

Clec12a Is an Inhibitory Receptor for Uric Acid Crystals that Regulates Inflammation in Response to Cell Death

Konstantin Neumann,¹ Mercedes Castiñeiras-Vilariño,¹ Ulrike Höckendorf,¹ Nicole Hanneschläger,¹ Simone Lemeer,² Danny Kupka,¹ Svenia Meyermann,¹ Maciej Lech,³ Hans-Joachim Anders,³ Bernhard Kuster,² Dirk H. Busch,⁴ Andreas Gewies,¹ Ronald Naumann,⁵ Olaf Groß,¹ and Jürgen Ruland^{1,*}

¹Institut für Klinische Chemie und Pathobiochemie, Klinikum rechts der Isar, Technische Universität München, 81675 Munich, Germany

²Lehrstuhl für Proteomik und Bioanalytik, Technische Universität München, Emil Erlenmeyer Forum 5, 85354 Freising, Germany

³Medizinische Klinik und Poliklinik IV, Klinikum der Universität München (LMU), 80336 München, Germany

⁴Institut für Medizinische Mikrobiologie, Immunologie und Hygiene, Technische Universität München, 81675 Munich, Germany

⁵Max Planck Institute of Molecular Cell Biology and Genetics, 01307 Dresden, Germany

*Correspondence: jruland@lrz.tum.de

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SUMMARY

Recognition of cell death by the innate immune system triggers inflammatory responses. However, how these reactions are regulated is not well understood. Here, we identify the inhibitory C-type lectin receptor Clec12a as a specific receptor for dead cells. Both human and mouse Clec12a could physically sense uric acid crystals (monosodium urate, MSU), which are key danger signals for cell-death-induced immunity. Clec12a inhibited inflammatory responses to MSU *in vitro*, and Clec12a-deficient mice exhibited hyperinflammatory responses after being challenged with MSU or necrotic cells and after radiation-induced thymocyte killing *in vivo*. Thus, we identified a negative regulatory MSU receptor that controls noninfectious inflammation in response to cell death that has implications for autoimmunity and inflammatory disease.

INTRODUCTION

Several families of germline-encoded pattern-recognition receptors (PRRs) can sense damage-associated molecular patterns (DAMPs), which are exposed or released upon cell death under sterile conditions. Subsequent immune signaling is important for the maintenance of tissue homeostasis but can also drive immune pathology when exacerbated (Kaczmarek et al., 2013; Kuraishy et al., 2011; Zitvogel et al., 2010). Although DAMPs can induce similar immune responses as microbial pathogen-associated molecular patterns (PAMPs), the positive and negative regulation of sterile inflammatory responses to cell death is not well understood.

One important class of PRRs that recognizes both DAMPs as well as PAMPs is the myeloid C-type lectin receptors (CLRs), which are organized in the Dectin-1 and Dectin-2 gene clusters located within the natural killer gene complex. These receptors are type II transmembrane proteins with single extracellular C-type lectin domains that can sense a diverse array of distinct ligands. A subset of these CLRs regulates immunity by activating

the tyrosine kinase Syk through immunoreceptor tyrosine-based activation motifs (ITAMs) or ITAM-like motifs. Subsequent Syk signaling can drive inflammation through the production of reactive oxygen species (ROS) and generation of cytokines and chemokines (Hardison and Brown, 2012; Sancho and Reis e Sousa, 2012, 2013; Wevers et al., 2013). Examples of Syk-activating CLRs that sense DAMPs under noninfectious conditions are Clec9a (DNGR-1), which detects filamentous actin exposed by dead cells (Ahrens et al., 2012; Zhang et al., 2012), and Mincle (Clec4E), which recognizes the spliceosome-associated protein SAP-130 (SF3B3) upon cell damage (Yamasaki et al., 2008).

To prevent inappropriate immune responses, cell-activating Syk signals are, in general, counterbalanced by negative regulatory receptors containing immunoreceptor tyrosine-based inhibitory motifs (ITIMs) (Long, 2008). Clec12a (M1CL, DCAL2, or CLL-1) is a poorly characterized ITIM-containing CLR encoded within the Dectin-1 cluster. This receptor binds unknown endogenous ligands and is widely expressed on innate immune cells, including neutrophils, monocytes, macrophages, and dendritic cells. Its intracellular ITIM sequence recruits the tyrosine phosphatases SHP-1 and SHP-2 to negatively regulate Syk signaling (Marshall et al., 2004). Because the expression of Clec12a can be downregulated under inflammatory conditions, this receptor might be involved in the control of myeloid cell activation (Marshall et al., 2006; Pyz et al., 2008). However, the physiological functions of Clec12a and the identities of its ligands are unclear.

Here, we report that Clec12a senses dead cells, and we identify uric acid crystals (MSU) as specific ligands of Clec12a. By using Clec12a-deficient mice, we demonstrate that Clec12a inhibited Syk-dependent neutrophil activation by MSU *in vitro* and immune cell recruitment after MSU challenge or sterile cell death *in vivo*. These findings provide a mechanism for the negative regulation of noninfectious inflammation and new insights into how the innate immune system responds to uric acid crystals.

RESULTS

Clec12a Is a Receptor that Recognizes Dead Cells

To identify Clec12a ligands, we created CLR fusion proteins consisting of murine or human Clec12a extracellular domains and

