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Interactions between CD47 and Thrombospondin Reduce Inflammation

Laurence Lamy,* Arnaud Foussat,† Eric J. Brown,‡ Paul Bornstein,§ Michel Ticchioni,*† and Alain Bernard²*¹

CD47 on the surface of T cells was shown in vitro to mediate either T cell activation or, in the presence of high amounts of thrombospondin (TSP), T cell apoptosis. We report here that CD47-deficient mice, as well as TSP-1 or TSP-2-deficient mice, sustain oxazolone-induced inflammation for more than four days, whereas wild-type mice reduce the inflammation within 48 h. We observe that prolonged inflammation in CD47-, TSP-1-, or TSP-2-deficient mice is accompanied by a local deficiency of T cell apoptosis. Finally, we show that upon activation normal T cells increase the expression of the proapoptotic Bcl-2 family member BNIP3 (Bcl-2/adenovirus E1B 19-kDa interacting protein) and undergo CD47-mediated apoptosis. This finding is consistent with our previous demonstration of a physical interaction between BNIP3 and CD47 that inhibits BNIP3 degradation by the proteasome, sensitizing T cells to CD47-induced apoptosis. Overall, these results reveal an important role in vivo for this new CD47/BNIP3 pathway in limiting inflammation by controlling the number of activated T cells. *The Journal of Immunology, 2007, 178: 5930–5939.

Lymphocyte programmed cell death plays a crucial role in controlling immune responses at several steps, notably in terminating peripheral T cell responses and achieving a return to homeostasis. Failure to eliminate inflammatory T cells results in harmful reactions, leading to tissue injury and disease. Indeed, multiple nonoverlapping mechanisms act to inactivate or to kill these activated cells. Although lymphocyte cell death can be mediated by cell surface signals through receptors from the TNFR superfamily (1), it can also be mediated by surface receptors that are not specialized in inducing cell death but are able to trigger other cell functions depending on the cell type, stimulus, and interacting ligand(s) (2–6).

CD47 is one of these equivocal initiators of lymphocyte death in both T cells and B chronic lymphocytic leukemia cells (5, 7). In addition to apoptosis, CD47 has been shown to trigger a wide variety of cellular functions in T cells, including activation, proliferation, arrest, and spreading on an inflamed vascular endothelium (8–11). Yet, under certain conditions it can also induce energy or generate regulatory T cells from CD4+CD25+ T cells (12, 13). Its role in T cell apoptosis is complex; on the one hand it has been shown to interact with Fas and to sensitize the cells to Fas-induced apoptosis by enhancing Fas clustering (14). On the other hand, CD47 can trigger direct death signals that lead to a rapid and caspase-independent cell death triggered only 1 h after cell stimulation in vitro (5, 7). These functional outcomes are actually related either to the cell activation state (because T cells must have received a prior TCR activation signal) or to the nature of the ligand for CD47 (5, 7).

Indeed, two families of proteins have been described to be the natural ligands for CD47: two members of the signal regulatory protein (SIRP) receptor family, SIRPα and SIRPγ, and two thrombospondin (TSP) family members. SIRPs are expressed on the surfaces of macrophages and endothelial and dendritic cells and inhibits tyrosine kinase-coupled signaling pathways via the phosphorylation of ITIM motifs in its cytoplasmic domain (15). SIRPγ, expressed mostly on peripheral blood leukocytes but also in many human tissues including the brain, lung, placenta, and liver, lacks the cytoplasmic ITIMs and is reported to mediate cell-cell adhesion and T cell costimulation (16, 17). However, neither SIRPα nor SIRPγ has been implicated in CD47-induced apoptosis in contrast to the TSP family members, which have been shown to bind CD47 specifically via their COOH-terminal cell-binding domain. The latter interactions subsequently induce CD47-dependent apoptosis in several cell lineages (7, 18–22).

