Protease-Activated Receptor-2 Signaling Triggers Dendritic Cell Development

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Dendritic cells (DC) are potent antigen-presenting cells that govern the effector cell responses of the immune system. DC are thought to continuously develop from circulating progenitors in a process that is accelerated by inflammatory stimuli. However, the physiological signals that regulate the development of DC from precursor cells have not been well defined. Here we show that a serine protease acting *via* protease-activated receptor-2 (PAR-2) stimulates the development of DC from bone marrow progenitor cells cultured in granulocyte-macrophage colony-stimulating factor and IL-4. DC fail to develop in bone marrow cultures treated with soy bean trypsin inhibitor, a serine protease inhibitor, but this inhibition is overcome by a PAR-2 agonist peptide. DC do not spontaneously develop from the bone marrow of PAR-2-deficient mice, but can be stimulated to do so by inflammatory mediators. These results suggest that endogenous serine proteases stimulate DC development *in vitro*. Thus, serine proteases may help trigger adaptive immune responses *in vivo*. (Am J Pathol 2003; 162:1817–1822)

Dendritic cells (DC) are present within almost all tissues as immature cells that take up and process antigens.^{1,2} Activation by danger signals, pathogens, or inflammatory mediators leads to the maturation of resident DC,³ their migration to lymphoid organs, and to the recruitment of additional DC to the site of inflammation.^{4,5} DC arise from circulating precursors in the form of pre-DC and, in some cases, monocytes.⁶ *In vitro*, DC can be derived from bone marrow cells, pre-DC in blood, or monocytes when these cells are cultured in the presence of granulocyte-macrophage colony-stimulating factor (GM-CSF) and interleukin (IL)-4.⁷ This process can be inhibited by the addition of cytokines such as IL-6⁸ but is strongly stimulated by inflammatory mediators such as tumor necrosis factor (TNF)- α and CD40L.⁹

DC maturation is critical for the initiation of an immune response.^{2,6,10} Immature DC (iDC) express low levels of surface major histocompatibility complex type II (MHC II)

and costimulatory molecules, have a round morphology and are highly phagocytic.^{11,12} These cells specialize in antigen capture, but are poor T-cell stimulators. Although antigen uptake and processing are integral steps of DC maturation, they alone do not complete the process. iDC must be activated by a “danger” signal^{5,13}; such signals, however, are not well characterized. These endogenous or exogenous mediators are released during “dangerous” situations such as infection, trauma, or tissue necrosis.¹⁴

Here we show that serine proteases can act as danger signals, serving as a maturation stimulus for iDC. Serine proteases are ideal candidates for this role because they are not expressed (or are quickly inactivated by serine protease inhibitors) in the extracellular space of healthy tissue but their levels increase during infection, trauma and tissue necrosis.^{13,15,16} Administration of partially purified bovine thrombin to mice induces autoimmunity, suggesting that serine proteases may have an immune stimulatory function.¹⁷ DC cultures are known to contain serine proteases.¹⁸ Busso and colleagues¹⁹ demonstrated that the administration of a serine protease inhibitor to mice with autoimmune arthritis ameliorated the disease. Further, we show that serine proteases act as danger signals by exerting their effects *via* the well-characterized protease activated receptors (PARs), a family of 7 membrane-spanning G-protein-coupled receptors. PARs are activated when the extracellular NH₂-terminus is cleaved by extracellular serine proteases. This exposes a new NH₂-terminus that is able to act as a “tethered ligand” and activate the receptor, triggering intracellular downstream signaling.^{20,21} Further, short peptide molecules modeled after the sequence of the tethered ligand can also activate PARs and serve as models of PAR activation.

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Materials and Methods

Mice

C57BL/6 mice (Harlan Sprague-Dawley, Indianapolis, IN) and PAR-2^{-/-} mice were housed at the Duke University Vivarium according to IACUC standards. PAR-2^{-/-} and wild-type control mice (kindly provided by Dr. Shaun Coughlin, University of California at San Francisco) were bred five generations into C57BL/6. Experiments that compared cultures derived from wild-type and PAR-2^{-/-} were performed blind to genotype.

