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A Strain of *Lactobacillus casei* Inhibits the Effector Phase of Immune Inflammation

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Some nonpathogenic bacteria were found to have protective effects in mouse models of allergic and autoimmune diseases. These “probiotics” are thought to interact with dendritic cells during Ag presentation, at the initiation of adaptive immune responses. Many other myeloid cells are the effector cells of immune responses. They are responsible for inflammation that accounts for symptoms in allergic and autoimmune diseases. We investigated in this study whether probiotics might affect allergic and autoimmune inflammation by acting at the effector phase of adaptive immune responses. The effects of one strain of *Lactobacillus casei* were investigated in vivo on IgE-induced passive systemic anaphylaxis and IgG-induced passive arthritis, two murine models of acute allergic and autoimmune inflammation, respectively, which bypass the induction phase of immune responses, in vitro on IgE- and IgG-induced mouse mast cell activation and ex vivo on IgE-dependent human basophil activation. *L. casei* protected from anaphylaxis and arthritis, and inhibited mouse mast cell and human basophil activation. Inhibition required contact between mast cells and bacteria, was reversible, and selectively affected the Lyn/Syk/linker for activation of T cells pathway induced on engagement of IgE receptors, leading to decreased MAPK activation, Ca²⁺ mobilization, degranulation, and cytokine secretion. Also, adoptive anaphylaxis induced on Ag challenge in mice injected with IgE-sensitized mast cells was abrogated in mice injected with IgE-sensitized mast cells exposed to bacteria. These results demonstrate that probiotics can influence the effector phase of adaptive immunity in allergic and autoimmune diseases. They might, therefore, prevent inflammation in patients who have already synthesized specific IgE or autoantibodies. *The Journal of Immunology*, 2011, 187: 2646–2655.

Inflammation is a physiological defense response. It associates destruction and repair mechanisms that are instrumental in wound healing and protection against infection. It involves the many myeloid cells that contribute to the effector phase of immune responses. The innate and adaptive immune systems indeed share cellular effectors. These can be activated by pathogens via TLR in innate immunity or by molecular effectors of adaptive immunity via cytokine, chemokine, and Fc receptors. Myeloid cells are also involved in the induction phase of adaptive immunity, for example, when dendritic cells present Ag to naive T cells. Normally, inflammation is tightly controlled and remains unapparent. It can be pathogenic if it escapes from regulation. Autoimmunity and allergy

are examples of pathological inflammation generated by adaptive immune responses. Both involve mast cells.

Mast cells express high-affinity IgE receptors (FcεRI) that have long been known to trigger allergic inflammation (1). They also express low-affinity IgG receptors (FcγR) (2) that have been recently recognized as being critical for Ab-dependent autoimmune inflammation (3). Both types of receptors activate mast cells when engaged by Ab and Ag. Activated mast cells release and secrete numerous proinflammatory molecules, which altogether increase vascular permeability, and attract and activate numerous cells, which generate inflammatory infiltrates. Expectedly, IgE-induced passive systemic anaphylaxis (IgE-PSA) was abrogated in mast cell-deficient mice (4). Unexpectedly, IgG-induced passive arthritis (induced by injecting serum of F1 hybrid mice obtained by crossing KRN transgenic [K] C57BL/6 mice [B] with NOD mice [N] into C57BL/6 mice) (K/BxN arthritis) (5), experimental allergic encephalomyelitis (6), bullous pemphigoid-like disease (7), and experimental glomerulonephritis (8) were also abrogated in mice lacking mast cells. K/BxN-arthritis and encephalomyelitis were restored by reconstituting mast cell-deficient mice with wild-type (wt) mast cells, but not with mast cells lacking activating FcR (9, 10). In humans, mast cells have been implicated in rheumatoid arthritis, Sjögren’s syndrome, multiple sclerosis, and thyroiditis (11). Besides FcR, mast cells express TLR (12, 13). They can be activated by pathogens and protect from bacterial infections (14–17). Mast cells are, therefore, effector cells of both adaptive and innate immunity.

Microorganisms are well-known to be controlled by the immune system. They are also increasingly understood to control the immune system. Intestinal bacteria control the formation and maturation of tertiary lymphoid tissues involved in adaptive immunity, which, in turn, regulates the commensal flora (18). Microbial products enable dendritic cells to present Ag (19), and they orient

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The online version of this article contains supplemental material.

Abbreviations used in this article: Akt, Rac-α serine/threonine-protein kinase; BMDC, bone marrow-derived mast cells; Gab2, Grb2-associated binding protein 2; GPI, glucose-6-phosphate isomerase; IgE-PSA, IgE-induced passive systemic anaphylaxis; K/BxN, F1 hybrid mice obtained by crossing C57BL/6 mice (B) expressing the KRN transgene (K) with NOD mice (N); LAT, linker for activation of T cells; MAR, mouse anti-rat Ig Abs; Nod1/2, nucleotide-binding oligomerization domain-containing proteins 1/2; PCMC, peritoneal cell-derived mast cells; PLCγ, phospholipase Cγ; PSA, passive systemic anaphylaxis; RAHE, rabbit anti-human IgE Abs; TRIF, TIR domain-containing adapter inducing IFN-β; wt, wild-type.

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T cell differentiation into Th1, Th2, Th17, and/or regulatory T cells of the various types. Some nonpathogenic bacteria referred to as probiotics were found to protect against allergic and/or autoimmune diseases in both humans and mice. Several strains of lactobacilli indeed reduced lung (20, 21) or skin (22, 23) allergic inflammation and experimental autoimmune arthritis (24) when administered orally. Similar effects were observed when lactobacilli were administered intranasally (25–27), s.c. (28), or i.p. (29). Cell wall molecules from *Lactobacillus casei* inhibited the secretion of IP-10 by an intestinal epithelial cell line induced by TNF- α (30). The protective effects of probiotics were proposed to result from an altered Ag presentation by dendritic cells, leading to decreased IgE responses (31), a skewed Th1/Th2 polarization (24), or the induction of regulatory T cells (21, 32). Because myeloid cells are also the effectors of adaptive immune responses, we wondered whether probiotics could affect the effector phase of adaptive inflammation in allergy and autoimmunity in vivo and mast cell activation in vitro.

