active sulfur oxyanions may directly inhibit the components of the electron transport chain or even the reductive dehalogenase, as observed in *Desulfomonile tiedjei* (*17*).

Comparative analysis of the nearly full-length 16*S* ribosomal DNA (rDNA) sequence of strain TCA1 and available 16*S* rDNA sequences revealed that strain TCA1 is related to gram-positive bacteria with low G+C content in their DNA. Strain TCA1, *Dehalobacter restrictus* (*18*, *19*), and three clones from trichlorobenzene- and 1,2-dichloropropane–dechlorinating consortia (*20*, *21*) form a phylogenetic cluster (Fig. 3) defined by 16*S* rDNA sequence similarities of 97% and higher. The closest relative of strain TCA1 is *D. restrictus* strain TEA, with a sequence similarity of 99%. *D. restrictus* strains PER-K23 and TEA are strict anaerobes capable of coupling PCE and TCE dechlorination to H_2 oxidation for growth in a respiratory process. However, we did not detect TCA dechlorination by strain PER-K23. These bacterial strains seem to be obligate H_2-oxidizing dechlorinators that only grow by reductive dechlorination of specific chlorinated ethanes or ethenes in anaerobic respiration. The physiology, morphology, and 16*S* rDNA sequence of strain TCA1 suggest that it is a *Dehalobacter* and perhaps represents a new species based on its unique features of TCA dechlorination and formate oxidation. The isolation of strain TCA1 further suggests an important role for *Dehalobacter* species in polluted anoxic environments.

To determine the potential of strain TCA1 to attenuate TCA in the natural environment, we bioaugmented anoxic aquifer sediments from Bachman and Schoolcraft (both in Michigan, USA) contaminant plumes. Both sites are contaminated with PCE and daughter products, and the Schoolcraft plume G site is also contaminated with TCA, DCA, and chromium. TCA was completely converted to CA within 2 months in both aquifer sediment samples amended with strain TCA1, whereas no dechlorination was observed in samples without the inoculum. These results suggest that bioaugmentation with strain TCA1 could ensure and speed the degradation of TCA, especially if naturally occurring populations are patchy or absent.

CA, rather than ethane, appears to be the terminal TCA product from our culture, and studies have shown that both DCA and CA can be degraded under aerobic conditions (*22*, *23*). Because the aerobic transformation of DCA is much slower than that of CA (*24*), complete conversion to CA would result in more reliable removal of chloroethanes on the aerobic fringes of a plume.

The discovery of an anaerobic dehalorespiring *Dehalobacter* that couples reductive dechlorination of TCA to growth not only may lead to a better understanding of the physiology, phylogeny, and biochemistry of dehalorespiring bacteria, but also suggests a strategy for bioremediation of TCA in soils and ground water, thereby aiding in the attenuation of this ozone-depleting compound.

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28. Supported by the Michigan Department of Environmental Quality and the Center for Microbial Ecology at Michigan State University.

Supporting Online Material

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Materials and Methods

4 June 2002; accepted 19 August 2002

Toll-Like Receptor 4–Dependent Activation of Dendritic Cells by β-Defensin 2

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β-Defensins are small antimicrobial peptides of the innate immune system produced in response to microbial infection of mucosal tissue and skin. We demonstrate that murine β-defensin 2 (mDF2β) acts directly on immature dendritic cells as an endogenous ligand for Toll-like receptor 4 (TLR-4), inducing up-regulation of costimulatory molecules and dendritic cell maturation. These events, in turn, trigger robust, type 1 polarized adaptive immune responses in vivo, suggesting that mDF2β may play an important role in immunosurveillance against pathogens and, possibly, self antigens or tumor antigens.

Activation of innate immunity through pattern recognition receptors for ligands derived from evolutionarily distant pathogens provides essential signals for initiation of the adaptive immune response (*1*–*3*). Microbial infection activates the TLR signaling cascade (*4*), which results in expression of various proinflammatory cytokines, chemokines, and large quantities of small antimicrobial peptides, such as defensins (*5*–*7*). Recently, it was reported that β-defensins, epithelial antibacterial peptides with six conserved cysteine residues, might have an additional function as potential chemoattractants of immature dendritic cells (iDCs) through chemokine receptor CCR6 (*8*, *9*). In the course of our studies using β-defensins and chemokines to target delivery of nonimmunogenic antigens to iDCs in vivo as vaccines, we unexpectedly observed that the resulting immune responses differed substantially depending on the type of chemoattractant moiety used. In particular, murine β-defensin 2 (mDF2β)–based vaccines elicited modest lev-

els of antigen-specific antibodies, but very potent cell-mediated responses and antitumor immunity (*8*, *10*). Therefore, we hypothesized that the ability of mDF2β to augment cell-mediated immune responses may be because of its specific effects on iDCs.

