TIM1 is an endogenous ligand for LMIR5/CD300b: LMIR5 deficiency ameliorates mouse kidney ischemia/reperfusion injury

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Leukocyte mono-immunoglobulin (Ig)–like receptor 5 (LMIR5)/CD300b is a DAP12-coupled activating receptor predominantly expressed in myeloid cells. The ligands for LMIR have not been reported. We have identified T cell Ig mucin 1 (TIM1) as a possible ligand for LMIR5 by retrovirus-mediated expression cloning. TIM1 interacted only with LMIR5 among the LMIR family, whereas LMIR5 interacted with TIM4 as well as TIM1. The Ig-like domain of LMIR5 bound to TIM1 in the vicinity of the phosphatidylserine (PS)-binding site within the Ig-like domain of TIM1. Unlike its binding to TIM1 or TIM4, LMIR5 failed to bind to PS. LMIR5 binding did not affect TIM1- or TIM4-mediated phagocytosis of apoptotic cells, and stimulation with TIM1 or TIM4 induced LMIR5-mediated activation of mast cells. Notably, LMIR5 deficiency suppressed TIM1-Fc–induced recruitment of neutrophils in the dorsal air pouch, and LMIR5 deficiency attenuated neutrophil accumulation in a model of ischemia/reperfusion injury in the kidneys in which TIM1 expression is up-regulated. In that model, LMIR5 deficiency resulted in ameliorated tubular necrosis and cast formation in the acute phase. Collectively, our results indicate that TIM1 is an endogenous ligand for LMIR5 and that the TIM1–LMIR5 interaction plays a physiological role in immune regulation by myeloid cells.

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Abbreviations used: BMMC, BM-derived mast cell; CHO, Chinese hamster ovary; ERK, extracellular signal-regulated kinase; FLMC, fetal liver–derived mast cell; IRI, ischemia/reperfusion injury; KIM-1, kidney injury molecule–1; LMIR, leukocyte mono-Ig–like receptor; PS, phosphatidylserine; TIM, T cell Ig mucin.

A growing number of studies have characterized a variety of paired activating and inhibitory receptors (Ravetch and Lanier, 2000; Klesney-Tait et al., 2006; Lanier, 2009). We have previously identified a leukocyte mono-Ig–like receptor (LMIR) mainly expressed in myeloid cells (Kumagai et al., 2003; Izawa et al., 2007; Yamanishi et al., 2008). The mouse LMIR family is also known as the CMRF-35–like molecule/myeloid-associated Ig-like receptor/dendritic cell–derived Ig-like receptor/CD300 family (Luo et al., 2001; Chung et al., 2003; Yotsumoto et al., 2003). LMIR1 and LMIR3 are immunoreceptor tyrosine-based inhibitory motif–containing inhibitory receptors, whereas other members are activating receptors that associate with immunoreceptor tyrosine-based activation motif–containing adaptor proteins (Luo et al., 2001; Chung et al., 2003; Kumagai et al., 2003; Yotsumoto et al., 2003; Izawa et al., 2007; Yamanishi et al., 2008). LMIR5 is a DAP12-coupled activating receptor predominantly expressed in myeloid cells (Yamanishi

The Rockefeller University Press $30.00
J. Exp. Med. Vol. 207 No. 7 1501-1511
www.jem.org/cgi/doi/10.1084/jem.20090581

The Journal of Experimental Medicine

et al., 2008). However, the ligands for LMIR remained unknown. In this study, we identified T cell Ig mucin 1 (TIM1) as a ligand for LMIR5 by retrovirus-mediated expression cloning (Kitamura et al., 2003).

TIM1–4 are characterized as important regulators of immune responses associated with autoimmunity and allergic diseases (McIntire et al., 2001; Kuchroo et al., 2003, 2008). The TIM molecules are type 1 cell-surface glycoproteins, consisting of an N-terminal IgV domain and a mucin domain. TIM1/hepatitis A virus cellular receptor 1 (Kaplan et al., 1996)/kidney injury molecule–1 (KIM-1; Ichimura et al., 1998) is expressed in activated T cells and delivers a signal that enhances T cell activation and proliferation (Meyers et al., 2005; Umetsu et al., 2005). TIM1 can also interact with itself (Santiago et al., 2007b). In addition, a soluble form of KIM-1/TIM1 is released by shedding (Bailly et al., 2002). On the other hand, TIM4 is expressed in macrophages and dendritic cells and is a natural ligand for TIM1 (Meyers et al., 2005). Interestingly, TIM1 and TIM4 recognize phosphatidylserine (PS) and are critical for the efficient clearance of apoptotic cells (Kobayashi et al., 2007; Miyanishi et al., 2007; Santiago et al., 2007a; Ichimura et al., 2008). Recent reports have demonstrated that the narrow cavity built by the CC' and FG loops of the Ig-like domain is a binding site for PS (Kobayashi et al., 2007; Santiago et al., 2007a,b). In addition, TIM1/KIM-1 expression is strongly induced in the injured kidney epithelial cells (Ichimura et al., 1998, 2008; Waanders et al., 2010), and confers a phagocytic phenotype on epithelial cells (Ichimura et al., 2008). TIM1 is also a marker for renal tubular damage (Waanders et al. 2010).

In the present study, using biological and biochemical analysis, we demonstrate that TIM1 and TIM4 are endogenous ligands for LMIR5. In addition, we generated LMIR5$^{-/-}$ mice and delineated the physiological significance of the LMIR5–TIM1 interaction by using an acute kidney injury model.

RESULTS

Cloning of the ligand for LMIR5

To identify the LMIR5 ligand, we generated an Fc fusion protein containing the extracellular domain of LMIR5 (LMIR5-Fc). Several hematopoietic cell lines were incubated with LMIR5-Fc, which stained A20 cells but not Ba/F3 cells, as determined by flow cytometric analysis, suggesting the expression of LMIR5 ligand in A20 cells (Fig. 1 A). To identify the surface protein bound by LMIR5-Fc, we used retrovirus-mediated expression cloning (Kitamura et al., 2003). A retrovirus cDNA library constructed from A20 cells was transduced via infection to Ba/F3 cells that were not stained by LMIR5-Fc (Fig. 1 A). The transfectants stained by LMIR5-Fc were sorted and expanded in culture. This cycle of sorting and expansion was repeated three times until LMIR5-Fc stained most cells (Fig. S1 A). After we obtained single-cell clones that were stained with LMIR5-Fc, we isolated TIM1 cDNA from most of these clones by PCR (Fig. S1 B and not depicted). We confirmed that a cell-surface glycoprotein TIM1 was expressed in A20 cells but not in Ba/F3 cells by using anti-TIM1 antibody (Fig. 1 A, bottom). When TIM1 was expressed in Ba/F3 cells, LMIR5-Fc strongly stained TIM1-transduced Ba/F3 but not parental Ba/F3 cells. In addition, LMIR5-Fc–staining levels were correlated with TIM1 expression at both surface protein and mRNA levels (Fig. 1, A and B). Collectively, these results indicated that LMIR5-Fc bound to TIM1. In accordance, pretreatment of LMIR5-Fc with 10 μg/ml anti-LMIR5 antibody, but not control antibody, abolished the binding of LMIR5-Fc to TIM1-expressing Ba/F3 cells (Fig. 1 C, left). Similarly, the preincubation of TIM1-expressing Ba/F3 cells with 100 μg/ml anti-TIM1 antibody (222414) suppressed this binding (Fig. 1 D, left). The binding of LMIR5-Fc to TIM1-expressing Ba/F3 cells was dose-dependently inhibited by anti-LMIR5 or anti-TIM1 antibody (Fig. 1, C and D, right). These observations strongly suggested that LMIR5 interacts directly with TIM1. Then, Fc fusion proteins containing the extracellular domains of LMIR1, 2, 3, and 4 (LMIR1/2/3/4-Fc) were generated. TIM1-expressing Ba/F3 cells were bound only by LMIR5-Fc among the LMIR-Fc fusion proteins (Fig. 1 E). When Ba/F3 cells transduced with Flag-tagged TIM1, 2, 3, and 4 were incubated with LMIR5-Fc, LMIR5-Fc bound to those cells transduced with TIM4 as well as TIM1 among the TIM family (Fig. 1 F). In support of this, coimmunoprecipitation experiments illustrated the physical interaction of LMIR5 with both TIM1 and TIM4 (Fig. 1 G). After generating Fc fusion proteins containing the extracellular domains of TIM1 or 4 (TIM1/4-Fc), we incubated LMIR5- or mock-transduced Ba/F3 cells with TIM1-Fc or TIM4-Fc, which stained LMIR5-transduced Ba/F3 cells more strongly as compared with parental Ba/F3 cells (Fig. S1 C), indicating that TIM1 or TIM4 bound to surface-expressed LMIR5. On the other hand, the fact that TIM1-Fc or TIM4-Fc stained parental Ba/F3 cells at significant levels suggested that ligands for TIM1 or TIM4 other than LMIR5 were expressed in Ba/F3 cells. Our results suggested that TIM1 and TIM4 are possible ligands for LMIR5.

