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CUTTING EDGE

Cutting Edge: Bradykinin Induces IL-12 Production by Dendritic Cells: A Danger Signal That Drives Th1 Polarization[1]

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Dendritic cells play a major role in the induction of both innate and acquired immune responses against pathogenic invaders. These cells are also able to sense endogenous activation signals liberated by injured tissues even in the absence of infection. In the present work, we demonstrate that kinins mobilize dendritic cells to produce IL-12 through activation of the B_2 bradykinin receptor subtype and that bradykinin-induced IL-12 responses are tightly regulated both by angiotensin-converting enzyme, a kinin-degrading peptidase, and by endogenous IL-10. Using a mouse model of allergic inflammation, we further show that addition of bradykinin to OVA during immunization results in decreased eosinophil infiltration on Ag challenge. The latter effect was demonstrated to be due to IL-12-driven skewing of Ag-specific T cell responses to a type 1 cytokine profile. Our data thus indicate that kinin peptides can serve as danger signals that trigger dendritic cells to produce IL-12 through activation of B_2 bradykinin receptors. *The Journal of Immunology,* **2003, 170: 5349–5353.**

The activation of dendritic cells (DC)[3] by microbes constitutes the bridge between innate and acquired immunity, because this APC population activates innate responses through the production of proinflammatory cytokines while at the same time priming naive T cells against invading pathogens. Innate immunity copes with microbial diversity by recognizing pathogen-derived products through Toll-like receptors (TLR), a receptor family comprising functionally distinct members (for review, see Ref. 1). TLR transduce activating signals that result in increased proinflammatory cytokine production and up-regulation of surface molecules, such as CD40, CD80, and CD86, as well as MHC class II molecules (1). In some cases, microbial products stimulate innate/proinflammatory responses through the activity of members of seven-transmembrane heterotrimeric G-protein-coupled receptors, such as the formyl peptide receptor for bacteria (2), or the CCR5 for the protozoan parasite *Toxoplasma gondii* (3). Despite the increased knowledge concerning the role of TLR in DC function, specifically in the setting of infection, there is limited information on the biochemical nature of endogenous danger (4–6) signals, i.e., those generated as a consequence of physical trauma, chemical toxicity, irradiation, or other types of lesions (7).

In the present study, we tested the possibility that activation of the kinin cascade system may likewise modulate DC function. The term "kinins" refers to a group of octa- to decapeptide metabolites structurally related to bradykinin (BK). Kinins were originally described as potent mediators of inflammation in light of their ability to promote vasodilatation, vascular permeability increases and pain sensations (8). Activation of the tightly regulated kinin system is usually initiated when plasma or tissue kallikreins liberate the kinin moiety from an internal segment of high or low molecular mass kininogens (9). Once released, kinins exert their biological activities by activating B_2R or B_1R, two pharmacologically distinct heterotrimeric G-protein coupled receptors. Physiological responses mediated by the constitutively expressed B_2R are induced by bradykinin (BK) or lysyl bradykinin (LBK), whereas the activation of B_1R, a receptor subtype up-regulated during inflammation is mediated by processed (i.e., [des-Arg]kinins) products generated by kininase I (10). Long range kinin effects in the vascular system are prevented by the action of other peptidases, e.g., the angiotensin-converting enzyme (ACE) (9, 11).

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[3] Abbreviations used in this paper: DC, dendritic cell; TLR, Toll-like receptor; BK, bradykinin; LBK, lysyl-BK; B_2R, BKR B2; B_1R, BKR B1; des-Arg-BK, [des-Arg^9]BK; ACE, angiotensin-converting enzyme; Hoe-140, D-Arg^0-Arg^1-Pro^2-Hyp^3-Gly^4-1,2,3,4-tetrahydro-3-isoquinolinecarboxylic acid^5-Ser^6-D-L-1,2,3,4-tetrahydroisoquinoline-3-carboxylic acid^7-L-octahydroindole-2-carboxylic acid^8-Arg^9; LOD, low density cell; WT, wild type.