To test whether β-defensins had any direct effect on DC function, we incubated bone marrow–derived iDCs (*11*) for 18 hours with various fusion proteins consisting of murine β-defensin 2 or -3 (mDF2β and mDF3β, respectively, fig. S1) linked to a nonimmunogenic lymphoma antigen (idiotype sFv) (*11*, *12*). The maturation status of DCs was determined by the expression of cell-surface markers such as B7.2, CD40, CD11c, and major histocompatibility complex (MHC) class II, as well as by the production of proinflammatory cytokines (*11*). In nature, β-defensins are produced in a functionally inactive prodefensin form, which is activated by cleavage of the prosequence (*13*). Therefore, recombinant murine pro–β-defensin fusion proteins (with sFv) were produced as controls (mproDF2β, fig. S1). Other controls were recombinant tumor antigen alone (sFv) or fused with chemokine MCP-3 (MCP3, fig. S1). All proteins were 95% pure and contained less than 0.5 U endotoxin (*11*). The proportion of CD11c-positive cells expressing both CD40 and B7.2 was not changed by the treatment of iDCs with pro–β-defensin 2 fusion protein, MCP-3, or sFv alone compared with complete medium (Fig. 1A). Similarly, expression of MHC class II was not increased in $CD11c^+$ cells by any of those agents (*14*). In contrast, iDCs treated with as little as 5 μg/ml mDF2β fusion protein expressed significantly higher levels of MHC class II (*14*) and $B7.2^+/CD40^+$ cells (Fig. 1A and fig. S2). Furthermore, two other recombinant fusion proteins of mDF2β, either with a short c-Myc peptide tag sequence (N21mDF2β) or fused with a different sFv

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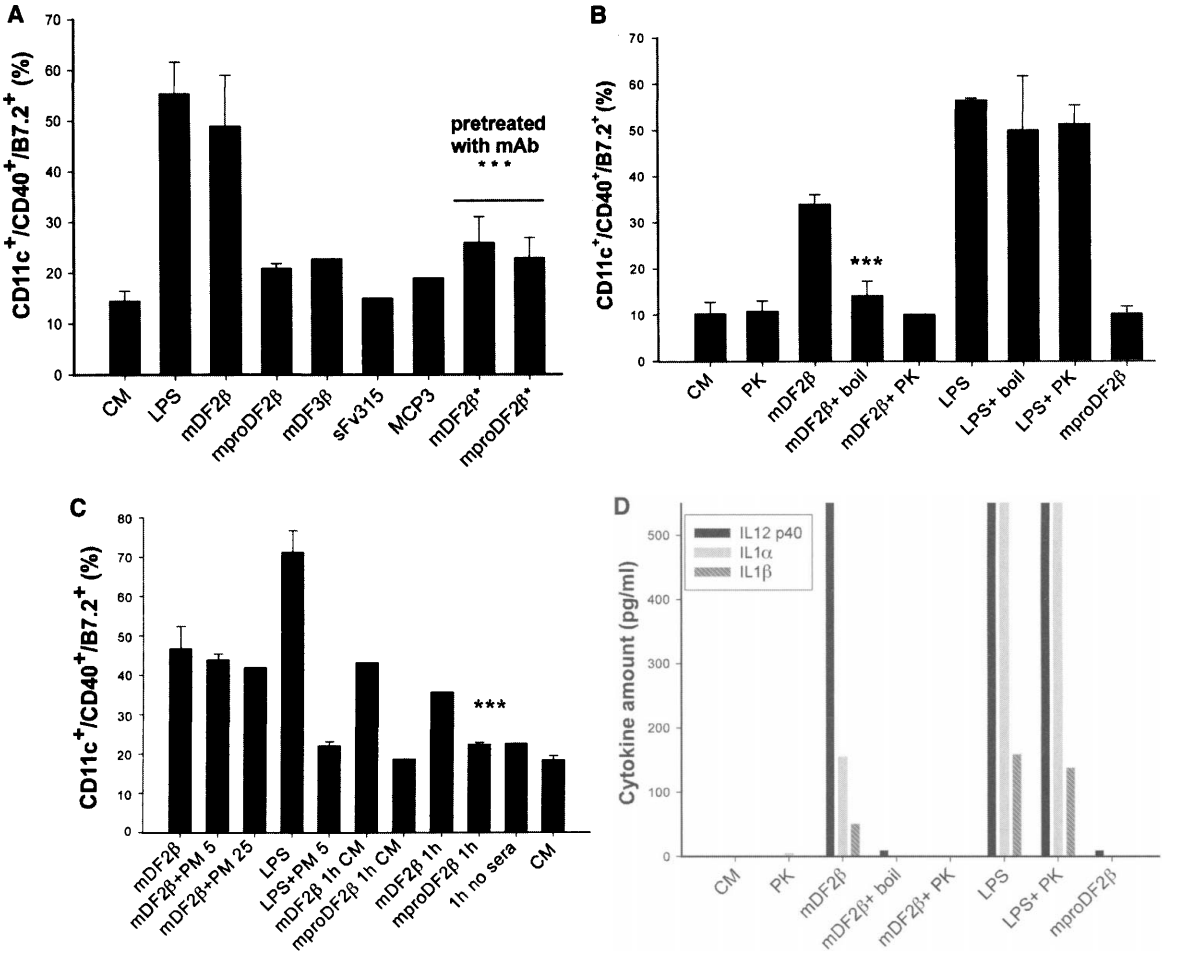