We found that one strain of *L. casei* could: 1) dampen IgE-PSA and K/BxN-arthritis, two mast cell-dependent in vivo models of inflammation that bypass the induction phase of the immune response; and 2) inhibit IgE- and IgG-induced mast cell activation in vitro. The in vitro effects of *L. casei* on mast cells could translate into an inhibition of adoptive anaphylaxis in vivo. *L. casei* could also reversibly inhibit IgE-dependent human basophil activation. These findings may have both fundamental and clinical implications.

Materials and Methods

Mast cells

Bone marrow-derived mast cells (BMMC) were obtained by culturing femoral bone marrow from C57BL/6 (Charles River, L'Arbresle, France), MyD88^{-/-} (33), TIR domain-containing adapter inducing IFN- β -deficient (TRIF^{-/-}) (34), MyD88/TRIF^{-/-}, nucleotide-binding oligomerization domain-containing protein 1 (Nod1)^{-/-} (35), Nod2^{-/-} (36), Nod1/Nod2, TLR2/4^{-/-} (37), TLR3^{-/-} (38), and corresponding littermate control mice in IL-3-containing medium as described previously (39). Peritoneal cell-derived mast cells (PCMC) were obtained by culturing peritoneal cells from C57BL/6 mice in stem cell factor-containing medium as described previously (39).

Blood samples

Blood from normal donors was obtained from the Centre Necker-Cabanel of the Etablissement Français du Sang (Paris, France).

Bacteria

L. casei (strain DN-114 001/CNCMI-1518) and *Streptococcus thermophilus* (strain DN-001 236/CNCM I-2773) were from the Danone library. They were cultured at 37°C in Mann–Rogosa–Sharpe or Elliker broth (Becton Dickinson, Mountain View, CA), respectively, then washed and resuspended in PBS before being added to cells or injected into mice. Unless otherwise specified, mast cells were incubated overnight at 37°C with live bacteria (100/cell) in culture medium containing IL-3 and washed before they were sensitized with IgE. A total of 5×10^7 live *L. casei* was injected i.p. into mice at the indicated days.

Mast cell stimulation

By IgE and Ag: BMMC or PCMC were sensitized with 1 μ g/ml IgE anti-DNP 2682-I (40) for 1 h and challenged with DNP-BSA. By immune complexes: PCMC were challenged with preformed immune complexes made with 100 μ g/ml glucose-6-phosphate isomerase (GPI; Sigma, St. Louis, MO) and the indicated dilutions of K/BxN serum. β -Hexosaminidase was quantitated in 20-min cell supernatants using an enzymatic assay (39). TNF- α was titrated in 3-h supernatants by a cytotoxicity assay on L929 cells (41). Twenty ILs, chemokines, or growth factors were also measured in 3-h supernatants using the mouse cytokine twenty-plex kit (Biosource, Nivelles, Belgium) and a Luminex 100IS System (Luminex, Austin, TX).

Human basophil activation

PBMC from normal donors were sensitized overnight with 3 μ g/ml rat IgE IR162 (IMEX; Université de Louvain, Bruxelles, Belgium) or not sensitized. Nonsensitized cells were challenged for 20 min at 37°C with F(ab')₂ fragments prepared by pepsine digestion of rabbit anti-human IgE Abs [RAHE F(ab')₂; Dako-Cytomation, Trappes, France]. Cells sensitized with rat IgE were challenged for 20 min at 37°C with F(ab')₂ fragments of mouse anti-rat Ig Abs [MAR F(ab')₂; Jackson ImmunoResearch, West Grove, PA]. Stimulation was stopped by placing cells on ice. Cells were stained with PE-conjugated anti-CD203c Abs (Immunotech, Marseille, France) and allophycocyanin-conjugated anti-Fc ϵ RI α Abs (eBioscience, San Diego, CA) or with matched isotype controls. Fluorescence was analyzed by flow cytometry with a FACSCalibur (Becton Dickinson). Basophils were identified as Fc ϵ RI⁺ CD203c⁺ cells. Basophil activation was monitored by the increased expression of CD203c.

Western blot analysis

BMMC sensitized with 1 μ g/ml IgE anti-DNP were challenged with 10 ng/ml DNP-BSA for 0, 3, 10, or 30 min and lysed in SDS at 95°C. Lysates were electrophoresed and Western blotted with indicated Abs. Rabbit anti-Rac- α serine/threonine-protein kinase (anti-Akt), phospho-Akt, phospho-Syk, phosphorylated Grb2-associated binding protein 2 (phospho-Gab2), NF- κ B, phospho-NF- κ B, p38, phospho-p38, Erk1/2, phospho-Erk1/2, JNK, and phospho-JNK were from Cell Signaling Technology (Beverly, MA); rabbit anti-linker for activation of T cells (anti-LAT), phospho-LAT, and Gab2 were from Upstate Biotechnology (Lake Placid, NY); and mouse anti-phospholipase C γ -1 (anti-PLC γ -1) and phospho-PLC γ -1, rabbit anti-Syk, and HRP-labeled goat anti-rabbit or mouse IgG were from Santa Cruz Biotechnology (Santa Cruz, CA). HRP was detected using an ECL kit (Amersham Biosciences, Buckinghamshire, U.K.).

Calcium mobilization

BMMC sensitized with 1 μ g/ml IgE anti-DNP were loaded with 0.5 μ M fluo-3-acetoxymethyl ester (Invitrogen, Carlsbad, CA) for 1 h at room temperature and analyzed by flow cytometry (Becton Dickinson) before and after stimulation with 10 ng/ml DNP-BSA.

PSA

Six- to 8-wk-old C57BL/6J mice (Charles River) were injected i.v. with 200 μ g IgE anti-OVA (mAb 2C6) (42) at day -1 and 1 mg OVA at day 0, or with 50 μ g IgE anti-DNP (mAb SPE-7; Sigma) at day -1 and 500 μ g DNP₁₅-BSA at day 0. Rectal temperature was measured using a Precision Digital Thermometer 4600 (YSI, Dayton, OH).

K/BxN arthritis

K/BxN mice were generated by crossing KRN transgenic mice on the C57BL/6 background (a gift from Drs. D. Mathis and C. Benoist, Harvard Medical School, Boston, MA, and Institut de Génétique et de Biologie Moléculaire et Cellulaire, Strasbourg, France) with NOD mice (Charles River). Six- to 8-wk-old C57BL/6J mice (Charles River) were injected i.v. with 50 μ l K/BxN serum at day 0. Arthritis was scored as described previously (43). Ankle joints were fixed, decalcified in Formal-4 (Decal Corporation, Tallman, NY) for 7 d, embedded in paraffin, and stained with hematoxylin/eosin.