TSPs are a family of five extracellular proteins composed of several structural domains known to bind extracellular matrix components and cell surface receptors (23). As a consequence of this modular structure, TSPs display diverse biological functions, including regulation of migration, adhesion, proliferation, differentiation, and apoptosis (23). Although TSP1 and TSP2 are widely expressed, TSP3, TSP4, and TSP5 show a more limited distribution and are unlikely to play a role in the events described here (24, 25). It should be noted that the TSP1 concentration is very low in plasma (<200 ng/ml) but increases locally within seconds when released by activated platelets and can reach concentrations of 10–20 mg/ml at these sites of platelet activation (26). It is also

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³Abbreviations used in this paper: SIRP, signal-regulatory protein; 7-AAD, 7-aminoactinomycin D; BNIP3, Bcl-2/adenovirus E1B 19-kDa interacting protein 3; DTH, delayed type hypersensitivity; LN, lymph node; MFI, mean fluorescence intensity; TSP, thrombospondin; WT, wild type.

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overexpressed by fibroblasts under mechanical stress and by endothelial cells following a disturbance in blood flow (18–20). TSP2 has also been shown to be up-regulated in the skin of mice subjected to cutaneous inflammation (27). All of these phenomena occur in damaged tissues where inflammation has developed.

We have shown in a previous article that the CD47 apoptotic pathway in T cells is peculiar (22). In a resting state, the Bcl-2 homology (BH3) domain-only protein BNIP3 (Bcl-2/adenovirus E1B 19-kDa interacting protein 3) is bound to CD47. Upon a pro-apoptotic signal, BNIP3 translocates to mitochondria and triggers mitochondrial death. BNIP3 regulates programmed cell death from mitochondrial sites by interacting selectively with Bcl-2 and Bcl-XL (28, 29). BNIP3 has a COOH-terminal transmembrane domain that is required for its association with the multiple membrane spanning domain of CD47 at the plasma membrane and also required for its mitochondrial localization and its homodimerization (22, 30). BNIP3 is overexpressed during hypoxia and in cytotoxic effector T cells and kills cells via the opening of the mitochondrial transition pore, which leads to profound mitochondrial dysfunction without caspase activation (31–34).

In this study, by using CD47−/− mice and an in vivo model of T cell-determined skin inflammation (35, 36) we show that CD47 plays a crucial role in limiting the course of an inflammatory immune response by eliminating activated lymphocytes. Our study also shows that TSP-1−/− and TSP-2−/− mice display a similar deficient phenotype to that displayed by CD47−/− mice in controlling cutaneous inflammation, implying a role for these two ligands of CD47 in T cell clearance. We demonstrate that the expression of BNIP3, which mediates CD47-induced cell death, is increased following CD3 stimulation, providing sensitization of T

FIGURE 1. Prolonged contact hypersensitivity elicited in CD47-deficient mice. A, Several groups of six mice, either CD47−/− or WT mice, were treated with a sensitizing dose of oxazolone (oxa) (2%) on the shaved abdomen followed by a challenging dose of oxazolone (0.4%) on the right ear. The left ear was treated with vehicle alone. Ear thickness was measured (displayed as the means ± SEM from six animals) in both types of mice at different time intervals after challenge (representative experiment, n = 3). B, Ear tissue sections were performed in CD47−/− mice and WT mice at 24 and 48 h after challenge. Ear sections were stained with H&E. Thirty hours after oxazolone challenge, cells were collected from the ears of CD47−/− or WT mice and the proportion of dead T cells was determined using an annexin V assay kit. Mouse T cells were identified by FITC-labeled CD3 mAb. Cell debris was electronically gated out based on forward light scatter (representative experiment, n = 3). D, Twenty-four hours after oxazolone challenge or treatment with the vehicle alone, cells were collected from the draining LNs of CD47−/− or WT mice and the proportion of dead T cells was determined using an annexin V assay kit. Naive and memory T cells were identified by PE-labeled CD3 mAb and FITC-labeled CD62L. Cell debris was electronically gated out based on forward light scatter (representative experiment, n = 3).
cells to CD47-induced apoptosis. Finally, we discuss a biphasic model for controlling T cell-dependent inflammation via CD47 based on a critical threshold of TSP ligands.

Materials and Methods

Reagents and antibodies

The FITC-conjugated CD47 mAb “B6H12” and an Ab against BNIP3 were from BD Pharmingen; the mAb against ubiquitin was from Sigma-Aldrich; the Ab against β-actin was from Cell Signaling Technology; the mAb against human CD3 (X3) was produced in our laboratory and described elsewhere (8); the mAb “Ab-4” against TSP-1 was from NeoMarkers; and the conjugated Abs anti-human CD25 (PE), anti-human CD4, and CD8 (PerCP), the anti-mouse CD3 (PE and FITC), and the anti-mouse CD31 were from BD Biosciences. The 4N1K (KRFYVVMWKK) and 4NGG (KRFYGGWMWKK) peptides were purchased from Genosys Biotechnologies. The proteasome inhibitor MG132 was purchased from Calbiochem.

