Bim–Bcl-2 Homology 3 Mimetic Therapy Is Effective at Suppressing Inflammatory Arthritis Through the Activation of Myeloid Cell Apoptosis

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Objective. Rheumatoid arthritis (RA) is a destructive autoimmune disease characterized by an increased inflammation in the joint. Therapies that activate the apoptotic cascade may have potential for use in RA; however, few therapeutic agents fit this category. The purpose of this study was to examine the potential of Bim, an agent that mimics the action of Bcl-2 homology 3 (BH3) domain–only proteins that have shown success in preclinical studies of cancer, in the treatment of autoimmune disease.

Methods. Synovial tissues from RA and osteoarthritis patients were analyzed for the expression of Bim and CD68 using immunohistochemistry. Macrophages from Bim–/– mice were examined for their response to lipopolysaccharide (LPS) using flow cytometry, real-time polymerase chain reaction analysis, enzyme-linked immunosorbent assay, and immunoblotting. Bim–/– mice were stimulated with thioglycollate or LPS and examined for macrophage activation and cytokine production. Experimental arthritis was induced using the K/BxN serum–transfer model. A mimetic peptide corresponding to the BH3 domain of Bim (TAT-BH3) was administered as a prophylactic agent and as a therapeutic agent. Edema of the ankles and histopathologic analysis of ankle tissue sections were used to determine the severity of arthritis, its cellular composition, and the degree of apoptosis.

Results. The expression of Bim was reduced in RA synovial tissue as compared with controls, particularly in macrophages. Bim–/– macrophages displayed elevated expression of markers of inflammation and secreted more interleukin-1β following stimulation with LPS or thioglycollate. TAT-BH3 ameliorated arthritis development, reduced the number of myeloid cells in the joint, and enhanced apoptosis without inducing cytotoxicity.

Conclusion. These data demonstrate that BH3 mimetic therapy may have significant potential for the treatment of RA.

Rheumatoid arthritis (RA) is a chronic inflammatory and destructive arthropathy of unknown cause. In RA, the synovial lining increases from 1–2 cell layers to as many as 10 layers thick, and it is composed of
macrophages and fibroblasts. Since there is a paucity of apoptotic cells in the RA joint, one potential mechanism responsible for the hyperplasia in the RA synovial lining may be attributed to a decrease in the death of fibroblasts and macrophages. However, the milieu of the RA joint contains noxious factors that are normally detrimental to the survival of the cell (1). Thus, an imbalance of apoptotic protectors and inducers may occur and may lead to the pathogenesis of RA. A variety of studies have shown that induction of synoviocyte apoptosis in animal models of inflammatory arthritis ameliorates both joint inflammation and joint destruction (1). These data suggest that the controlled induction of apoptosis locally in the inflamed joint may be therapeutically beneficial.

There are 2 distinct apoptotic pathways, an extrinsic pathway that requires binding of death ligands to their cognate receptors on the cell surface and an intrinsic pathway in which mitochondria play a critical role. Extrinsic apoptosis is mediated through caspases 8/10, which may activate the downstream caspases 3/7 directly or through an amplification step that requires the intrinsic apoptotic pathway. During intrinsic apoptosis, the mitochondrial intermembrane protein cytochrome c is released into the cytosol, where it binds to Apaf-1, resulting in activation of the initiator caspase 9 and the effector caspases 3 and 7. Caspases 3 and 7 are responsible for the downstream degradative events in apoptosis (2).

The Bcl-2 protein family is divided into antiapoptotic (Bcl-2, Bcl-xL, and Mcl-1]) and proapoptotic (Bak, Bax, and Bim) members; these proteins are central mediators of the intrinsic apoptotic pathway (2). Bcl-2-related proteins contain Bcl-2 homology (BH) domains BH1–BH4, which are critical for homodimer and heterodimer formation between the family members. While the antiapoptotic Bcl-2-like proteins contain 3 or 4 BH domains, the proapoptotic Bcl-2-related proteins are subdivided into 2 categories: the multi-BH domain (e.g., Bak and Bax) and the BH3-only domain (e.g., Bim) proteins (2). Recent studies have shown that BH3-only domain proteins are also subdivided into 2 categories based on their ability to sequester antiapoptotic Bcl-2 family members, bind to and activate Bak or Bax, and/or induce apoptosis (3–6). Apoptosis signaling through the intrinsic pathway is inhibited by overexpression of any of the Bcl-2-like prosurvival members or by loss of both of the multi-BH domain proteins Bak and Bax (2).