Guidelines for animal experiments

All mouse protocols were approved by the Animal Care and Use Committees of Paris, Ile de France, France.

Statistical analyses

Data were analyzed for statistical significance using Student *t* test. The *p* values >0.05 were not considered statistically significant.

Results

L. casei inhibits IgE-PSA and mast cell activation

To investigate whether probiotics can affect the effector phase of immune inflammation, we first studied the effects of one strain of *L. casei* that was previously found to exert various anti-inflammatory effects (44) on IgE-PSA. IgE-PSA was induced by injecting i.v. C57BL/6 mice with IgE Abs at day -1 and specific Ag at day 0. The systemic shock induced by Ag challenge was monitored by assessing the body temperature decline that developed over the

following 30 min. Mice injected i.p. with *L. casei* at days -2 and -1 displayed significantly reduced shocks compared with mice injected i.p. with PBS at the same days. *L. casei* reduced IgE-PSA induced by OVA in mice sensitized with IgE anti-OVA (Fig. 1A) or by DNP-BSA in mice sensitized with IgE anti-DNP (Fig. 1B).

IgE-PSA was demonstrated to depend on the engagement of FcεRI on mast cells, leading to the rapid release of vasoactive and proinflammatory mediators. We therefore investigated whether *L. casei* could affect mast cell activation in vitro. When sensitized with IgE anti-DNP Abs and challenged with DNP-BSA, BMMC

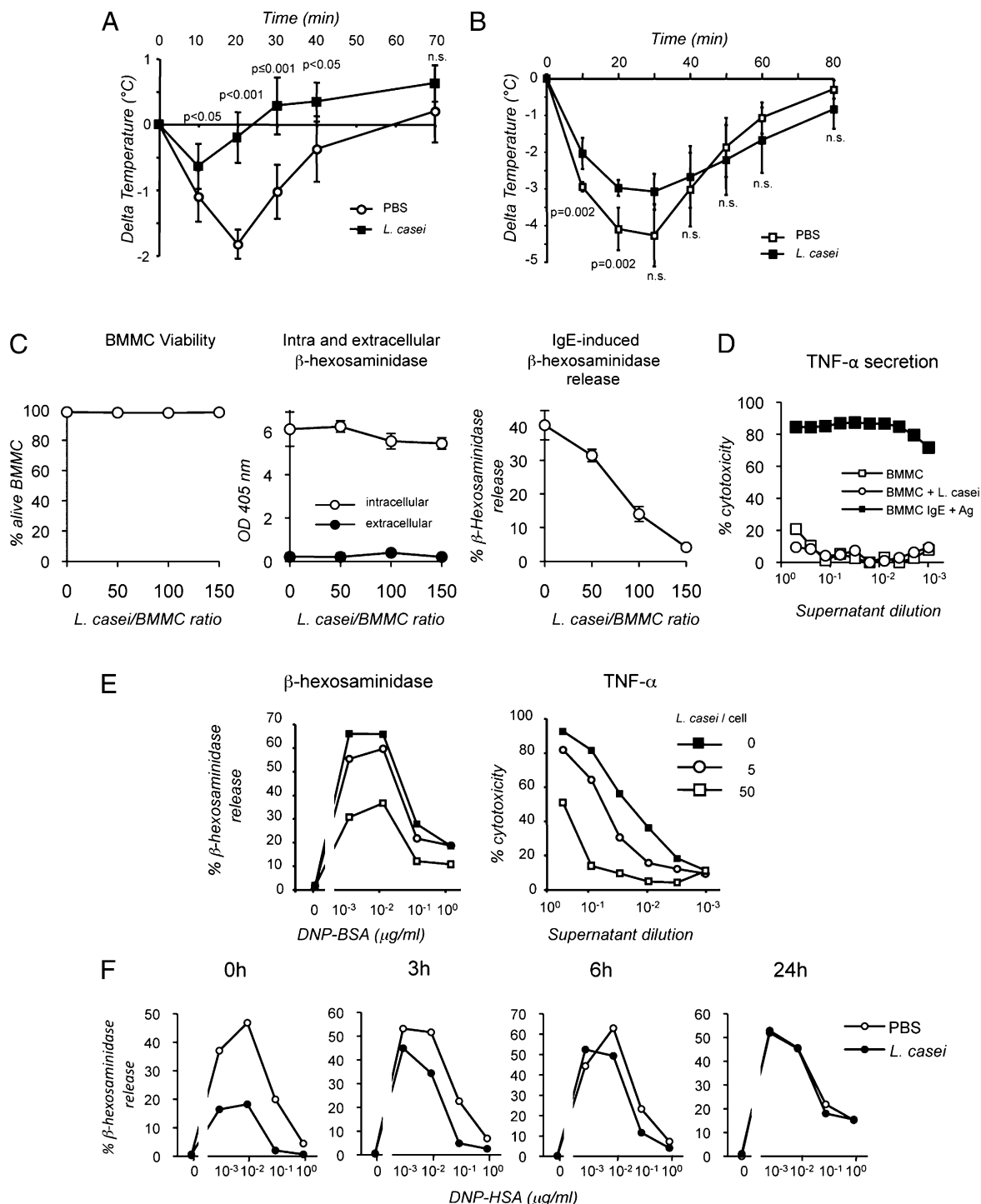


FIGURE 1. *L. casei* inhibits IgE-PSA and IgE-dependent mast cell activation. A and B, C57BL/6 mice, injected i.p. with PBS or *L. casei* at days -2 and -1, were injected i.v. with IgE anti-OVA at day -1 and OVA at day 0 (A), or with IgE anti-DNP at day -1 and DNP-BSA at day 0 (B). Body temperature was recorded as a function of time. Data are the means \pm SEM of 5 mice/group. C, BMMC were incubated for 16 h with different concentrations of *L. casei*. Viability of BMMC was assessed with Topro 3. Extracellular and intracellular β -hexosaminidase were measured. BMMC were then sensitized with IgE, challenged with Ag for 20 min, and β -hexosaminidase was measured in supernatants. D, BMMC were incubated for 16 h with *L. casei* or PBS, or they were sensitized with IgE and challenged with Ag for 3 h. TNF- α was measured in supernatants. E, BMMC incubated overnight with PBS or indicated numbers of *L. casei* and sensitized with IgE anti-DNP were challenged with DNP-BSA. β -Hexosaminidase and TNF- α were measured in supernatants, 20 min and 3 h later, respectively. F, BMMC incubated overnight with PBS or *L. casei* were washed, incubated for the indicated periods without bacteria in fresh medium, sensitized with IgE, and challenged with Ag. β -Hexosaminidase was measured in supernatants 20 min later. Experiments shown are representative of two to five similar experiments.

