Phosphatidylserine receptor Tim-4 is essential for the maintenance of the homeostatic state of resident peritoneal macrophages

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Edited by Rafi Ahmed, Emory University, Atlanta, GA, and approved March 30, 2010 (received for review September 23, 2009)

Tim-4 is a phosphatidylserine (PS) receptor that is expressed on various macrophage subsets. It mediates phagocytosis of apoptotic cells by peritoneal macrophages. The in vivo functions of Tim-4 in phagocytosis and immune responses, however, are still unclear. In this study, we show that Tim-4 quickly forms punctate caps on contact with apoptotic cells, in contrast to its normal diffused expression on the surface of phagocytes. Despite its expression in marginal zone and tingible body macrophages, Tim-4 deficiency only minimally affects outcomes of several acute immune challenges, including the trapping of apoptotic cells in the marginal zone, the clearance apoptotic cells by tingible body macrophages, and the formation of germinal centers and elicitation of antibody responses against sheep red blood cells (SRBCs). In addition, Tim-4$^{-/-}$ resident peritoneal macrophages (rPMs) phagocytose necrotic cells and other opsonized targets normally. However, their ability to bind and engulf apoptotic cells is significantly compromised both in vitro and in vivo. Most importantly, Tim-4 deficiency results in increased cellularity in the peritoneum. Resting rPMs produce higher TNF-α in culture. Their response to LPS, on the contrary, is dampened. Our data support an indispensible role of Tim-4 in maintaining the homeostasis of rPMs.

phagocytosis | apoptotic cells | receptor dynamics | TNF-α

Tim-4 is a member of the T-cell immunoglobin mucin (Tim) family of proteins (1, 2). The first recognized function of Tim-4 was its role as a costimulatory molecule regulating T-cell activation (3), largely through its interaction with Tim-1 (3). Recently, Miyanishi et al. (4) demonstrated that Tim-4 is a receptor recognizing apoptotic cells through its binding to phosphatidylserine (PS) on the surface of apoptotic cells. This finding has been independently confirmed by Kobayashi et al. (5). Structural studies of Tim-4 interaction with PS indicated that the hydrophilic head of PS penetrates into the metal-ion-dependent ligand binding site formed between the characteristic CC loop and FG loop of the Ig domain on Tim-4 (6). Interestingly, other Tim family proteins, Tim-1 and Tim-3 (but not Tim-2), have been identified to interact with PS and mediate the phagocytosis of apoptotic bodies by various cell types as well (4, 5, 7, 8).

Apoptotic cells use PS as an "eat-me" signal (9). Multiple receptors have been identified to detect this eat-me signal, including stabilin-2 (10), BAI (11), and PSR (12). In addition, a soluble protein, MFG-E8, binds to PS and brings apoptotic cells to phagocytes through its interaction with integrin αvβ3 (13). Deficiency of MFG-E8 in mice resulted in the development of autoimmunity (14). Given the redundancy of these receptors on macrophages, it is not known whether there is any unique role of Tim-4 in mediating the phagocytosis of apoptotic cells. Tim-4 is highly expressed on resident peritoneal macrophages (rPMs), marginal zone macrophages (MZMs), and other tissue-associated macrophages (15). The functions of Tim-4 in these organs are still largely unaddressed. Importantly, it is unclear whether the expression of Tim-4 is essential to maintain the homeostatic status of these tissue-infiltrating macrophages.

Here, we demonstrate that dynamic Tim-4 distribution is involved in both the binding and engulfing steps during phagocytosis of apoptotic cells. By generating Tim-4-deficient mice, we show that the phagocytic function of Tim-4 is only indispensable for certain subsets of macrophages, particularly the rPMs. Tim-4, however, is essential to maintain the homeostatic state of rPMs. The number of macrophages in the peritoneum of Tim4$^{-/-}$ mice increases significantly. In addition, in the absence of any stimulation, these macrophages produce more basal TNF-α in culture.

Results

Tim-4 Is Involved in Both the Adhesion and Ingestion Processes of Phagocytosis of Apoptotic Cells. Phagocytosis of apoptotic cells is a highly dynamic process that requires coordinated regulation of phagocytic receptors and the engulfing machineries over time and space. To determine the dynamic localization of Tim-4 during phagocytosis, we assessed the distribution of Tim-4 in rPMs by immunofluorescence microscopy with an anti-Tim-4 antibody (2H3). In the absence of apoptotic cells, Tim-4 (Fig. 1*A*, *Upper*, red) normally localizes to the plasma membrane in a diffused manner. When apoptotic thymocytes are present, this diffused pattern changes to punctate staining that resides at junctions between the apoptotic cells and macrophage at 30 min after incubation (Fig. 1*A*, *Lower*, arrows). Fig. 1*B* shows the formation of Tim-4 clusters (red) around the apoptotic cells (nuclei stained in blue) in three dimensions.