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Herein, we demonstrate that injection of mice with synthetic kinins activates DC to produce IL-12 through the activation of B_2 kinin receptors. Using a mouse model of allergic inflammation, we show that OVA Ag emulsions supplemented with BK skewed OVA-specific T cell responses from a Th2 to a Th1 type of response and prevent eosinophil infiltration in the pleura of challenged mice. These results suggest that the fine tuning of the Th1/Th2 balance may be influenced by levels of endogenous kinins generated in injured tissues.

Materials and Methods

Animals

B6, 129 F_1, C57BL/6, BALB/c; B6, 129 B_2 kinin-receptor $(B_2R)^{-/-}$; C57BL/6 IL-$10^{-/-}$, and BALB/c IL-12p$40^{-/-}$ were obtained from The Jackson Laboratory (Bar Harbor, ME) and maintained at a National Institute of Allergy and Infectious Diseases American Association of Laboratory Animal Care-accredited animal facility. The animals were equally distributed between both sexes and were used at 5–6 wk of age.

Reagents

LBK, BK, [des-Arg^9]BK (des-Arg-BK), D-Arg^0-Arg^1-Pro^2-Hyp^3-Gly^4-Thi^5-Ser^6-D-Tic^7-Oic^8-Arg^9 (Hoe 140), des-Arg^{10}-HOE 140 (des-Arg-HOE 140), carrageenin, and OVA were purchased from Sigma-Aldrich (St. Louis, MO). Captopril was purchased from Calbiochem (San Diego, CA).

Splenic DC purification

Splenic DC were purified as previously described (3). Briefly, spleens were digested with Liberase CI (Roche Biochemicals, Indianapolis, IN), and the resulting cell suspensions were centrifuged in a dense BSA (Sigma) gradient, low density cells (LOD) were then collected from the interface. After a washing, the cells were resuspended in RPMI medium supplemented with 10% FCS (heat-inactivated).

Cytokine ELISA

IL-12p40, IL-10, and IFN-γ ELISAs were performed as previously described (3). Both IL-12p70 and IL-4 ELISA were performed using the Quantikine M kit from R&D Systems (Minneapolis, MN).

Flow cytometry

For surface staining, spleen cell suspensions were incubated with anti-CD11c-FITC, anti-CD4-PE, and anti-CD8α-PerCP (BD Biosciences, San Diego, CA) for 30 min at 4°C. For intracellular IL-12 staining, cells were then washed in PBS, fixed, and incubated with anti-IL-12p40-APC (BD Biosciences) for 30 min at 4°C. Cells were acquired via FACSCalibur (BD Biosciences), and data were analyzed using FlowJo software (ThreeStar, San Carlos, CA) as previously described (3). For B_2R staining, cells were incubated with rabbit anti-B_2R (Santa Cruz Biotechnology, Santa Cruz, CA), followed by washing and incubation with anti-CD11c-PE and anti-rabbit IgG-FITC (Santa Cruz Biotechnology).

Pleurisy model

Animals were immunized by injecting OVA (10 μg/mouse) in alum (Sigma-Aldrich) emulsions in the footpad (0.05 ml/footpad). Seven days after the first injection of immunogen, the animals received a second injection of OVA (10 μg/mouse) in their footpads. Five days later, the mice were given an intrapleural injection of OVA (10 μg/mouse); after 24 h, the pleural cavity was washed, and the exudate cells were counted, cytospun onto slides, and Giemsa-stained for differential leukocyte determinations.