The Ig-like domain of LMIR5 binds to that of TIM1 in the vicinity of the PS-binding site

To determine which region of LMIR5 was required for the interaction with TIM1, we generated LMIR5 deletion mutants (LMIR5 del1/2/3/4/5–Fc; Fig. 2, A and B). Notably, like LMIR5-Fc, LMIR5 del1/2/3–Fc bound to TIM1-expressing Ba/F3 cells, whereas LMIR5 del4/5–Fc lacking the C terminus of the Ig-like domains did not bind at all (Fig. 2 C). These results suggested that the intact Ig-like domain of LMIR5 is indispensable for the LMIR5–TIM1 interaction.

According to recent reports, TIM1 and TIM4 bind to PS through the highly conserved binding cleft (FG–CC' cleft) of the Ig-like domains (Kobayashi et al., 2007; Miyanishi et al., 2007; Santiago et al., 2007a,b; Ichimura et al., 2008). To clarify the involvement of this region in LMIR5–TIM1 binding, we generated the TIM1 mutants W115A/F116A (WF/AA) or N117A/D118A (ND/AA), because these mutations in the metal ion–dependent ligand-binding site were reported

to dampen TIM1–PS binding (Kobayashi et al., 2007; Santiago et al., 2007a). Interestingly, these substitutions completely abolished the binding of LMIR5-Fc to TIM1-expressing Ba/F3 cells (Fig. 2 D). Collectively, these findings indicated that LMIR5 bound to TIM1, presumably through the interaction of the Ig-like domain of LMIR5 with the structural