To study the dynamic localization of Tim-4 throughout the process of phagocytosis further, we transfected 293T cells with an expression plasmid that encodes mouse Tim-4 C-terminally fused with YFP (mTim-4-YFP) and monitored the localization of YFP using time-lapse fluorescence microscopy (Fig. 1*C* and Movie S1). The expression and function of Tim-4 have been confirmed in 293T cells (Fig. 1 *D–F*). Before the addition of apoptotic thymocytes, Tim-4-YFP signal is mainly found diffusely throughout the cell membrane (Fig. 1*C*, *Right*). Live imaging of the same cell shows that shortly after the addition of apoptotic cells, Tim-4-YFP signal reorganizes. At 10 min, ring-like structures can be found at sites that coincide with apoptotic cell contacts (Fig. 1*C*, arrow). Immunofluorescence microscopy study of these cells at 90 min after incubation with apoptotic cells shows a similar distribution: Tim-4 signals aggregate to puncta adjacent to apoptotic cells (Fig. 1*D*, *Upper Right*, arrow). To determine whether the short cytoplasmic tail of Tim-4 is required for this dynamic tethering process, a Tim-4 construct containing a deletion of its cytoplasmic domain (Tim-4ΔC-YFP) was tested in the same system. Tim-4ΔC-YFP aggregates at contact sites with

Author contributions: K.W. and W.O. designed research; K.W., P.A.V., and S.Y. performed research; K.W., P.A.V., C.T., and J.-A.H. contributed new reagents/analytic tools; K.W. and W.O. analyzed data; and K.W. and W.O. wrote the paper.

Conflict of interest statement: All authors are employees of Genentech, Inc.

This article is a PNAS Direct Submission.

Freely available online through the PNAS open access option.

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This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.0910929107/-/DCSupplemental.

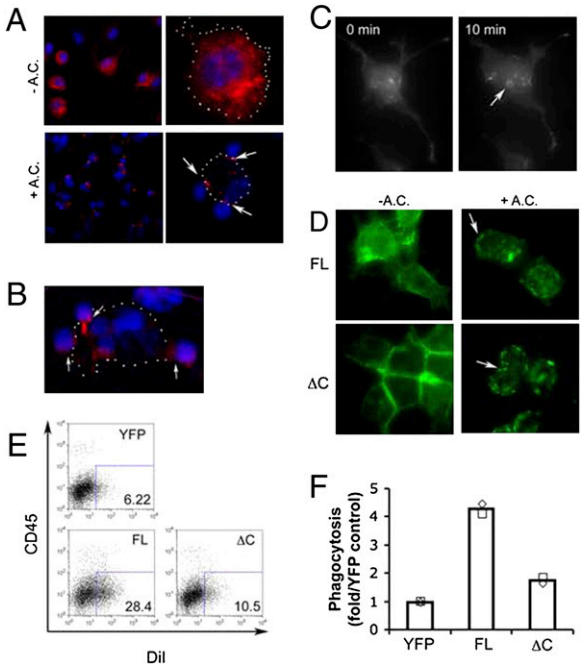

Fig. 1. Tim-4 undergoes dynamic localization, and its cytoplasmic tail is required during the phagocytosis of apoptotic cells. (*A*) Immunofluorescence images of rPMs from WT mice stained for Tim-4 (red) and nuclei (blue) without (*Upper*) or with (*Lower*) the addition of apoptotic cells (A.C.). Thymocytes are identified by their DAPI-stained nuclei with round morphology. The dotted line outlines the cell periphery. Arrows point to contact sites between macrophage and apoptotic cells with enriched Tim-4 signal. (*B*) 3D image of a macrophage in contact with apoptotic cells (stained blue with nuclear dye) revealing the concentrated Tim-4 (red) distribution to these contact sites (arrows). (*C*) Time-lapse images of a 293T cell expressing Tim-4-YFP. Apoptotic thymocytes were added at 0 min. Rings of YFP-rich structure (arrows) began to appear at 10 min after apoptotic cell addition. (*D*) Surface expression of full-length mTim-4-YFP (FL) and mTim-4ΔC-YFP (ΔC). 293T cells transfected with either construct were stained for cell surface Tim-4 without detergent permeabilization. Representative images are shown with or without the addition of apoptotic cells (A.C.). Arrow points to aggregate of surface Tim-4 signal. (*E*) FACS plot of YFP$^+$ cells from 293T cells expressing YFP alone, full-length mTim-4-YFP (FL), or mTim-4ΔC-YFP (ΔC). After a 90-min incubation with DiI-labeled apoptotic thymocytes, extracellular thymocytes were stained for CD45. YFP$^+$ cells that have phagocytosed apoptotic cells are DiI$^+$ CD45$^-$ (gated). (*F*) Graph of phagocytic YFP$^+$ cells relative to 293T cells expressing YFP alone. Each experiment was performed at least three times. Representative data are shown.

apoptotic cells with similar dynamics as those of full-length Tim-4-YFP when overexpressed in 293T cells, suggesting that the intracellular tail of Tim-4 is not essential to recruit apoptotic cells by these 293T cells (Fig. 1*D*, *Lower*).

We examined the requirement of the cytoplasmic tail of Tim-4 for the ingestion step in 293T cells. Expression of full-length Tim-4-YFP increases the ability of these cells to phagocytose apoptotic cells compared with cells expressing YFP alone (Fig. 1 *E* and *F*). Cells expressing mTim-4ΔC-YFP show reduced phagocytosis compared with those expressing full-length mTim-4-YFP at a level comparable to that of YFP-expressing cells (Fig. 1 *E* and *F*). Our data demonstrate that the Tim-4 cytoplasmic tail is required for the ingestion process in 293T cells.

Tim-4 Is Present in a Subpopulation of MOMA-1$^+$ MZMs and Tingible Body Macrophages in the Spleen. To understand the role of Tim-4 in phagocytosis and in immune responses in vivo further, we generated Tim-4-deficient mice on a pure 129 background (Fig. S1*A*). Tim-4 KO mice are born in normal Mendelian ratios and develop normally. Tim-4 deficiency has been confirmed at the genomic DNA, mRNA, and protein expression levels (Fig. S1 *B–D*). The expression of Tim-1, Tim-2, and Tim-3, which reside with Tim-4 in the same locus, is comparable in WT and Tim-4 KO mice, however (Fig. S1 *E–G*).