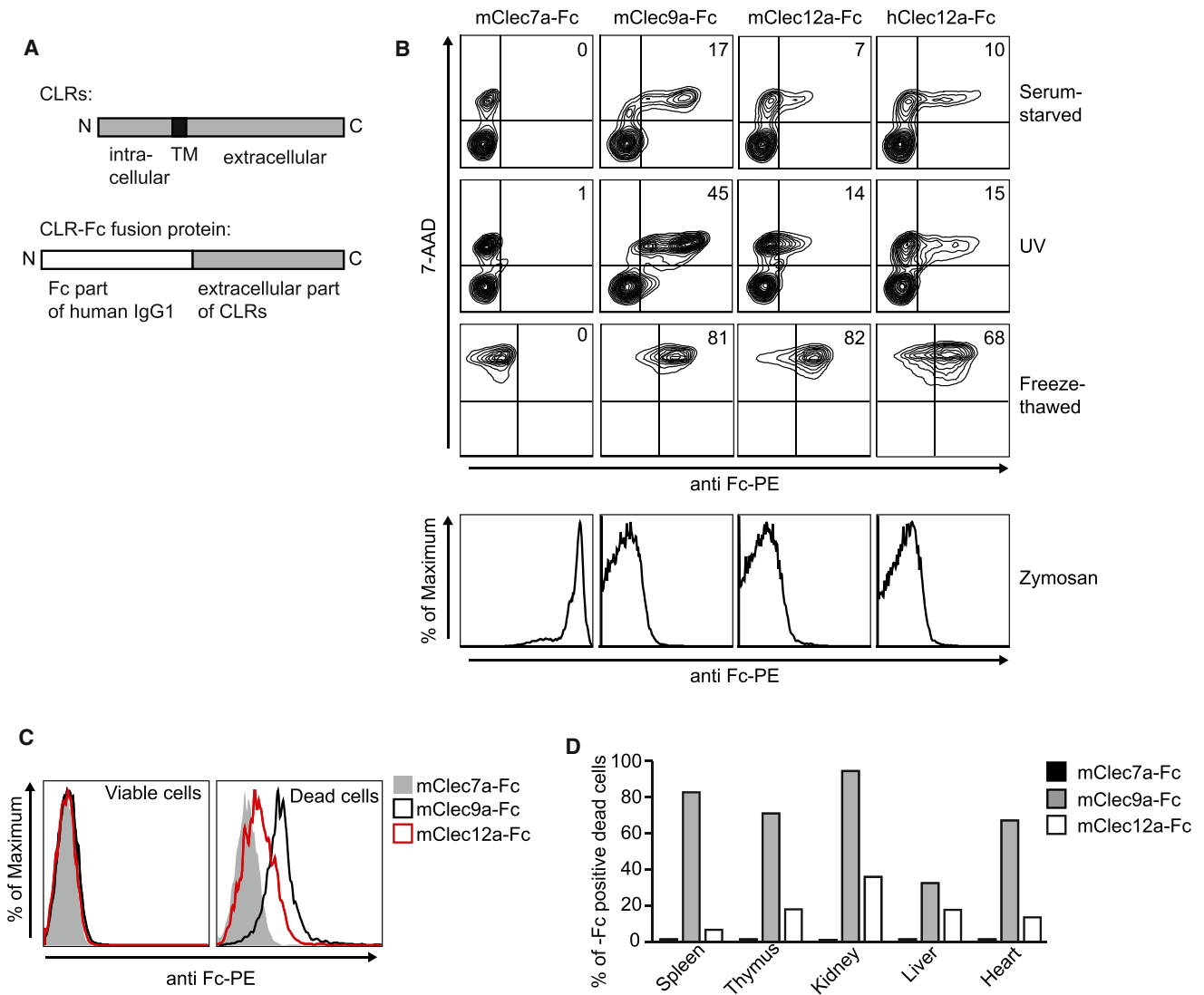


Figure 1. Clec12a Binds to Dead Cells

(A) Schematic representation of recombinant CLR-Fc fusion proteins.

(B) HEK293 cells were killed by serum starvation or UV light, followed by incubation for 16 hr at 37°C or freeze thawing, and stained with indicated CLR-Fc fusion proteins and anti-human Fc-PE and 7-AAD and were then analyzed by flow cytometry.

(C) Mouse thymocytes were left untreated or subjected to a freeze-thaw cycle, stained, and analyzed as in (B). Untreated (viable) cells were gated on 7-AAD-negative cells, and freeze-thawed (dead) cells were gated on 7-AAD-positive cells.

(D) The indicated tissues were mechanically disrupted using a cell strainer, and thymocytes and splenocytes were subjected to a freeze-thaw cycle to kill the cells and then stained and analyzed as in (B). The fraction of dead cells (7-AAD⁺) binding to CLR-Fc fusion proteins is shown. All data are representative of at least three independent experiments.

the Fc portion of human immunoglobulin G (IgG) (mClec12a-Fc, hClec12a-Fc) (Figure 1A). Analogous fusions were created for the β -glucan receptor, Clec7a (Dectin-1) (Brown and Gordon, 2001), or the dead cell receptor, Clec9a (Sancho et al., 2009). To test whether Clec12a would also bind to dead cells, we killed HEK293 cells by serum starvation, UV radiation, or a freeze-thaw cycle and then stained these cells with CLR-Fc proteins with 7-AAD to discriminate dead cell. Flow cytometry analysis revealed that both murine and human Clec12a-Fc specifically recognized 7-AAD⁺ cells but did not bind to viable HEK293 cells (Figure 1B). As expected, Clec9a-Fc also bound to dead cells but not to

viable cells, and Clec7a-Fc bound only to the β -glucan-rich fungal cell wall preparation, zymosan.

Next, we costained primary thymocytes with the CLR-Fc proteins and 7-AAD. Similar to Clec9a-Fc, the Clec12a-Fc fusions again specifically bound 7-AAD-positive dead cells, whereas Clec7a-Fc did not recognize dead or viable thymocytes (Figure 1C). Next, we meshed murine spleens, kidneys, livers, and hearts and then stained the cell suspensions with the CLR-Fc fusions and 7-AAD. We observed that Clec12a recognized dead cells from all of the tested tissues, with the highest levels of binding observed for kidney tissue (Figure 1D). Thus,