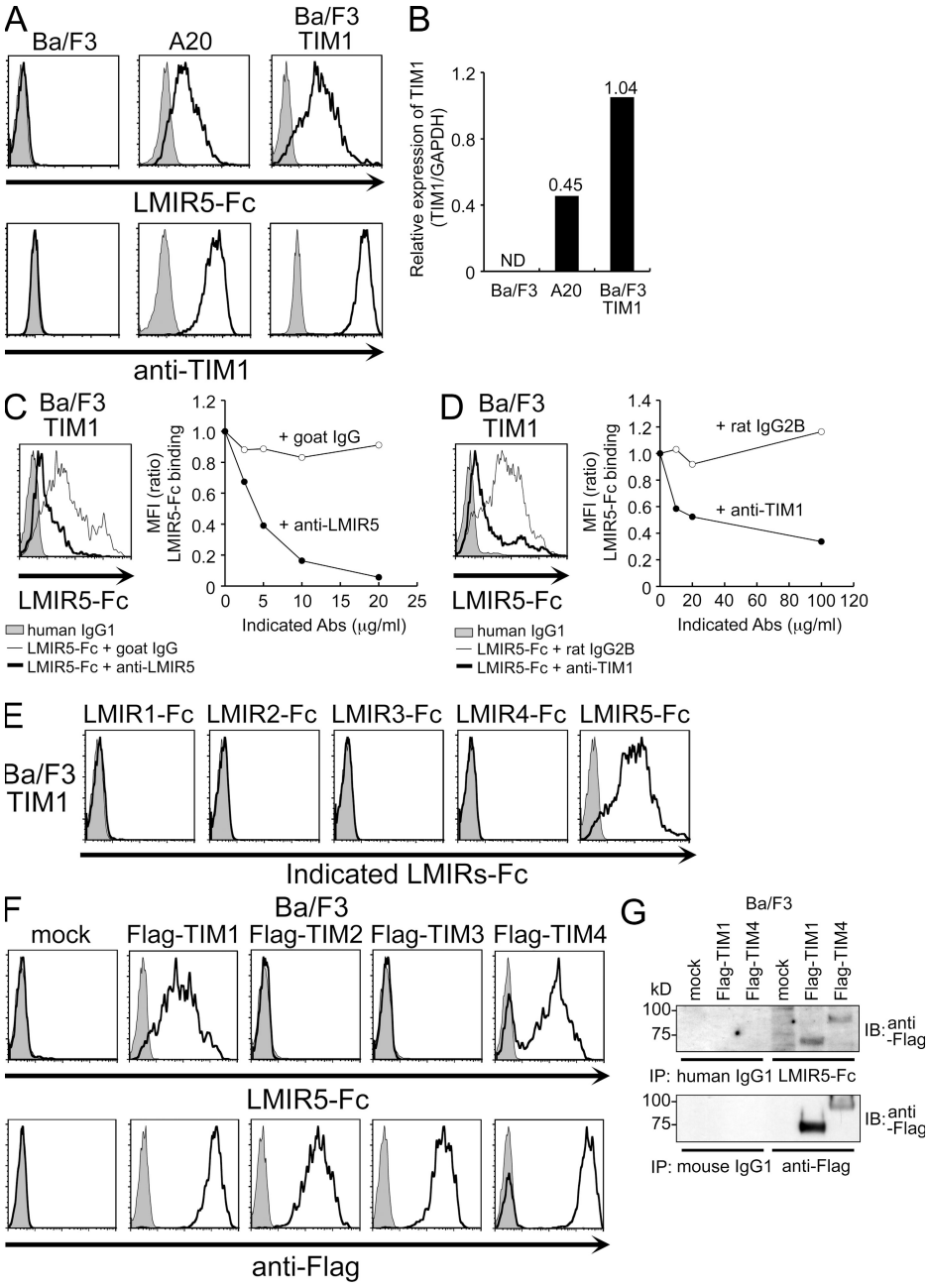

Figure 1. Specific binding of LMIR5–Fc to TIM1–expressing cells. (A) The indicated cells or TIM1-transduced Ba/F3 cells were stained with LMIR5-Fc (top) or with anti-TIM1 antibody (RMT1-4; bottom). (B) Relative gene expression levels of TIM1 were estimated by using real-time PCR. (C) LMIR5-Fc was pretreated with the indicated concentrations (left, 10 μg/ml) of anti-LMIR5 antibody or goat IgG. (D) TIM1-transduced Ba/F3 cells were preincubated with the indicated concentrations (left, 100 μg/ml) of anti-TIM1 antibody (222414) or rat IgG2b. TIM1-transduced Ba/F3 cells were then stained with LMIR5-Fc. The mean fluorescent intensity (MFI) of LMIR5-Fc staining is shown (C and D, right). (E) TIM1-transduced Ba/F3 cells were stained with LMIR1/2/3/4/5-Fc (continuous line histograms). Control staining with human IgG1 is shown (shaded histograms). (F) Ba/F3 cells transduced with Flag-tagged TIM1/2/3/4 or mock transduced were stained with LMIR5-Fc (top, continuous line histograms) or with anti-Flag antibody (bottom, continuous line histograms). Control staining with human (top, shaded histograms) and mouse IgG1 (bottom, shaded histograms) is shown. (G) TIM1 and TIM4 proteins were detected by immunoblotting (IB) with anti-Flag antibody in the immunoprecipitates (IP) of lysates derived from Flag-tagged TIM1-, TIM4-, or mock-transduced Ba/F3 cells incubated with LMIR5-Fc (top) or anti-Flag antibody (bottom). All data are representative of three independent experiments. ND, not detected.

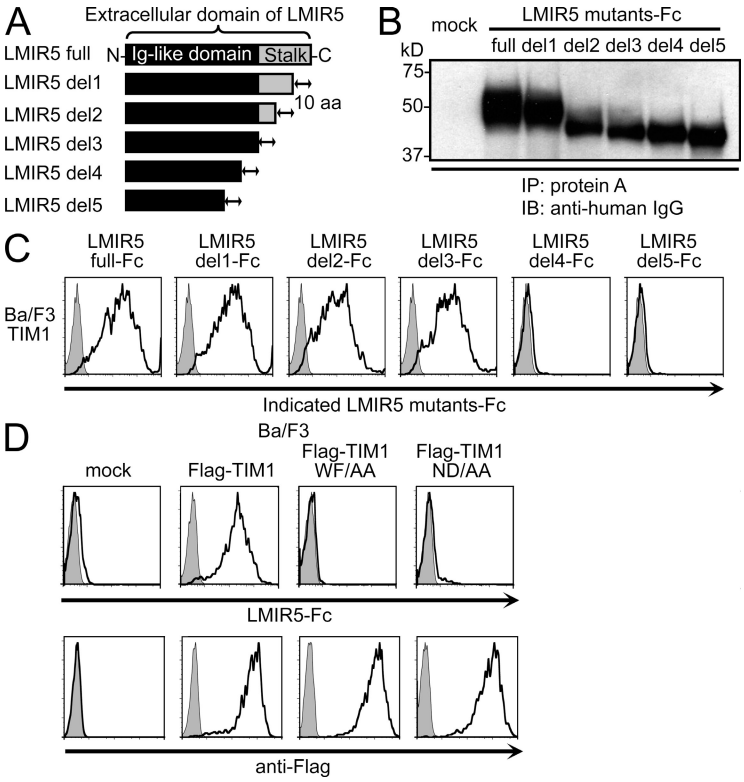

Figure 2. Ig-like domains of both LMIR5 and TIM1 are required for the LMIR5–TIM1 interaction. (A) Structures of LMIR5 extracellular domain in LMIR5-Fc and its deletion mutants. (B) The culture supernatants from 293T cells transduced with LMIR5-Fc or LMIR5-Fc mutants were immunoprecipitated (IP) with protein A, and then immunoblotted (IB) with anti-human IgG antibody. (C) TIM1-transduced Ba/F3 cells were stained with LMIR5-Fc or LMIR5-Fc mutants (continuous line histograms). Control staining with human IgG1 is shown (shaded histograms). (D) Ba/F3 cells transduced with Flag-tagged TIM1, TIM1 (WF/AA), TIM1 (ND/AA), or mock were stained with LMIR5-Fc (top, continuous line histograms) or anti-Flag antibody (bottom, continuous line histograms). Control staining with human (top, shaded histograms) and mouse IgG1 (bottom, shaded histograms) is shown. All data shown are representative of three independent experiments.

domain formed by FG loops of TIM1. Related to this, we found different capabilities of anti-TIM1 antibodies to recognize TIM1 epitopes. Anti-TIM1 antibody (222414) detected TIM1 WT and the ND/AA mutant but not the WF/AA mutant, whereas anti-TIM1 antibody (RMT1-10) detected TIM1 WT and the mutants WF/AA and ND/AA (Fig. S2 A). Thus, anti-TIM1 antibody (222414) presumably recognizes the FG loop structure that is critically maintained by W115/F116 but not N117/D118, whereas anti-TIM1 (RMT1-10) antibody reacts outside this area of TIM1. Then, we compared the inhibitory effect of these antibodies on LMIR5–TIM1 binding. Unlike anti-TIM1 antibody (RMT1-10) or anti-Flag antibody, anti-TIM1 antibody (222414) pretreatment did inhibit LMIR5–TIM1 binding (Fig. S2 B). Collectively, these results suggested that the Ig-like domain of LMIR5 interacted with TIM1 in the region formed by the FG loop of the Ig-like domain, the structure of which was similar or close to the FG–CC' cleft bound by PS, and that it was recognized by anti-TIM1 antibody (222414).

LMIR5 neither binds to PS nor affects TIM1- or TIM4-mediated phagocytosis of apoptotic cells

Given the close proximity of the PS- and LMIR5-binding regions in TIM1, we tested whether LMIR5 interacts with TIM1 through PS. To this end, we performed a protein–lipid overlay assay; although TIM1-Fc bound specifically to PS, as previously reported (Kobayashi et al., 2007; Miyanishi et al., 2007), we found no binding of LMIR5-Fc to PS or to other phospholipids (Fig. 3 A). Consistently, NIH3T3 cells transduced with TIM1 but not LMIR5 promoted phagocytosis of the apoptotic cells through recognition of PS (Fig. 3 B). Next, we asked if the LMIR5–TIM1 interaction affected the TIM1-mediated phagocytosis of the apoptotic cells. As expected, coincubation with anti-TIM1 antibody or TIM1-Fc significantly suppressed phagocytosis of the apoptotic cells in TIM1-expressing NIH3T3 cells (Fig. 3 C). In contrast, coincubation with LMIR5-Fc did not inhibit this phagocytosis (Fig. 3 C). Similarly, the interaction of LMIR5 with TIM4 did not affect TIM4-mediated phagocytosis of the apoptotic cells in peritoneal macrophages (Fig. 3, D and E). Collectively, interaction of neither TIM1 nor TIM4 with LMIR5 affected the phagocytosis of apoptotic cells through its recognition of PS, despite the finding that LMIR5 bound to TIM1 or TIM4 at close proximity to the PS-binding site.