Culture Media

Complete media (CM) consisted of RPMI-1640 (Invitrogen, Carlsbad, CA) supplemented with 10% heat-inactivated fetal calf serum (FCS; Hyclone, Logan, UT), 1 mmol/L nonessential amino acids, 1 μ mol/L sodium pyruvate, 2 mmol/L fresh L-glutamine, 100 μ g/ml streptomycin, 100 U/ml penicillin, and 50 μ mol/L 2-mercaptoethanol (all from Invitrogen). DC media consisted of CM plus 10 ng/ml of both murine GM-CSF (mGM-CSF, specific activity of 5×10^6 U/mg) and murine IL-4 (mIL-4, specific activity of 1×10^7 U/mg) (both from Peprotech, Rocky Hill, NJ). Protease-inhibited DC media consisted of DC media supplemented with soy bean trypsin inhibitor (SBTI, 0.2 to 100 μ mol/L), lima bean trypsin inhibitor (LBTI, 0.2 to 100 μ mol/L), 4-(2-aminoethyl)-benzylsulfonylfluoride (AEBSF, 20 to 500 μ mol/L), bovine pancreatic trypsin inhibitor (BPTI, 3 to 100 μ mol/L), leupeptin (10 to 100 μ mol/L), bestatin (6 to 50 μ mol/L), or E-64 (4 to 20 μ mol/L) (all from Sigma, St. Louis, MO). These inhibitors offer broad specificity for the inhibition of serine, cysteine, and aspartic proteases.²²

Dendritic Cell Generation

Spleen- and bone-marrow-derived DC were generated as previously described.¹¹ Briefly, for bone-marrow-derived DC, femurs and tibias were removed, rinsed briefly with 70% ethanol, and placed in phosphate-buffered saline (PBS). The bones were then immediately placed in a sterile Petri dish where the ends were cut off and the marrow cavity flushed using PBS. The cellular solution was then passed through a 70- μ m cell strainer (BD Biosciences). RBC were lysed by ammonium chloride solution and the resulting cells were resuspended at 10^6 cells/ml in DC media with or without protease inhibition and cultured for 4 days at 37°C in 5% CO₂. In some experiments, DC were exposed to one of the following conditions for 24 hours before harvest: 10 ng/ml TNF- α (Peprotech), a crosslinking anti-mouse-CD40 antibody (HM40-3; BD PharMingen, San Diego, CA),²³ PAR-2 agonist peptide²⁴ (PAR-2: SLIGRL; SynPep, Dublin, CA), or PAR-2 agonist control peptide^{24,25} (PAR-2: LSLIGRL; SynPep). Final DC preparations were obtained by passing loosely and nonadherent cells over a 14.5% metrizamide (Sigma) solution as previously described.¹¹ The resulting

band cells were harvested and used in all further experiments.

Briefly, for spleen-derived DC, spleens were removed, minced with sterile scissors, filtered over sterile 100 nylon mesh (Nytex; TETKO Inc., Briarcliff Manor, NY) and washed once in PBS. RBC were lysed and the resulting cell suspension was purified over a Percoll (Pharmacia Biotech, Uppsala, Sweden) gradient. The resulting band was harvested and washed twice in PBS. Cells were then incubated for 90 minutes at 37°C. Cultures were then vigorously pipetted and nonadherent cells were removed and discarded. Fresh CM was added to the adherent cells, and cultures were incubated for 24 hours. After gentle pipetting, nonadherent cells were harvested, and represent the spleen DC preparation referred to in subsequent experiments. All results, for bone-marrow- and spleen-derived DC, are representative of at least two independent experiments.