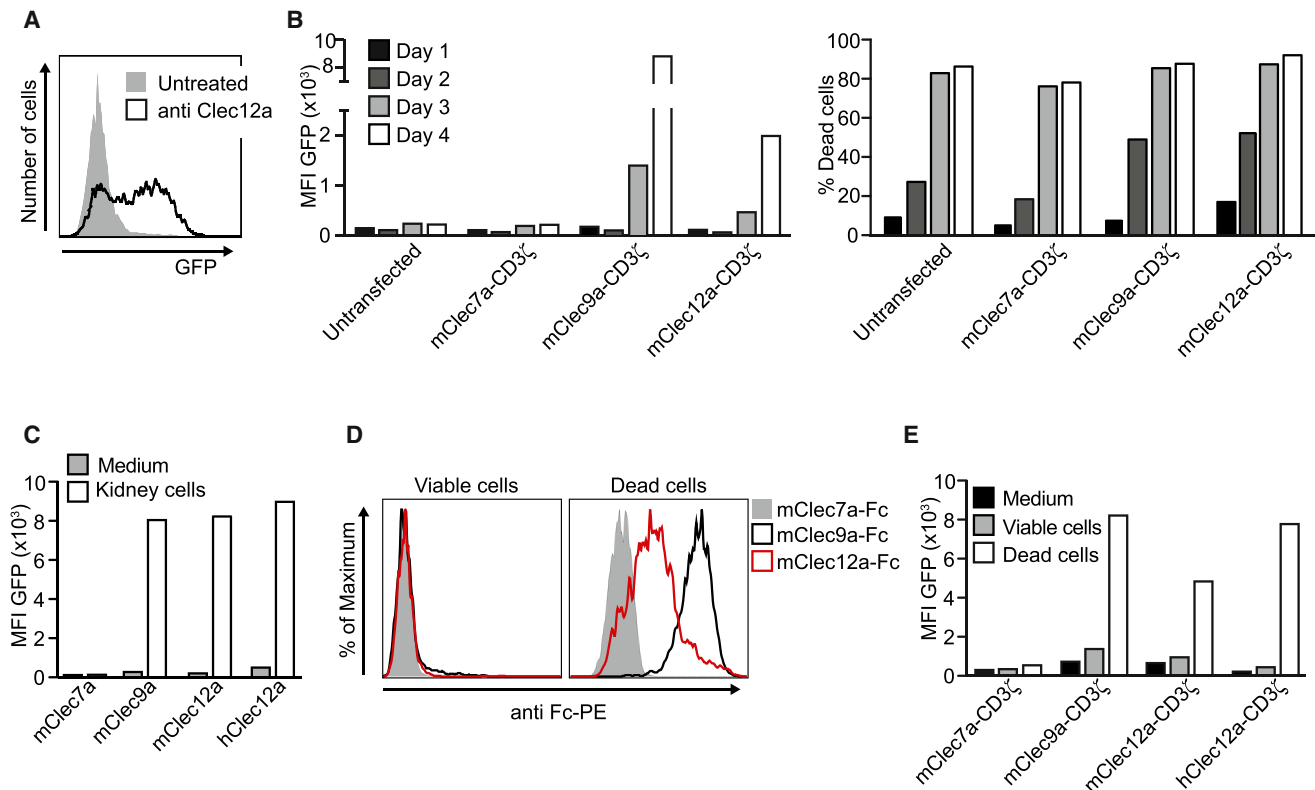


Figure 2. Dead Cells Activate Clec12a

(A) Clec12a-CD3 ζ -expressing A5 NFAT-GFP reporter cells were incubated with or without polyclonal anti-mouse Clec12a rabbit serum. Eighteen hours later, the viable cells (7-AAD⁻) were analyzed for GFP expression by flow cytometry. (B) A5 reporter cells expressing the indicated CLR-CD3 ζ fusion proteins were cultured for 4 days without changing the growth medium. GFP fluorescence of the live cells (left panel) and the fraction of dead (7-AAD⁺) cells (right panel) were analyzed each day by flow cytometry. MFI, mean fluorescence intensity. (C) A5 reporter cells expressing the indicated CLR-CD3 ζ fusion proteins were incubated with mouse kidney cells, which were isolated by mechanical disruption using a cell strainer. Twenty hours later, the GFP expression of viable (7-AAD⁻) reporter cells was analyzed by flow cytometry. (D) Mouse kidney cells were isolated by collagenase digestion of kidney tissue. The cells were either left untreated or killed by freeze-thawing. The cells were stained with indicated CLR-Fc fusion proteins and anti-human Fc-PE and 7-AAD and analyzed by flow cytometry. (E) Both cell preparations from (D) were added to A5 reporter cells expressing the indicated CLR-CD3 ζ fusion proteins. Twenty hours later, the A5 reporter cells were analyzed by flow cytometry for GFP expression. All data are representative of at least three independent experiments.

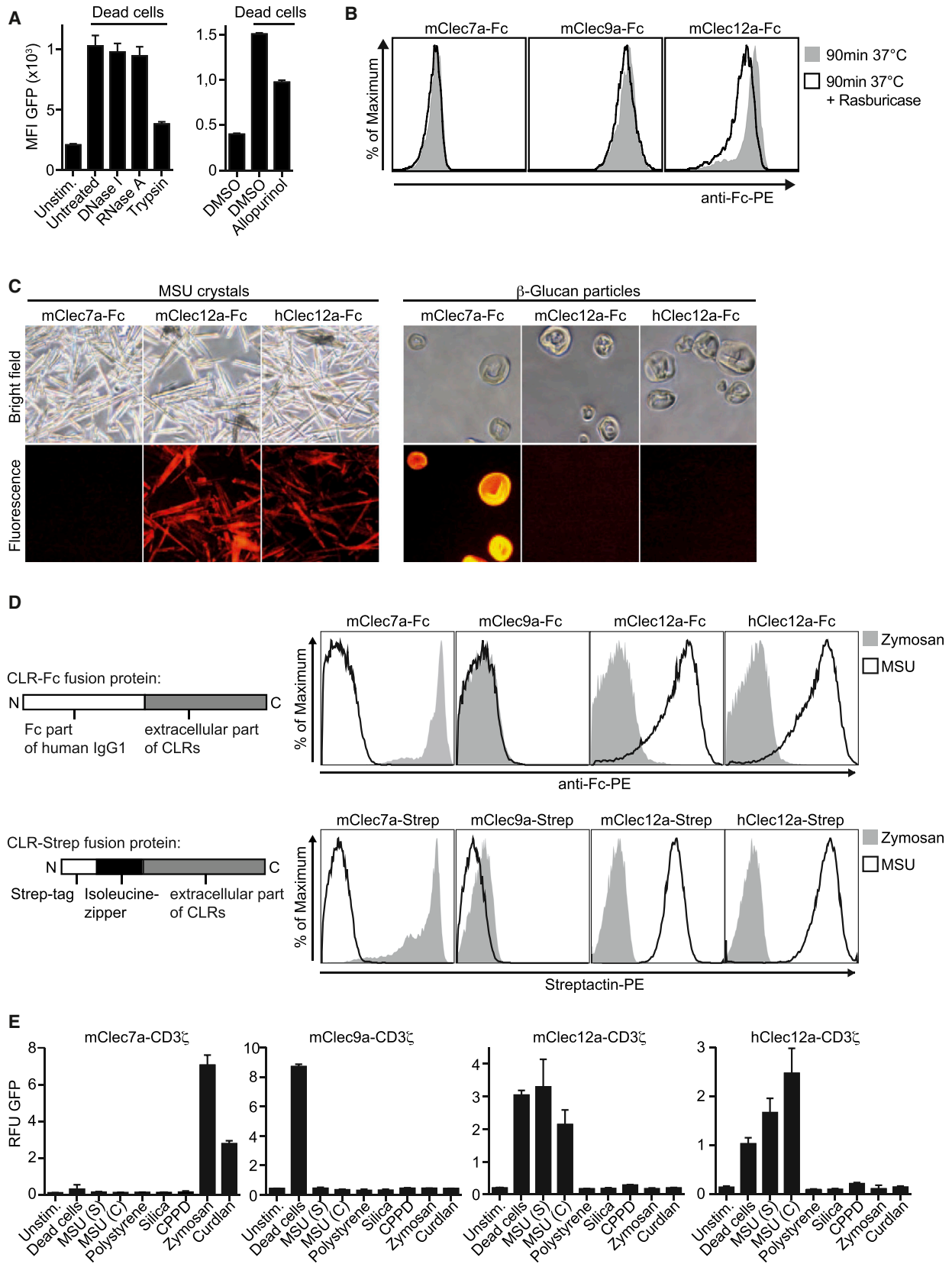
Clec12a binds structures that are formed or exposed after cell death.

Dead Cells Can Crosslink Clec12a

After identifying the specific binding of Clec12a to dead cells, we determined whether dead cells would also be able to crosslink Clec12a on the surface of cells. Therefore, we created cellular reporter systems based on the T cell hybridoma A5 (Bot et al., 1996), which contains NFAT-controlled green fluorescent protein (GFP) expression cassettes, that were stably transfected with fusions of the indicated CLR extracellular and transmembrane domains (Clec7a, Clec9a, mClec12a, or hClec12a) coupled to the cytoplasmic signaling tail of CD3 ζ . The ligation of the CLR-CD3 ζ fusion proteins signals NFAT activation, which subsequently results in GFP expression (Andersen et al., 2001). We stimulated mClec12a-CD3 ζ reporter cells as a positive control with a crosslinking mClec12a antibody. This treatment induced GFP fluorescence in the reporter cells (Figure 2A). Culturing reporter cell lines for a prolonged period of time without media exchange also induced GFP fluorescence in the Clec12a-

CD3 ζ - and Clec9a-CD3 ζ -expressing cells but not in the Clec7a-CD3 ζ reporters. The increase in the number of GFP-positive cells paralleled the increasing numbers of dead cells in the cultures (Figure 2B), suggesting that dead cells triggered Clec12a crosslinking.