To date, almost all of the studies that have examined the potential of altering the molecular rheostat that governs the Bcl-2 family through the use of BH3 mimetics have focused on xenograft tumor models (3,7–10). In the present study, we found that the expression of the BH3-only protein Bim is reduced in RA synovial tissue and that its reduction affects not only macrophage survival, but also the state of activation. The expression of Bim was found to be decreased in macrophages in RA synovial tissue. Macrophages from Bim−/− mice displayed increased activation as compared with control cells. Arthritic mice treated with a Bim-BH3 mimetic peptide (TAT-BH3) displayed reduced edema of the ankle, markedly lower histologic scores for arthritis, fewer neutrophils and macrophages in the joints, and enhanced numbers of TUNEL-positive synoviocytes. These findings are the first to document the therapeutic potential of systemic delivery of a BH3 mimetic to ameliorate inflammatory arthritis.

MATERIALS AND METHODS

Patients and tissue preparation. Synovial tissue was obtained at the time of arthroplasty from patients with a diagnosis of RA (n = 8) or osteoarthritis (OA; n = 8) and was fixed in 10% neutral buffered formalin. All patients met the American College of Rheumatology classification criteria for RA (11) or OA (12). All experiments on human tissues were approved by the internal review boards at St. Louis University, University of California San Diego, Northwestern University, and University of Michigan. Synovial sections were stained for Bim and CD68 and were scored by a pathologist who was blinded to the study group (GKH). At least 3 fields per section were examined, with a minimum of 3 sections per tissue, as described previously (13–17).

Mice. Bim−/− (18,19), nonobese diabetic (NOD), homozygous KRN T cell receptor–transgenic, C57BL/6, and C57BL/6:129 mice were maintained at St. Louis University and at Northwestern University. Mice were injected intraperitoneally with lipopolysaccharide (LPS; 10 mg/kg of body weight) from Escherichia coli O111:B4 (Sigma, St. Louis, MO) (20). Peritonitis was induced by intraperitoneal injection of 4% aged thioglycollate. All experiments on mice were approved by the Animal Care and Use Committee at St. Louis University and at Northwestern University.

Cell culture. Bone marrow cells were isolated from mice, as previously described (20,21). To induce activation, macrophages were treated with 10 ng/ml of LPS. Interleukin-1β (IL-1β) maturation was induced by stimulating LPS-treated macrophages with 5 mM ATP (Sigma), and brefeldin A (5 µg/ml) was used to inhibit the release of IL-1β.

IL-1β synthesis. RNA isolation and real-time polymerase chain reaction for IL-1β and GAPDH have previously been described (22). Data were normalized to the housekeeping gene GAPDH and were analyzed using the ∆∆Ct method to obtain the fold increase over the level in the untreated control for each genotype. For detection of IL-1β in cell supernatants, sandwich enzyme-linked immunosorbent assays (ELISAs) were performed as previously described (20). All ELISA data (expressed in pg/ml) were normalized according to the number of cells per well.
Flow cytometry. Phenotyping of macrophages, splenocytes, peripheral blood leukocytes, bone marrow cells, or peritoneal cells was performed as previously described (19,21,23,24). Apoptosis was measured by staining with allolymphocyanin-labeled annexin V. Cells were acquired on a BD LSR II flow cytometer (BD Biosciences, San Jose, CA) at the St. Louis University Core Flow Cytometry Facility or at the Northwestern University Translational Medicine Flow Cytometry Core Facility. All analyses were performed using FlowJo software (Tree Star, Ashland, OR). Total leukocyte numbers were determined using an automated hematology analyzer (ABX Pentra 60; Diamond Diagnostics, Holliston, MA).