released granular mediators such as β -hexosaminidase and secreted cytokines such as TNF- α (Supplemental Fig. 1A). *L. casei* induced no detectable β -hexosaminidase release (Supplemental Fig. 1A) and no detectable secretion of cytokine (including TNF- α), chemokine, or growth factor (Supplemental Fig. 1A, 1B) when incubated with BMMC for 20 min or 3 h, respectively, that is, when corresponding IgE-induced responses are maximal. An overnight incubation of BMMC with *L. casei* affected neither mast cell viability nor the intracellular content of β -hexosaminidase (Fig. 1C). No β -hexosaminidase release (Fig. 1C) and no TNF- α secretion (Fig. 1D) over background was detected in supernatants of mast cells incubated overnight with *L. casei*.

An overnight incubation of BMMC with *L. casei* before sensitization with IgE Abs, however, dose-dependently inhibited Ag-induced β -hexosaminidase release (Fig. 1C, 1E) and TNF- α secretion (Fig. 1E). Inhibition of β -hexosaminidase release observed when mast cells were sensitized with IgE and challenged with Ag immediately after the overnight incubation with *L. casei* decreased with time if cells were incubated without bacteria for increasing periods before they were sensitized and challenged. β -Hexosaminidase release was fully restored after 24 h (Fig. 1F). *L. casei*, therefore, both decreased the intensity of IgE-PSA in vivo and reversibly inhibited IgE-induced mast cell secretory responses in vitro.

L. casei inhibits IgG-induced passive arthritis and mast cell activation

We next investigated the effect of the same strain of *L. casei* on IgG-dependent passive arthritis (K/BxN-arthritis). K/BxN-arthritis was induced by injecting C57BL/6 mice with K/BxN serum i.v. at day 0. Like serum from rheumatoid arthritis patients (45), K/BxN serum contains anti-GPI autoantibodies. Arthritis was monitored by assessing joint inflammation that developed over the following week. Joint inflammation was markedly reduced in mice injected i.p. with *L. casei* daily between days -2 and +4, compared with mice injected i.p. with PBS at the same days (Fig. 2A), and abrogated in mice injected daily with *L. casei* between days -9 and +2 (Supplemental Fig. 2). Synovitis and neutrophil infiltration observed 6 d after K/BxN serum injection in PBS-treated mice (46) were also abrogated in these mice (Supplemental Fig. 2). Noticeably, *L. casei* also inhibited inflammation when administered 2 d after K/BxN serum (Fig. 2B).

K/BxN arthritis was reported to involve the engagement of mast cell Fc γ RIIIA by GPI-IgG anti-GPI immune complexes. We

therefore investigated whether *L. casei* would affect IgG-induced mast cell activation. Unlike BMMC, which can be activated by IgE Abs but not or very poorly by IgG Abs, but like peritoneal mast cells, PCMC degranulate not only in response to IgE and Ag, but also in response to IgG immune complexes, and degranulation depends on Fc γ RIIIA (39). IgG-induced β -hexosaminidase release was inhibited at least as efficiently as IgE-induced β -hexosaminidase release, when PCMC were incubated overnight with *L. casei* before challenge with preformed immune complexes made with GPI and K/BxN serum or before sensitization with IgE, respectively (Fig. 2C).

L. casei, therefore, markedly inhibited inflammation in an IgG-induced passive model of autoimmune arthritis in vivo. It could also inhibit in vitro mast cell activation induced by the same IgG Abs that induced K/BxN arthritis.

L. casei, but not *S. thermophilus*, inhibits mast cell activation

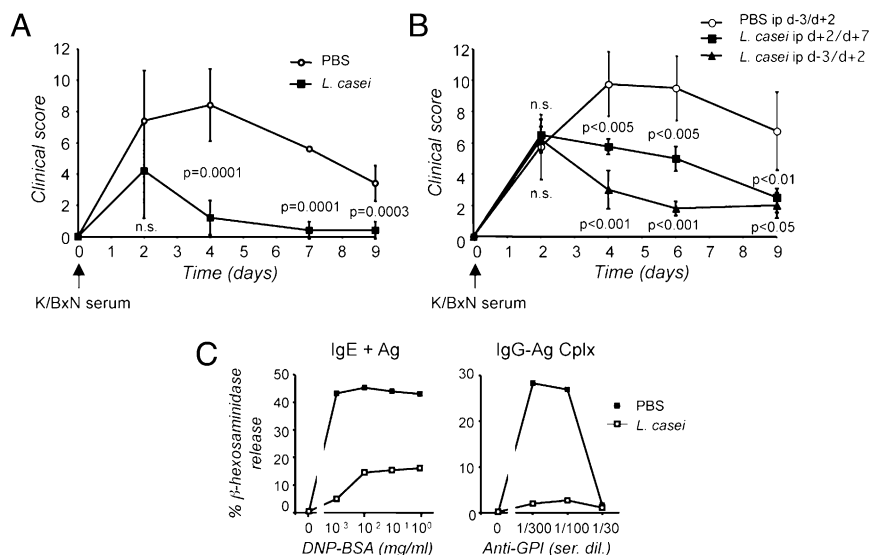
To investigate whether other lactic bacteria would have similar inhibitory properties as *L. casei*, we examined a strain of *S. thermophilus* (DN-001 236). We found that K/BxN serum-induced arthritis, which was markedly reduced by *L. casei*, was not affected by the same number of *S. thermophilus* when administered i.p. (Fig. 3A).