The TIM1–LMIR5 interaction induces LMIR5-mediated activation of mast cells

As previously reported (Kitaura et al., 2003; Yamanishi et al., 2008), LMIR5-mediated activation of mast cells depended on LMIR5 expression levels (Fig. 4 A, left). Notably, stimulation with TIM1-Fc, but not human IgG1 as a control, induced significant levels of extracellular signal-regulated kinase (ERK) activation in LMIR5-transduced BM-derived mast cells (BMMCs), whereas no detectable levels of ERK activation were induced by TIM1-Fc in mock-transduced BMMCs (Fig. 4 A, right). Similarly, stimulation with TIM1-Fc or TIM4-Fc induced cytokine production of fetal liver–derived mast cells (FLMCs) transduced with LMIR5, but not mock-transduced cells (Fig. 4 B; and Fig. S3, A and B). In addition, DAP12 deficiency completely dampened cytokine production of LMIR5-transduced FLMCs stimulated by TIM1-Fc, but not PMA as a control (Fig. 4 B). These results indicated that TIM1-Fc or TIM4-Fc activated mast cells through interaction with LMIR5, which was dependent on the expression levels of LMIR5. As previously reported (Nakae et al., 2007), we found high expression levels of TIM3 as well as

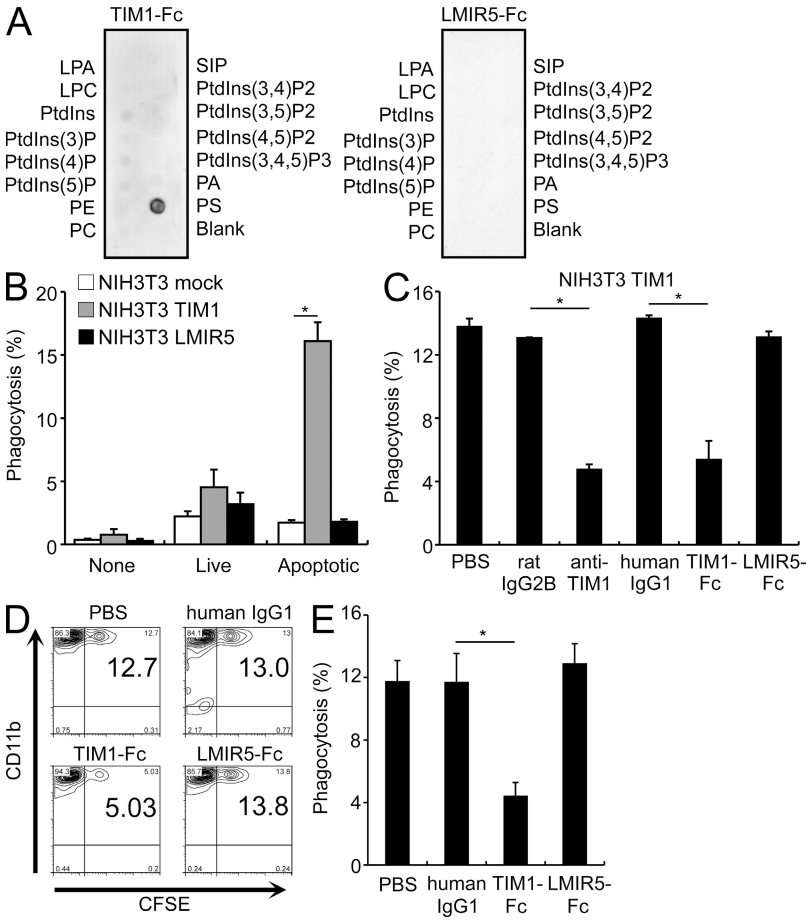

Figure 3. LMIR5 is not involved in TIM1- or TIM4-mediated phagocytosis of apoptotic cells through recognition of PS. (A) PIP strips spotted with the indicated phospholipids were incubated with TIM1-Fc or LMIR5-Fc. (B and C) NIH3T3 cells transduced with either TIM1, LMIR5, or mock were co-cultured with CFSE-labeled live or apoptotic U937 cells in the presence (C) or absence (B) of the indicated antibodies or Fc fusion proteins. The percentage of NIH3T3 cells containing CFSE-labeled U937 cells was determined. (D) Peritoneal macrophages were co-cultured with CFSE-labeled apoptotic thymocytes for 30 min in the presence of 10 µg/ml TIM1-Fc, LMIR5-Fc, or human IgG1. After removal of nonadherent cells, peritoneal macrophages were stained with PE-conjugated anti-CD11b antibody. The percentage of CFSE/CD11b double-positive cells was determined by flow cytometric analysis. (E) Based on the flow cytometric analysis in D, the percentage of phagocytosis is shown. All data points correspond to the means ± SD of triplicate samples. Data are representative of three independent experiments. *, $P < 0.05$.

no detectable expression levels of TIM2 and TIM4 in mast cells (Fig. S4). However, TIM1 expression in mast cells was not confirmed in either protein or transcript levels (Fig. S4). Therefore, we reasoned that the involvement of the TIM1–TIM1/4 interaction in TIM1-Fc–induced activation of mast cells was negligible. We then performed co-culture of LMIR5- or mock-transduced FLMCs with TIM1-transduced Chinese hamster ovary (CHO) cells, demonstrating higher levels of IL-6 released into the supernatants of the former co-culture as compared with the latter (Fig. 4 C, left). In addition, we found higher levels of IL-6 released into the supernatants in the co-culture of LMIR5-expressing FLMCs with TIM1-transduced CHO cells as compared with mock-transduced CHO cells (Fig. 4 C, right). These results indicated that interaction of LMIR5 with surface-expressed TIM1 as well as soluble TIM1 induced the LMIR5-mediated activation of mast cells.

In vivo evidence that the LMIR5–TIM1 interaction induces the accumulation of neutrophils

To investigate the physiological role of TIM1 as a ligand for LMIR5, we generated LMIR5-deficient mice (Fig. S5 A). We confirmed gene targeting by genomic PCR (Fig. S5 B) and the complete absence of LMIR5 expression in BM neutrophils by flow cytometry (Fig. 5 C). Semiquantitative RT-PCR analysis demonstrated no significant difference of LMIR1–4 transcript levels in BM cells between WT and LMIR5$^{-/-}$ mice (Fig. S5 C). LMIR5$^{-/-}$ mice were born at the expected Mendelian ratio and showed no obvious abnormalities. In addition, WT and LMIR5$^{-/-}$ mice did not reveal major differences in the myeloid and lymphoid development of the BM, spleen, thymus, peripheral blood, and peritoneal cells (Fig. S6).

To address the physiological significance of the LMIR5–TIM1 interaction, we chose a model for kidney ischemia/reperfusion injury (IRI; Kelly et al., 1996; Ichimura et al., 1998, 2008; Wu et al., 2007; Lech et al., 2009; Waanders et al., 2010), which is the known in vivo model for TIM1 induction. Consistent with previous reports (Ichimura et al., 1998, 2008; Waanders et al., 2010), TIM1 mRNA levels rapidly increased at day 1 after IRI and diminished through days 2–4 in the IRI kidneys of WT mice, whereas they were maintained at low levels before and after IRI in contralateral kidneys (Fig. 5 A, left). TIM4 mRNA was undetectable in IRI or contralateral kidneys (unpublished data). Notably, LMIR5 mRNA levels increased through days 1 and 2, and thereafter decreased through days 3 and 4 in IRI but not control kidneys of WT mice (Fig. 5 A, right). Further examination showed that in IRI kidneys of WT mice, TIM1 was highly expressed in CD45$^-$ (nonhematopoietic) cells as compared with CD45$^+$ (hematopoietic) cells, whereas LMIR5 was predominantly expressed in CD45$^+$ cells (Fig. 5 B). Together with previous findings (Kelly et al., 1996; Ichimura et al., 1998, 2008; Wu et al., 2007; Lech et al., 2009; Waanders et al., 2010), these results indicated that LMIR5-expressing neutrophils were recruited in IRI kidneys after the up-regulation of TIM1 expression in the renal tubular cells. We then