K/BxN serum-transfer arthritis. K/BxN serum was collected from mice at ages 7–8 weeks and pooled, and then divided for storage. At the time of injection, serum was again pooled and then divided appropriately for injections. One hundred fifty microliters of K/BxN serum was injected intra-peritoneally into each flank of 6–8-week-old mice, as previously described (21,24–26). In all studies, mice were matched prior to addition of the serum or peptide and were coded. For the prophylactic study, 2 mg/kg of TAT-BH3 peptide was injected intra-peritoneally 1 hour before injection of serum and on days 2 and 4 after serum injection. For the therapeutic study, 10 mg/kg of TAT-BH3 peptide was injected intra-peritoneally on days 2, 3, 4, 5, and 6 after serum injection.

The variant TAT sequence is composed of D-amino acids and has a glutamine-to-ornithine substitution, which has been shown to markedly enhance (10-fold) cellular uptake of the peptides (27). The peptide from the BH3 domain of Bim was constructed as follows. TAT-BH3 consisted of Ac-RKKRR-Orn-RRR-EIWIAQELRRIGDEFNAYYAR-OH, and TAT-BIM inactive (TAT-inactive BH3) consisted of acetyl (Ac)-RKKRR-Orn-RRR-EIWIAQELRRIGDEFNAYYAR-OH or Ac-RKKRR-Orn-RRR-DMPETWIEOEARRIE-AEFNAYYARR-OH. Underlined residues are mutated residues. These were purchased from the Peptide Synthesis group at Tufts University (Boston, MA). In addition, a fluoroeocin-conjugated TAT-BH3 peptide was generated.

At each time point and prior to euthanasia, the degree of arthritis was measured (calculated as the increase in ankle circumference) (21,24–26). The change in ankle circumference at each time point was defined as the difference from the circumference as measured on day 0. Following euthanasia, peripheral blood was obtained by cardiac puncture, and serum was isolated. Ankle joints were removed, fixed in 10% neutral buffered formalin, decalcified in EDTA, embedded in paraffin, and sectioned. To examine toxicity due to systemic delivery of TAT-conjugated peptide, levels of alkaline phosphatase, alanine aminotransaminase, and blood urea nitrogen were measured (Department of Comparative Medicine, St. Louis University).

Immunohistochemistry. Ankle sections were stained and scored by a pathologist who was blinded to the study group (GKH), as previously described (21,24–26,28). Photographs were taken on a Nikon microscope equipped with a Nikon digital camera (DMX1200; Nikon, Melville, NY).

Statistical analysis. Results are expressed as the mean ± SEM. Differences between groups were analyzed using Student’s t-test or analysis of variance.

RESULTS

Bim expression is decreased in RA synovial tissue. Previous studies have focused on the expression pattern of the prosurvival Bcl-2 family members (1) in order to define a potential mechanism responsible for resistance to apoptosis in RA synovial tissue. In the present study, we focused on determining the expression level of Bim, a known Bcl-2 proapoptotic family member that is vital for leukocyte survival and inflammation (18).

Bim showed a granular cytoplasmic staining pattern consistent with localization in the cytoplasm and mitochondria. There was no positive staining in control RA and OA synovial tissue (data not shown) stained with IgG. Fewer RA synovial lining cells were positive for Bim (mean ± SEM 48 ± 10.4%; versus OA synovial lining cells (80 ± 7.2%; P < 0.02) (Figures 1A and B). Further examination of adjacent sections stained for CD68 (macrophages) and Bim revealed a decrease in Bim expression in the macrophage population in the synovial lining and sublining of RA as compared with OA synovial tissues (Figures 1A and B). There was no difference in expression of Bim in lymphocytes, fibroblasts, endothelial cells, or blood vessels in RA versus OA synovial tissue (data not shown). Since there was an increase in the average synovial lining thickness (mean ± SEM 2.4 ± 0.2 versus 1.7 ± 0.4; P < 0.06) and inflammation score (mean ± SEM 3.4 ± 0.5 versus 1.6 ± 0.3; P < 0.01) in RA versus OA synovial tissue, the decreased expression of Bim was associated with the increase in inflammatory score and synovial lining thickness.