Likewise, *L. casei* inhibited Ag-induced β -hexosaminidase release by IgE-sensitized BMMC, when incubated overnight with mast cells, but not *S. thermophilus* (Fig. 3B). When challenged for 3 h with Ag, BMMC sensitized with IgE-secreted TNF- α , but also IL-5, IL-6, IL-13, MCP-1, and MIP-1 α as assessed by Luminex but not IL-1 α , IL-1 β , IL-2, IL-4, IL-10, IL-12, IL-17, IP-10, keratinocyte-derived chemokine, monokine induced by γ IFN, vascular endothelial growth factor, fibroblast growth factor, GM-CSF, or IFN- γ (data not shown). The secretion of all Ag-induced cytokines and chemokines was inhibited when BMMC were incubated overnight with *L. casei*, but not with *S. thermophilus*, before sensitization with IgE Abs (Fig. 3C). *L. casei*, therefore, has inhibitory properties on mast cell activation that other lactic bacteria such as *S. thermophilus* do not have.

Inhibition requires contact between mast cells and *L. casei*, but it requires neither TLR nor Nod1/2

L. casei secretes lactic acid and other metabolites that could possibly account for inhibition. An overnight incubation of BMMC with concentrations of lactic acid as high as 2 mg/ml, however, did not affect IgE-induced responses (data not shown). To confirm

FIGURE 2. *L. casei* inhibits K/BxN-arthritis and IgG-dependent mast cell activation. **A**, C57BL/6 mice, injected i.p. daily with PBS or *L. casei* from day -2 (d-2) to day +4 (d+4), were injected i.v. with K/BxN serum at day 0. Clinical score was recorded as a function of time. Data are the means \pm SEM of 5 mice/group. **B**, C57BL/6 mice, injected daily with PBS or *L. casei* i.p. either from day -3 (d-3) to day +2 (d+2) or from d+2 to day +7 (d+7), were injected with K/BxN serum i.v. at day 0. Data are the means \pm SEM of 5 mice/group. **C**, PCMC incubated overnight with PBS or *L. casei* were sensitized with IgE and challenged with Ag, or not sensitized and challenged with preformed IgG immune complexes. Experiments shown were repeated twice.



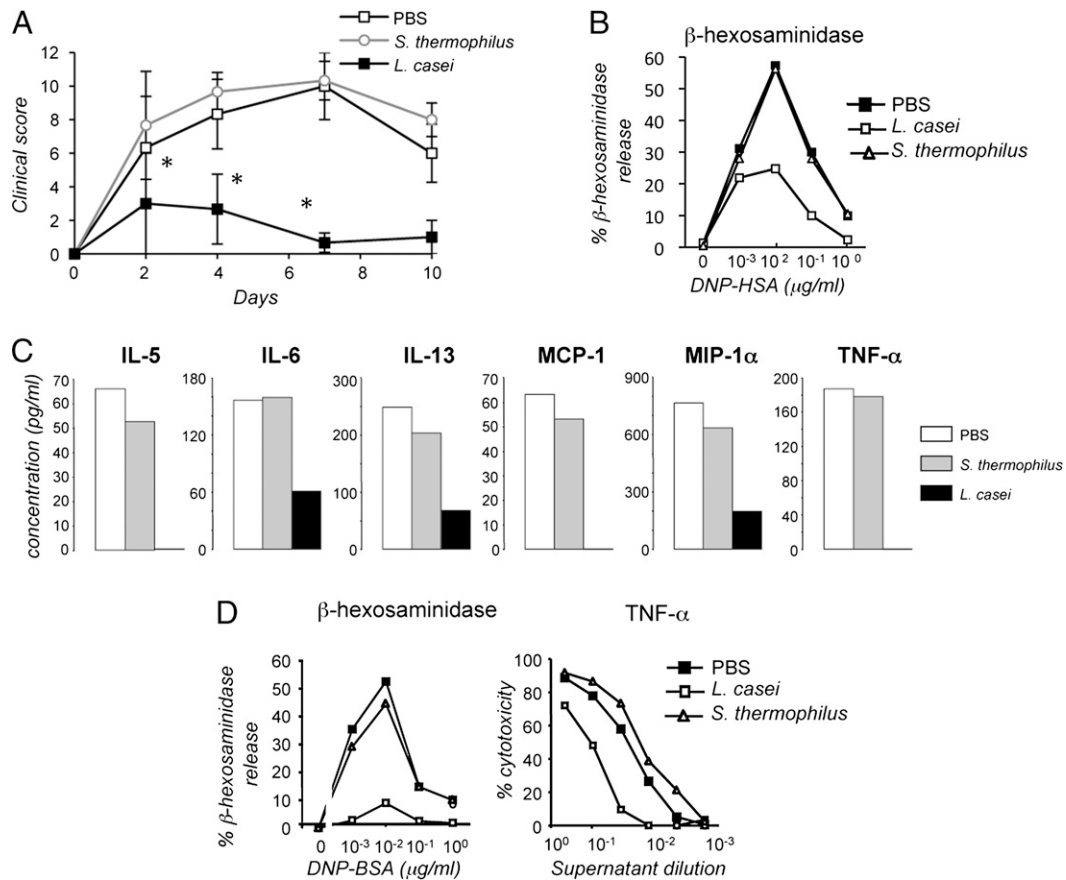


FIGURE 3. *L. casei*, but not *S. thermophilus*, inhibits inflammatory responses in vivo and in vitro. **A**, C57BL/6 mice, injected i.p. daily with PBS, *L. casei*, or *S. thermophilus* from day -3 (d-3) to day +2 (d+2), were injected i.v. with K/BxN serum at day 0. Clinical score was recorded as a function of time. Results are representative of two experiments. Data are the means \pm SEM of 5 mice/group. * $p < 0.05$ (PBS versus *L. casei*). **B**, BMMC incubated overnight with PBS, *L. casei*, or *S. thermophilus* and sensitized with IgE were challenged with Ag. β -Hexosaminidase was measured in supernatants 20 min later. Results are representative of two experiments. **C**, BMMC incubated overnight with PBS, *L. casei*, or *S. thermophilus* and sensitized with IgE were challenged with Ag. Cytokines were measured using Luminex, 3 h later. **D**, BMMC incubated overnight with PBS, irradiated *L. casei*, or irradiated *S. thermophilus* were sensitized with IgE and challenged with Ag. β -Hexosaminidase and TNF- α were measured in supernatants, 20 min and 3 h later, respectively. Results are representative of more than five experiments.

this observation, we examined irradiated bacteria that do not secrete metabolites. Although it did not markedly alter the morphology of *L. casei* when examined under the microscope (Supplemental Fig. 3A), irradiation abrogated the ability of bacteria to form colonies (Supplemental Fig. 3B), and it rendered them permeable to the vital dye Topro 3 (Supplemental Fig. 3C). Irradiated *L. casei*, however, but not irradiated *S. thermophilus*, inhibited IgE-induced β -hexosaminidase release and TNF- α secretion as live *L. casei* (Fig. 3D). Higher numbers of irradiated bacteria than of live bacteria were required for inhibition (Supplemental Fig. 3D).