Flow Cytometry

Cell-surface staining used direct immunofluorescence and was analyzed by flow cytometry (FACScan; BD PharMingen). Staining was performed with the following mouse antibodies: I-A^b, CD11c, CD80, and CD86 (PharMingen). Primary antibodies were directed toward a panel of cell surface markers and were compared with the appropriate isotype-matched controls (PharMingen).

Phagocytosis Assay

Phagocytosis assays were carried out as described previously^{11,26} with the following modifications. Five hundred thousand fresh DC in 250 μ l DC media were incubated with 100 μ l of a freshly prepared 10 mg/ml solution of 40,000 MW fluorescein isothiocyanate (FITC)-labeled dextran (Molecular Probes, Eugene, OR). Cells were incubated at 37°C or on ice for 15, 30, or 60 minutes. Cells were washed five times with cold PBS, fixed in 1% paraformaldehyde and analyzed on a FACScan flow cytometer (BD PharMingen). Data are represented as the geometric mean channel fluorescence of 20,000 cell counts with data points shown as (fluorescence measured at 37°C) – (fluorescence measured on ice).

Mixed Leukocyte Reactions

One hundred thousand allogeneic (BALB/c) or syngeneic (C57BL/6) fresh splenocyte responder cells were placed into each well of a round-bottom 96-well plate (Costar, Cambridge, MA) in CM. Stimulator DC were treated with 15 μ g/ml of mitomycin C (Sigma) and added to each well at various stimulator-to-responder ratios. Each well contained a final volume of 0.2 ml. After 72 hours of culture, 10 μ g/ml of concavalin A (Sigma) was added to wells of splenocytes alone as a positive control. After 5 days of culture, cell growth proliferation was determined using 3-(4,5-dimethyl thiazolyl-2)-2,5-diphenyltetrazolium bromide (MTT; Sigma).^{27,28} Briefly, 15 μ l of a 5 mg/ml solu-

Figure 1. Inhibition of serine proteases in DC media blocks DC development. Cells cultured in GM-CSF and IL-4 demonstrate high levels of DC surface markers (**a**) and show multiple cytoplasmic extensions and abundant cytoplasm (**b**, **c**). Cells cultured in GM-CSF and IL-4 supplemented with SBTI demonstrate low levels of DC markers (**d**), large nuclei, and multiple vacuoles (**e**, **f**). **g**: DC surface markers (■ = I-Ab, ▲ = CD11c, ▼ = CD80, ◆ = CD86) decrease dose-dependently when SBTI is added to cultures. Results are shown as percent-positive expression by flow cytometry based on 20,000 cell counts. **h**: Cells not treated with SBTI (■) demonstrate an increased ability to stimulate a mixed leukocyte reaction when compared to SBTI treated cells (■). Data were significant ($P < 0.03$) in all stimulator-to-responder ratios. **i**: SBTI treated cells (■) showed an increased ability to take up FITC-labeled dextran when compared to non-SBTI treated cells (▲). Scale bars, 15 μm; **b** and **e** and **c** and **f** are equivalent in magnification.

tion of MTT was added to each well. The plate was then incubated for 4 hours at 37°C. The resultant absorbance at a wavelength of 562 nm was determined using a microplate spectrophotometer. Results are shown as (allogeneic response) – (syngeneic response + background) ± SEM