We assessed various lymphoid and myeloid cell populations in spleens from Tim-4 WT and KO animals by FACS analysis. The total number or frequency of various immune subsets, including T cells, B cells, natural killer cells, and myeloid cells, is comparable in the spleens of WT and KO animals (Fig. S2*A*). Similar results are obtained from analyses on lymph nodes and blood samples from WT and Tim-4$^{-/-}$ mice. Furthermore, the activation states of splenic dendritic cells (DCs) and T and B cells are similar in WT and Tim-4 KO mice (Fig. S2*B*). Quantification of the serum antibody isotype of naive mice shows that Tim-4 KO animals exhibit increased IgG2a at 6 months in comparison to their age-matched WT counterparts (Fig. S2*C*).

Immunofluorescence study confirms that Tim-4 is expressed in the marginal zone and germinal center (Fig. 2*A*). In particular, Tim-4 signal mostly overlaps with that of the MOMA-1$^+$ metal-

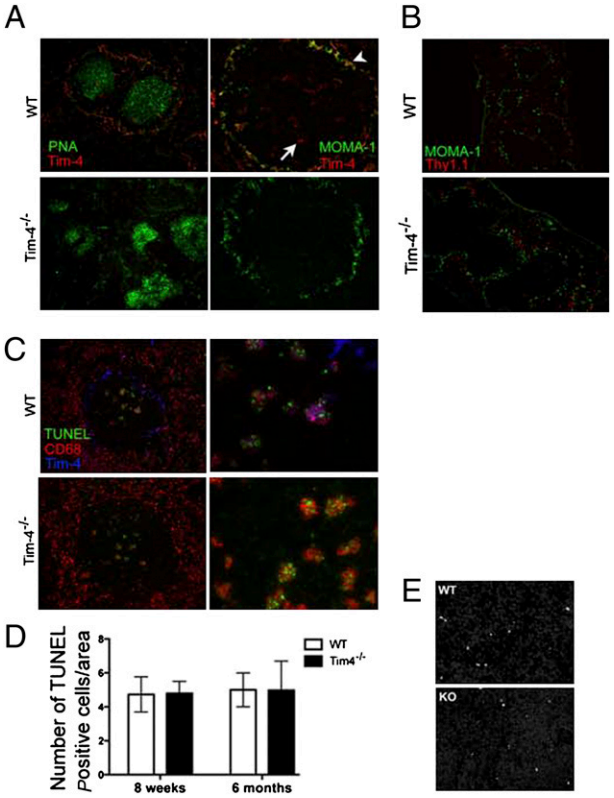

Fig. 2. Tim-4 in the marginal zone and germinal center of the spleen is not required for apoptotic cell trapping and clearance or anti-SRBC antibody responses. (*A*) Tim-4 is expressed in and inside the marginal zone. Cryosections from WT and Tim-4$^{-/-}$ spleens 6 days after SRBC i.p. injection stained for peanut agglutinin (PNA) (green) and Tim-4 (red) (*Left*) or costained for the marginal metallophilic macrophage marker, MOMA-1 (green) and Tim-4 (red) (*Right*). The arrowhead indicates Tim-4$^+$ MOMA-1$^+$ cells in the marginal zone. Inside the marginal zone, Tim-4 can be detected in cells that are MOMA-1$^-$ (arrow). (*B*) Fluorescence images of a spleen section from animals injected with Thy1.1$^+$ apoptotic thymocytes. Trapping of Thy1.1 apoptotic thymocytes (red) by MOMA-1 (green) is observed for both WT and Tim-4$^{-/-}$. (C) Tingible bodies in spleens from WT and Tim-4$^{-/-}$ mice after primary SRBC challenge. Apoptotic bodies are shown in green by TUNEL staining. Tingible body macrophages are stained with CD68. Tim-4 is shown in blue. (*D*) Graph of average number of TUNEL$^+$ cells per imaging field in the spleens of WT and Tim4$^{-/-}$ mice at 8 weeks and 6 months of age. Error bars: ±SEM. (*E*) Representative images of TUNEL$^+$ (white dots) splenic sections from WT (*Upper*) and Tim4$^{-/-}$ (*Lower*) animals (n = 3). DAPI counterstain is shown (gray).

lophilic macrophage population in the marginal zone (Fig. 2*A*, arrowhead). Some MOMA-1$^-$ MZMs also express Tim-4. In addition, we observed Tim-4$^+$ cells inside the germinal centers (Fig. 2*A*, arrow). Further detailed analysis shows that these cells correspond to tingible body macrophages (Fig. 2*C*). Consistent with these results, FACS analysis using macrophage and DC markers shows that Tim-4$^+$ cells are mainly in DC and MOMA-1$^+$ populations (Fig. S3).

Minimal Roles of Tim-4 in Apoptotic Cell Trapping and Clearance in the Spleen. Splenic MZMs play major roles in trapping foreign antigen, whereas tingible bodies are sites in the germinal centers at which nonproductive apoptotic B cells are phagocytosed by macrophages. To assess the requirement for Tim-4 in trapping apoptotic thymocytes, we injected apoptotic thymocytes from Thy1.1$^+$ AKR mice into WT and Tim-4$^{-/-}$ mice. Thirty minutes after injection, spleen sections were stained for Thy1.1 and MOMA-1. Thy1.1$^+$ thymocytes are found in the red pulp alongside MOMA-1$^+$ cells in both Tim-4 WT and KO spleen (Fig. 2*B*), indicating that lack of Tim-4 does not compromise the ability of MZMs to trap apoptotic cells. To test the involvement of Tim-4 in the function of tingible macrophages, we determined the localization of apoptotic cells and their association with tingible body macrophages in spleen sections after sheep red blood cell (SRBC) immunization. Using immunofluorescence microscopy, we found that Tim-4 (blue) is expressed in germinal centers on CD68$^+$ cells (red) that associate with TUNEL$^+$ cells (green) in WT spleens (Fig. 2*C*, *Top*). No apparent difference is observed between WT and Tim-4$^{-/-}$ spleens (Fig. 2*C*), implying a redundant role of Tim-4 for the phagocytic function in tingible body macrophages. Consistently, there is no increased accumulation of apoptotic cells in the spleens of both 8-week-old and 6-month-old Tim-4$^{-/-}$ mice (Fig. 2 *D* and *E*).