Bim functions to limit the activation of macrophages. Since macrophages in the RA joint express lower levels of Bim, are highly activated, secrete high levels of tumor necrosis factor α (TNFα) and IL-1β, and their numbers correlate with disease outcome (29), we examined whether Bim may play a role in the activation of macrophages. Bone marrow–derived macrophages isolated from wild-type (WT) and Bim−/− mice were stimulated with the Toll-like receptor 4 agonist LPS, and the expression of CD86, CD69, CD16/CD32, CD40, and class II MHC was examined by flow cytometry.

There was no change in viability in response to stimulation with LPS in any of the treated macrophages as compared with the untreated cells. (Data obtained from analysis of the effect of LPS on viability of bone marrow–derived macrophages from WT and Bim−/− mice are available upon request from the author.) While LPS induced the expression of CD86, CD69, CD16/CD32, and CD40 on WT and Bim−/− macrophages as
compared with untreated cells, there was an increase in the mean fluorescence intensity (MFI) of CD86 (44%; \( P < 0.003 \)), CD69 (32%; \( P < 0.0003 \)), and CD40 (23%; \( P < 0.02 \)) in macrophages from Bim–/– mice as compared with WT mice (Figure 2A).

To determine whether Bim–/– macrophages also display elevated expression of markers of inflammation in vivo, Bim–/– and WT mice were injected intraperitoneally with aged 4% thioglycollate. There was an increase in both the expression (Figure 2B) and the number of cells positive for CD86 (3.0-fold), CD69 (2.0-fold), CD16/CD32 (4.8-fold), and class II MHC (1.3-fold) in Bim–/– macrophages as compared with WT macrophages at 48 hours following thioglycollate stimulation. These data suggest that Bim may function not only to induce apoptosis, but also to limit the extent of the inflammatory response in macrophages.

**Bim deficiency leads to increased production of IL-1β in LPS-stimulated macrophages.** We have shown that Bim−/− mice display increased levels of IL-1β in arthritic joints (25). Furthermore, we found that the increased secretion of IL-1β in the joint was associated with more macrophages in the pannus in Bim−/− mice (data not shown). Expression of IL-1β transcripts was undetectable under resting conditions in isolated macrophages from WT and Bim−/− mice. However, there was a 6-fold (\( P < 0.001 \)), 11-fold (\( P < 0.006 \)), and 25-fold (\( P < 0.0002 \)) increase in the level of IL-1β mRNA in Bim−/− as compared with WT macrophages at 1, 3, and 6 hours, respectively, following LPS stimulation (Figure 3A).

Figure 1. Bim expression in synovial tissue from patients with rheumatoid arthritis (RA) and patients with osteoarthritis (OA). A, Bim expression in tissue sections from RA and OA patients, showing decreased expression in RA synovial tissue. Shown are representative photomicrographs of adjacent RA (n = 8) and OA (n = 8) synovial tissue sections stained with anti-Bim (brown) or anti-CD68 (brown) and then counterstained with hematoxylin (blue). (Original magnification × 100.) B, Quantitative analysis of Bim expression in RA and OA synovial tissue. Bim expression in the synovial lining and macrophages in adjacent sections of synovial tissue stained with anti-Bim and anti-CD68 were scored as described in Materials and Methods. Values are the mean and SEM.

Figure 2. Bim deficiency and increased activation of macrophages. A, Increased lipopolysaccharide (LPS)–induced expression of activation markers on bone marrow–derived macrophages from Bim−/− mice as compared with wild-type (WT) mice. WT and Bim−/− macrophages were stimulated with LPS and examined by flow cytometry. Results are representative of 4 independent experiments. B, Enhanced expression of activation markers in thioglycollate (thio)–elicited peritoneal macrophages from Bim−/− mice. Peritoneal macrophages from WT (n = 4 per time point) and Bim−/− (n = 4 per time point) mice were isolated before and after thioglycollate injection and were analyzed by flow cytometry.
Since IL-1β is synthesized as a precursor and requires active caspase 1 for processing, we performed immunoblot analysis on WT and Bim−/− macrophages that had been stimulated with LPS and activated with ATP. ATP is required to activate the inflammasome, a multiprotein complex including caspase 1. Analysis of the secreted isoform of IL-1β showed that there was a 6.0-fold increase \( (P < 0.01) \) in IL-1β (Figure 3B) in supernatants isolated from Bim−/− macrophages as compared with WT macrophages following stimulation with LPS and then ATP. In addition, there was a 4.0-fold increase \( (P < 0.02) \) in serum IL-1β (Figure 3C) in Bim−/− as compared with WT mice injected intraperitoneally with LPS (10 mg/kg of body weight). Collectively, these data suggest that the reduced expression of Bim may contribute to increased macrophage activation.