RNA Preparation and RT-PCR

Total RNA was isolated using TRIzol reagent (Invitrogen). One microgram of total RNA was reverse transcribed into cDNA using MuLV reverse transcriptase (Roche Diagnostics, Basel, Switzerland) by incubating reagents at room temperature for 10 minutes, followed by 15 minutes at 42°C. The cDNA products were then used as PCR templates for the amplification of a 614 bp PAR-1 fragment (PAR-1 sense: 5'-CTGACGCTCTTCATCCCCTCCGTG, PAR-1 antisense: 5'-GACAGGAACAAAGCCCGCGACTTC), a 599 bp PAR-2 fragment (PAR-2 sense: 5'-GGTCTTTCTTCCGGTCGTCTACAT, PAR-2 antisense: 5'-GCAGTTATGCAGTCAGGC), a 601 bp PAR-3 fragment (PAR-3 sense: 5'-GAGTCCCTGCCACACAGTC, PAR-3 antisense: 5'-TCGCCAAATACCCAGTTGTT), a 492 bp PAR-4 fragment (PAR-4 sense: 5'-GAGCCGAAGTCCTCAGACAA, PAR-4 antisense: 5'-AGGCCACCAACAGAGATCCA), and a 300 bp CCR-7 fragment (CCR7 sense: 5'-CATCAGCATTGACCGCTACGT, CCR-7 antisense: 5'-GGTACGGATGATAATGAGGTAGCA). The PCR consisted of 25 to 40 cycles between 95°C (15 seconds) and 55°C (45 seconds). Controls included reactions without template, without reverse transcriptase, and water alone. Primers for glyceraldehydes phosphate dehydrogenase

(GAPDH; sense: 5'-GACCCCTTCATTGACCTCAAC, antisense: 5'-CTTCTCCATGGTGGTGAAGA) were used as controls. Reaction products were resolved on a 1.2% agarose gel and visualized using ethidium bromide.

Results

We first examined the effect of serine proteases on DC function. Standard cultures of bone marrow progenitors^{11,29} were supplemented with SBTI. Bone marrow cells cultured in GM-CSF and IL-4 alone gave rise to a predominant population of typical mature DC, expressing CD11c, MHC class II, and costimulatory molecules (Figure 1a) and displaying numerous dendritic processes (Figure 1, b and c). Strikingly, addition of SBTI led to a complete absence of such DC. Cells arising from SBTI-treated progenitors lack all DC surface markers (Figure 1d) and display no dendritic processes (Figure 1, e and f). The effect of SBTI on surface phenotype was dose-dependent from 0.2 to 100 μmol/L (Figure 1g).

Functionally, cells cultured with SBTI had a markedly reduced ability to stimulate allogeneic T-cell proliferation when compared to cells cultured without SBTI (Figure 1h). Cells cultured with SBTI demonstrated a three-fold increased capability to take up FITC-dextran over cells cultured without SBTI (Figure 1i). In the presence of SBTI and without the addition of exogenous stimuli, the morphology, surface phenotype, and functional capabilities of these cells did not change for up to 7 days, including after centrifugation and/or replating (data not shown).

Figure 2. Stimulation of SBTI-treated bone marrow progenitor cells triggers DC development. When either $\text{TNF-}\alpha$ (a) or a crosslinking CD40 antibody (b) are added to SBTI treated cultures 24 hours before harvest, high levels of all surface markers and DC morphology are observed. Micron bar, 15 μm ; all micrographs are equivalent in magnification.

Taken together, these results suggest that the inhibition of serine proteases leads to an arrest in DC differentiation before the development of mature DC. The fact that the resultant cells do not express CD11c, a marker present on all known murine DC subsets, suggests that this arrest occurs at an early stage of DC development.

To determine whether SBTI-treated DC progenitors are still capable of developing into DC, these cells were provided with an additional stimulus for DC development. Addition of $\text{TNF-}\alpha$ (Figure 2a) or CD40 crosslinking (Figure 2b), during the last 24 hours of culture in the presence of SBTI leads to the development of characteristic mature DC. This would suggest that an intrinsic SBTI-sensitive protease normally stimulates DC development, but that this function can be replaced by other stimuli. To investigate if these results were limited to SBTI-sensitive proteases, the effects of other protease inhibitors were examined. LBTI and 4-(2-aminoethyl)-benzylsulfonylfluoride (AEBSF), which have similar inhibitory profiles but differ structurally and mechanistically from SBTI³⁰ also blocked DC development in a dose-dependent manner (data not shown). However, typical mature DC developed in the presence of BPTI, aprotinin, and hirudin, indicating that not all serine protease inhibitors are able to exert this effect. Other classes of protease inhibitors (acid-, cysteine-, and metallo-protease inhibitors) failed to have any effect on DC development (data not shown). These results suggest that specific serine proteases are involved in DC development.