Next, we directly stimulated fresh reporter cells with mechanically disrupted mouse tissue. We also observed the activation of GFP expression in Clec12a-CD3 ζ and Clec9a-CD3 ζ reporter cells but not in Clec7a-CD3 ζ reporter cells (Figure 2C; data not shown). Again, the most robust GFP activation was observed upon stimulation with kidney tissue. To directly test whether the presence of dead cells was responsible for the stimulation of Clec12a, we prepared viable kidney cell suspensions by treating the organs with collagenase. Then, we killed a fraction of the cells in vitro. Consistent with above findings, both Clec12a and Clec9a only bound to dead cells (Figure 2D). Subsequent reporter cell stimulation demonstrated that only the dead cell fraction, but not the viable cell fraction, specifically activated mClec12a-CD3 ζ and hClec12a-CD3 ζ signaling, as well as Clec9a-CD3 ζ signaling (Figure 2E). Together, these experiments



(legend on next page)

demonstrate that Clec12a functions as an immune receptor that recognizes endogenous ligands that are generated or exposed upon cell death and that Clec12a therefore shares some similarities with Clec9a and Mincle.

Clec12a Is a Receptor for Uric Acid Crystals

Several structurally unrelated molecules alert the immune system to dying cells. These DAMPs include nucleic acids and proteins, including HMGB1, as well as uric acid crystals (Kaczmarek et al., 2013; Zitvogel et al., 2010). To get insights into identities of Clec12a ligands, we treated killed kidney cells with DNase or RNase to degrade nucleic acids, with trypsin to digest proteins, or with allopurinol to inhibit uric acid synthesis. Interestingly, only the protein digestion or pharmacological inhibition of uric acid synthesis reduced Clec12a-CD3 ζ -mediated GFP induction (Figure 3A). To test the role of uric acid for Clec12a binding to dead cells we incubated freeze-thawed cells with Rasburicase, an enzyme that oxidizes uric acid to allantoin. This treatment specifically reduced Clec12a-Fc binding to dead cells, while Clec9a-Fc binding was not affected (Figure 3B), indicating that Clec12a binding to dead cells is at least in part dependent on uric acid.

The enzyme xanthine oxidase produces uric acid during purine catabolism in a process that continues after cell injury (Kono et al., 2010). Uric acid is soluble within intact cells, but when cells lose their membrane integrity and high uric acid concentrations come into contact with high extracellular concentrations of sodium ions, monosodium urate (MSU) microcrystals can form. To test the hypothesis that Clec12a could directly bind MSU crystals, we incubated mClec12a-Fc, hClec12a-Fc, or mClec7a-Fc fusion proteins with MSU crystals or β -glucan particles (curdlan) as a control. Surprisingly, by using fluorescent microscopy, we observed that both human and murine Clec12a-Fc fusions bound specifically to uric acid crystals, whereas mClec7a-Fc selectively recognized β -glucan, as expected (Figure 3C).

Next, we utilized a flow cytometer to further characterize the MSU-Clec12a interaction. Again, both mClec12a-Fc and hClec12a-Fc specifically bound to MSU crystals, whereas mClec7a-Fc selectively interacted with zymosan, and Clec9a-Fc did not bind to MSU or zymosan (Figure 3D). To validate these results, we created an additional set of independent CLR fusion proteins by coupling the ligand-binding domains of hClec12a, mClec12a, mClec7a, or mClec9a to strep-tagged isoleucine zipper motifs (CLR-Strep) (Stark et al., 2005). We also observed

that hClec12a-Strep and mClec12a-Strep bound specifically to MSU crystals, whereas mClec7a-Strep bound to zymosan, and mClec9a-Strep did not bind MSU or zymosan (Figure 3D), demonstrating that the extracellular domains of both human and murine Clec12a can specifically bind MSU. We tested monoclonal antibodies raised against Clec12a and found a clone that interferes with binding of recombinant Clec12a to MSU (see Figure S1 available online), suggesting that Clec12a contains a specific binding site for MSU close to the epitope of this antibody.

To test whether MSU binding leads to Clec12a crosslinking, we stimulated CLR-CD3 ζ -expressing NFAT-GFP reporter cells (Figure 3E). As anticipated, Clec7a-CD3 ζ reporters activated GFP expression only upon stimulation with zymosan or curdlan, which is a selective Clec7a agonist, whereas Clec9a-CD3 ζ induced GFP fluorescence only upon stimulation with dead cells. In contrast, mClec12a-CD3 ζ and hClec12a-CD3 ζ induced high NFAT-GFP expression not only upon dead cell stimulation but also upon selective stimulation with MSU crystals from two different sources (Figure 3E). We also stimulated the cells with other crystalline structures, such as silica and calcium pyrophosphate dihydrate (CPPD), which are known to activate immune cells (Martinon et al., 2006). In contrast to MSU, these crystals did not activate GFP expression in any of the reporter cells. We therefore conclude that both murine and human Clec12a can act as specific immune receptors for MSU and that MSU can trigger Clec12a crosslinking in vitro.

Clec12a Downmodulates Neutrophil Activation in Response to MSU

To investigate the physiological functions of Clec12a in MSU sensing and dead cell recognition, we generated Clec12a-deficient (*Clec12a*^{-/-}) mice (Figure S2; Figure 4A). These animals were viable, and they exhibited regular myeloid and lymphoid immune cell compartments in the bone marrow, spleen, and lymph nodes. There were no significant differences in the frequencies and numbers of B and T cells, neutrophils, monocytes, or macrophages between wild-type (WT) and *Clec12a*^{-/-} mice, and these animals exhibited no obvious signs of pathology under homeostatic conditions (Figure 4B; data not shown).

MSU can activate Syk signaling in immature myeloid cells, neutrophils, or dendritic cells via a direct lipid membrane interaction leading to ITAM receptor crosslinking or through a still-undefined mechanism that involves the receptors CD16 and CD11b

Figure 3. Clec12a Is a Receptor for Uric Acid Crystals

(A) Freeze-thawed mouse kidney cells were incubated with RNase A (10 μ g/ml), DNase I (50 U/ml), trypsin (100 μ g/ml), or 1 mM allopurinol for 1 hr at 37°C and subsequently added to Clec12a-CD3 ζ reporter cells. Twenty hours later, the GFP fluorescence of live (7-AAD⁻) A5 reporter cells was analyzed by flow cytometry. The means of three dead cell preparations + SD are shown.

(B) HEK293 cells were killed by freeze-thawing and incubated with or without 0.3 mg/ml Rasburicase, stained with indicated CLR-Fc fusion proteins and anti-human Fc-PE and analyzed by flow cytometry.

(C) The indicated Fc fusion proteins were incubated with uric acid crystals (MSU) or curdlan (β -glucan) particles. Bound CLR-Fc fusion proteins were stained with anti-human Fc-CF568, and the particles were analyzed by fluorescence microscopy.

(D) MSU or zymosan was stained with CLR-Fc fusion or CLR-Strep fusion proteins, as indicated. Bound Fc fusion proteins were detected with anti-human Fc, and Strep-tagged proteins were detected with streptactin-PE using a flow cytometer.