A peptide corresponding to the BH3 domain of Bim suppresses the clinical severity of arthritis. Our data suggest that Bim may play a central role in limiting the development of inflammatory arthritis; therefore, a mimic of Bim may be a potential therapy for the disease. To mimic the expression of Bim, a peptide corresponding to the BH3 domain of Bim, which has been shown to duplicate the functions of Bim (3,4,6), was generated. A polycationic peptide derived from human immunodeficiency virus type 1 (HIV-1) TAT (9) was fused to the BH3 peptide of Bim (TAT-BH3) or to an altered, inactive BH3 peptide (TAT-inactive BH3). Mice were injected intraperitoneally with K/BxN serum, which induces the effector phase of inflammatory arthritis. For the prophylactic study, mice were injected intraperitoneally with saline, TAT-inactive BH3, or TAT-BH3 peptide at 1 hour before and on days 2 and 4 after injection of serum.

Saline-treated mice and TAT-inactive BH3 peptide–treated mice had a 1.2-mm \( (P < 0.007) \) and a 1.6-mm \( (P < 0.001) \) change in ankle circumference on day 2, respectively, while TAT-BH3 peptide–treated mice had only a 0.1-mm change in ankle circumference (Figure 4A). TAT-BH3 peptide dramatically reduced the development of arthritis by 80% \( (P < 0.02) \) and 72% \( (P < 0.001) \), respectively, as compared with saline or TAT-inactive BH3 peptide treatment on day 4. Furthermore, there was a 55% \( (P < 0.003) \) and a 61% \( (P < 0.001) \) inhibition of arthritis in the TAT-BH3 peptide–treated group as compared with the saline-treated or the TAT-inactive BH3 peptide–treated group, respectively, on day 7 following injection of K/BxN serum (Figure 4A). There was no statistical difference between saline and TAT-inactive BH3 peptide treatments.

In addition, a therapeutic study was performed to test the efficacy of TAT-BH3 in mice with established arthritis (Figure 4B). Mice were injected intraperitoneally with K/BxN serum, and then on day 2 following serum injection, a time point at which all mice displayed overt characteristics of inflammatory arthritis, daily injections of saline, TAT-inactive peptide, or TAT-BH3 peptide were administered (days 2–6; \( n = 5 \) injections). A higher dose of the TAT-BH3 peptide as compared with that in the prophylactic study was used for the therapeutic regimen, since lower doses or fewer injections had minimal effects on development of arthritis (data not shown). TAT-BH3 peptide–treated mice exhibited a 63% \( (P < 0.001) \) and a 46% \( (P < 0.07) \)
decrease in ankle circumference on day 4 and a 52% (P < 0.0001) and a 63% (P < 0.008) decrease on day 7 as compared with the saline-treated mice and with the TAT-inactive BH3 peptide–treated mice, respectively (Figure 4B). The TAT-BH3 peptide was nontoxic and had no consistent effect on renal or hepatocyte function. (Data on the toxicity in TAT BH3-peptide–treated mice are available on request from the author.)

To accurately assess the degree of inflammation and destruction of cartilage and bone, ankle sections were evaluated according to an established histopathologic scoring system (21,24–26,28). When used prophylactically, TAT-BH3 peptide suppressed pannus formation by 79% as compared with saline or TAT-inactive BH3 peptide treatment (Figures 4C and D). The lack of pannus formation in TAT-BH3 peptide–treated mice was associated with a 71% decrease in the bone erosion score as compared with saline or TAT-inactive BH3 peptide treatment (Figures 4C and D). Furthermore, there was a 72% reduction in inflammation in TAT-BH3 peptide–treated mice as compared with saline-treated or TAT-inactive BH3 peptide–treated mice (Figures 4C and D). There was no statistical difference in histologic scores in the saline or the TAT-inactive BH3 peptide treatment groups (Figure 4D).