Normal Delayed-Type Hypersensitivity and Anti-SRBC Antibody Responses in Tim-4-Deficient Mice. To assess whether Tim4$^{-/-}$ mice can mount normal immune responses, we injected WT and Tim-4 KO mice with an emulsion of ovalbumin (OVA) peptide and complete Freud's adjuvant and measured the delayed-type hypersensitivity (DTH) response 7 days later by challenging the right ears with a low dose of OVA. The change in ear thickness in Tim-4$^{-/-}$ animals elicited by OVA is comparable to that in WT animals, suggesting that general T-cell responses are normal in Tim-4$^{-/-}$ mice (Fig. 3*A*).

Next, we i.p. immunized 8–10-week-old Tim-4-deficient mice and their WT littermates with SRBCs and analyzed lymphoid follicles in the spleens and anti-SRBC level in sera at day 6. Cryosections of spleens from WT and Tim-4$^{-/-}$ mice were costained for peanut agglutinin (PNA), CD35, and B220 for germinal centers, follicular DCs, and B cells, respectively. Tim-4-deficient mice show more variability in the size and shape of this lymphoid structure (Fig. 3*B*). Nevertheless, mice lacking Tim-4 are equally capable of mounting an SRBC-specific antibody response, as evidenced by the comparable serum levels of anti-SRBC IgM and IgG after primary (Fig. 3*C*) and secondary (Fig. 3*D*) challenges as those from WT mice.

Miyanishi et al. (4) showed that treatment of C57/Bl6 mice with an anti-Tim-4 antibody for 5 weeks results in increased anti-dsDNA antibody. We measured the level of anti-dsDNA in aged WT and Tim-4 KO mice but failed to detect any significant difference in age- and gender-matched WT and Tim-4$^{-/-}$ animals (Fig. 3*E*). When apoptotic cells were injected every 2 weeks for five times to induce the production of autoantibody (16), the level of anti-dsDNA increased over time in both WT and Tim-4 KO mice in a similar manner (Fig. 3*F*).

Tim-4 Is Uniquely Required for rPMs to Phagocytose Apoptotic Cells. rPMs from WT and Tim-4 KO animals were assessed for their ability to phagocytose in vitro by fluorescence microscopy (Fig. 4 *A* and *B*) and FACS (Fig. S4), as described (5) in *SI Materials and Methods*. Using this technique, macrophages from Tim-4$^{-/-}$ mice show

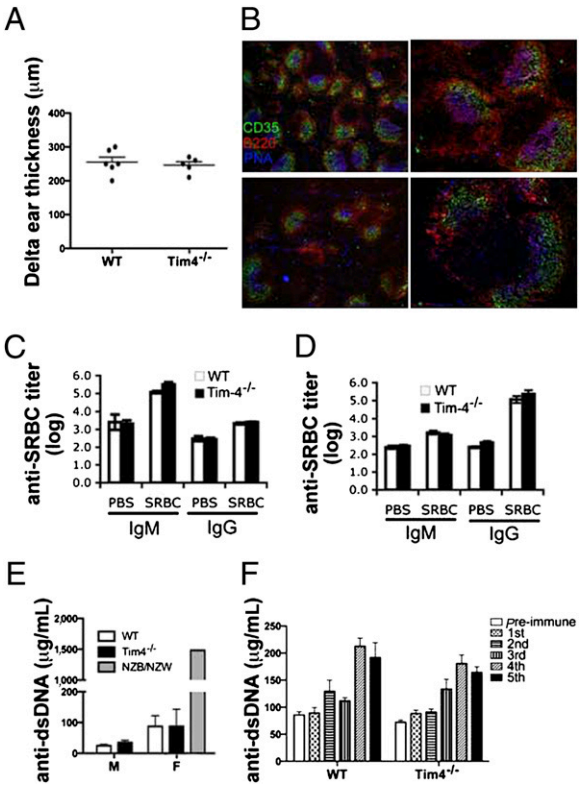

Fig. 3. Unaltered immune function in Tim4$^{-/-}$ mice. (*A*) Change in ear thickness in WT and Tim4$^{-/-}$ mice DTH response induced by OVA (n = 6). (*B*) Structure of lymphoid follicles in spleens after SRBC challenge. Sections from Fig. 2*A* were stained with CD35 (green), B220 (red), and peanut agglutinin (PNA) (blue) for follicular DCs, B cells, and germinal center B cells, respectively. Serum IgM and IgG levels of anti-SRBCs after primary (*C*) and secondary (*D*) challenges are shown. The serum level of anti-dsDNA from aged (6–8 months old) WT and Tim4$^{-/-}$ mice (*E*) or from mice after each biweekly injection of apoptotic thymocytes (*F*) ($n \geq 7$). The serum level of anti-dsDNA from the autoimmune-prone NZB/NZW F1 mouse strain is shown as a reference (gray bar in *E*). Error bar: ±SEM. Each immunization experiment was repeated once with n = 5.

a drastic decrease in their ability to phagocytose apoptotic cells compared with their WT counterparts (Fig. 4 *A* and *B*). We also noticed that Tim-4-deficient macrophages seldom associate with apoptotic cells in contrast to WT macrophages (Fig. 4 *A* and *B*). These results are confirmed by independent FACS analysis (Fig. S4).