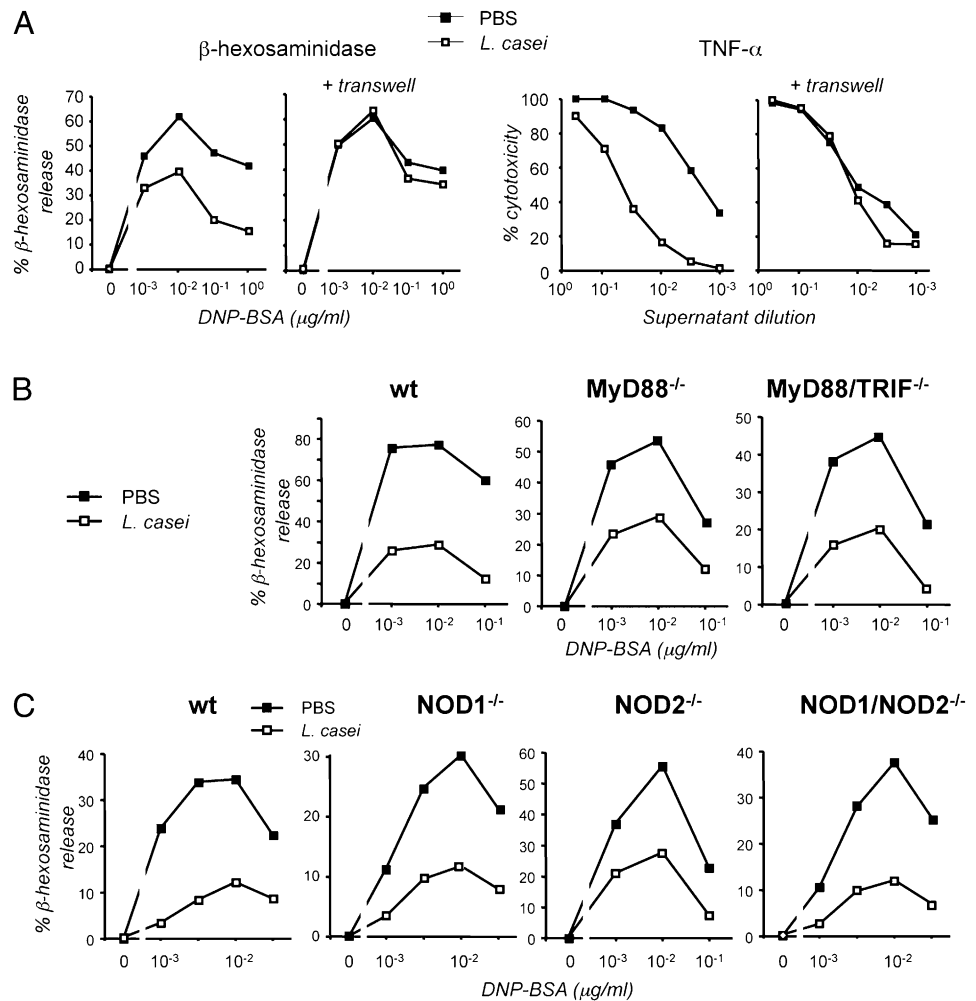
Inhibition was abrogated when BMMC were separated from live *L. casei* by a porous membrane (pore size, 0.4 μ m) during incubation, confirming this observation (Fig. 4A). *L. casei*, however, was not detectably phagocytosed by BMMC as assessed by a gentamicin-protection assay (Supplemental Fig. 3E).

To investigate mast cell receptor(s) possibly involved in inhibition, we generated BMMC from knockout mice lacking receptors or signaling molecules involved in the recognition of microbial products and from corresponding wt controls, and we examined the responses of these cells to IgE and Ag, after an incubation with *L. casei* or without. *L. casei*-induced inhibition was unaffected by the deletion of either MyD88 or MyD88+TRIF (Fig. 4B). All known TLR use MyD88 and/or TRIF to transduce signals. Inhibition was also unaffected by the deletion of Nod1,

Nod2, or Nod1+Nod2 (Fig. 4C). Neither TLR nor Nod1/2 are, therefore, mandatory for inhibition. *L. casei*-induced inhibition of mast cell activation therefore requires a contact of bacteria with an unknown receptor on mast cells but not internalization.

Inhibition of mast cell activation by L. casei does not result from altered Fc ϵ RI expression

E. coli was reported to decrease Fc ϵ RI expression (3). We did observe also some reduction of Fc ϵ RI expression (not shown) on BMMC exposed to *L. casei* overnight, which, in some experiments, resulted in a significant decreased IgE sensitization (Fig. 5A). To investigate whether this effect could account for *L. casei*-induced inhibition, BMMC were sensitized with 0.2 μ g/ml IgE, a concentration that yielded a similar binding as when cells were sensitized with 1 μ g/ml IgE after exposure to *L. casei* (Fig. 5A). As expected, cells sensitized with 0.2 μ g/ml IgE concentration released similar amounts of β -hexosaminidase on challenge as cells sensitized with 1 μ g/ml IgE, but they required a higher concentration of Ag. No such shift in optimal Ag concentration was observed when cells were exposed to *L. casei* and sensitized with 1 μ g/ml IgE. Instead, β -hexosaminidase release was decreased at every Ag concentration used for challenge (Fig. 5A). A decreased Fc ϵ RI expression and/or sensitization therefore does not account for inhibition of IgE-induced mast cell activation by *L. casei*.



L. casei selectively inhibits the Lyn/Syk/LAT pathway in Fc ϵ RI signaling

BMMC were incubated overnight with bacteria, sensitized with IgE, challenged with Ag for various periods of time, lysed, and Western blotted with Abs anti-phosphorylated and anti-nonphosphorylated signaling molecules, to investigate molecular mechanisms by which *L. casei* inhibits mast cell activation. Fc ϵ RI aggregation activates two Src kinase-dependent signaling pathways (47) (Supplemental Fig. 4A). *L. casei* inhibited both proximal and distal signals in the Lyn-initiated pathway. The phosphorylation of Syk, LAT, and PLC γ -1 was reduced, and that of the three MAPKs Erk1/2, JNK, and p38 was more transient. NF- κ B phosphorylation was, however, not detectably affected. By contrast, neither proximal (Gab2 phosphorylation) nor distal (Akt phosphorylation) events were impaired in the Fyn-initiated pathway (Fig. 5B). Ultimately, IgE-induced Ca²⁺ mobilization was reduced in BMMC exposed to *L. casei* (Fig. 5C). *L. casei*, therefore, inhibits Fc ϵ RI signaling by affecting selectively the Lyn/Syk/LAT pathway.