Fig. 1. Murine β-defensin 2 induces maturation of bone marrow–derived immature DCs. (**A**) Proportion of cells positive for CD11, CD40, and B7.2 after incubation of iDCs with recombinant β-defensin 2 (5 μg/ml) for 18 hours (see fig. S1) was increased from 14.51 ± 1.88% to 49 ± 8.15%. Control DCs were incubated in culture medium (CM), with 5 μg/ml sFv alone or fused with pro–β-defensin (mproDF2β), murine β-defensin 3 (mDF3β), MCP-3 (MCP3), or LPS (10 ng/ml). Proportion of $CD11^+/CD40^+/B7.2^+$ in untreated group was, on average, 14.51 ± 1.88%. To confirm specificity, iDCs were incubated with supernatants from the mDF2β or mproDF2β samples pretreated with 9E10 mAb, specific for Myc tag, coupled with protein A–Sepharose beads (mDF2β*, or mproDF2β*, pretreated with mAb, repeated twice). $***P < 0.001$ is the significance of a comparison of mDF2β (pooled data), treated with mAb or untreated, from five independent experiments. (**B**) Effects of mDF2β were abrogated by pretreatment of samples with proteinase K (PK), or by boiling for 15 min before DC incubation (boil). $***P < 0.005$ is the significance of a comparison between mDF2β and mDF2β+boil (pooled data). Representative data from three independent experiments. (**C**) Specific inhibitors of LPS such as polymixin B at 5 and 25 μg/ml (mDF2β+PM 5 and mDF2β+PM 25, respectively) do not inhibit mDF2β-induced maturation of iDCs treated for 18 hours. The experiment was repeated three times. Protein-pulsing experiment DCs were washed in Dulbecco's phosphate-buffered saline (PBS) after 1 hour incubation with mDF2β in CM (mDF2β 1h CM), or in serum-free medium (mDF2β 1h). $***P < 0.004$ is the significance of a comparison of pooled data for mDF2β and mproDF2β. (**D**) mDF2β-matured DCs produce proinflammatory cytokines IL-12, IL-1α, and IL-1β. Conditioned medium from DCs incubated for 18 hours with mDF2β or mproDF2β, with or without proteinase K (PK) or boiled (mDF2β+boil) was measured by enzyme-linked immunosorbent assay (ELISA). Control groups were treated with LPS (10 ng/ml, boiled or not boiled), with or without PK pretreatment. Representative data from three independent experiments. DCs were isolated from BALB/c mice. A representative recombinant protein N24mDF2β (fig. S1) was used as a source of mDF2β.