(E) A5 reporter cells expressing the indicated CLR-CD3 fusions were incubated with self-crystallized (s) or commercial (c) MSU crystals (250 μ g/ml), zymosan (250 μ g/ml), curdlan (500 μ g/ml), silica (500 μ g/ml), calcium pyrophosphate dihydrate (CPPD) (250 μ g/ml), polystyrene beads (250 μ g/ml), or dead kidney cells. GFP fluorescence was measured 16 hr later using a plate reader. Means + SD of triplicates are shown. RFU, relative fluorescence units. All data are representative of at least three independent experiments.

See also Figure S1.

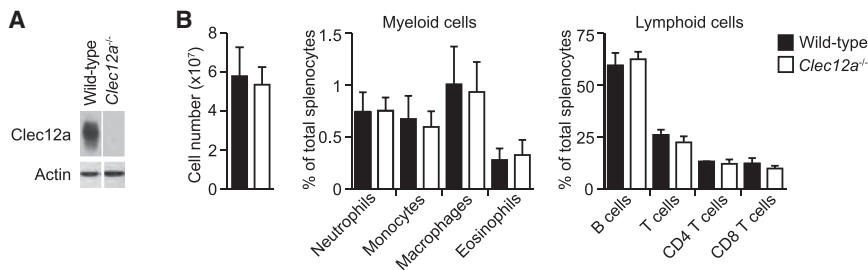


Figure 4. Generation of Clec12a Deficient Mice

(A) Bone marrow from WT or Clec12a-deficient (*Clec12a*^{-/-}) mice was subjected to immunoblot analysis using anti-Clec12a and anti-actin antibodies.

(B) Splenocytes from WT or Clec12a-deficient mice were stained for myeloid (Gr-1, Ly-6b.2, CD11b and F4/80) and lymphoid markers (B220, TcR, CD4 and CD8) and analyzed by flow cytometry. The means of three mice per genotype + SD are shown.

See also Figure S2.

(Barabé et al., 1998; Desaulniers et al., 2001; Landis et al., 2002; Ng et al., 2008). Consistently, pharmacological Syk inhibition blocked the generation of ROS by MSU, as well as by zymosan in bone-marrow cells (Figure 5A; data not shown). Because Clec12a, as an ITIM receptor, has been shown to downmodulate Syk signals (Marshall et al., 2004), we next tested the Syk-mediated activation of Clec12a-deficient bone-marrow cells by using ROS generation as a functional readout. *Clec12a*^{-/-} bone-marrow cells produced regular dose-dependent amounts of ROS upon Dectin-1 triggered Syk activation after zymosan or curdlan treatment (Figures 5B and 5C), which do not bind to Clec12a, as shown above. In contrast, MSU-induced ROS generation was substantially increased in the absence of Clec12a (Figures 5B and 5C), which is consistent with the engagement of Clec12a by MSU and the negative regulatory role of this receptor. To further characterize the role of Clec12a in the negative regulation of MSU-mediated ROS production, we purified neutrophils from *Clec12a*^{-/-} and control animals. In line with the results above, we observed a specific increase in MSU-triggered ROS production, but not in zymosan-induced ROS generation in purified *Clec12a*^{-/-} neutrophils (Figure 5D; data not shown), demonstrating that Clec12a specifically downmodulates this MSU response in vitro. ROS production in neutrophils requires phosphorylation of the NADPH oxidase subunit p40^{phox} (also called NCF4) (Chessa et al., 2010). We tested and found enhanced phosphorylation of p40^{phox} specifically after MSU treatment, but not after zymosan stimulation of Clec12a-deficient cells (Figure 5E; Figure S3), demonstrating that Clec12a inhibits MSU-induced signaling to p40^{phox} phosphorylation.

Clec12a Counteracts Inflammatory Reactions to MSU and Cell Death In Vivo

To study the function of Clec12a signaling in MSU-controlled inflammatory pathways in vivo, we injected MSU crystals into the peritoneum of *Clec12a*^{-/-} mice. Compared with WT animals, the *Clec12a*^{-/-} animals experienced a significantly increased neutrophil influx upon treatment, demonstrating negative regulatory functions for Clec12a after MSU sensing in the intact organism. The fact that LPS-induced neutrophil recruitment was not significantly altered by Clec12a disruption excludes nonspecific neutrophil recruitment defects in these mice (Figure 6A). Subsequently, we investigated the function of Clec12a in the inflammatory responses to dead cells by first injecting killed kidney cells into Clec12a-deficient mice. We observed that these animals had an increased inflammatory reaction, which was measured by a greatly increased number of infiltrating

neutrophils compared to the WT mice (Figure 6B). In an alternative model of dead-cell-induced sterile inflammation, we X-ray irradiated mice with 1 Gy to induce double-positive thymocyte killing. In WT animals, this treatment results in the infiltration of neutrophils into the thymus, which is positively regulated by the Syk-coupled CLR MinCLE (Uchimura et al., 2000; Yamasaki et al., 2008). In the absence of Clec12a, we found that the infiltration of neutrophils was significantly enhanced (Figure 6C). Moreover, the expression of the chemokines CXCL1 and CXCL10 and the inflammatory cytokine tumor necrosis factor alpha (TNF- α) was increased in Clec12a-deficient animals (Figure 6D), collectively demonstrating a negative regulatory role for Clec12a in these sterile inflammatory reactions in vivo.

DISCUSSION

Our biochemical and genetic results define Clec12a as an inhibitory innate immune receptor for dead cells that recognizes the danger signal MSU and regulates inflammatory responses. While searching for specific Clec12a ligands, we observed that the activation of Clec12a signaling can be downmodulated by a proteinase pretreatment of killed cells before reporter cell stimulation. Subsequently, we discovered that allopurinol also inhibits the response and that both human and murine Clec12a can physically bind to MSU crystals. Because the generation of uric acid after cell death is an active process that requires xanthine oxidase and that continues after cell injury (Kono et al., 2010), it is likely that a proteinase treatment of killed cells would inhibit this mechanism. However, we predict that Clec12a, in addition to MSU, also binds protein ligands that are exposed on dead cells. However, to date, we have failed to identify specific protein agonists that can stimulate Clec12a (data not shown). Because the extracellular domains of both human and murine Clec12a can bind to dead cells or MSU and the signaling active fusion proteins of both human and mouse Clec12a induce reporter cell activation after stimulation by dead cells or MSU, this function of Clec12a is considered to be conserved among species.

The identification of Clec12a as a specific receptor for MSU is, to our knowledge, the first description of a transmembrane immune receptor for which the direct binding to a crystal has been shown. However, crystal-binding proteins have been known for a long time; the most astounding are the antifreeze proteins that are expressed in a variety of organisms, from bacteria to fish (Raymond and DeVries, 1977). The antifreeze proteins adsorb to ice crystals, thereby blocking crystal growth.