L. casei-induced inhibition of mast cell activation can prevent systemic anaphylaxis

Because *L. casei* could inhibit mast cell-dependent IgE-PSA in vivo and IgE-induced mast cell activation in vitro, we investigated whether inhibition of the latter could be seen in vivo. To this aim, we devised a model of adoptive anaphylaxis in which mice were injected i.v. with BMMC previously sensitized in vitro with IgE. When challenged by an i.v. injection of Ag 15 min later, all mice underwent a severe shock. An i.v. injection of an equal

number of BMMC that had been incubated overnight with *L. casei* before IgE sensitization, however, induced no shock on Ag challenge (Fig. 6A). Injected cells had the same viability, whether incubated with *L. casei* or not (Fig. 6B), and BMMC incubated with *L. casei* released less β -hexosaminidase than BMMC incubated with PBS, when challenged with Ag in vitro (Fig. 6C).

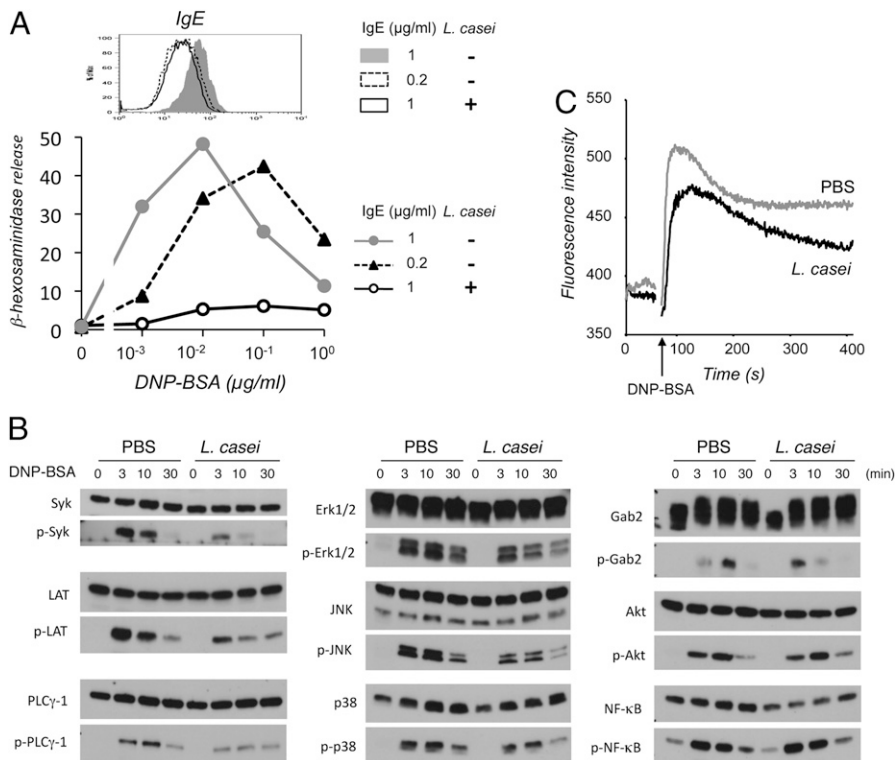
L. casei inhibits IgE-dependent activation of human basophils

Finally, we investigated whether *L. casei* could affect IgE-dependent activation of human basophils. Basophils were identified in PBMC from normal donors as CD203c⁺, Fc ϵ RI⁺ cells (48). Because they carry constitutively Fc ϵ RI-bound human IgE (not shown), normal basophils could be activated by F(ab')₂ fragments of RAHE Abs. Basophil activation was monitored by the upregulation of CD203c as described previously (49). CD203c upregulation induced by RAHE F(ab')₂ was dose-dependently inhibited if PBMC were incubated overnight with *L. casei* (Fig. 7A).

Because only a fraction of Fc ϵ RI are occupied by human IgE, human basophils from normal donors could be passively sensitized with rat IgE and challenged with F(ab')₂ fragments of MAR. When added to PBMC during sensitization with rat IgE, *L. casei* dose-dependently inhibited MAR F(ab')₂-induced basophil activation (Fig. 7B).

Like IgE-dependent mouse mast cell activation, IgE-dependent human basophil activation was inhibited by *L. casei*, but not by *S. thermophilus* (Fig. 7C). Because bacteria were added to human PBMC, and not to purified cells, we wondered whether the effect of *L. casei* on basophils could be indirect, that is, through the

FIGURE 5. *L. casei* inhibits IgE-induced intracellular signaling. **A**, BMMC were incubated overnight with PBS or *L. casei* and sensitized with 0.2 or 1 μg/ml IgE. Cells were stained with FITC-labeled anti-IgE and analyzed by flow cytometry (*top panel*) or challenged with Ag, and β-hexosaminidase was measured in supernatants 20 min later (*bottom panel*). Results are representative of two experiments. **B**, BMMC were incubated overnight with PBS or *L. casei*, sensitized with IgE, and challenged with Ag for the indicated periods. Cell lysates were electrophoresed and Western blotted with the indicated Abs. Results are representative of two experiments. **C**, BMMC incubated overnight with PBS or *L. casei* were sensitized with IgE, loaded with fluo-3-acetoxymethyl ester, and analyzed by flow cytometry before and after stimulation with Ag. Results are representative of two experiments.



release of a soluble product by cells other than basophils, in response to bacteria. To test this possibility, we incubated PBMC either with PBS or with bacteria. Overnight supernatants were harvested, added to PBMC incubated with PBS, and cells were challenged with RAHE F(ab)₂ 1 h later. Inhibition was induced by none of the supernatants (Fig. 7D). Like for mouse mast cells, inhibition is, therefore, likely to be the consequence of a direct interaction between *L. casei* and human blood cells.