In the therapeutic study, TAT-BH3 peptide reduced pannus formation by 34% (P < 0.03) and 22% (P not significant), respectively, and reduced bone erosion scores by 39% (P < 0.01) and 38% (P < 0.06), respectively, as compared with saline or TAT-inactive BH3 peptide (Figure 4E).

These data demonstrate that systemic delivery of TAT-BH3 peptide leads to decreased arthritis and reduced bone destruction.

TAT-BH3 peptide induces apoptosis of myeloid cells in vivo. To determine the mechanism of TAT-BH3 peptide–mediated suppression of arthritis, we treated single cell suspensions of splenocytes with medium, DMSO, TAT-BH3, or TAT-inactive BH3 and analyzed leukocyte viability 24 hours later. Only the TAT-BH3 peptide induced apoptosis in the myeloid cell populations, but it had no effect on the lymphocytes. (Data on the apoptosis of splenocytes are available upon request from the corresponding author.) There was no difference between the level of apoptosis in TAT-inactive BH3 peptide–treated cells and the DMSO-treated control cells. These data suggest that the myeloid cells may be more susceptible to TAT-BH3 peptide and that the inactive peptide is relatively inert.

In addition to the cell culture studies, apoptosis was examined in K/BxN serum–treated mice. One hour prior to intraperitoneal injection of K/BxN serum, an FITC-conjugated TAT-BH3 peptide was injected intraperitoneally into mice. The cells in the peritoneal cavity were examined at 6 and 24 hours after K/BxN serum injection for FITC positivity, annexin V staining, and cell surface expression of F4/80 and Gr-1. At 6 hours following serum injection, 44% of the peritoneal cells were positive for the FITC-labeled TAT-BH3 peptide. Surprisingly, 88% of the macrophages incorporated the peptide, while only 46% of the neutrophils and 53% of the lymphocytes were positive for the TAT-BH3 peptide. Furthermore, 50% of the macrophages that incorporated the peptide were also positive for annexin V.
Figure 5. TAT-BH3 peptide induction of apoptosis in myeloid cells. Peritoneal cells from mice treated according to the prophylactic regimen were harvested at 6 hours following injection of K/BxN serum, stained with allophycocyanin (APC)–labeled annexin V, anti-F4/80 antibody, or anti-Gr-1 antibody, and analyzed by flow cytometry. Shown are the cells gated for the total population, the fluorescein isothiocyanate (FITC)–labeled TAT-BH3 cells, or the APC-labeled annexin V cells. Numbers in each compartment are the percentage of positive cells. SSC = side scatter.

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(Figure 5). (Data on the quantitative analysis of TAT-BH3 peptide induction of apoptosis in macrophages and neutrophils are available upon request from the author.) Similar results were obtained at 24 hours following K/BxN serum transfer (data not shown). These data demonstrate that the macrophages preferentially take-up the TAT-BH3 peptide and are sensitive to the death-inducing effects of the BH3 peptide.

TAT-BH3 peptide treatment reduces the number of myeloid cells in arthritic joints. Since macrophages and neutrophils are required for the development of K/BxN serum–transfer arthritis (25,30,31) and since TAT-BH3 peptide has a substantial effect on the fate of myeloid cells in the peritoneum, we quantified the numbers of macrophages (F4/80+) and granulocytes (Gr-1+ F4/80–; polymorphonuclear neutrophils [PMNs]) in the peripheral blood (data available upon request from the author) and joints of arthritic mice.

While there was no decrease in the total number of circulating monocytes in the peripheral blood of TAT-BH3 peptide–treated mice, there was a 77% (P < 0.001) and a 68% (P < 0.02) decrease in the numbers of macrophages within the synovial lining and pannus region of TAT-BH3 peptide–treated versus saline-treated arthritic mice, respectively (Figures 6A and B). Analysis of bone marrow and peripheral blood from saline-treated and TAT-BH3 peptide–treated arthritic mice revealed a 40% (P < 0.05) decrease in the numbers of granulocytes in TAT-BH3 peptide–treated mice as compared with saline-treated mice (data available upon request from the author). Furthermore, TAT-BH3 peptide–treated mice showed a 69% reduction in the PMN score in the joints as compared with saline-treated mice (Figure 6C). In addition, there were increased numbers of TUNEL+ cells in the joints of TAT-BH3 peptide–treated mice as compared with saline-treated mice from the therapeutic regimen (Figure 6D).