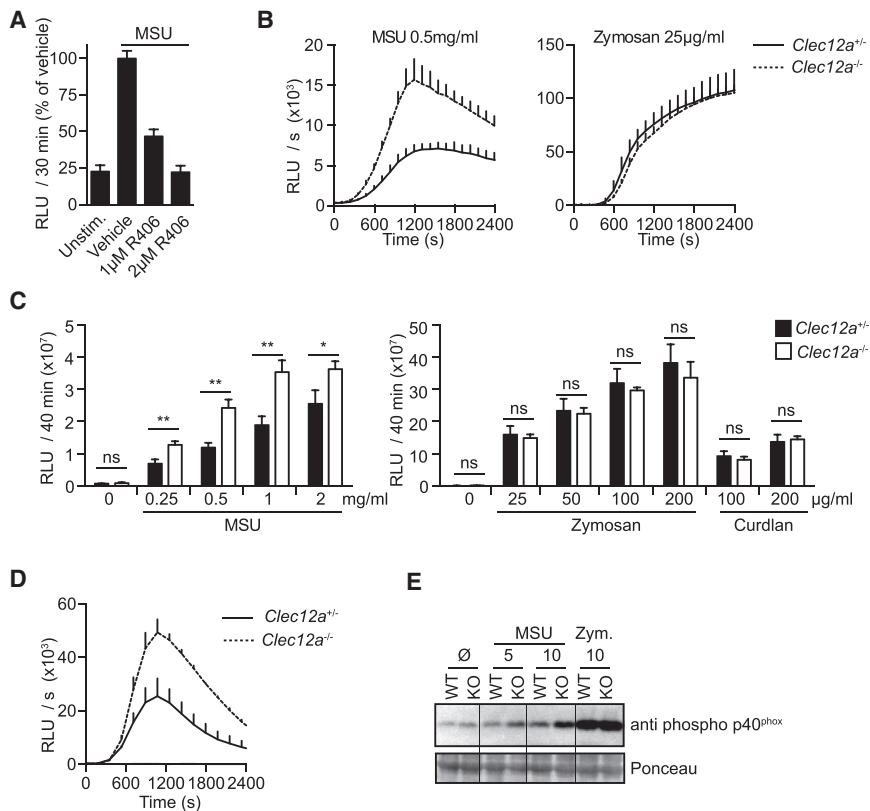


Figure 5. Clec12a Inhibits Neutrophil Activation by MSU

(A) Bone-marrow cells from WT mice were pre-incubated with vehicle, 1 or 2 μM R406, and then stimulated with 500 $\mu\text{g}/\text{ml}$ MSU crystals in the presence of 50 μM luminol. Emitted light was integrated over 30 min. The means of triplicates + SD from one experiment, representative of three independent experiments is shown. RLU, relative light units.

(B) Clec12a-expressing (*Clec12a*^{+/+}) and Clec12a-deficient bone-marrow cells were incubated with 500 $\mu\text{g}/\text{ml}$ MSU crystals (left panel) or 25 $\mu\text{g}/\text{ml}$ zymosan (right panel) in the presence of 50 μM luminol. Emitted light was recorded in 2 min intervals for 40 min. The means of cells from three individual mice per genotype + SD are shown.

(C) Integrated light units emitted within 40 min from bone-marrow cells left unstimulated or stimulated with the indicated concentrations of MSU (left panel) or the indicated concentrations of zymosan or curdlan (right panel). The means of three mice per genotype + SD are shown. Data are representative of two independent experiments.

(D) Purified neutrophils from *Clec12a*^{+/+} or *Clec12a*^{-/-} mice were stimulated with 500 $\mu\text{g}/\text{ml}$ MSU and analyzed as in (B). The means of triplicates + SD are shown. Data are representative of three independent experiments.

(E) Purified neutrophils from *Clec12a*^{+/+} or *Clec12a*^{-/-} mice were stimulated with 500 $\mu\text{g}/\text{ml}$ MSU or 200 $\mu\text{g}/\text{ml}$ Zymosan for the indicated times. Lysates were subjected to immunoblot analysis by using anti phospho-p40^{phox} (Thr154). Equal loading was verified by Ponceau staining. See also Figure S3.

Intriguingly, several of these antifreeze molecules consist of a single C-type lectin domain that is similar to mammalian CLRs (Zelensky and Gready, 2005). These results indicate that this domain can, in principle, bind crystalline surfaces. In the future, it will be important to identify the exact structural determinants within Clec12a that mediate MSU binding. Such results would provide important general insights into the mechanisms of how the innate immune system interacts with crystalline surfaces.

Prior to the identification of uric acid as a DAMP that is released by or associated with killed cells (Shi et al., 2003), MSU was long recognized as the causative agent of gout. High systemic uric acid levels are also frequently associated with cardiovascular disease or diabetes mellitus (Rock et al., 2013). In gout, relatively large MSU crystals form in the synovial fluid of joints and in other locations, such as the kidney, leading to inflammation. The phagocytosis of MSU particles and subsequent lysosomal damage triggers the activation of the NLRP3 inflammasome, which induces the maturation and secretion of the proinflammatory cytokine IL-1 β (Hornung et al., 2008; Martinon et al., 2006). In experiments with isolated neutrophils and macrophages, we did not observe the effects of the Clec12a deletion on MSU-induced IL-1 β production (data not shown), indicating that the NLRP3 inflammasome pathway is not directly affected by Clec12a signaling. Thus, the mechanisms that drive NLRP3 activation after the phagocytosis of MSU particles presumably operate in parallel to other pathways emanating from Syk (Kool et al., 2011).

As indicated above, MSU activates proinflammatory responses not only by triggering NLRP3 but also by directly activating Syk at the cell surface of innate immune cells through ITAM-coupled CD11b/CD16 on neutrophils or induced lipid sorting and the subsequent formation of cholesterol rich regions that recruit ITAM-containing transmembrane adapters on dendritic cells (Barabé et al., 1998; Ng et al., 2008). In vivo, MSU is also found opsonized by complement components and antibodies, which can trigger Syk-coupled complement receptors and Fc receptors, respectively (Cherian and Schumacher, 1986; Terkeltaub et al., 1983). Our results demonstrating that Clec12a inhibits Syk-dependent ROS production induced by MSU suggest that Clec12a can counterbalance these Syk activating ITAM signals. Consistent with this finding and our discovery that Clec12a directly binds MSU crystals, a current independent study reported enhanced tyrosine phosphorylation, Ca²⁺ signaling and IL-8 production upon MSU stimulation in a human neutrophil cell line after siRNA knockdown of Clec12a (Gagné et al., 2013). Similarly, down-regulation of Clec12a with a monoclonal antibody enhanced the MSU-induced activation of human neutrophils. We found that this antibody (clone 50C1) also reduces Clec12a binding to MSU crystals (Figure S1), indicating that this antibody enhances neutrophil responses not merely by down-regulating Clec12a but directly interferes with Clec12a ligand binding.