(N24mDF2β), also induced DC maturation (fig. S3A). This effect was abrogated completely when mDF2β proteins were absorbed beforehand with c-Myc–specific monoclonal antibody (mAb) (pretreated with mAb, Fig. 1A and fig. S3A).

Lipopolysaccharide (LPS) is known to induce DC maturation and, consistent with this, was able to induce activation of $CD11c^{+}$/$B7.2^{+}$/$CD40^{+}$ cells (Fig. 1A). It is unlikely that the effects of mDF2β were due to contaminating LPS, because the amount of endotoxin in the proteins was well below the threshold level of 1 ng/ml LPS (fig. S2). Moreover, DC maturation was abrogated completely by treatment of mDF2β, but not LPS, with proteinase K (mDF2β+PK, Fig. 1B) or by boiling for 15 min (mDF2β+boil); these findings suggest that the component responsible for inducing DC activation was a protein. Efficiency of protein denaturation by boiling was confirmed by lack of chemoattraction of CCR6-expressing HEK 293 cells to boiled mDF2β (*11*, *14*). Furthermore, the activity of LPS on DC activation could be completely blocked by microbial peptides, such as polymixin B (LPS+PM, Fig. 1C) (*15*), or by diphosphoryl lipid A from the nontoxic LPS of *Rhodobacter sphaeroides* (LPS+RsDPLA, fig. S3, B and C), both of which are able to compete with LPS for binding to LPS-binding protein and CD14, respectively (*16*). In contrast, neither of these inhibitors affected mDF2β-induced maturation (Fig. 1C and fig. S3, B and C). In addition, the DC maturation could be induced by brief (1 hour) treatment with mDF2β (mDF2b 1h CM on Fig. 1C), even in serum-free medium (mDF2b 1h), which suggests that mDF2β could directly activate iDCs in the absence of serum accessory proteins, such as LPS-binding protein, which is needed for LPS activity. Finally, DC maturation required fully functional β-defensin 2, as mproDF2β or denatured mDF2β (mDF2b+boil, Fig. 1B), neither of which show chemoattraction with DCs (via CCR6), failed to induce maturation.

CCR6 is unlikely to be the signaling receptor of mDF2β-induced DC maturation, because DCs isolated from CCR6-deficient mice (CCR6 KO) were still capable of being activated by treatment with either mDF2β or LPS, but not with control MCP-3 fusion protein (Fig. 2A). The CCR6 KO phenotype was verified by polymerase chain reaction (PCR) and by the inability of splenocytes from these mice to migrate in response to the macrophage-inflammatory protein MIP3α (*14*). In addition, a homologous antimicrobial peptide, mDF3β, which is also capable of acting as a chemoattractant for iDCs via CCR6 (*8*), failed to induce maturation of DCs (Fig. 1A).

Treatment of iDCs with mDF2β and LPS generated similar expression profiles for proinflammatory chemokines and cytokines, including RANTES, macrophage-derived chemokine (MDC), interferon-γ–inducible protein (IP-10), MIP1α and MIP/β, tumor necrosis factor–α (TNF-α), and interleukins IL-1β, and IL-12, as well as the expression of receptors, such as CCR7, which is also associated with the maturation state of DCs (Table 1). mRNA for cell-surface receptors associated with the iDCs, such as CCR2 and CCR5, mannose receptor, and macrophage scavenger receptor 2, were all down-regulated (Table 1). Furthermore, DC maturation induced by both mDF2β- and LPS was similarly inhibited by coincubation with various pharmacologic inhibitors of signal transduction molecules (*11*, *14*), which suggests that mDF2β and LPS share signal transduction pathways and possibly the same receptor, namely, Toll-like receptor 4 (TLR-4) (*17*, *18*). Consistent with this hypothesis, neither mDF2β nor LPS induced maturation of DCs isolated from either TLR-4 mutant mice or mice lacking the TLR-4 locus (C3H/HeJ and C57BL10ScNcr strains, respectively), whereas they induced maturation of DCs from control C3H/HeN mice (Fig. 2B). Finally, mDF2β, but not control antigen (sFv315), activated TLR-4 expressed by HEK 293 cells transiently transfected with murine TLR-4 and MD2 plasmids (Fig. 2C). Overall, these data strongly indicate that mDF2β is an endogenous signaling ligand for TLR-4.