One potential mechanism by which DC progenitors might respond to serine proteases is *via* cleavage of a protease-activated receptor. RT-PCR analysis of fresh bone marrow progenitor cells and bone marrow progenitor cells cultured with and without SBTI demonstrate that PARs are expressed by all populations (Figure 3a). PAR-1 is expressed in fresh bone marrow progenitor cells

Figure 3. Stimulation of the PAR-2 receptor on SBTI-treated bone marrow progenitor cells triggers DC development. **a:** The PAR-2 receptor is expressed on SBTI-treated DC, but not on CCR7 high DC cultured from bone marrow and harvested from spleen (and further stimulated with $\text{TNF-}\alpha$). RT-PCR expression of CCR7, PAR-1, -2, -3, -4, and GAPDH in SBTI- and non-SBTI-treated day 4 DC, spleen DC, fresh bone marrow progenitor cells, and control tissues (spleen for CCR7 and PAR-2, heart muscle for PAR-1, -3, and -4, and GAPDH). Results were similar across all cycle reactions. **b** and **c:** SBTI-treated cells display DC surface phenotype and morphology in response to PAR-2 stimulation. SBTI treated cells were treated with 10 $\mu\text{mol/L}$ PAR-2 agonist (**b**) or PAR-2 agonist control (**c**) peptide 24 hours before harvest.

but shows very low-level expression in cultured cells. PAR-2 is expressed by fresh bone marrow progenitor cells and in cells whose development is arrested by SBTI; however, its expression is reduced markedly in both bone marrow derived DC and in DC isolated from spleen. PAR-3 and -4 are expressed in all populations of cells.

Because PAR-2 expression appears to be regulated during DC development, the effect of PAR-2 activation in this process was examined. Addition of the PAR-2 agonist peptide SLIGRL to SBTI-treated cultures resulted in the restoration of DC development as assessed by I-Ab, CD11c, CD80, and CD86 expression (Figure 3b) and cell morphology (data not shown) in a manner similar to that seen with inflammatory stimuli such as $\text{TNF-}\alpha$ (Figure 2a). The scrambled PAR-2 peptide LSIGRL (which does not activate PAR-2) failed to induce any changes in surface

Figure 4. Generation of DC from bone marrow progenitor cells of PAR-2^{-/-} mice is impaired. **a:** Culturing bone marrow progenitor cells from PAR-2^{+/+} mice in GM-CSF and IL-4 results in mature DC surface phenotype and morphology. **b:** Culturing bone marrow progenitor cells from PAR-2^{-/-} mice does not result in mature DC surface phenotype or morphology. **c** and **d:** The addition of TNF- α (**c**) or a crosslinking CD40 antibody (**d**) to PAR-2^{-/-} cultures 24 hours before harvest triggers DC development as indicated by surface phenotype and morphology. Micron bar, 15 μ m; all micrographs are equivalent in magnification.

phenotype (Figure 3c). This result indicates that activation of PAR-2 can stimulate DC development, and SBTI inhibits an endogenous serine protease in DC cultures that stimulates DC development through PAR-2.

To confirm the role of PAR-2 in DC development, we prepared bone-marrow-derived DCs from PAR-2^{-/-} mice. Unlike wild-type mice (Figure 4a), PAR-2^{-/-} mice failed to develop DC (Figure 4b) with or without SBTI. PAR-2^{-/-} cultured cells had the same characteristics of cells cultured from the bone marrow of wild-type mice in the presence of SBTI (Figure 1d-f). Stimulating PAR-2^{-/-} cultures with TNF- α (Figure 4c) or crosslinking of CD40 (Figure 4d) triggered mature DC development, demonstrating that these cells are capable of becoming DC. The failure of bone marrow progenitor cells from PAR-2^{-/-} mice to yield DC in standard culture conditions in the absence of an exogenous stimulus strongly argues that signaling through PAR-2 is involved in the process of DC development.