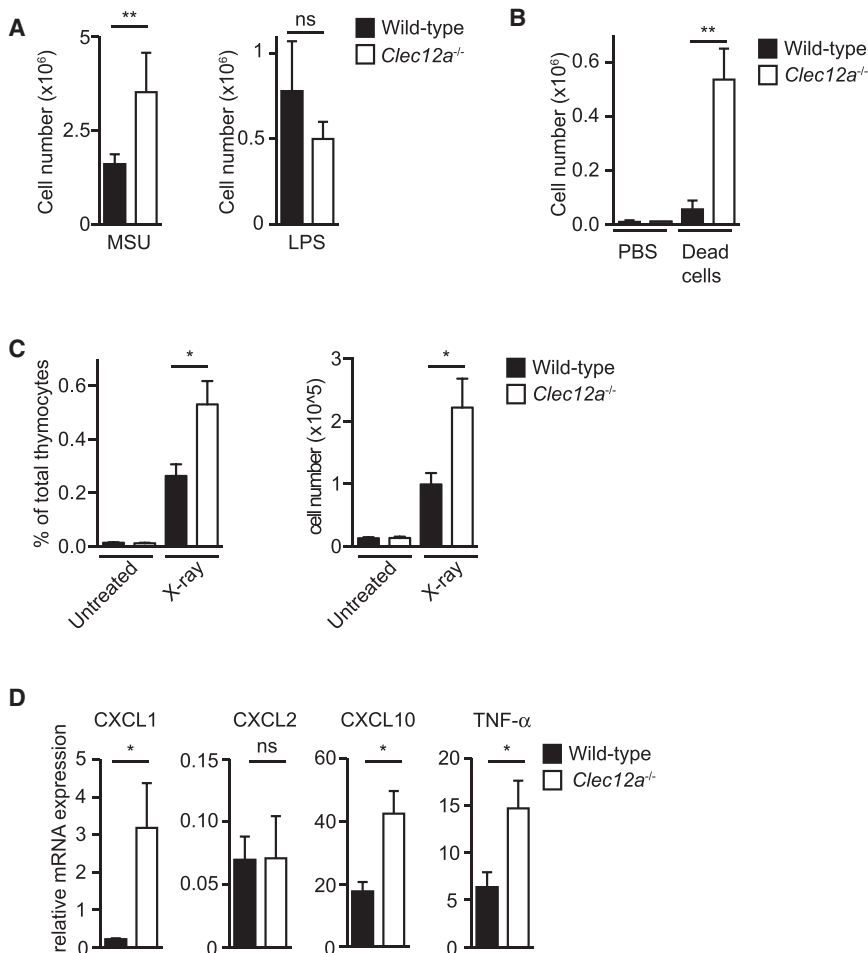


Figure 6. Clec12a Regulates Sterile Inflammation in Response to MSU or Cell Death In Vivo

(A) WT and *Clec12a*-deficient mice were injected with 400 μ g of MSU or 1 μ g of LPS into the peritoneum. Fourteen hours later, the numbers of CD11b⁺Gr-1^{hi} neutrophils in the peritoneum were analyzed by flow cytometry. Data are the means + SD of five mice per genotype and are representative of two independent experiments.

(B) WT and *Clec12a*-deficient mice were injected with PBS or freeze-thawed kidney cells into the peritoneum. Fourteen hours later, the numbers of CD11b⁺Gr-1^{hi} neutrophils in the peritoneum were analyzed by flow cytometry. Data are means + SD of five mice per group and are representative of two independent experiments.

(C) WT and *Clec12a*-deficient mice were total body-irradiated with 1 Gy of X-rays. Fourteen hours later, the fraction and total cell number of CD11b⁺Gr-1^{hi} neutrophils in the thymus was analyzed by flow cytometry. Data are means + SEM of 12 mice per group for irradiated and 5 mice per group for untreated.

(D) Thymus mRNA from the irradiated mice was isolated and quantified by qPCR with primers specific for the indicated chemokines and TNF- α and normalized to the level of 18S RNA. (C) and (D) Data are the means + SD of six mice per group and are representative of two independent experiments.

It has been shown that ligands from dead cells can trigger the Syk-activating CLR Mincle and Clec9a (Sancho et al., 2009; Yamasaki et al., 2008). Here, we reveal Clec12a as the first negative regulatory ITIM containing CLR for dead cell. These data extend the importance of CLR sensing and signaling in innate immune responses to cell death. In natural killer (NK) cells, the balance between ITAM and ITIM receptor signaling dictates the NK cell response and can serve as a paradigm for the regulation of innate immune recognition (Long, 2008). Our findings, together with the established functions for CLRs, such as Mincle and Clec9a, indicate that myeloid CLRs constitute a similar balanced ITAM/ITIM receptor system that regulates inflammatory responses in response to dead cells. Indeed, the activation of Mincle in response to radiation-induced sterile thymocyte death mediates the infiltration of neutrophils (Uchimura et al., 2000; Yamasaki et al., 2008). We found that this response is enhanced in the absence of *Clec12a*, indicating the first in vivo relevance of this ITAM/ITIM balance during sterile inflammation. Enhanced sterile inflammatory responses were also observed after the intraperitoneal injection of killed cells in the absence of *Clec12a*. Whether these reactions are also dependent on Mincle or other activating CLRs or direct Syk activation and whether *Clec12a* ligands in addition to MSU are involved remains to be defined.

CLRs are not the only PRRs that control cell death-induced inflammation. Other receptors, including TLRs, are also

involved, and the distinct types of cell death and subsequent release of a specific combinations of DAMPs is expected to dictate the subsequent immune response, which is important to maintain tissue homeostasis after damage (Kaczmarek et al., 2013; Zitvogel et al., 2010). However, the same pathways are thought to be key mechanisms of inflammatory pathology in cases such as ischemic reperfusion injury or toxic chemically induced tissue damage. In these cases, TLRs and inflammasomes seem to cooperate in inducing tissue-damaging inflammation (Eltzschig and Eckle, 2011; Imaeda et al., 2009). It will be important to investigate the role of *Clec12a* in these pathologies, as crosstalk of Syk-coupled receptors and TLRs have been described (Ivashkiv, 2009). Furthermore, it is widely believed that autoantibody production in systemic lupus erythematosus is driven by defects in dead cell clearance (Shao and Cohen, 2011). Moreover, given that cell death is also a hallmark of infection by pathogens in vivo (Matzinger, 1994), it is conceivable that *Clec12a* is also involved in immune regulation under infectious conditions. The fact that uric acid crystals are found in malaria in the *Plasmodium falciparum*-infected red blood cells (van de Hoef et al., 2013) and the finding that uric acid regulates adaptive immune responses to this pathogen (Guermonez et al., 2013) suggest that *Clec12a* could be involved in such immune responses. Our identification of *Clec12a* as an MSU and dead cell receptor will provide a starting point for investigating the mechanisms and contributions of *Clec12a* functions in host defense and immune pathology.

EXPERIMENTAL PROCEDURES

Reagents

MSU crystals were from InvivoGen or crystallized from uric acid (U0881, Sigma-Aldrich) under pyrogen-free conditions, as previously described (Schlitz et al., 2002). All assays were performed with crystals from both sources. Rasburicase was from Sanofi-Aventis. CPPD crystals and Ultrapure LPS from *E. coli* 0111:B4 were from InvivoGen. Polystyrene microspheres (1 μ m) were from Polysciences. Silica crystals (1.5 μ m) were from Alfa Aesar. Allopurinol and zymosan were from Sigma. Curdlan was from Wako. Clec12a antibody for Western Blot analysis was from R&D Systems, human Clec12a monoclonal antibody (50C1) was from BioLegend. Phospho-p40phox (Thr154) antibody was from Cell Signaling Technology. All of the antibodies for FACS analysis were from eBiosciences, except anti-mouse Ly-6b.2 clone 7/4, which was from AbD Serotec. R406 was from Selleck Chemical. All of the cell culture reagents were from Life Technologies. Fetal calf serum (FCS) was used without heat inactivation.