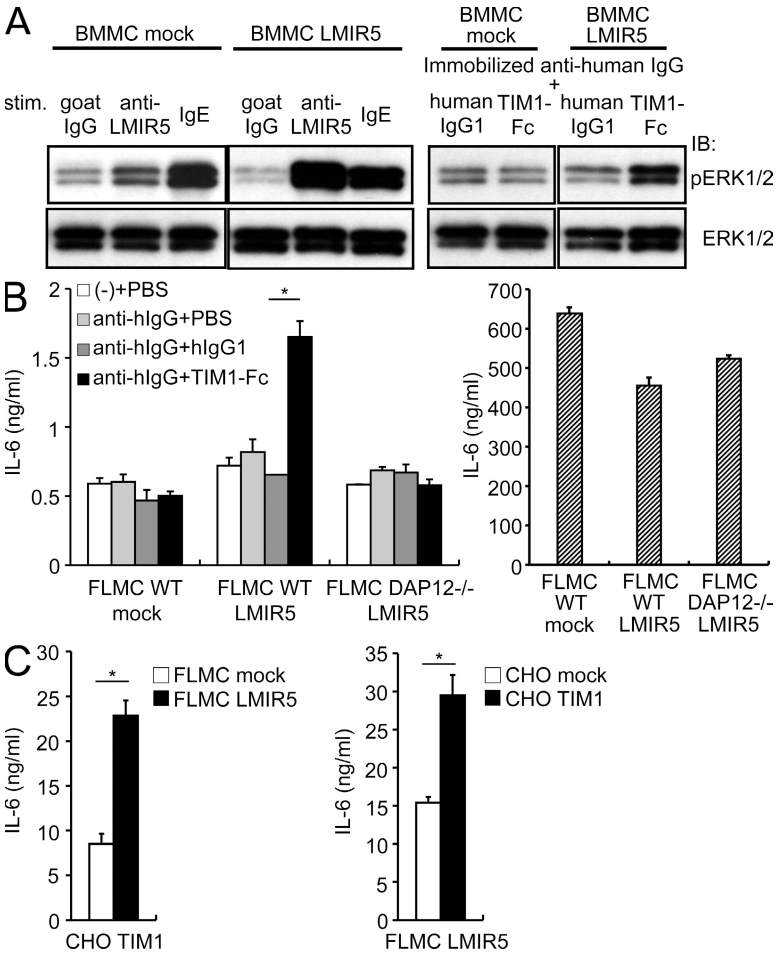

Figure 4. The binding of TIM1 to LMIR5 induced LMIR5-mediated mast cell activation. (A) BMMCs transduced with LMIR5 or mock were stimulated with anti-LMIR5 antibody, goat IgG, or SPE-7 IgE (left), or with TIM1-Fc or human IgG1 (right). Cell lysates were subjected to immunoblotting (IB) with anti–phospho-p44/42 mitogen-activated protein kinase (pERK1/2). One representative out of three independent experiments is shown. (B) Either WT or DAP12-deficient FLMCs transduced with LMIR5 or mock were stimulated with 100 nM PMA (right) or with TIM1-Fc, human IgG1 (hIgG1), or PBS (left). (C) FLMCs transduced with LMIR5 or mock were co-cultured with TIM1-expressing CHO cells (left), or LMIR5-expressing FLMCs were co-cultured with CHO cells transduced with TIM1 or mock for 24 h (right). IL-6 released into the culture supernatants was measured by ELISA. Data are means ± SD of four triplicate samples. One representative out of four independent experiments is shown. Statistically significant differences are shown. *, $P < 0.05$.

compared the IRI kidneys from WT mice with those from LMIR5$^{-/-}$ mice. Flow cytometric analysis delineated that the percentage of neutrophils in IRI kidneys was lower in LMIR5-deficient mice compared with WT mice (Fig. 5 D). On the other hand, in vitro migration assays demonstrated a comparable ability of WT and LMIR5$^{-/-}$ neutrophils to migrate toward chemoattractants such as MIP-2, C5a, KC, and fMLP (Fig. S7 A). To further test if the LMIR5–TIM1 interaction was involved in the neutrophil accumulation in the kidney IRI, we used dorsal air pouch experiments as an in vivo neutrophil recruitment model. Importantly, both TIM1-Fc–induced neutrophil migration and cytokine production in dorsal air pouches were dampened by LMIR5 deficiency, although the response to LPS was comparable between both mice (Fig. 5, E and F). These results suggested that the LMIR5–TIM1 interaction contributed to the accumulation of neutrophils in kidney IRI.

LMIR5 deficiency ameliorates renal tubular damage induced by kidney IRI

Next, we examined if LMIR5 deficiency attenuated the renal damage induced by IRI. As previously reported (Kelly et al., 1996; Wu et al., 2007; Lech et al., 2009), higher amounts of IL-6 and MCP-1 were produced in IRI kidneys compared with contralateral kidneys of WT mice (Fig. 6 A and Fig. S7 B). Notably, LMIR5 deficiency attenuated the increase of cytokine production in IRI kidneys, whereas it did not affect TIM1 expression in IRI kidneys (Fig. 6 A and Fig. S7 B). In WT mice, IL-6 transcripts were equivalently expressed in CD45$^+$ and CD45$^-$ cells of IRI kidneys, whereas MCP-1 transcripts were highly expressed in CD45$^+$ cells (Fig. 6 B). These results indicated a contributory role of CD45$^+$ cells in cytokine/chemokine production of IRI kidneys. Histological analysis of IRI kidneys showed severe tubular damage of WT mice, as indicated by widespread tubular necrosis in the outer medulla and cast formation in the inner medulla at 1 d after IRI (Fig. 6 C). On the other hand, tubular damage was significantly ameliorated by LMIR5 deficiency (Fig. 6 C). Related to this, the number of neutrophils infiltrating the interstitial compartments of IRI kidneys was lower in LMIR5-deficient mice compared with WT mice (Fig. 6, D and E). We found no tubular damage as well as negligible numbers of neutrophils in contralateral kidneys of WT or LMIR5$^{-/-}$ mice (Fig. 6, C and E; and not depicted). Importantly, immunohistological examination displayed the frequent colocalization of LMIR5-expressing neutrophils and TIM1-expressing tubular epithelial cells in IRI kidneys of WT mice (Fig. 6 F). Altogether, LMIR5 deficiency ameliorated ischemia-induced renal tubular damage associated with neutrophil accumulation.

DISCUSSION

Identification of ligands for immune receptors is indispensable for delineation of their biological functions. Recent studies led us to postulate that paired immune receptors have acquired the ability to recognize both endogenous and exogenous ligands (Shiratori et al., 2004; Satoh et al., 2008). The same hypothesis could be applied to LMIR/CD300 family members, but their ligands remained unknown.