Results and Discussion

Kinins mobilize and activate splenic DC in vivo

To test whether kinins stimulate innate immunity, we injected LBK into $B_2R^{+/+}$ and $B_2R^{-/-}$ mice and monitored serum levels of IL-12p70. LBK injection induced a B_2R-dependent increase in IL-12p70 levels in vivo. The response was rapid, peaking at 1 h postinjection and declining precipitously thereafter (Fig. 1). The brief response observed on LBK injection in vivo was not unexpected, because kinins are quickly degraded in the circulation by the action of ACE (9). To verify whether IL-12 production is modulated by ACE, LBK was injected into mice that were pretreated with captopril, a well-known inhibitor of this enzyme (12), and IL-12p70 levels were monitored. As shown in Fig. 1*A*, the duration of the IL-12 responses was significantly increased, suggesting that this intervention increases the half-life of LBK in the circulation, thus allowing for a more prolonged stimulation of IL-12-responsive cells.

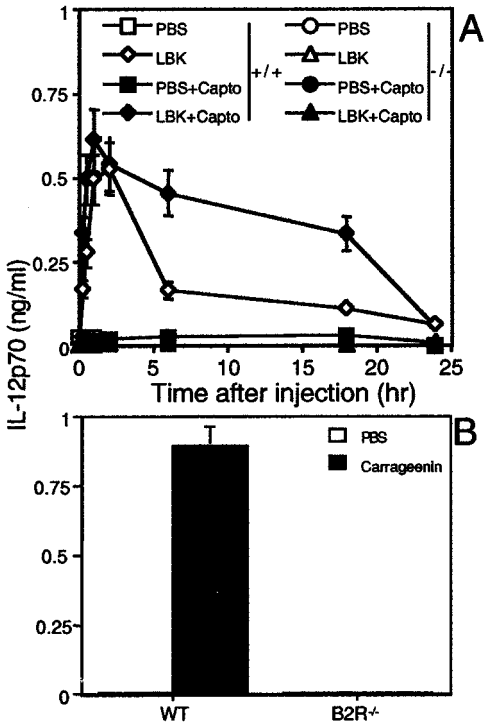

FIGURE 1. LBK induces DC activation and mobilization in vivo. $B_2R^{+/+}$ (B6, 129 F_1 (□, ■, ◇, ◆)) or $B_2R^{-/-}$ (B6, 129 $B_2R^{-/-}$ ○, ●, △, ▲)) mice were injected i.p. with PBS (0.2 ml/mouse (□, ■, ○, ●)) or LBK (10 μg/mouse (◇, ◆, △, ▲)). Before injection (2 h), the mice were treated with (■, ◆, ●, ▲) or without (□, ◇, □, ○) captopril (Capto, 10 mg/kg). At different times thereafter, the animals were bled, and serum IL-12p70 was measured by ELISA (*A*). *B,* $B_2R^{+/+}$ or $B_2R^{-/-}$ mice were injected i.p. with PBS (0.2 ml/mouse (□)) or carrageenin (10 μg/mouse (■)). At 6 h postinjection, spleens were harvested, and LOD cells were purified and incubated for 18 h at 37°C. IL-12p70 levels were then assayed in the supernatants by ELISA. Each point or column represents mean ± SD of triplicate samples for two independent experiments.

We next asked whether endogenous release of kinins (i.e., resulting from proteolysis of natural kininogens) would have effects on DC activation comparable with those observed after injection of the synthetic peptide homologues. To answer this question, WT or $B_2R^{-/-}$ mice were injected with carrageenin, a well-known in vivo activator of the kinin system (13). Analysis of the cytokines secreted by WT DC indicated that carrageenin induced IL-12 and IL-10 production ex vivo and promoted the migration of $CD11c^+$ DC to the periarteriolar lymphoid sheath area of the spleen (Fig. 1*B* and data not shown). These results likely reflect the stimulatory activity of endogenously released kinins on cell types that express the constitutive B_2R receptor subtype because animals deficient for this receptor failed to produce IL-12 in response to carrageenin injection (Fig. 1*B*). Consistent with these observations, in vivo injection of either LBK or carrageenin induced CD40, CD80, and CD86 up-regulation by WT DC (data not shown).