Production of Recombinant CLR Extracellular Domains

The cDNAs encoding the extracellular domains of mouse Clec7a (NCBI: NP_064392.2 amino acids [aa]: 75–244), mouse Clec9a (NCBI: NP_001192292.1 aa: 63–264), mouse Clec12a (NCBI: NP_808354.1 aa: 67–267) and human Clec12a (NCBI: NP_612210 aa 78–265aa) were amplified by PCR and verified by sequencing. The cDNAs were fused to the C terminus of human IgG1-Fc in the expression vector pFuse-hlgG1-Fc2 (InvivoGen).

CLR-Strep fusion constructs were generated by replacing the Fc part by a Strep-tag II (IBA Life Sciences) Isoleucine Zipper cassette (Watzl, 2006) with the protein sequence IHHHHHHGLSAWHPQFEKGGGGGGGGSSWSHPQFEKGRMKQIEDKIEILSKIYHIENIARIKKLIGERGIS.

Constructs were transfected into 293T cells, and secreted fusion proteins were harvested 48 hr after transfection and adjusted to 5 μ g/ml (Fc-fusion proteins) or 0.5 μ g/ml (Strep-tagged proteins) in DMEM with 10% FCS.

Generation of Reporter Cell Lines

The cDNA encoding the transmembrane and extracellular domains of mouse Clec7a (NCBI: NP_064392.2 aa 44–244), mouse Clec9a (NCBI: NP_001192292.1 aa 31–264), mouse Clec12a (NCBI: NP_808354.1 aa 41–267) and human Clec12a (NCBI: NP_612210 aa 40–265) were amplified and fused to the C terminus of the intracellular domain of murine CD3 ζ (NP_001106862.1, aa 52–164). A5 T cell hybridoma cells that contain an NFAT-GFP expression cassette (Andersen et al., 2001; Bot et al., 1996) were transfected with CLR-CD3 ζ fusion proteins in pEF1 (Life Technologies) and selected for G418 resistance and CLR surface expression.

Generation of Clec12a-Deficient Mice

A targeting vector was generated from 129J genomic DNA and was electroporated into R1 embryonic stem cells. Homologous recombination replaced the genomic fragment containing the first two exons encoding the intracellular and transmembrane domain of Clec12a with a neomycin-resistance gene (Figure S1). Clones resistant to G418 were selected, and correctly recombined clones were identified with a Southern blot. Injection of two independent clones into C57BL/6J blastocysts generated chimeric mice. Clec12a^{+/-} mice were identified by PCR and crossed to C57BL/6J for four to eight generations. Control mice on the same mixed genetic background were used. Experimental groups of sex and age matched mice were cohoused for at least 3 weeks and used at an age of 8–12 weeks. Mice were housed in the Zentrum für Präklinische Forschung under specific pathogen-free conditions, according to Federation of European Laboratory Animal Science Associations (FELASA) guidelines. All animal experiments were approved by the Regierung von Oberbayern.

FACS Analysis of Splenocytes

Single-cell suspensions of spleen tissues were preincubated with Fc-block (eBiosciences) and subsequently stained with propidium iodide and fluorescently labeled antibodies. Lymphoid cells were identified with antibodies against B220, CD19, TCR- β , CD4, and CD8. Myeloid cells were identified using antibodies against CD11b, F4/80, Gr-1, and Ly-6b.2 (7/4), as previously described (Taylor et al., 2003). FACS data were acquired on a BD FACS Canto II (BD Biosciences) and analyzed with FlowJo software (Tree Star).

Staining with Recombinant CLR Fusion Proteins

All cells and particles were incubated with individual CLR-Fc fusion proteins for 2 hr at 4°C in DMEM containing 10% FCS, washed in PBS with 3% FCS, incubated with anti-human IgG-PE (eBiosciences) in PBS with 3% FCS, and analyzed with a flow cytometer (FACS Canto II, BD Biosciences). CLR-Strep fusion proteins were used as Fc fusion proteins but detected with streptactin-PE (IBA Life Sciences). For flow cytometric analysis, MSU crystals were disrupted by ultrasound to create <5 μ m crystals. To kill cells by freeze thawing, we flash froze suspended cells in liquid nitrogen and thawed them on ice. To degrade uric acid, dead cells were incubated with 0.3 mg/ml Raburicase for 90 min at 37°C.

A5 Reporter Cell Stimulation

A5 reporter cells (Andersen et al., 2001; Bot et al., 1996) were grown in RPMI 1640 with 5% FCS and 50 μ M β -MeOH and were stimulated with dead cell preparations in complete growth medium for 18–20 hr, harvested, and stained with anti-CD4-APC and 7-AAD (eBiosciences) to identify live A5 reporter cells. Flow cytometry was used to examine GFP expression in the live (7-AAD⁻) reporter cells (CD4⁺). To analyze the GFP expression in response to particulate stimuli, we washed cells in PBS and lysed them in PBS containing 0.1% NP-40, and green fluorescence was analyzed with a Mithras LB 940 plate reader (Berthold Technologies).

In Vitro Activation of Bone-Marrow Cells and Neutrophils

Untouched neutrophils were purified from bone marrow with a neutrophil isolation kit (Miltenyi Biotec), according to the manufacturer's recommendations. Neutrophils or bone-marrow cells were resuspended at 0.5×10^7 or 1×10^7 cells per ml, respectively, in Opti-Mem I (GIBCO) containing 5% FCS and they were plated in 96-well Luminometer plates (Nunc) blocked in 5% FCS overnight. Cells were adjusted to 37°C for 15 min and stimulated with 500 μ g/ml MSU or 100 μ g/ml zymosan in the presence of 50 μ M luminol (Sigma-Aldrich). Emitted light was recorded with a Mithras LB940 plate reader every 3 min for 40 min.

Dead Cell- and MSU-Induced Peritonitis

Experiments were performed as previously described (Chen et al., 2006; Yamasaki et al., 2008). Kidney cells were prepared by the digestion of WT mouse kidneys with 200 U/ml of collagenase IV (GIBCO) for 45 min. Cells were washed 2 \times with PBS, resuspended in 5 volumes of PBS, and killed by freezing in liquid nitrogen and were then thawed at room temperature. Kidney cells, 400 μ g of MSU crystals, or 1 μ g of LPS in 150 μ l of PBS were injected into the peritoneum of 8- to 12-week-old WT and Clec12a-deficient sex- and age-matched mice. Fourteen hours later, the mice were sacrificed, and their peritoneal cells were counted and analyzed by flow cytometry with propidium iodide (PI), anti-CD11b, anti-Gr-1, and anti-Ly-6b.2 (clone 7/4). Viable neutrophils were identified as PI⁻, Gr-1^{hi}, Ly-6b.2^{int}, and CD11b⁺.

Neutrophil Infiltration after Total Body Irradiation

Analysis of neutrophil infiltration into the thymus of total body-irradiated (1 Gy) mice was performed as previously described (Yamasaki et al., 2008). The expression of inflammatory genes was analyzed with real-time PCR as previously described (Lassen et al., 2010).

Statistical Analysis

An unpaired two-tailed Student's t test was used for all of the statistical analyses. *p < 0.05, **p < 0.01, ns: not significant.

SUPPLEMENTAL INFORMATION

Supplemental Information includes three figures and can be found with this article online at <http://dx.doi.org/10.1016/j.immuni.2013.12.015>.

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