Mice

Wild-type (WT) C57BL/6 mice were purchased from Charles River Laboratories-Iffa Credo. C56BL/6 mice deficient in CD47 were generated as previously described (37). WT 129/SvJ mice and TSP-1- and TSP-2-deficient mice on a mixed 129SvJ/129SvEms+Ter background were previously described (38, 39). Female mice between 7 and 12 wk of age were used. Mice were maintained in our animal facility. All mice used were cared for in accordance with the Institut National de la Sante et de la Recherche Médicale (INSERM) guidelines. Studies were conducted after full approval of the ethical committee of INSERM.

Induction of cutaneous inflammation

Cutaneous inflammation was induced in the skin of 10-wk-old female WT C57BL6 mice (n = 6 for each group) as previously described (40). The mice were sensitized by topical application of a 2% oxazolone (4-ethoxymethylene-2-phenyl-2-oxazoline-5-one; Sigma-Aldrich) solution in acetone/olive oil (4:1; v/v) to the shaved abdomen (25 μl). Five days after sensitization the right ears were challenged by topical application of 16 μl of a 0.4% oxazolone solution, whereas the left ears were treated with vehicle alone. The extent of inflammation was measured daily using the mouse ear swelling test (40).

Cell preparation

Murine blood was collected from the retro-orbital venous plexus in citrate, and PBMCs were isolated by Ficoll-Isopaque density gradient centrifugation (Nycomed/ALTANA Pharma). Splenocytes were harvested by gently forcing them through a 70-μm cell strainer (Falcon, catalog no. 2350; BD Biosciences) into 3.0 ml of HBSS containing 2% FBS and then depleted of RBC using ammonium chloride (ACK) lysis buffer. The draining lymph nodes (LN) of mouse ears (3 to 5 mice per experiment) were collected 24 h after oxazolone challenge or treatment with vehicle alone (n = 3 per time point and genotype). Cells were isolated from the nodes by gently forcing them through a 70-μm cell strainer into 3.0 ml of HBSS containing 2% FBS.

Blood from healthy adult donors was obtained by venipuncture in citrate and PBMCs were isolated by Ficoll-Isopaque density gradient centrifugation (Nycomed/ALTANA Pharma). Donors did not take any drugs during the previous 10 days. Approval for this study was obtained from the institutional review board of the French National Institute of Health and Medical Research; informed consent was provided according to the Declaration of Helsinki.

Flow count fluorospheres (Beckman Coulter) were used to quantify the number of living cells by flow cytometry. Under each condition, 20,000 microbeads were added just before the analysis, 2000 microbeads were collected, and the number of lymphocytes in region R1 was determined using a FACScan flow cytometer (BD Biosciences).

Immunofluorescence analysis

Three-color flow cytometry analysis was performed on a FACScan device. PBMCs were incubated at 4°C for 30 min in the dark in 100 μl of PBS and 0.1% BSA with saturating concentrations of directly conjugated mAbs. Cells were washed twice with PBS and 0.1% BSA. Forward and right-angle scatter gatings were set to include lymphocytes. Mean fluorescence intensity (MFI) values were computed from 10,000 gated cells and are presented as the difference between the MFI of tested cells and the MFI of background staining. Experiments were repeated three times.

Assay for apoptosis

Mouse ears were excised 30 h after oxazolone challenge, diced, and digested in 1 mg/ml collagenase D (Sigma-Aldrich) in HBSS medium for 1 h at 37°C. Cells were washed in HBSS medium with 5% FCS and resuspended in PBS. Cells were then labeled with a FITC-labeled CD3 Ab and
the degree of cell death was quantified using annexin V-PE/7-aminoactinomycin D (7-AAD) staining as described above. The percentage of apoptotic cells was calculated by scoring 7-AAD and annexin V-PE-binding cells (as described by the manufacturer, Boehringer Mannheim) after backgating on CD3+ cells in the third color. The cell suspensions obtained from the draining LNs were stained with PE-labeled CD3 and cell death was quantified using annexin V-allophycocyanin/7-AAD staining.