Functionally, mDF2β-activated DCs exhibited T_H1-polarized responses, such as the production of proinflammatory cytokines IL-12, IL-1α, IL-1β, and IL-6 (Fig. 1D and fig. S3D). In addition, the proliferative response of splenocytes from C57BL/6 mice in a mixed-lymphocyte reaction was significant-

Table 1. mRNA expression profiles of DCs incubated with either mDF2β or LPS for 6 and 24 hours (*11*). Representative data from mRNA expression arrays of genes are shown. Numerical values (stimulation index) indicate specific mean fluorescence intensity after subtraction of background fluorescence from untreated DCs.

Gene/treatment	For 6 hours		For 24 hours	
	mDF2β	LPS	mDF2β	LPS
	Chemokine			
RANTES	1.7	3.9	8.8	9.5
MDC	2.4	1.7	2.2	2.8
IP-10	1.7	1.8	1.6	2.6
MIP-1α	3.3	2.8	1	1.2
MIP-1β	2.6	2.9	0.9	1.2
	Chemokine receptor			
CCR2	0.5	0.3	0.2	0.2
CCR5	1	0.7	0.3	0.4
CCR7	1.8	2.3	2	2.6
	Proinflammatory cytokine			
IL-1β	7.2	6.2	4.7	6.9
TNFα	3.5	2.5	1.2	1.2
IL12 (p40)	2.4	5	2.1	3.1
	Costimulatory molecule			
CD40	1.9	2.4	1.4	1.4
4-1BB	1.5	1.5	2.7	3.1
CD24a	0.4	0.3	0.2	0.1
	Surface receptors			
Mannose receptor, C type 1	0.4	0.3	0.2	0.3
Macrophage scavenger receptor	0.4	0.3	0.4	0.3
Prolactin receptor	0.3	0.4	0.8	0.8
	Other genes			
IGF binding protein 4	0.4	0.5	1.4	1.3
Serum amyloid A3	7.3	8.2	3	3.4
Superoxide dismutase 2 (Mitochondrial)	3.8	6.3	2	2
Nitric oxide synthase 2	1.4	1.6	4.7	6.2
Caspase 12	6.4	1.2	1.1	1.1
Metallothionein 1	1.4	1.4	2.4	3.4
Metallothionein II	1.5	1.4	4.4	6.2
SOCS-3	2.3	3.8	1.9	2.3
Transcription factor 2	1.6	2.7	3.4	5.8
IκB-β	2.3	2.7	1.5	1.7
VEGF-A	1.1	1.1	1.9	2.7
Thrombospondin 1	1.1	1.1	2.2	4.8
Pre-B cell colony-enhancing factor	1.8	2.5	1.5	2.2

ly increased by pretreatment of DCs from BALB/c mice with mDF2β or LPS, but not with control mproDF2β, mDF3β, or MCP3 proteins (Fig. 3A), which suggests that mDF2β augmented primary T cell immune responses by activating DCs.

Previously, we demonstrated that mDF2β-based vaccines elicited therapeutic, T-cell dependent, antitumor immunity in vivo [mDF2β, Fig. 3B and (*8*)]. However, interferon-γ–deficient (IFN-γ KO) mice immunized with these mDF2β fusion constructs failed to reject tumors (mDF2β IFN-γ KO, Fig. 3B), which suggests that IFN-α is required and provides an important association between mDF2β and type 1 immunity in vivo. Furthermore, the vaccine required that tumor antigen was physically linked with fully functional mDF2β, whereas unlinked free peptide mixture or fusion antigens with an inactive pro–β-defensin 2 did not elicit any