Discussion

The involvement of proteases in DC antigen processing and presentation has been well described. Mellman and colleagues³¹ demonstrated that an intracellular cysteine protease was critical for MHC II loading with antigenic peptides. Furthermore, Santambrogio and colleagues¹⁸ demonstrated that primary DC cultures contain serine proteases, which were proposed to be involved in extracellular antigen processing. These proteases were not present in either culture media or serum alone or cultures of other immune cells. Thus, serine proteases are specifically present in primary cultures of bone-marrow derived DC.

Here we extend the role of proteases in DC by showing that SBTI-sensitive serine proteases can induce DC development through activation of PAR-2. From a practical standpoint, these observations have significant implications for the *in vitro* study of DC. In the presence of SBTI, cultured bone marrow progenitor cells do not develop into DC, even following physical manipulation (centrifugation and replating), a process known to trigger DC development.¹⁴ However, when SBTI is removed from these cultures, DC begin to differentiate within 24 hours with minimal effect on viability (data not shown). Therefore, cells cultured in GM-CSF, IL-4 and SBTI provide a source of DC progenitor cells. These cells could be used to identify key regulatory steps of DC development, including the characterization of other signals that trigger DC development. These observations also have significant implications for DC vaccine development.²⁹ For example, it may be possible to achieve higher levels of target antigen presentation on DC by pulsing SBTI-treated progenitor cells with antigen before driving DC development. This could allow DC to more efficiently capture antigen, providing a stronger immune response against a particular antigen and improving vaccine efficacy.

The consequences of the observations in this study could prove to be important in understanding the generation of a primary immune response and in differentiating harmful antigens from benign, self antigens. *In vivo*, proteases released or generated at sites of tissue damage may serve as danger signals that promote DC to switch from a sentinel, antigen-capturing mode to a mature, antigen-presenting mode, thus initiating a primary immune response. PAR-2 can be activated by mast cell tryptase³² and by the coagulation factor Xa,³³ especially when the latter is presented to PAR-2 on the cell surface by the tissue factor/factor VIIa complex.³⁴ In this fashion, DC generate an immune response to harmful stimuli. The same or related mechanisms might also contribute to autoimmune responses or transplant rejection by enhancing the probability of mounting an adaptive immune response in the setting of tissue damage related to surgical injury.

The ability of the immune system to distinguish between benign and harmful antigens is central to maintaining the overall health of an organism. However, it is difficult to envision a system where a single cell determines the nature of an antigen based solely on primary amino acid sequence. By presenting all exogenous anti-

gens on MHC II molecules, DC display a snapshot of their environment to the immune system. However, an immune response is not initiated against all antigens. Only those antigens presented in the context of costimulation incite an immune response. Factors that influence costimulation have been studied in detail because of their ability to influence the immune response. Here we show that proteases, namely those that can activate the PAR-2 transmembrane protein, can up-regulate costimulatory molecules on DC and initiate an immune response. Once activated, PAR-2 initiates a number of intracellular events, including G_{α} and $G_{\beta\gamma}$ signaling. Whether this signaling cascade results directly in the above observations or whether it acts indirectly and requires certain intermediate steps remains to be seen and is currently under investigation in our laboratory. Whatever the mechanism, the ability of proteases to influence DC biology, and possibly the generation of a primary immune response, opens a new area of investigation.

The ability of proteases to influence DC development and maturation represents a novel pathway of affecting immune responses. This finding will stimulate further work to characterize the immunostimulatory role of proteases and may provide a new approach to therapeutically modulate the immune system.

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