Taken together, these data suggest that TAT-BH3 peptide may modulate the immune response, presumably through decreasing the activation potential of,
Figure 6. Recruitment of myeloid cells to the joints of TAT-BH3 peptide–treated arthritic mice. A, Representative photomicrographs of macrophages in ankle sections from the prophylactic study, showing that fewer myeloid cells are recruited to the joints of TAT-BH3 peptide–treated mice. P = pannus; B = bone; SL = synovial lining; C = cartilage. The image at the far right is a higher-magnification view of the boxed area of the adjacent image. Arrowheads represent F4/80-positive cells. (Original magnification × 100.) B and C, Percentages of macrophages (F4/80+ cells) (B) and polymorphonuclear neutrophil (PMN) scores (C) in mice treated with saline (n = 8 and 6, respectively), TAT-inactive BH3 (n = 7 and 6, respectively), or TAT-BH3 peptide (n = 16 and 6, respectively). The TAT-BH3 peptide–treated mice have decreased macrophages and PMNs. Values are the mean and SEM. * = P < 0.05 versus saline-treated mice; † = P < 0.05 versus TAT-inactive BH3–treated mice. D, Representative photomicrographs of TUNEL-stained and Hoechst 33258–stained ankle sections from the therapeutic study (original magnification × 200).
and/or the induction of apoptosis in inflammatory cells, leading to the suppression of arthritis.

**DISCUSSION**

In the rheumatoid joint, there is a shift in the balance of proapoptotic and antiapoptotic Bcl-2 factors toward increased expression of the antiapoptotic Bcl-2 members. There are elevated numbers of cells positive for Bcl-2, Mcl-1, and Bcl-xL in RA synovial tissue compared with control synovial tissue (13,14,32,33). Although the expression of Bax has been shown to be elevated in RA synovial tissue as compared with healthy controls, the activated form of Bax was not examined in these tissues (34). Recently, the expression of p45 up-regulated modulator of apoptosis (PUMA) was shown to be localized to the sublining, and not to the synovial lining, region (35). Since there are few apoptotic cells in the joint, these data suggest that the increased expression of Bax and PUMA is insufficient to induce apoptosis in the RA joint. Our study is the first to show that expression of Bim is reduced in RA synovial tissue as compared with OA controls. Thus, these data are consistent with the concept that the molecular rheostat that governs the survival of cells in the RA joint is shifted toward the Bcl-2 prosurvival proteins.

Bim deficiency results in enhanced activation of dendritic cells (36) and macrophages (Figures 2 and 3), which are known to be major contributors to RA. The transcription of IL-1β in Bim+/− mouse macrophages was elevated in response to LPS as compared with WT mouse cells under parallel conditions, and loss of Bim resulted in a marked increase in the secretion of IL-1β by LPS-treated macrophages and mice.

Since IL-1β production is a multistep process, Bim may affect the synthesis of IL-1β at various levels. One potential pathway that may be affected by Bim is the inflammasome, a multiprotein complex including active caspase 1 (37) that is responsible for the processing of proIL-1β. Recently, Bcl-2 and Bcl-xL were shown to interact with the NALP1 inflammasome (38), which responds to the bacterial ligand muramyldipeptide to activate the synthesis and release of IL-1β (39). Interestingly, preliminary studies revealed that the TAT-BH3 peptide led to a decrease in the release of IL-1β in Bim−/− macrophages, indicating that the BH3 mimetic may partially compensate for the loss of full-length Bim (data not shown). Further studies are therefore required to determine whether Bim may interact with the NALP3 inflammasome, which is responsible for the activation of IL-1β in response to LPS (39).

