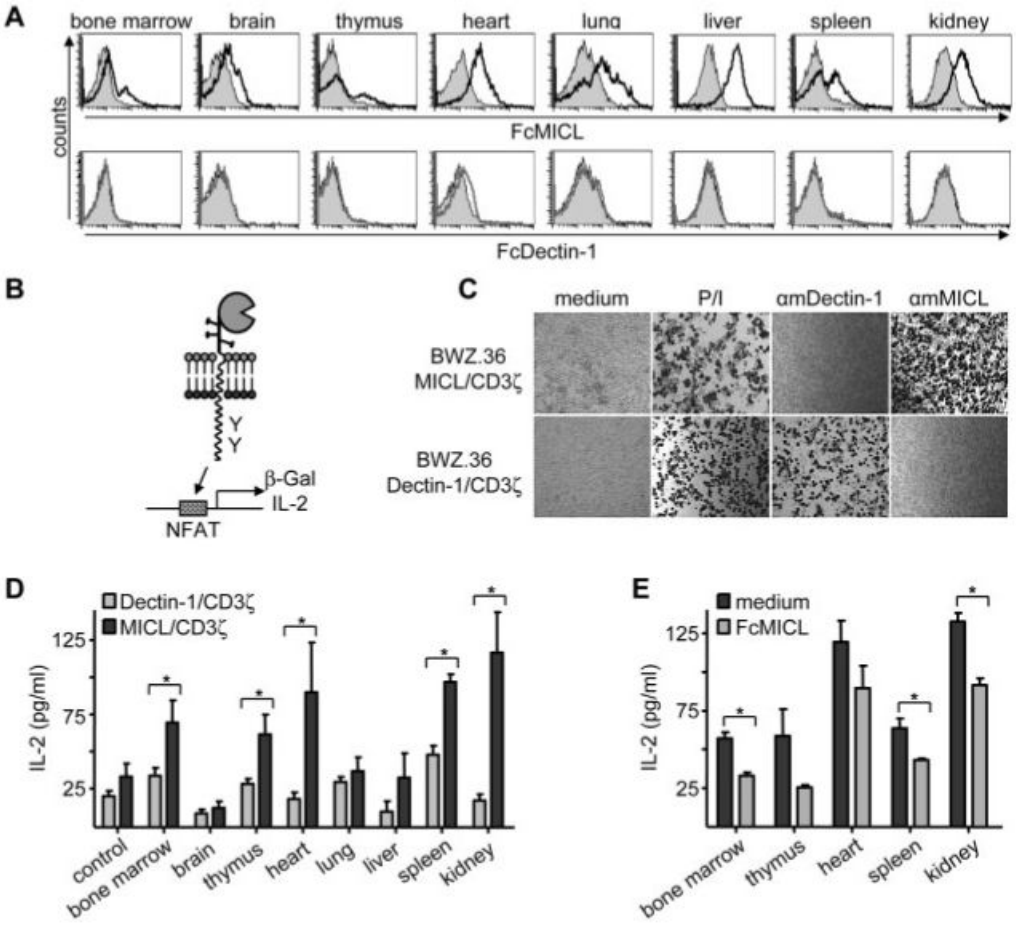

Figure 3.

mMICL recognises an endogenous ligand. (A) Flow cytometric analysis of single-cell suspensions isolated from various murine tissues, as indicated, and stained with Fc MICL (dark line, upper panels) or FcDectin-1 (grey line, lower panels). Secondary only staining is indicated by grey-filled histograms. The data are representative of three independent experiments. (B) Cartoon representation of the mMICL/CD3ζ chimaeric receptor and signal transduction pathway in BWZ.36 reporter cells. (C) X-gal staining of cells stimulated with immobilised anti-Dectin-1 or anti-mMICL antibodies, or PMA and ionomycin (P/I), as indicated, demonstrating the functionality of the reporter cell system. (D) IL-2 production from BWZ.36 reporter cells after co-culture with single-cell suspensions isolated from various murine organs, as indicated. (E) Inhibition of IL-2 production by inclusion of soluble FcMICL protein. The data are presented as mean ± SEM of data pooled from three (D) and two (E) independent experiments. *$p<0.05$.

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