To test the in vivo requirement of Tim-4 during apoptotic cell clearance, DiI-labeled apoptotic thymocytes are injected i.p. into Tim-4 WT and KO animals. Cells from the peritoneum are harvested 30 min after injection and stained with Thy1.2 to label thymocytes that remain outside the macrophages. FACS analysis shows reduced frequency of DiI$^+$ F4/80$^+$ macrophages from Tim-4-deficient mice (Fig. 4*C*), suggesting a reduced binding of apoptotic cells to Tim-4$^{-/-}$ macrophages. Within the F4/80$^+$ cells, the frequency of DiI$^+$ Thy1.2$^-$ cells is also significantly reduced in the Tim-4$^{-/-}$ sample (Fig. 4 *C–E*, *Right*), indicating a lower phagocytic level compared with WT. These results support an indispensable role for Tim-4 in phagocytosing apoptotic cells by rPMs.

Thioglycollate-elicited macrophages in the peritoneum lack Tim-4 expression (4). We confirmed the finding that the Tim-4$^+$ population is dramatically reduced (from 70 to 10%) upon thioglycollate administration in the peritoneum (Fig. S5 *A* and *B*). In addition, the level of Tim-4 expression in the remaining positive population is decreased (mean fluorescence intensity: 1,539 ± 95 in WT vs. 1,077 ± 62.5 in KO) (Fig. S5 *A* and *B*). In vitro treatment of plated rPMs in culture with proinflammatory cytokines, such as TNF-α and IFN-γ,

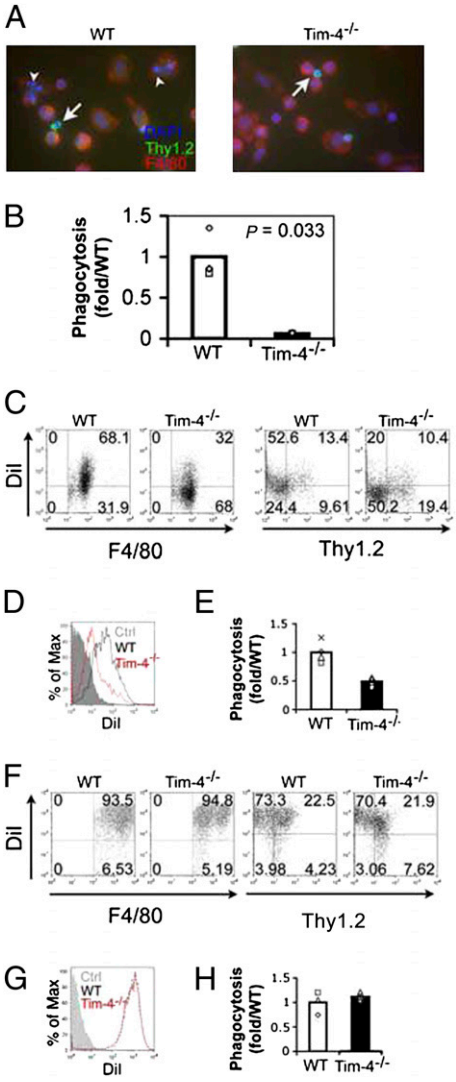

Fig. 4. Specific requirement of Tim-4 in the phagocytosis of apoptotic cells by rPMs. (*A*) rPMs fed with apoptotic thymocytes were stained for Thy1.2 (green), F4/80 (red), and DAPI (blue). Apoptotic cells internalized by macrophages appear as round nuclear-stained dots devoid of green staining inside red F4/80$^+$ cells (arrowheads). Arrows depict extracellular apoptotic cells. (*B*) Quantification of internalized thymocytes per 100 macrophages relative to WT. (*C–H*) In vivo phagocytosis by rPMs. DiI-labeled apoptotic thymocytes were injected i.p. into WT or Tim-4$^{-/-}$ mice, and peritoneal cells were harvested at 30 min and stained with Thy1.2 to identify extracellular thymocytes. FACS plots from peritoneal cells from animals injected with apoptotic cells (*C*) and the corresponding histogram of DiI intensity in the F4/80$^+$ population (*D*) are shown. Filled gray curves are macrophages without apoptotic cells. F4/80$^+$ macrophages that were DiI$^+$ Thy1.2$^-$ were scored as phagocytic. (*E*) Percent of phagocytic macrophages that engulfed apoptotic cells were expressed relative to WT. (*F–H*) Phagocytosis of apoptotic thymocytes by thioglycollate-elicited peritoneal macrophages from WT and Tim-4$^{-/-}$ mice. (*F*) FACS plots of DiI (*Left*) and DiI and Thy1.2 (*Right*) within the F4/80$^+$ population. (*G*) Histogram of DiI intensity in the F4/80$^+$ population. The filled gray curve represents macrophages without apoptotic thymocytes. (*H*) Graph of phagocytic thioglycollate-elicited peritoneal macrophages relative to WT. Each experiment was repeated at least twice.

does not alter Tim-4 expression. However, LPS can moderately repress the Tim-4 expression level on these macrophages (Fig. S5*C*), suggesting that a Toll-like receptor (TLR) pathway may participate in the regulation of Tim-4. Finally, in contrast to rPMs from Tim-4$^{-/-}$ mice, thioglycollate-activated macrophages from Tim-4$^{-/-}$ mice maintain their ability to phagocytose apoptotic cells (Fig. 4 *F–H*), further supporting that the role of Tim-4 is limited by the state and compartment of macrophages.