Kinins induce DC activation in vitro

To investigate the role of kinins in the activation of DC, we isolated total splenic $CD11c^+$ DC and incubated the cells for 18 h with different concentrations of LBK. Analysis of the culture supernatants (Fig. 2*A*) indicated that addition of LBK (0.01–10 μM) stimulated IL-12p40 production by WT splenic DC. Similar results were observed when we used DC from endotoxin-hyporesponsive C3H/HeJ mice (not shown). The IL-12p40 response induced by LBK was dependent on the B_2R activation pathway, because DC purified from $B_2R^{-/-}$ mice failed to respond to this agonist (Fig. 2*A,* filled triangles) while responding normally to microbial stimulation, such as LPS or CpG-oligo-DNA (not shown). As seen in Fig. 2*A* (open symbols), des-Arg-BK, a B_1R agonist, did not induce detectable levels of IL-12p40 secretion over a wide range of ligand concentration. Furthermore, a B_2R (HOE 140), but not a B_1R antagonist (des-Arg-HOE 140), blocked IL-12p70 and p40 (not shown) production by DC treated in vitro with LBK (Fig. 2*B*). Together, these data suggest that kinins stimulate DC to produce IL-12 by signaling through the B_2R constitutive receptor. In support of this mechanism, B_2R was detected by FACS staining on ~20% of the $CD11c^+$ cells in resting spleen (Fig. 2*C*).