Western blot analysis
Freshly isolated PBMCs and splenocytes were activated for 24, 48, or 72 h with anti-CD3 or incubated with medium alone. Cells were then washed twice in cold PBS and lysed in 2% Triton X-100 isotonic buffer with 10 mM EDTA, 10 mM sodium orthovanadate, 100 mM NaF, 2% Triton X-100, 10 µg/ml aprotinin, 10 µg/ml leupeptin, and 0.5 mM PMSF. Cell debris was removed by centrifugation and 50 µg of lysate per lane, separated by electrophoresis on 10% SDS-polyacrylamide gels, transferred to nitrocellulose membranes, and immunoblotted with rabbit polyclonal anti-BNIP3 (28), anti-CD47 "B6H12", anti-ubiquitin, or anti-β-actin Abs. The immune complexes were detected by HRP-conjugated secondary Abs (DakoCytomation) and developed using ECL (Amersham Biosciences). Densitometric assessment of Western blots has been performed using NIH Image 1.61 software (http://rsb.info.nih.gov/nih-image).

RNA isolation, cDNA synthesis, and PCR amplification
RNA was prepared from human PBMC and murine splenocytes using an RNeasy mini kit (Qiagen, Germany). cDNA was prepared from RNA using the SuperScript first strand synthesis system (Invitrogen Life Technologies) for RT-PCR. RT-PCR was typically performed for 35 cycles (denaturation at 95°C for 20 s, annealing at 68°C for 1 min, and extension at 72°C for 1 min). The primers used in PCR were as follows: 5' primer (5'-GATCAGCTCAGCTACTAT-3') and 3' primer (5'-ACAATGACAG TGATCACT-3') for human CD47; the 5' primer (5'-GGTCCAGCTCACC TACTGT-3') and 3' primer (5'-CTCTTTATTCGATGCGCTG-3') for murine CD47; and the 5' primer (5'-GAACCTGCACCTCAATGCTG-3') and 3' primer (5'-AA CCTGAAAGCTC-3') for murine BNIP3. β-Actin was used as loading control.

Real-time quantitative RT-PCR Assay
BNIP3 mRNA expression was measured by real-time quantitative PCR, using the SYBR Green PCR core reagents kit in an ABI PRISM 5700 sequence detection system (Applied Biosystems) according to the manufacturer’s instructions. Primers (MWG Biotechnik) were designed using the computer program Primer Express (Applied Biosystems) to span exon-intron junctions to prevent the amplification of genomic DNA and to amplify DNA fragments in the size range of 100–250 bp that is optimal for real-time RT-PCR. Reaction data were expressed as cycle thresholds, which are the PCR cycle numbers at which the fluorescent signal in each reaction reaches a threshold above background. Because the precise amount of total RNA added to each reaction mix and its quality were difficult to assess, we also quantified the level of four different housekeeping genes (β-actin, ubiquitin, GAPDH, and hypoxanthine-guanine-phosphoribosyltransferase) as endogenous RNA controls. The expression of target genes was measured after normalization of RNA concentrations with the four different housekeeping genes, and values were expressed as fold increased expression above a theoretical negative sample, as previously described (41).

Histology and immunohistochemistry
Tissues were fixed in 10% neutral buffered formalin, processed routinely, and embedded in paraffin. They were then sectioned at 6-µm thickness and stained with H&E. Frozen sections of ears measuring 5 µm, prepared on a cryostat, were fixed in acetone for 10 min at 4°C and then air dried. After blocking with 3% BSA for 20 min, tissue sections were incubated with Abs against CD31 or biotinylated Abs against TSP-1 for 30 min. After washing, bound Abs were detected by incubation with HRP-conjugated secondary
Abs and HRP-conjugated streptavidin and revealed with diaminobenzidine (DakoCytomation). Control stainings were performed by omitting the primary Abs.

Results

CD47-deficient mice suffer prolonged cutaneous inflammation

To investigate the role of CD47 in inflammation in vivo, we elicited delayed-type hypersensitivity (DTH) reactions in WT and in CD47−/− mice by using oxazolone (36). The resulting inflammation is known to develop and resolve quickly and is associated with marked interstitial edema, vascular enlargement, and mononuclear cell infiltration (36). CD47−/− and WT mice (six for each group) were treated as indicated and the extent of inflammation was measured daily using the mouse ear swelling test (40) (Fig. 1A). Remarkably, ear thickness increased to the same extent, maximizing at 24 h in both types of mice. Nevertheless, WT mice displayed a sharp decrease in ear thickness during the second day, with the ear tissue returning to normal by approximately day 3. By contrast, CD47−/− mice retained their thick and inflamed ears for >4 days followed by a slow recovery (Fig. 1A). Forty-eight hours after challenge, histological examinations revealed that edema and mononuclear infiltration were markedly resorbed in WT mice but still elevated in CD47−/− mice (Fig. 1B).