Tim-4 Deficiency Results in Increased Cellularity in the Peritoneum but Does Not Alter Fc or Complement Receptor-Mediated Phagocytosis. The defects of Tim-4$^{-/-}$ rPMs in the clearance of apoptotic cells in vivo prompted us to examine the homeostatic states of the peritoneal macrophages further. First, we found that there is a significant increase in the number of total cells in the peritoneum of Tim-4 KO mice without any challenge (Fig. 5*A*). Both lymphoid and myeloid populations are expanded in the Tim-4-deficient mice (Fig. 5*B*). In contrast to the lack of expression of Tim-4 (Fig. S1*D*), expression of other phagocytic receptors examined, namely, CD11b, Fcγ-RII and III, and CRIg (17), is not altered in Tim-4$^{-/-}$ mice (Fig. S6). In addition, the expression of MHCII and costimulatory molecules CD80 and CD86 is unchanged.

Fc receptors and complement receptors mediate phagocytosis of opsonized targets by macrophages. It is unclear whether Tim-4 plays a role in these processes. Using previously established methods (18, 19), we tested these possibilities by assaying rPMs from Tim-4 WT and KO mice for their ability to engulf beads coated with either rabbit IgG or complement C3, respectively. After phagocytosis, the cultures were stained for anti-rabbit IgG or anti-C3 to differentiate beads associated extracellularly with the cell surface of macrophages from those that have been internalized. Fluorescence microscopy revealed that F4/80$^+$ rPMs from Tim-4$^{-/-}$ mice show a slight decrease in Fc receptor-mediated uptake of IgG-coated beads (Fig. S7 *A* and *B*), whereas the engulfment of C3-coated beads is modestly increased (Fig. S7 *C* and *D*).

Next, we injected i.p. SRBCs coated with complement C3 and measured the degree of their uptake mediated by the complement receptor pathway in vivo, as detailed in *SI Materials and Methods*. F4/80$^+$ macrophages that have internalized C3-coated SRBCs are IgM$^+$ and C3$^-$. Unlike the phagocytosis of apoptotic cells, the frequency of IgM$^+$ in an F4/80$^+$ population is similar between WT and KO populations, suggesting that macrophages from WT and Tim-4$^{-/-}$ animals associate equally well with C3-coated SRBCs (Fig. S7*E*, *Left*). Moreover, the frequencies of F4/80$^+$ macrophages that have internalized C3-coated SRBCs (IgM$^+$ C3$^-$) from WT and KO populations are comparable (Fig. S7*E*, *Right*, and Fig. S7 *F* and *G*). We infer from these results that Tim-4 is not necessary for C3-mediated phagocytosis in vivo.

Necrotic cells also expose PS on their surface but are thought to engage distinct phagocytic machinery for their clearance. To test whether Tim-4 plays a role in this process, we assessed the ability of Tim-4-deficient F4/80$^+$ cells to uptake thymocytes that have been labeled with DiI and induced to undergo necrosis by heating. Extracellular thymocytes were identified by Thy1.2 staining, as described above. Microscopy analysis revealed that fewer necrotic cells associate with Tim-4-deficient macrophages (Fig. S7*H*), in contrast to their WT counterparts. When phagocytosis was measured by counting DiI$^+$ Thy1.2$^-$ cells inside F4/80$^+$ macrophages, both Tim-4 WT and KO macrophages showed a similar level of necrotic cell uptake (Fig. S7*I*). Taken together, these data dem-

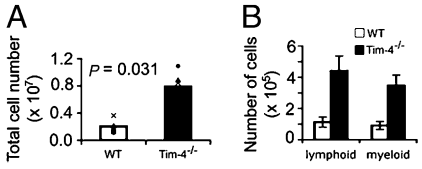

Fig. 5. Tim-4 deficiency causes increased peritoneal cell number. Peritoneal cells from WT and Tim-4$^{-/-}$ mice were collected by lavage (n = 3). (*A*) Total number of peritoneal cells from WT and Tim-4$^{-/-}$ mice. (*B*) Number of cells in lymphoid and myeloid compartments was determined by gating based on forward and side scatter. Error bar: ±SEM.

onstrate that Tim-4 is not essential for phagocytosis of necrotic cells and other opsonized targets.

Tim-4 Deficiency Results in Dysregulation of TNF-α Production from rPMs. Engulfment of apoptotic cells suppresses TNF-α production induced by LPS from peritoneal macrophages. We hypothesized that this repression of TNF-α production by apoptotic cells was compromised in Tim-4-deficient peritoneal macrophages because of their failure to engulf apoptotic cells. rPMs were allowed to adhere to plates for 2 h; they were then washed and followed by various culture conditions. We first confirmed that LPS induces TNF-α production from peritoneal macrophages in culture and that the induction is inhibited by adding apoptotic cells concurrently (Fig. 6*A*, open bars). To our surprise, LPS failed to induce comparable TNF-α production from peritoneal macrophages cultured from Tim-4 KO mice after 4 h of stimulation. Adding apoptotic cells does not inhibit TNF-α production further (Fig. 6*A*, filled bars). Interestingly, without LPS stimulation, Tim-4-deficient peritoneal macrophages in culture have a higher basal level of TNF-α when compared with that from WT macrophages (Fig. 6*A*).