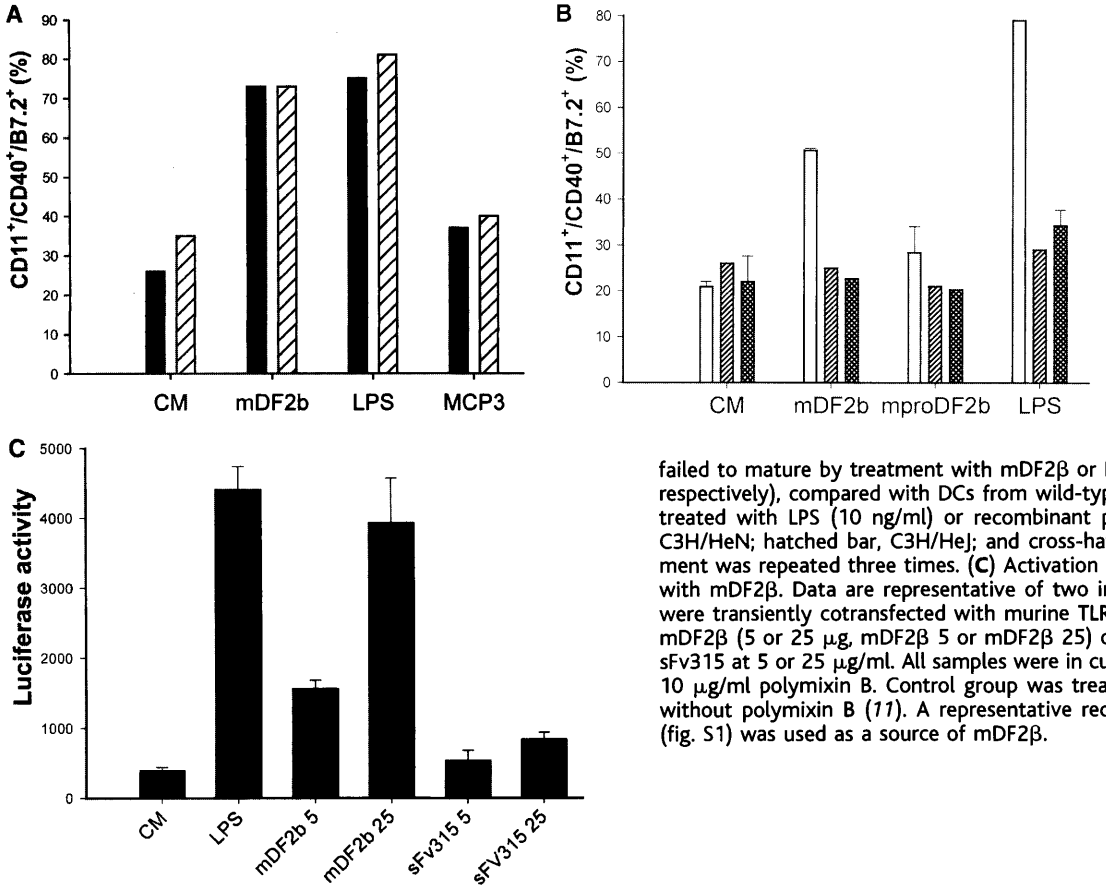

Fig. 2. Although murine β-defensin 2 is attracted to iDCs via CCR6 (*8*), TLR-4 is the receptor for DC activation. Both mDF2β and LPS, but not MCP-3 fusion protein (MCP3), induce maturation of iDCs from CCR6 KO mice (**A**). The CCR6 KO phenotype was verified by PCR analysis. Black bar, $CD11c^+$ / $B7.2^+$ / $CD40^+$; hatched bars, $CD11c^+$/ $B7.2^+$/$I\text{-}A^{high}$ (MHC class II). Data are representative of two independent experiments. (**B**) iDCs from the mice with TLR-4 mutation or TLR-4 locus deletion failed to mature by treatment with mDF2β or LPS (C3H/HeJ and C5710ScNr, respectively), compared with DCs from wild-type mice (C3H/HeN). DCs were treated with LPS (10 ng/ml) or recombinant proteins (5 μg/ml). Open bar, C3H/HeN; hatched bar, C3H/HeJ; and cross-hatched bar, C5710ScNr. Experiment was repeated three times. (**C**) Activation of the luciferase reporter gene with mDF2β. Data are representative of two independent experiments. Cells were transiently cotransfected with murine TLR-4 and MD2 and treated with mDF2β (5 or 25 μg, mDF2β 5 or mDF2β 25) or control recombinant protein sFv315 at 5 or 25 μg/ml. All samples were in culture medium (CM) containing 10 μg/ml polymixin B. Control group was treated with 10 ng/ml LPS in CM without polymixin B (*11*). A representative recombinant protein N24mDF2β (fig. S1) was used as a source of mDF2β.