Because contact hypersensitivity is a T cell-mediated cutaneous inflammatory reaction (36), we recovered the cells infiltrating the ears after 30 h and measured CD3+ T cell viability. The percentage of CD3-positive cells measured 30 h after challenge represents 25–35% of total cells, with the remaining cells being monocytes and neutrophils (results not shown). As expected, CD47−/− mice had a higher proportion of living inflammatory T cells compared with WT mice, suggesting that a delay in T cell death occurs in CD47−/− mouse-inflamed tissues (Fig. 1C). It should be noted that the amount of cell death in WT and CD47−/− mice could have been overestimated as a consequence of the digestion procedure used to isolate infiltrated T cells. Nevertheless, this isolation process was the same for CD47−/− mice and WT mice, and the differences in cell viability are still significant. To investigate whether T cell apoptosis could begin earlier during the immune response at another location, we collected the draining LNs 24 h after challenge and looked for cell death (Fig. 1D). In contrast to the inflammatory infiltrate, we could not see any difference in the viability of the cells isolated from the WT or the CD47−/− LNs. Taken together, these results suggest that the prolonged cutaneous inflammation in CD47−/− mice results from a defect in T cell apoptosis in peripheral tissues.

TSP-1- and TSP-2-deficient mice also suffer prolonged cutaneous inflammation

Members of the TSP family have been described as natural ligands for CD47, and TSP-1 has been shown to induce T cell apoptosis (22, 42). TSP-2 has previously been shown to be highly up-regulated in inflamed skin 24 h after oxazolone challenge and is found prominently in blood vessels and dermal cells (27). We have found that in this inflammation model the expression of TSP-1 is also enhanced. Twenty-four hours after oxazolone challenge, an extensive vascular thrombosis was observed in ear tissue sections (Fig. 2A). This was identical in CD47−/− mice and their normal counterparts. A strong staining for TSP-1 was detected not only in areas where leukocyte infiltration has developed (27).

FIGURE 4. A TSP peptide induces cell death of CD3-activated but not resting T cells via CD47. A, PBMCs from WT or CD47−/− mice (1 × 10^6 cells/ml) were stimulated for 24 h with medium alone or with a soluble anti-CD3ε mAb (1 μg/ml). Cells were then treated for 1 h with the 4N1K peptide (400 μM), the 4NGG peptide (400 μM), or vehicle only (co). Tubes containing a known number of flow count fluorospheres were used to determine by flow cytometry the relative numbers of lymphocytes following treatment. The region representing living lymphocytes was defined as R1, and the number of cells in R1 was determined following a sampling of 2000 reference beads (population R2) (representative experiment, n = 3). The values represent means ± SEM. B, A representative dot plot of forward light scatter (FSC) vs side light scatter (SSC) of human PBMCs treated as described in A. The number of living lymphocytes is indicated above each plot (representative experiment, n = 3).

TSP-1−/− and TSP-2−/− mice were subjected to DTH reactions as described in Fig. 1A. Twenty-four hours after oxazolone challenge, the increase in ear thickness in WT mice was similar to that observed in TSP-1−/− and TSP-2−/− mice (Fig. 3, A and B). In contrast, the two types of mutant mice were slower to resolve compared with WT animals after 24 h, as indicated by the prolonged ear swelling (Fig. 3, A and B). These findings suggest that TSP-1 and TSP-2 are both implicated in the control of inflammation. Moreover, TSP-1−/− mice suffer more prolonged inflammation than TSP-2−/− mice, with inflammation lasting 10 days for TSP-2−/− mice and >13 days for TSP-1−/− mice. Finally, we recovered the cells infiltrating the ears after 30 h and observed, as for CD47−/− mice (Fig. 1C), a decrease in inflammatory T cell apoptosis in TSP-1−/− and TSP-2−/− mice (Fig. 3C).