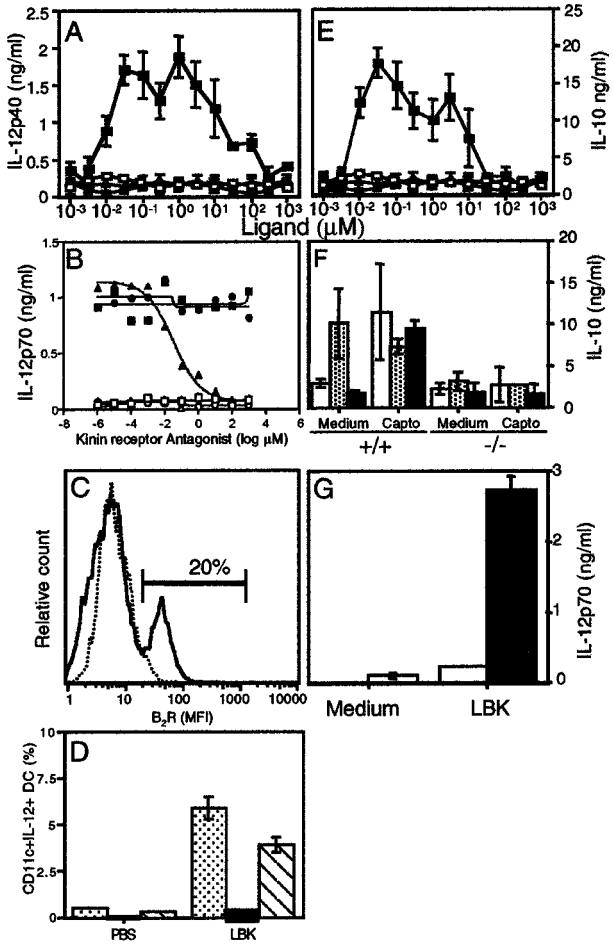

FIGURE 2. LBK induces IL-12 production from murine splenic DCs in vitro through activation of the B_2 kinin receptor. LOD spleen cells were purified from $B_2R^{+/+}$ (B6, 129 F_1 (□, ■)) or $B_2R^{-/-}$ (△, ▲) mice. The cells were incubated with increasing concentrations of LBK (■, ▲) or des-Arg-BK (□, △), and 18 h later the culture supernatants assayed for IL-12p40 (*A*) and IL-10 (*E*) by ELISA. Each point represents the mean ± SD of triplicate samples from a representative experiment of three independent analysis. *B,* WT LOD cells were incubated with medium alone (□, △) or with LBK (100 nM; ■, ▲) together with vehicle (PBS (□, ■) increasing concentrations of HOE 140 (△, ▲) or des-Arg-HOE 140 (○, ●) for 18 h. The culture supernatants were then harvested and assayed for IL-12p70. *C,* LOD spleen cells stained for CD11c and B_2R were analyzed by FACS. Histograms show B_2R expression of naive $B_2R^{+/+}$ (——) or $B_2R^{-/-}$ (·····) from cells gated for $CD11c^+$. Data are representative of at least two experiments with similar results. MFI, mean fluorescence intensity. *D,* WT LOD cells were incubated overnight with LBK and stained for surface CD11c, CD4, and CD8α and intracellular IL-12p40. Bars, Frequency of $CD11c^+$IL-12^+ cells gated on $CD8\alpha^+$ (▨), $CD4^+$ (■), or $CD8\alpha^-CD4^-$ (▧) cells. Values are the mean ± SD of the frequencies of each subset in two independent experiments. *F,* $B_2R^{+/+}$ or $B_2R^{-/-}$ splenic DC were incubated with medium alone (□), LBK (▨), or des-Arg-BK (■) (all at 1 μM) with or without pretreatment with captopril (1 h before stimulation) for 18 h at 37°C. Culture supernatants were harvested and assayed for IL-10 levels by ELISA. Data are representative of two independent experiments. *G,* LOD cells from IL-$10^{+/+}$ (□) or IL-$10^{-/-}$ (■) mice were stimulated in vitro with 1 μM LBK and incubated for 18 h. Culture supernatants were harvested and assayed for IL-12p70 by ELISA.

To further characterize the role of DC in kinin-induced IL-12 production, we stimulated splenic DC in vitro with LBK and then performed surface and IL-12 intracellular staining. Subsequent FACS analysis gating on specific DC subpopulations revealed that $CD11c^+CD8\alpha^+CD4^-$ (CD8) and $CD11c^+CD8\alpha^-CD4^-$ (DN), but not $CD11c^+CD8\alpha^-CD4^+$ (CD4), DC respond as assessed by the significantly higher frequencies of IL-12^+ cells produced after stimulation with LBK (Fig. 2*D*). Furthermore, using a similar approach, we found that the costimulatory molecules CD40, CD80, and CD86 as well as MHC class II are up-regulated on kinin-activated DC (not shown).

In addition to IL-12, we also measured levels of the anti-inflammatory cytokine, IL-10, in cultures stimulated with LBK or des-Arg-BK. Similar to the B_2R-dependent induction of IL-12, IL-10 production by DC was increased in response to LBK, but not by des-Arg-BK, the B_1R agonist (Fig. 2*E*). Even in the absence of exogenous LBK, addition of captopril to WT DC resulted in higher levels of IL-10 (Fig. 2*F*). In the same culture setting, captopril alone failed to stimulate IL-10 production by DC from $B_2R^{-/-}$ mice. Together, these results suggest that in the presence of ACE inhibition, WT DC preferentially produce IL-10 in response to low levels of endogenous kinins released in culture. Consistent with this hypothesis, DC from IL-$10^{-/-}$ mice produced higher amounts of IL-12 than WT DC when stimulated with LBK, arguing that IL-10 counteracts the proinflammatory activity of BK by down-regulating IL-12 production (Fig. 2*G*).

LBK induces IL-12-dependent Th1 priming for OVA Ag in vivo and prevents eosinophilic infiltration in a pleurisy model

Given the IL-12-stimulatory activity of LBK in vivo, we whether synthetic kinins (LBK or BK) can serve as adjuvants for induction of immune responses to protein Ag. To analyze the potential benefits of LBK as a Th1-skewing mediator, mice were immunized with alum-based OVA emulsions that contained fixed amounts of synthetic LBK. A Th2-dependent pleurisy model was then used to evaluate whether the LBK adjuvant could prevent induction of the predominant Th2 responses which in this model are characterized by high levels of IL-4 secretion and lower levels of IFN-γ (Fig. 3*B*). When immunized with OVA Ag in the absence of LBK, an eosinophil-enriched infiltrate was observed in the pleural cavity on OVA challenge (Fig. 3*A*). In contrast, mice immunized with alum-based emulsions containing a mixture of LBK and OVA displayed reduced