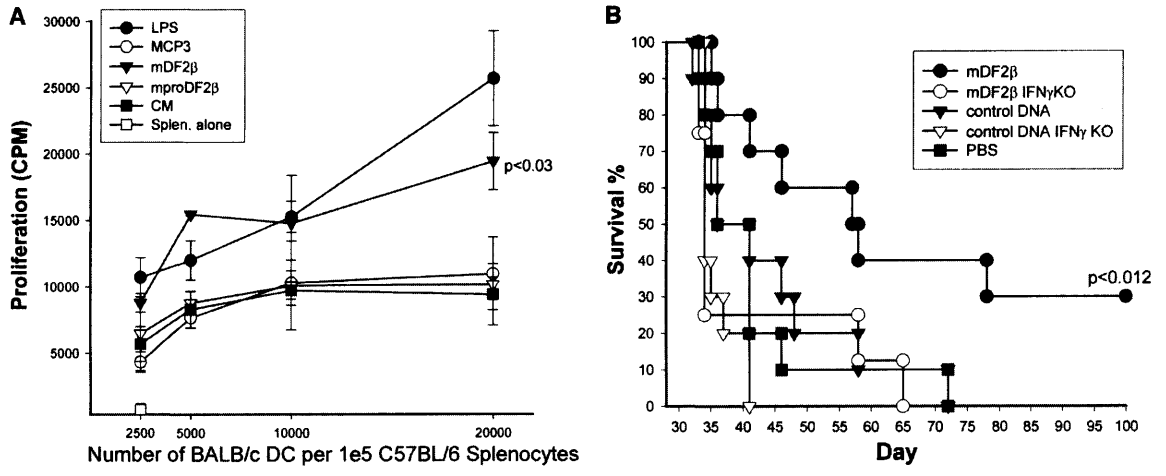

Fig. 3. DCs treated with murine β-defensin 2 elicit augmented T cell responses (**A**). $CD11c^+$ iDCs from BALB/c mice were irradiated at 3000 rad after overnight incubation with 5 μg/ml of mDF2β or 10 ng/ml LPS, and washed three times with cold PBS to remove soluble stimulants. 1e5 untreated splenocytes from C57BL6 mice were cultured alone (splen. alone) or mixed with titrated amounts of irradiated DCs. Proliferation of splenocytes was measured by uptake of $[^3H]$thymidine after four days. *P* value is comparison between mDF2β and MCP3 treated samples. Data are representative of two experiments. (**B**). The effect of mDF2β fusion to render nonimmunogenic self-tumor antigens immunogenic and elicit therapeutic antitumor immunity requires IFN-γ activity. IFN-γ gene knockout (IFN-γ KO) or w.t. BALB/c mice were inoculated intraperitoneally with 2.5×10^5 syngeneic A20 lymphoma cells on day 0. Then, on days 1, 4, 8, and 18, mice were immunized with 2 μg DNA constructs expressing sFv20, A20 tumor derived idiotype, fused with mDF2β (mDF2). Control groups were treated with PBS, or with constructs expressing an irrelevant idiotype sFv38, derived from the 38C13 lymphoma, fused to MIP3α (control DNA). Logrank *P* value is for comparison with control DNA immunization. Results shown are representative of three experiments with 10 mice per group.

antitumor immunity (*8*). Thus, linkage of tumor antigens with mDF2β enabled not only efficient APC targeting, but presumably also activated DC maturation in vivo. The importance of DC maturation in induction of adaptive immune responses has been recently suggested by a similar observation that linkage with agonistic DEC205-specific antigen facilitated efficient antigen uptake and processing by DCs, yet this construct induced tolerance unless DCs were first activated by CD40 engagement (*19*).

We report here that mDF2β, which has hitherto been considered a peptide with direct antimicrobial effects, modulates adaptive immune response not only by recruiting iDCs to the site of inflammation through chemokine receptor CCR6 (*8*, *9*) but also by activating signaling for DC maturation through a microbial pattern recognition receptor, TLR-4. Our data suggest that mDF2β could be considered a so-called endogeneous ligand of TLR-4 signaling as proposed, for example, for heat shock antigens Hsp60 and Hsp70 expressed during stress and/or necrosis (*20*, *21*). Formally, the possibility remains that mDF2β may act as a potentiator of subthreshold amounts of LPS, tightly bound to it in a complex during defensin purification (*14*). The biological relevance of our finding remains to be elucidated. It is tempting to speculate that some β-defensins may function to counter suppressive microbial factors by generating more robust host inflammatory and T_H1 responses. Furthermore, we do not know yet whether mDF2β activates other subsets of immune cells, such as mature DCs, although our preliminary data suggest that it may activate the macrophage cell line RAW267 (*14*). Finally, the natural adjuvant property of mDF2β may also be utilized for the development of more effective vaccines and immunotherapeutics, for example, by targeting and/or recruiting iDCs in vivo, and at the same time, activating them to elicit potent T cell immunity (*8*, *10*).