Death of CD3-activated, but not resting T cells, is induced by an active peptide derived from a cell-binding domain of TSP

The COOH-terminal cell-binding domain of TSP-family members has been identified as the site of their interaction with CD47 (43, 44), and the peptide 4N1K, which is conserved in all TSP isoforms...
and mimics the activity of this domain, has been used extensively in place of TSP1, although the crystal structures of recombinant COOH-terminal regions of TSP have shown that this region is not solvent exposed on the protein surface (45–47). We have shown that this domain induces apoptosis in Jurkat cells (22). Thus, we investigated whether it affects normal peripheral T cell survival. Previous studies have shown that CD3 ligation was required to induce the sensitization of normal human peripheral T cells to CD47-induced cell death (48). Therefore, in this study we tested in a murine model whether TSP also requires normal peripheral T cell survival. For this, PBMCs obtained from either type of mice as assayed by annexin V binding and propidium iodide uptake (data not shown). After 24 h, cells were incubated with either the CD47-binding agonist 4N1K peptide or with the 4NGG control peptide (Fig. 4A) (49). We found comparable numbers of living lymphocytes among unstimulated PBMCs (gated in R1) in CD47−/− and control mice, regardless of whether they were incubated with 4N1K or 4NGG peptides. In contrast, when T cells were prestimulated for 24 h with a CD3 mAb and then further incubated for 1 h with 4N1K peptide, we observed a marked decrease in the number of living lymphocytes in normal mice as compared with cells left unstimulated or stimulated with the 4NGG control peptide. In contrast, we observed no proapoptotic effect of the 4N1K peptide on T cells from CD47−/− mice. Notably, we obtained similar results to those obtained in the mouse studies when we used PBMC obtained from healthy volunteers (Fig. 4B). Thus, these results show that TSP induces the death of activated, but not resting T cells via a CD47-dependent mechanism.

Expression of CD47 and BNIP3 during T cell activation

Recently it has been observed that the expression of CD47 increases in several cell types (e.g., fibroblasts and endothelial cells) following appropriate stimulations. Moreover, this overexpression triggers apoptosis when CD47 is allowed to interact with its ligand (18–20). We thus assessed whether such up-regulation of CD47 would occur on T cells upon their stimulation via the TCR. As shown in Fig. 5A, RT-PCR analysis detected no increase in CD47 mRNA expression following CD3 stimulation, indicating that CD47 is not induced during T cell activation. To confirm this result, we monitored the surface expression of CD47 on T cells after CD3 stimulation. Fig. 5B shows that CD47 was constitutively expressed on resting T cells (MFI, 63.2 ± 4.22) and CD3 did not affect significantly CD47 surface expression at 24 h (MFI, 72.1 ± 4.82) and at 48 h (MFI, 71.7 ± 5.03). The induction of CD25 expression was used as a control for CD3-induced T cell activation. These observations demonstrate that the sensitization of activated T cells to apoptosis is not due to an increase of CD47 at the cell surface, in contrast to the reported effects in fibroblasts and endothelial cells.
We have shown in a previous article, using coimmunoprecipitation, that CD47 is physically associated with the proapoptotic molecule BNIP3 and that CD47 ligation induces the apoptosis of Jurkat T cells via BNIP3 (22). Because it has been shown that BNIP3 expression increases in effector CTLs (33), we next examined the effect of T cell stimulation on the expression of BNIP3. Human and murine PBMCs were incubated with soluble CD3 mAb for different periods of time, and the cells were examined for BNIP3 mRNA levels by RT-PCR. As shown in Fig. 5C, CD3 ligation increased BNIP3 mRNA, which was apparent after 12 h. To quantify these data, cDNA from unstimulated and stimulated cells was subjected to a real-time quantitative PCR assay (Fig. 5D). As a control, we compared gene expressions of BNIP3 and Bax, another member of the Bcl2 family, which is known to be
unaffected during T cell activation (33). As expected, no significant difference was observed in the gene expression of Bax following CD3 ligation. In contrast, BNIP3 gene expression increased >8-fold after 24 h of induced activation. Western blot analysis also revealed higher levels of BNIP3 protein expression after 48 and 72 h in CD3-stimulated cells compared with unstimulated cells (Fig. 5E). Notably, cell incubation with the 4N1K peptide did not alter BNIP3 expression regardless of whether or not they were activated by CD3 (data not shown).