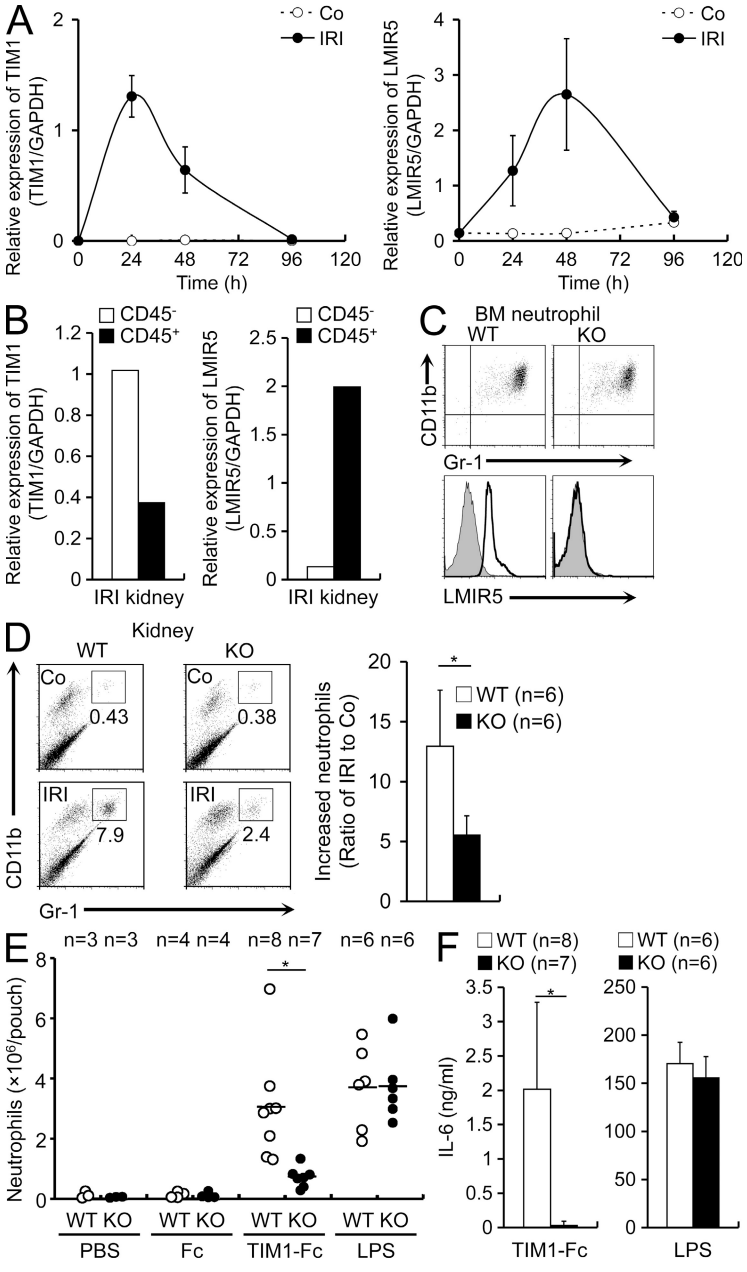

Figure 5. In vivo evidence that the LMIR5–TIM1 interaction induced accumulation of neutrophils. (A and B) Relative gene expression levels of TIM1 (left) or LMIR5 (right) in the IRI or contralateral (Co) kidneys from WT mice at different time intervals after surgery (A) or in the $CD45^-$ or $CD45^+$ cells sorted from the IRI kidneys of WT mice at 24 h after surgery (B). Data are means ± SD ($n = 6$ mice in each group). Data are representative of three independent experiments. (C) Surface expression levels of LMIR5 (bottom, continuous line histograms) as well as CD11b and Gr-1 (top) were examined in BM neutrophils from WT or $LMIR5^{-/-}$ (KO) mice. Control staining with goat IgG is shown (shaded histograms). Data are representative of five independent experiments. (D) Percentages of $CD11b^+Gr\text{-}1^+$ neutrophils in either contralateral or IRI kidney cells from WT or KO mice at 24 h after surgery (left). The ratio of neutrophil counts in IRI kidneys to those in contralateral kidneys was determined. Data are means ± SD ($n = 6$ mice in each group; right). Two independent experiments were performed. (E) Either 100 µg TIM1-Fc or control Fc, or 1 mg LPS was injected into the air pouches of WT or $LMIR5^{-/-}$ mice. At 4 h after injection, neutrophils recruited into the pouches were counted. Each symbol represents an individual mouse. The number of mice in each group is shown. Two independent experiments were performed. (F) IL-6 released into the dorsal air pouches (ELISA). The number of mice in each group is shown. Two independent experiments were performed. Data in D–F are means ± SD. Statistically significant differences are shown. *, $P < 0.05$.

In the present study, we identified TIM1 and TIM4 as endogenous ligands for LMIR5. Because TIM1 and TIM4 play an important role in mediating uptake of apoptotic cells through recognition of PS, we were curious about the specific functions of LMIR5 in a similar context. In fact, the Ig-like domain of LMIR5 bound to TIM1 in the vicinity of the PS-binding site within the Ig-like domain of TIM1 (Fig. 2). However, the LMIR5–TIM1/4 interaction did not hamper the TIM1/4-mediated clearance of apoptotic cells (Fig. 3, C–E). One possible explanation is that TIM1 or TIM4 binds to PS at a higher affinity in comparison to LMIR5. Alternatively, the binding site of TIM1 or TIM4 to LMIR5 might be close, but not identical, to that to PS. In addition, several lines of evidence (Fig. 3, A and B) led us to conclude that LMIR5 is not directly involved in the clearance of apoptotic cells. Because LMIR5 is a DAP12-coupled activating receptor, it is plausible that the LMIR5–TIM1/4 interaction induces activation of LMIR5-expressing mast cells. However, in in vitro experiments, myeloid cells were not activated by the interaction of endogenous LMIR5 and TIM1. As one possible explanation, we assumed that biological events induced by the LMIR5–TIM1 interaction require in vivo environmental factors such as cytokines/chemokines, cell–cell interaction, or cell–extracellular matrix interaction. We also postulated that the biological outcomes induced by the LMIR5–TIM1 interaction might be evident in a pathological situation where soluble TIM1 and/or surface-expressed LMIR5 increase at high levels. To test this, we generated LMIR5-deficient mice and used a mouse model of acute kidney injury, IRI, where TIM1 is up-regulated in the IRI kidney. Intriguingly, LMIR5 deficiency attenuated the acute kidney damage, characterized by tubular necrosis and cast formation. In addition, the recruitment of neutrophils in IRI kidneys, reportedly associated with tissue damage (Kelly et al., 1996; Wu et al., 2007; Bolisetty and Agarwal, 2009; Lech et al., 2009), was suppressed in LMIR5-deficient mice. Notably, IRI induced the marked up-regulation of TIM1 expression in epithelial tubular cells, followed by the recruitment of neutrophils to IRI kidneys. Considering that a large amount of soluble TIM1 is released in the ischemic kidneys, it was possible that LMIR5-expressing myeloid cells interacted with soluble TIM1 or surface TIM1 in