Detailed kinetic studies reveal that the compromised TNF-α production from Tim-4$^{-/-}$ macrophages is consistent at all time points examined (Fig. 6*B*). Lower TNF-α production from Tim-4$^{-/-}$ macrophages can be detected as early as 2 h and lasts at least until 72 h after LPS stimulation (Fig. 6*B*). The elevated basal level of TNF-α production, however, is most evident at 2 h after the start of the experiment (Fig. 6*D*). This basal TNF-α level gradually drops after 2 h in culture, presumably attributable to the consumption and degradation of TNF-α in the culture system. Despite the changes in TNF-α, the levels of IL-6, IL-10, and IL-12(p40) in macrophage cultures from WT and Tim-4$^{-/-}$ animals remain comparable with or without LPS (Fig. 6 *C* and E, respectively).

The diminished LPS response prompted us to examine the expression of the LPS receptor TLR-4 on rPMs from Tim-4$^{-/-}$ mice. The level of TLR-4 on the resting rPMs from Tim4$^{-/-}$ mice is reduced when compared with that of WT mice (Fig. S8*A*). As a control, the expression of TLR-2 is comparable in both WT and Tim-4$^{-/-}$ macrophages (Fig. S8*B*). However, after 2 h of culture, macrophages from WT and Tim4$^{-/-}$ mice show a similar increase in TLR-4 expression (Fig. S8*C*), suggesting that TLR-4 alone is insufficient to account for the dampened LPS-induced TNF-α production from Tim-4$^{-/-}$ macrophages.

To assess whether Tim-4-deficient mice are more prone to inflammation in the peritoneum, we measured cytokine levels in lavage fluids from WT and Tim-4 KO mice at a basal state and after *Escherichia coli* i.p. injection. At a basal state, the levels of IL-17, IFN-γ, TNF-α, and keratinocyte-derived chemokine (KC) from both WT and Tim-4 KO mice fall below the detection limits, although their levels increase comparably after *E. coli* challenge (Fig. 6 *F–I*).

In conclusion, our data demonstrated that Tim-4 deficiency results in an alteration of the homeostatic state of rPMs, characterized by increased cell numbers, elevated basal TNF-α production, and dampened responsiveness to LPS.

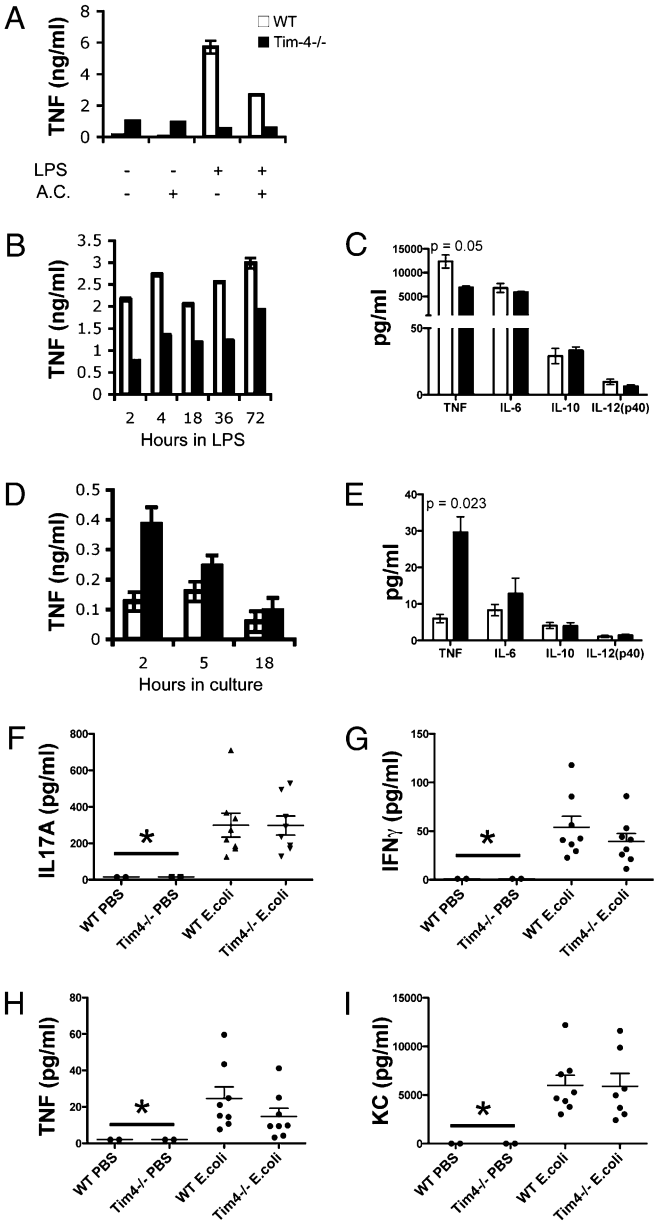

Fig. 6. Tim-4$^{-/-}$ rPMs produce elevated basal TNF-α and exhibit dampened TNF-α production when stimulated with LPS. (*A*) TNF-α level in supernatant from rPMs cultured for 4 h without or with 100 ng/mL LPS in the absence or presence of apoptotic thymocytes (A.C.) measured by ELISA. The graph shows representative data from two independent experiments. TNF-α concentration in supernatant from rPMs cultured in the presence (*B*) or absence (*D*) of 100 ng/mL LPS for the indicated time. Levels of TNF-α, IL-6, IL-10, and IL-12(p40) in supernatant of resident peritoneal cell culture at 4 h in the presence (*C*) or absence (*E*) of LPS. Shown are representative data from three independent experiments. (*F–I*) Levels of IL-17, IFN-γ, TNF-α, and keratinocyte-derived chemokine (KC) in peritoneal lavage from WT and Tim4$^{-/-}$ mice injected i.p. with PBS or *E. coli*. *, measurement below detection limit. Error bar: ±SEM.