**CD47 allows BNIP3 to accumulate in the cell by preventing its degradation by the proteasome**

BNIP3 mRNA expression has been shown to increase in response to different stimuli, but the protein accumulates only after a prolonged exposure because of its degradation by the proteasome (33). Cell death has been shown to occur in 3 or 4 days after a sufficient amount of BNIP3 accumulates in the cytoplasm (33). To determine whether the inability to regulate degradation of BNIP3 could explain the failure of apoptosis in CD47−/− mice, we studied the effects of the proteasome inhibitor MG132 on BNIP3 protein expression in CD47−/− and WT splenocytes after CD3 stimulation. Fig. 6A shows that in CD47−/− cells the amount of BNIP3 protein is much lower than in WT cells after 48 h of CD3 stimulation but increases after 3 h of MG132 treatment. Of note, this short exposure to MG132 did not cause a detectable increase in the BNIP3 level already present in WT cells stimulated by CD3. An anti-ubiquitin antiserum showed the accumulation in the cells of ubiquitinated proteins after 3 h of MG132 treatment. As expected, MG132 had no effect on the mRNA levels of CD47 and BNIP3 (Fig. 6B). The BNIP3 mRNA induction and the accumulation of the protein were also detected in TSP1−/− cells after CD3 stimulation and were similar to the ones observed in WT cells (Fig. 6, C and D). The quantification of the Western blots in Fig. 6, A and C, were confirmed by the measurement of intracellular BNIP3 and Bax using flow cytometry with specific Abs as an indication of their intracellular accumulation (our unpublished data). Together, these data indicate that BNIP3 induction is independent of the presence of CD47 or TSP but that its accumulation is highly dependent on its association with CD47.

**Discussion**

The exact significance of the various and sometime opposite functional effects that have been reported to be triggered via CD47 requires clarification, and in vivo studies should shed some light on the role(s) played by CD47 in physiological and pathophysiological conditions. CD47−/− mice are viable and healthy when raised under normal conditions; however, when subjected to bacterial infections they suffer from a defect in neutrophil migration and phagocytic activation and are more prone to succumb than their WT relatives (37). In this study, by using a CD8+ T cell-mediated skin inflammation model (35) we demonstrate in vivo that CD47 on peripheral T cells is a powerful regulator of tissue inflammation. Indeed, experiments conducted on mice deficient for CD47 or its ligands, TSP1 and TSP2, show a similar defect in controlling the length of inflammation and in apoptosis of infiltrating T cells. We assume that the similarity observed in these null mouse phenotypes after the induction of DTH reactions can be attributed, at least in part, to a defect in the TSP-CD47 interactions. Notably, TSP-1−/− mice suffer from a somewhat longer period of inflammation than TSP-2−/− mice, suggesting that TSP1 is a more powerful anti-inflammatory molecule than TSP2 and is potentially the major in vivo proapoptotic ligand for CD47. This supposition is consistent with the more restricted expression of TSP-2 compared with TSP-1 and with the delayed up-regulation of TSP-2, at least in the case of wound healing (50, 51).

However, given the diversity of TSP receptors, the delayed inflammation observed in TSP1 and TSP2−/− mice probably reflects a more complex model. Indeed, other receptors on the T cell surface, such as the αβ integrin or heparan sulfate proteoglycan receptors, could also influence the extent and duration of inflammation (52, 53). In addition, the anti-inflammatory activity of TSP1 can be driven by mechanisms other than apoptosis, such as inhibition of the production of proinflammatory cytokines by dendritic cells through interactions with CD47 and CD36 (54) or facilitation of the clearance of damaged tissues and cells (55). Finally, through its interaction with CD47, TSP1 induces the conversion of CD4+CD25+ T cells into CD4+CD25+ regulatory T cells in LNs and reduces effector T cell immune responses (13). This model is further complicated by the fact that SIRPs, another CD47 ligand, is an inhibitory receptor on macrophages and the lack of CD47 might affect phagocytosis (56). These considerations highlight the fact that, even if this inflammatory model is principally mediated by CD8+ T cells, the manifestation of the reaction depends on a much more complex cooperation between the cells participating in the inflammatory process, including endothelial cells, platelets, dendritic cells, and macrophages, and emphasize the limitations of in vitro studies. Thus, our data reveal that a deficiency, either in CD47 or in TSP, reduces the rate of T cell apoptosis in inflamed tissues, and it seems reasonable to assume that the prolonged inflammation observed in these animals is driven in part by a disrupted CD47/TSP proapoptotic pathway.