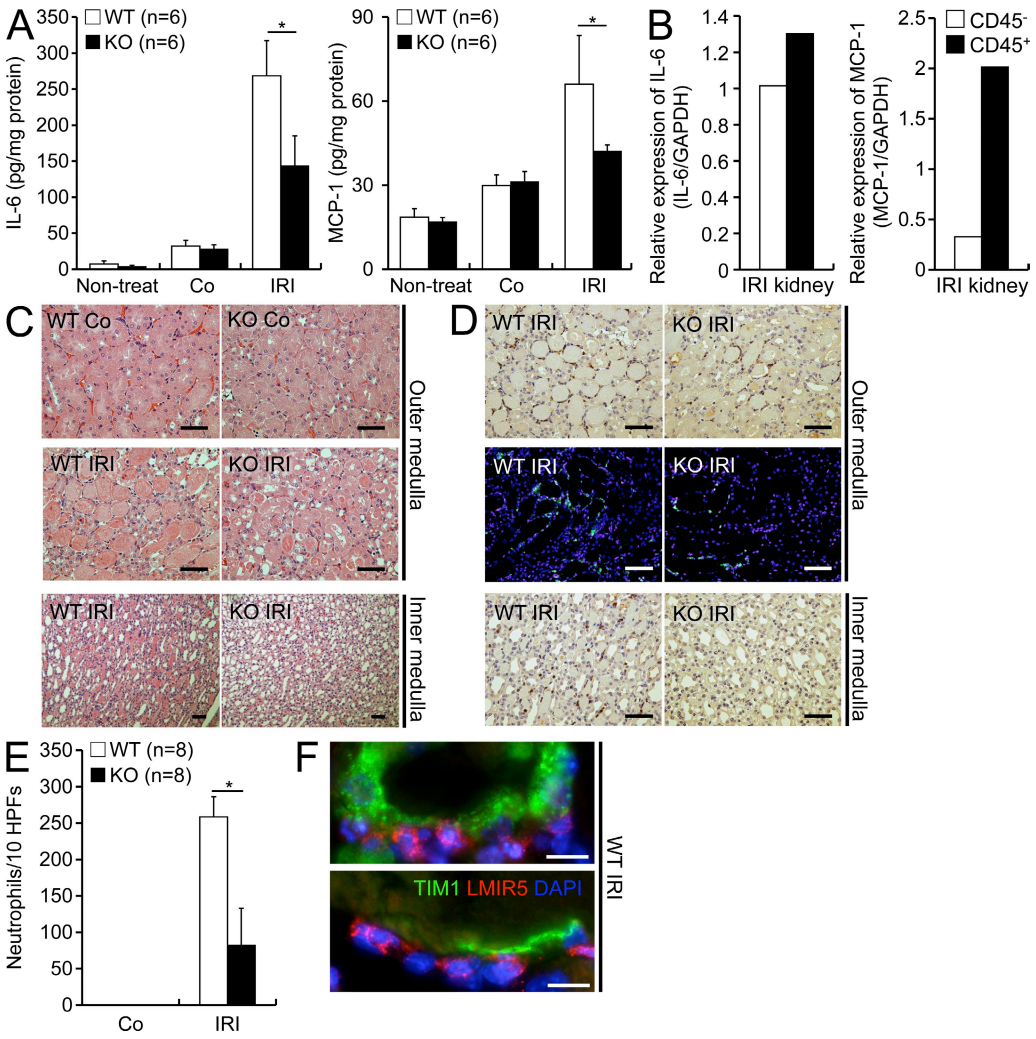

Figure 6. LMIR5$^{-/-}$ mice were protected from renal IRI. (A and B) IL-6 or MCP-1 protein expression (ELISA) in contralateral (Co) or IRI kidneys from WT or LMIR5$^{-/-}$ mice at 24 h after surgery or in nontreated kidneys (A), and relative gene expression levels of IL-6 or MCP-1 (real-time PCR) in CD45^{-} or CD45^{+} cells sorted from IRI kidneys at 24 h after surgery (B). Data are means ± SD (n = 6 mice in each group). Statistically significant differences are shown. *, $P < 0.05$. Data are representative of three independent experiments. (C and D) Tubular injury (C) or neutrophil accumulation within the interstitium in IRI kidneys (D) of WT and LMIR5$^{-/-}$ mice at 24 h after surgery. Representative sections of the outer and inner medulla from contralateral or IRI kidneys from WT and LMIR5$^{-/-}$ mice at 24 h after surgery. (C) Hematoxylin and eosin staining. Bars, 100 µm. Three independent experiments were performed. (D) Immunohistochemical identification of neutrophils (brown) in the outer and inner medulla (top and bottom, respectively). Immunohistochemical identification of neutrophils (green) in the outer medulla (middle). The nuclei were counterstained with DAPI (blue). Bars, 100 µm. Three independent experiments were performed. (E) Neutrophil counts of 10 high-power fields (HPFs) from each section from the outer medulla. Data shown are means ± SD (n = 8 mice in each group). Statistically significant differences are shown. *, $P < 0.05$. Three independent experiments were performed. (F) Immunohistochemical identification of LMIR5-expressing neutrophils (red) and TIM1-expressing epithelial cells (green) in the outer medulla of IRI kidneys of WT mice. The nuclei were counterstained with DAPI (blue). Bars, 10 µm. Three independent experiments were performed. ND, not detected.

epithelial cells. In support of this, histological examination displayed the frequent colocalization of LMIR5-expressing neutrophils and TIM1-expressing epithelial cells in IRI kidneys. Taking these observations together, we assume the relevant mechanism to be as follows. First, soluble TIM1 released from or surface TIM1 expressed by renal tubular cells activates LMIR5-expressing myeloid cells, including resident macrophages/monocytes, mast cells, neutrophils, and dendritic cells through the LMIR5–TIM1 interaction. Then, production of cytokines/chemokines and/or soluble TIM1 promotes the neutrophil recruitment, leading to the renal tubular damage. This scenario was supported by the finding that the neutrophil accumulation induced by TIM1-Fc, a mimic form of soluble TIM1, was dampened by LMIR5 deficiency in the dorsal air pouch experiments. Because the migration of BM neutrophils toward chemoattractants was not affected by LMIR5 deficiency in the in vitro assay (Fig. S7 A), we reasoned that the impaired recruitment of neutrophils in LMIR5-deficient mice was presumably caused by the lack of LMIR5–TIM1 interaction, but not to the

defective migratory function of LMIR5-deficient neutrophils. Of note, neutrophils failed to migrate toward TIM-Fc alone in the in vitro assay (unpublished data), suggesting the requirement of additional signals supplied by the surrounding cells in vivo. We concluded that soluble TIM1 induced the neutrophil accumulation via LMIR5 under in vivo conditions through both direct and indirect mechanisms. However, TIM1-Fc also binds to LMIR5-deficient neutrophils as well as parent Ba/F3 cells (Fig. S1 C and not depicted), suggesting the existence of unidentified ligands for TIM1. Although recent studies implied multiple mechanisms in the pathology of kidney IRI (Kelly et al., 1996; Wu et al., 2007; Bolisetty and Agarwal, 2009; Jang and Rabb, 2009; Lech et al., 2009), the present paper shows that the LMIR5–TIM1 interaction was involved in the tubular damage in the acute phase after IRI.

In conclusion, we provide evidence that TIM1 is a physiological ligand for LMIR5 and that the LMIR5–TIM1 interaction is pivotal in neutrophil accumulation related to tissue damage in kidney IRI. Blocking the LMIR5–TIM1 interaction might be a novel therapeutic strategy for acute renal tubular damage.

MATERIALS AND METHODS

Cells and mice. *DAP12*$^{-/-}$ mice were used as previously described (Kaifu et al., 2003). Animal experiments were conducted in accordance with the guidelines of and with permission provided by the Animal Care and Use Committee of the University of Tokyo (approval no. 20-8). *LMIR5*$^{-/-}$ mice were generated as previously described (Murata et al., 2004). We used *LMIR5*$^{-/-}$ mice that had been backcrossed for at least eight generations with C57BL/6 mice (Charles River).

BM-derived cells, FLMCs, and peritoneal macrophages were generated as previously described (Izawa et al., 2007; Kobayashi et al., 2007; Miyanishi et al., 2007; Yamanishi et al., 2008). Single-cell suspensions of kidney cells were obtained by using Liberase Research Grade Enzyme (Roche).