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22. We thank R. Gress (NCI/NIH), for critical discussion of the manuscript and helpful comments; N. Qureshi (University of Missouri) for the gift of RsDPLa; and B. Reis and C. Green (SAIC-Frederick) for proofreading. This publication has been funded in part with federal funds from the National Cancer Institute and National Institutes of Health, under contract No. NOL-CO-12400.

Supporting Online Material

www.sciencemag.org/cgi/content/full/298/5595/1025/DC1
Materials and Methods
Figs. S1 to S3

28 June 2002; accepted 30 August 2002

Cytoprotective Role of Ca^{2+}-Activated K^+ Channels in the Cardiac Inner Mitochondrial Membrane

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Ion channels on the mitochondrial inner membrane influence cell function in specific ways that can be detrimental or beneficial to cell survival. At least one type of potassium (K^+) channel, the mitochondrial adenosine triphosphate–sensitive K^+ channel (mitoK_{ATP}), is an important effector of protection against necrotic and apoptotic cell injury after ischemia. Here another channel with properties similar to the surface membrane calcium-activated K^+ channel was found on the mitochondrial inner membrane (mitoK_{Ca}) of guinea pig ventricular cells. MitoK_{Ca} significantly contributed to mitochondrial K^+ uptake of the myocyte, and an opener of mitoK_{Ca} protected hearts against infarction.

Sustained adenosine triphosphate (ATP) production by mitochondria requires maintenance of a large electrochemical gradient for protons across the mitochondrial inner membrane. This proton motive force is established by active proton pumping by the electron transport chain, producing both a pH gradient (ΔpH) and a mitochondrial transmembrane potential ($\Delta\Psi_m$). Because $\Delta\Psi_m$ can be depolarized by energy-dissipating ion flux, the mitochondrial inner membrane was earlier assumed to have a low resting permeability to cations (*1*). However, it is well established that both divalent (*2*) and monovalent cation transport pathways (uniporters) are present on the inner membrane and that K^+ conductance can be substantial in energized mitochondria (*3*, *4*).

A growing body of evidence indicates that mitochondrial ATP-sensitive K^+ channels (mitoK_{ATP}) are important determinants of resistance to ischemic damage (*5*, *6*) and apoptosis (*7*) and may be clinically recruitable to prevent or mitigate cardiac or neuronal ischemic injury (*8*). To determine whether other K^+ channel subtypes are also present on the cardiac mitochondrial inner membrane, here we use direct single channel patch-clamp recordings of cardiac mitoplasts and mitochondrial K^+ flux measurements to identify mitochondrial Ca^{2+}-activated K^+ channels (mitoK_{Ca}) as a component of the mitochondrial background K^+ conductance, and we test whether mitoK_{Ca} confers protection against infarction in the intact heart.

Mitoplasts prepared from isolated cardiac myocytes were patch-clamped (*9*) to identify the major single channel conductances of the inner membrane. In K^+ solutions (150 mM K^+) containing 512 nM Ca^{2+}, single channel currents with a full unitary conductance of 307 ± 4.6 pS (n = 4 of 17 single channel patches) were often observed, with openings frequently interrupted by transitions to subconductance states ranging from 24 to 161 pS (Fig. 1, A to C). When pipettes were backfilled with the K^+ channel toxin charybdotoxin (ChTx; 200 nM) to permit slow diffusion of the toxin into the pipette tip, channel activity disappeared within 30 min, indicative of the probable presence of K_{Ca} channels (Fig. 1D). In some patches in 512 nM Ca^{2+}, and particularly at higher bath Ca^{2+} concentrations, channel activity was too great to identify individual channel openings; in these cases, ensemble average patch currents were analyzed and shown to be reversibly increased by raising Ca^{2+} in the medium (Fig.

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