Discussion

Through a dynamic process, Tim-4 is involved in both the adhesion and ingestion steps during phagocytosis of apoptotic cells. Tim-4 is normally dispersedly expressed on the cell surface of rPMs (Fig. 1), which may increase its chance of detecting surrounding apoptotic cells. On contact with apoptotic cells, Tim-4 quickly forms structures that encapsulate the apoptotic bodies, resembling phagocytic cups (Fig. 1). The extracellular domain of Tim-4 is sufficient in mediating this adhesion step, because deletion of the intracellular domain of Tim-4 does not disrupt the formation of these ring-like structures (Fig. 1*D*). Interestingly, we note that the engulfment of apoptotic cells is significantly reduced when cytoplasmic tail-truncated Tim-4 is expressed in 293T cells (Fig. 1 *E* and *F*), suggesting that the cytoplasmic tail of Tim-4 is important in mediating the engulfment step in these cells. The cytoplasmic tail of Tim-4 may directly or indirectly interact with cytoskeleton proteins, similar to other PS receptors (10, 11).

Timely removal of apoptotic cells is critical to maintain the integrity and functions of the surrounding tissues (9, 20). Myeloid cells expressing different PS receptors, such as Tim-4 and MFG-

E8, may exert distinct functions in the clearance of apoptotic cells during various immune challenges. We show here that the function of Tim-4 in the clearance of apoptotic cells is limited to rPMs. Under immune challenges, such as thioglycollate stimulation, inflammatory macrophages are recruited into the peritoneal cavity. These macrophages express MFG-E8 but not Tim-4. In addition, Tim-4 expression on the rPMs is down-regulated (Fig. S5 *B* and *C*). It is thus not surprising that the phagocytosis of apoptotic cells is mediated by MFG-E8 but not by Tim-4 under this condition (4, 14). Similar redundancy may also occur under other acute immune challenges, because there are no obvious defects in Tim-4 KO mice during *E. coli* challenge, DTH response, and SRBC-elicited antibody responses. However, despite the redundancy, we cannot completely exclude a role of Tim-4 in these responses. Generation of mice deficient in both Tim-4 and MFG-E8 may be required in the future to address this issue further. Finally, the levels of anti-dsDNA in aged or apoptotic cell-injected mice do not differ significantly between WT and Tim-4 KO animals (Fig. 3 *E* and *F*). This is in contrast to previous data showing that blocking Tim-4 with an antibody leads to autoimmunity (4). We think that these different results are likely attributable to the different genetic backgrounds of mice.

Tim-4 plays a crucial role in mediating the phagocytosis of apoptotic cells by rPMs both in vitro and in vivo. In addition, we noticed that there is elevated TNF-α production during the attachment of rPMs to the plate in culture. Although we have not detected a similar elevation of TNF-α in vivo, presumably because of its relatively low level, a similar mechanism could take place when rPMs adhere to other tissue cell types or extracellular matrix. This low level of TNF-α production, as well as a defect in the clearance of apoptotic cells by these rPMs, may represent a low degree of the inflammatory state, which could alter the homeostatic status in the peritoneal cavity. In supporting this premise, we found an increased number of both myeloid and lymphoid cells in the peritoneum of Tim-4 KO mice. Furthermore, rather than play a direct role in acute inflammatory responses, Tim-4 may be essential in some chronic autoimmune diseases, such as type I diabetes and lupus, in which defects in clearance of apoptotic cells have been reported (21, 22).

The responses to LPS by Tim-4$^{-/-}$ rPMs are consequently compromised in vitro, as measured by TNF-α production (Fig. 6*B*). The dampened TNF-α response to LPS might be attributable to desensitization as a result of the elevated basal TNF level (23). It is still unclear why loss of Tim-4 on the rPMs causes increased cellularity and higher basal TNF-α production. Because Tim-4 has been shown to play a role in adhesion (24) and increased basal TNF-α is observed in supernatant from in vitro culture of adherent rPMs, Tim-4 may modulate secretion of TNF-α by regulating adhesion. It is thus tempting to speculate that Tim-4, by maintaining the ability of rPMs to engulf apoptotic cells, constrains the recruitment and activation of these cells in vivo.

Materials and Methods

Mice, RT-PCR, Real-Time PCR, DNA Constructs, and Transfection. TIM-4 KO mice were generated by homologous recombination, as described in *SI Materials and Methods*. RT-PCR, real-time PCR, DNA constructs, and transfection were done by standard methods, as described in *SI Materials and Methods*. Splenocytes were polarized to T helper subtypes, as described elsewhere (25).

Antibodies, Immunofluorescence Staining, and Microscopy. Immunostaining and imaging were done using standard protocols, and antibodies used are described in *SI Materials and Methods*.

Phagocytosis Assays. Phagocytosis assays on peritoneal macrophages (5) and 293T cells were performed and analyzed as described in *SI Materials and Methods*.

In vivo phagocytosis assay was performed by i.p. injecting DiI-labeled apoptotic thymocytes or C3-coated SRBCs. Thirty minutes following injection, peritoneal cells were collected by lavage, blocked, and stained as described above for in vitro assay on peritoneal macrophages.

In Vitro Macrophage Culture and Quantification of Cytokines. Macrophages were isolated and cultured, and TNF-α and other cytokines produced were measured as detailed in *SI Materials and Methods*.

In Vivo Models for Immune Responses. Models for SRBC immunization, i.p. *E. coli* challenge, DTH, and apoptotic cell injection-induced autoantibody are detailed in *SI Materials and Methods*.

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