Antibodies and other reagents. Anti-LMIR5 polyclonal antibody and anti-TIM1 mAb (222414) were obtained from R&D Systems. Biotinylated anti–mouse TIM1 (RMT1-10 or RMT1-4) mAbs were obtained from eBioscience. Anti–mouse TIM1 (RMT1-17), TIM2 (RMT2-14), TIM3 (RMT3-23), and TIM4 (RMT4-54) mAbs have been previously described (Nakayama et al., 2009).

Gene expression analysis. Real-time PCR or RT-PCR was performed using gene-specific primers (Table S1) as previously described (Yamanishi et al., 2008).

Generation of Fc fusion proteins. The cDNA fragments corresponding to the extracellular domains of mouse LMIR1/2/3/4/5, LMIR5 deletion mutants, TIM1, or TIM4 were used. The Fc fusion proteins were purified as previously described (Satoh et al., 2008).

Flow cytometry. In some experiments, cells were stained with 1 μg/ml of Fc fusion proteins or human IgG1 followed by 10 μg/ml of PE-conjugated $F(ab')_2$ donkey anti–human IgG. Flow cytometric analysis was performed with a FACSCalibur (BD) equipped with CellQuest software (BD) and FlowJo software (Tree Star, Inc.), as previously described (Izawa et al., 2007; Yamanishi et al., 2008).

Retrovirus-mediated expression cloning. Expression cloning was performed as previously described (Kitamura et al., 2003). In brief, plasmids (provided by H. Arase, Osaka University, Osaka, Japan) from the cDNA library were transfected into PLAT-E packaging cells (Morita et al., 2000). Infected Ba/F3 cells were stained by LMIR5-Fc. After three rounds of enrichment using a FACSAria (BD), single-cell clones were obtained. The integrated cDNA was recovered by PCR and sequenced.

PIP-strip assay. PIP-strip assay was performed as previously described (Kobayashi et al., 2007). PIP strips were purchased from Echelon Bioscience.

Phagocytosis assay. Phagocytosis assay was performed as previously described (Kobayashi et al., 2007; Miyanishi et al., 2007). CFSE-labeled apoptotic thymocytes were coincubated with peritoneal macrophages in the presence of 10 μg/ml of the Fc fusion proteins indicated in the figures for 30 min. Alternatively, CFSE-labeled live or apoptotic U937 cells were coincubated with NIH3T3 transfectants in the presence of 20 μg/ml of the antibodies or Fc fusion proteins indicated in the figures for 45 min. The percentage of $CSFE^+$ cells was measured by flow cytometric analysis.

Immunoprecipitation and Western blotting. Transfected Ba/F3 cells were incubated with 20 μg/ml LMIR5-Fc, human IgG1, anti-Flag mAb, or mouse IgG1. Cell lysates were immunoprecipitated by using protein A–sepharose. To detect phosphorylation of ERK1/2, transfected BMMCs preincubated with 20 μg/ml TIM1-Fc or human IgG1 for 30 min on ice were stimulated in anti–human IgG antibody–coated plates at 37°C for 7 min, as previously described (Kumagai et al., 2003; Izawa et al., 2007; Yamanishi et al., 2008).

Measurement of cytokines. For co-culture assay, 5×10^4 CHO cells transduced with TIM1 or mock transduced were co-cultured with 2×10^5 FLMCs transduced with LMIR5 or mock transduced for 24 h. Concentrations of IL-6 in the culture supernatants and those of IL-6 and MCP-1 in the renal extract were measured by ELISA, as previously described (Izawa et al., 2007; Wu et al., 2007; Yamanishi et al., 2008). Protein levels of cytokines/chemokines in the renal extract were corrected for the total amounts of protein.

Neutrophil infiltration into mouse dorsal air pouch. Air pouches were formed on the dorsum of mice as previously described (Sin et al., 1986). In brief, 7 ml of sterile air was injected subcutaneously into the back of mice on days 0 and 3. On day 6, 100 μg TIM1-Fc or of control Fc, or 1 mg LPS dissolved in 1 ml of PBS was injected into the air pouches. 4 h after the injection, the air pouches were lavaged. Total cells in the lavage fluid were counted and the percentages of $Gr\text{-}1^+/CD11b^+$ neutrophils were estimated by FACS analysis. IL-6 levels in the lavage fluid were measured by ELISA.

Induction of renal IRI. In brief, the left renal pedicle of male mice was exposed and clamped for 45 min with a microaneurysm clamp via flank incision, as previously described (Lech et al., 2009). Mice were sacrificed at 24, 48, and 96 h after reperfusion. The IRI and contralateral kidneys were collected for histological analysis, flow cytometry analysis, and measurements of cytokines and chemokines.

Histological analysis. Formalin-fixed kidneys were embedded in paraffin and stained with hematoxylin and eosin by standard methods. Neutrophils were detected using rat anti–mouse neutrophil mAb (clone 4/7; AbD Serotec), as previously described (Wu et al., 2007). Neutrophils in the outer medulla were counted in 10 consecutive high-power fields (400×) from each section. Immunofluorescence staining was performed as previously described (Morikawa et al., 2004). In brief, 6-μm-thick frozen sections were stained with primary antibodies: anti-LMIR5 antibody, anti-TIM1 (RMT1-17) mAb, rat anti–mouse neutrophil mAb, and the appropriate secondary antibodies (Jackson ImmunoResearch Laboratories, Inc.). All sections were counterstained with DAPI (Invitrogen).

Statistical analysis. Data are shown as means ± SD. Statistical significance was determined by the Student *t* test, with $P < 0.05$ considered statistically significant.

Online supplemental material. Fig. S1 shows that TIM1 was identified as the ligand for LMIR5 by retrovirus-mediated expression cloning. Fig. S2 shows differential blocking effects of anti-TIM1 antibodies on the TIM1–LMIR5 interaction. Fig. S3 shows that TIM4-Fc as well as TIM1-Fc induced LMIR5-mediated activation of mast cells. Fig. S4 shows no detectable expression levels of TIM1 and TIM4 in BMMCs, FLMCs, or neutrophils. Fig. S5 shows gene targeting of *LMIR5*. Fig. S6 shows normal development of myeloid cells and lymphocytes in LMIR5$^{-/-}$ mice. Fig. S7 shows relative gene expression levels of IL-6, MCP-1, TIM1, or LMIR5 in the postischemic kidneys from WT or LMIR5$^{-/-}$ mice. Table S1 shows the gene-specific primers used in this study. Online supplemental material is available at http://www.jem.org/cgi/content/full/jem.20090581/DC1.

We thank Dr. H. Arase for providing plasmids. We also thank Drs. N. Watanabe and Y. Ishi for cell sorting, and K. Kimura for histological analysis. We are grateful to Dr. D. Wylie for her excellent language support.

This study was supported by grants from the Ministry of Education, Culture, Sports, Science, and Technology (MEXT), Japan, and was in part supported by the Global Center of Excellence Program "Center of Education and Research for the Advanced Genome-Based Medicine–for personalized medicine and the control of worldwide infectious diseases," MEXT, Japan. Y. Yamanishi is supported by a postdoctoral fellowship from the Japan Society for the Promotion of Science.

Dr. T. Kitamura serves as a consultant for R&D Systems. The authors declare no further financial conflicts of interest.

Submitted: 13 March 2009
Accepted: 21 May 2010

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