It has been shown recently that endogenous TSP1 is also expressed on the membrane of blood T cells within minutes following CD3 stimulation, provided the cells are in contact with an extracellular matrix substrate (57). It would be interesting to investigate whether this endogenous TSP1 contributes to the induction of death signals via cis interactions with CD47. Clearly, triggering apoptosis via CD47 is critically dependent on the surface densities of CD47 and TSPs in many cell types as shown by stimulating fibroblasts with mechanical tension (an event occurring during wound healing) or by stimulating endothelial cells with a disturbance in blood flow (18–20). In both cases, the surface density of CD47 increases, resulting in cell apoptosis. However, on T cells CD47 expression remains constant regardless of whether or not they have been activated through the TCR. We have thus hypothesized that, during T cell activation, another mechanism might take place, such as the lowering of the apoptotic threshold, that is required for T cell apoptosis via CD47. Indeed, BNIP3 expression increases greatly during this period of time in which T cells, under the influence of an activation stimulus, become sensitive to the proapoptotic effect of CD47. This is quite consistent with the decisive role played by this protein in the proapoptotic intracellular pathway triggered via CD47 (22). Accordingly, BNIP3 has been shown recently to be up-regulated at the transcriptional level in effector CTLs, leading to an increased susceptibility to activation-induced cell death (33). In the present study we show that this accumulation of BNIP3 in activated T cells is facilitated by its physical association with CD47, which prevents its degradation by the proteasome. Indeed, in T cells obtained from CD47−/− mice BNIP3 mRNA increases following CD3 stimulation, but the overexpression of the protein is reduced. In these CD47-deficient cells the overexpression of BNIP3 is restored by the use of a proteasome inhibitor. Recently it has been shown that CD47 enhances Fas-mediated cytotoxicity (14). Our data fit well with these results because it is likely that the absence of CD47 might, on the one
hand, suppress BNIP-3 up-regulation and thus susceptibility to activation-induced cell death and, on the other, decrease Fas-dependent mechanisms involved in cutaneous hypersensitivity (58, 59). Notably, these two mechanisms are not mutually exclusive but might take place simultaneously in the model we describe here.

When inflammation develops in a tissue subjected to mononuclear/T cell infiltration, vascular thrombosis occurs in small vessels, leading to the release of increasing amounts of TSPs, particularly TSP-1. Concomitantly, activation leads to an increase in BNIP3 levels in T cells, allowing them to become more sensitive to apoptotic signals. In the second phase, when TSP-1 reaches a critical level an apoptotic signal is delivered via CD47, leading to massive and rapid T cell apoptosis.

There are several consequences of a biphasic mode of CD47 stimulation in terms of the functional outcomes. First, it provides a powerful negative control of inflammation, because after being triggered inflammation will be dampened when both TSPs and BNIP3 reach a critical threshold. Second, this model suggests that there are physiologic mechanisms that can prevent undue apoptosis because, under basal conditions, two major components are lacking, namely TSPs in tissues and BNIP3 within T cells. Should an interaction of TSPs with CD47 occur for some reason other than inflammation, T cells are in a resting state and conditions for apoptosis are not achieved. Third, the apoptotic process should spare innocent unactivated bystander T cells, avoiding uncontrolled apoptosis. Fourth, in CD47−/− mice delayed apoptosis will finally occur due to the slow accumulation of BNIP-3 in mitochondrial membranes, as is seen under hypoxic conditions (31, 32, 60). Additionally, it was observed that TSP-1−/− mice suffer from acute and chronic inflammations in various tissues, which is unexpected in view of the supporting role of TSP-1 in neutrophil adhesion and chemotaxis (38, 39) but is consistent with the present model.

In summary, our results emphasize that the CD47 molecular pathway can, in vivo, potently control the inflammation linked to toxic T lymphocytes. Notably, these two mechanisms are not mutually exclusive but additively, it was observed that TSP-1 and CD47 interaction: a pathway to generate regulatory T cells from human CD4+ CD25+ T cells in response to inflammation. J. Immunol. 177: 3534–3541.

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Disclosures

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References