The role that multi-BH domain proteins and BH3-only domain proteins play in the development of inflammatory arthritis has been investigated recently. Mice deficient in Bak or Bax develop inflammatory arthritis similar to that in control mice (25). In contrast, mice lacking Bim develop a more aggressive and severe form of inflammatory arthritis (25) even as compared with Bid−/− mice (26). These data are consistent with the notion that a decrease in the expression of Bim may enhance the progression of inflammatory arthritis.

In the present study, we showed that systemic delivery of a peptide corresponding to the BH3 domain of Bim was dramatically effective at preventing and suppressing the progression of K/BxN serum-transfer arthritis, which closely resembles the effector phase of RA. However, in the prophylactic study, it appeared that at later time points, the TAT-BH3–treated mice showed the development of a mild inflammatory arthritis, as indicated by joint edema. A potential explanation for this observation may be attributed to the half-life of the peptide, since in the prophylactic study, the TAT-BH3 peptide was administered at a lower concentration and with fewer injections as compared with the therapeutic study protocol. Interestingly, in the therapeutic study, the TAT-BH3 peptide maintained and even partially decreased the joint edema but had minimal effect on the inflammatory infiltrate. The TAT-BH3 peptide was, however, effective at reducing pannus and bone destruction scores. Thus, the TAT-BH3 peptide may not be sufficient to inhibit the inflammation in the synovium but may render the cells that are destroying the joint ineffective by inducing apoptosis. Future studies will be required to examine potential modifications of the BH3 peptide (i.e., using hydrocarbon-stapled BH3 peptide [8]) and the utility of the TAT-BH3 peptide as a dual agent (i.e., coadministration with a disease-modifying antirheumatic drug).

Levels of IL-1β, but not TNFα (data not shown), were reduced in the joints of TAT-BH3 peptide–treated mice. A decrease in the numbers of macrophages and neutrophils appeared to account for this decline in IL-1β. It is not surprising that neutrophils were found to be susceptible to the BH3 peptide, since it was shown to have a high affinity for Mcl-1, unlike other BH3 mimetics (3,4,6), and Mcl-1 is required for the development of neutrophils, but not monocytes or macrophages (40).

Previous investigations have suggested a staging model for the development of K/BxN serum-transfer arthritis (41), in which neutrophils are required for one of the early steps in the K/BxN serum-transfer arthritis model (31). A decrease in the numbers of neutrophils in
the circulation and in the joint may also contribute to a reduction in the numbers of macrophages recruited to the joint by the lowering the levels of secreted chemo-
tactic factors. Our data also indicate that macrophages are more susceptible to TAT-BH3 peptide as compared with lymphocytes. The decreased numbers of macro-
phages in the joint is consistent with studies showing the importance of macrophages in the K/BxN serum-
transfer arthritis model (20,30). While the K/BxN model of arthritis closely resembles the effector phase of RA, future studies will be required to examine the broad
impact of BH3 peptide on different models of inflam-
matory arthritis.

BH3 mimetic therapy has gained considerable
momentum over the past several years (4). Almost all of
the studies using BH3 mimetic therapy have focused on
the feasibility of treating oncogenesis (4). The most well
characterized BH3 mimetic is ABT-737 (4,42–48). Re-
cently, ABT-737 was shown to induce lymphocytopenia
and thereby suppress the development of collagen-
induced arthritis (49), a model which requires lympho-
cytes (50). While ABT-737 mechanistically behaves sim-
ilarly to the sensitizer class of BH3-only domain proteins
(Bad, Noxa), TAT-BH3 functions like Bim, a promiscu-
ous BH3-only initiator protein. These data suggest that
TAT-BH3 has a broader range of antiapoptotic targets
and, thus, may prove to be a more effective therapy than
other BH3 mimetics. Although the TAT-BH3 peptide
resulted in a minimal, but statistically significant, reduction in platelet counts (data available upon request from
the author), ABT-737 has been shown to have a dra-
matic effect on platelet numbers (51). These data also
indicate that TAT-BH3 may have fewer adverse side
effects than ABT-737. Thus, these data suggest that a
Bim-BH3 mimetic has therapeutic value in autoimmune
diseases such as RA, in which a failure to delete the
autoreactive cells leads to the pathogenesis of this
disease.

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