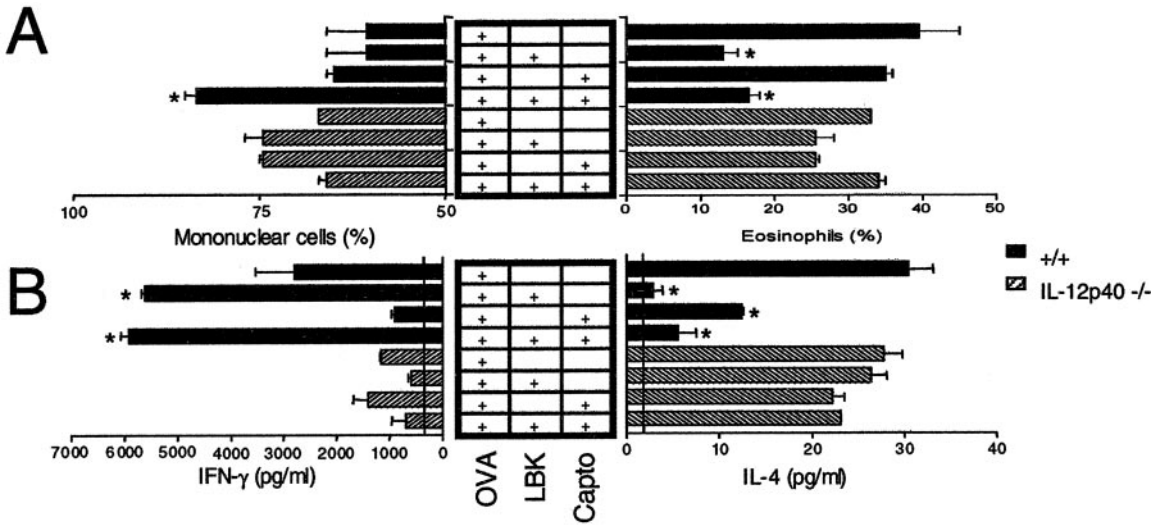

FIGURE 3. LBK induces Th1 priming in vivo and prevents eosinophil infiltration in pleural cavity. BALB/$c^{+/+}$ (■) or BALB/cIL-12p$40^{-/-}$ mice (▨) were injected in the footpad with OVA (10 μg/mouse) with or without LBK (10 μg/mouse) in alum (0.05 ml/mouse), preceded or not by treatment with captopril (Capto; 10 mg/kg) 2 h before immunization. After 1 week, the animals received a second injection in the footpad of OVA alone; 5 days later, the animals received an intrapleural challenge with OVA; and after 24 h, the pleural cavity was washed and the frequencies of leukocytes were evaluated by Giemsa staining of cytospin slides (*A*). Popliteal and inguinal lymph nodes from the same mice used in *A* were harvested, pooled, and incubated for 4 days with syngeneic splenic DC previously primed with OVA (10 μg/ml). IL-4 and IFN-γ levels was then evaluated in culture supernatants by ELISA (*B*). Data are means ± SD of the values obtained from five animals per group. *, Statistically significant differences between groups treated with OVA plus $LBK^{+/-}$ captopril and cells treated with OVA alone ($p < 0.05$).

eosinophil infiltration in response to the same challenge in the pleura (Fig. 3*A, right*). Consistent with these findings, analysis of OVA recall responses in vitro indicated that immunization with the OVA and LBK mixture has led to reduced IL-4 and increased IFN-γ levels (Fig. 3*B*). Pretreatment with captopril or LBK peptide alone (without OVA) had no effect on background OVA-specific responses (not shown). Pretreatment of mice immunized with OVA alone with captopril failed to inhibit pleural eosinophil infiltration or result in Th1 skewing (Fig. 3). However, captopril optimized Th1 skewing in animals immunized with OVA plus LBK and resulted in the production of high levels of OVA-induced IFN-γ by T cells (Fig. 3*B*) as well as increased numbers of mononuclear cells (Fig. 3*A*).

To further characterize the mechanism by which LBK affects OVA T cell priming in vivo, IL-12p$40^{-/-}$ animals were subjected to the same immunization protocols. This experiment showed that, in the absence of LBK, OVA-immunized IL-12p$40^{-/-}$ mice challenged by injection of Ag in the pleural cavity develop an intense eosinophil infiltration and augmented levels of IL-4 (Fig. 3, *A* and *B, right*, striped bars). Significantly, in these IL-12p$40^{-/-}$ animals, addition of BK to the OVA immunogen, with or without captopril, failed to induce the Th1-type responses previously described in WT mice. Moreover, the IL-12p$40^{-/-}$ animals that received LBK had marked eosinophil infiltration in the pleural cavity, suggesting that the major mechanism by which BK immunization results in reduced eosinophilic infiltration is through the induction of IL-12 by APCs, which in turn promotes the development of Ag-specific IFN-γ-producing T cells in vivo.

The data presented here demonstrate that exogenous or endogenous kinins acting through their cognate receptors on DC can initiate innate immunity as well as influence the subsequent adaptive immune response. Initially, we demonstrated that splenic DC secrete IL-12 in response to kinin stimulation both in vitro and in vivo and that this response is dependent on B_2R, but not B_1R. In further support of a role for B_2R in DC activation, we found that carrageenin, a kinin system activator, stimulates B_2R-dependent IL-12 production in vivo. Although the mechanism responsible for endogenous release of kinins by systemically administered carrageenin was not examined in the present study, it is likely to reflect contact phase activation of plasma kallikrein (14). In other settings, e.g., infection, DC may respond to B_2R agonists that are released from kininogens by tissue kallikrein or by unique microbial cysteine proteases (15–18).

Tight regulatory constraint on kinin activity is imposed to prevent excessive edema formation, hypotension, and hyperalgesia. In addition to B_2 receptor desensitization (10), physiological control of short-lived kinins is exerted by the degrading activity of ACE and other peptidases (9). Consistent with the latter mechanism, IL-12 levels in LBK-stimulated mice were significantly increased in captopril-treated animals. In addition, we found that LBK-driven IL-12 responsiveness is also modulated by the anti-inflammatory cytokine IL-10, because the latter cytokine is produced concomitantly with IL-12 after LBK stimulation, and LBK-induced IL-12 responses are significantly enhanced in IL-$10^{-/-}$ mice.

Our observation that activation of innate immunity through the B_2R pathway is held in check by kinin-degrading peptidases and by IL-10-mediated down-regulation underscores the importance of tight regulatory constraints on kinin homeostasis. To limit the action of BK spatially and temporally, we added synthetic LBK to alum-based OVA-containing emulsions and tested whether this strategy results in preferential priming of Th1 cells. LBK addition during immunization suppressed Th2-dependent pleurisy in an OVA injection model, while skewing the cytokine response toward a Th1 pattern. Interestingly, the addition of an ACE inhibitor was not essential for revealing the effects of kinins in this model, suggesting that the levels of BK added to the OVA-alum emulsions were sufficiently high to

reach the kinin receptor activation threshold required for induction of a Th2-Th1 switch. Together, the above findings suggest that synthetic BK homologues are worth investigating as potential adjuvants in vaccine formulations designed to trigger Th1 responses, either for the development of protective immunity against intracellular pathogens or for the prevention/treatment of allergic disease.

In summary, our findings establish the kallikrein-kinin system as an important example of a pathway by which tissue injury can modulate both innate and adaptive immunity. As such, it may provide a useful paradigm for studying a chemically defined danger signal distinct from those triggered by infectious non-self.

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