L. casei-induced inhibition of basophil activation slowly decreased with time if challenge with RAHE F(ab)₂ was delayed

after the overnight incubation of PBMC with bacteria. Basophil activation was fully restored at 24 h (Fig. 7E). These results altogether indicate that anti-IgE-induced activation of human basophils can be reversibly inhibited by *L. casei*.

Discussion

We show in this article that one strain of *L. casei* can inhibit both the effector phase of immune inflammation *in vivo* and the activation of effector cells involved in immune inflammation such as mouse mast cells and human basophils *in vitro*. These findings

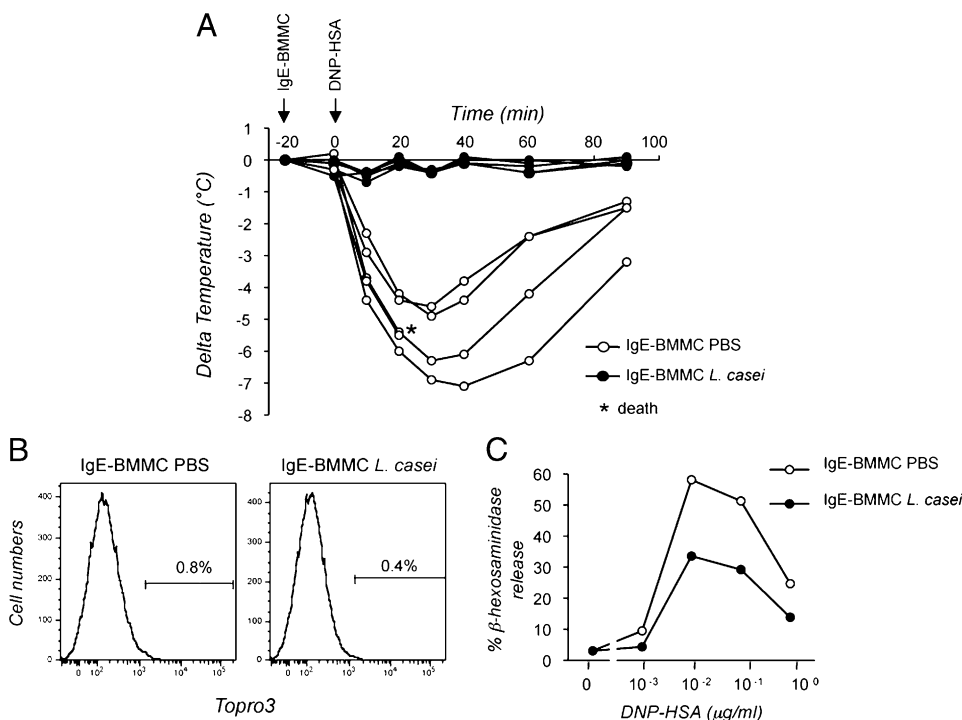
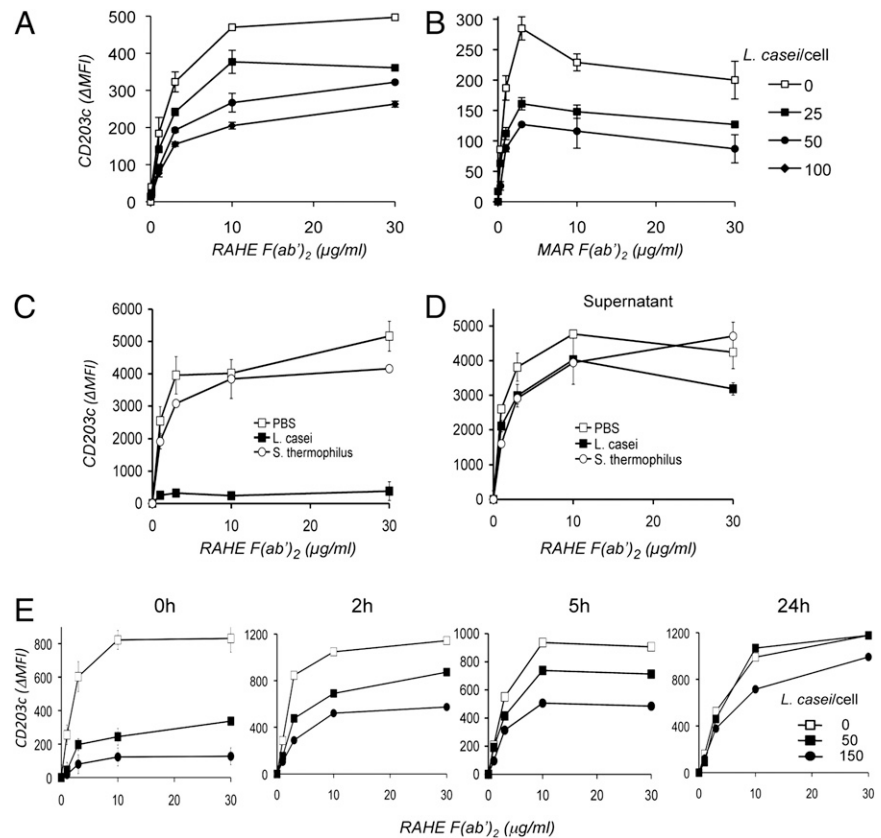


FIGURE 6. *L. casei* inhibits IgE-induced adoptive systemic anaphylaxis. **A**, A total of 3 × 10⁶ BMMC, incubated overnight with PBS or *L. casei* and sensitized with IgE anti-DNP, were injected into C57BL/6 mice *i.v.* Mice were challenged 15 min later with DNP-HSA *i.v.* Body temperature was recorded as a function of time. Curves show the temperature of individual mice (5 mice/group). Results are representative of three experiments. **B**, The viability of BMMC incubated with *L. casei* or without and sensitized with IgE was checked by Topro 3 staining before they were injected. Results were from the cells used in **A**. **C**, β-Hexosaminidase release by the same cells as in **B**, 20 min after challenge with Ag. Results were from the cells used in **A**.

FIGURE 7. *L. casei* inhibits IgE-dependent human basophil activation. *A* and *B*, PBMC from normal donors were incubated overnight with PBS or indicated numbers of *L. casei* in the absence (*A*) or presence (*B*) of rat IgE and challenged with the indicated concentrations of RAHE F(ab')₂ or MAR F(ab')₂. Basophil activation was monitored by the upregulation of CD203c expression (Δ MFI). Results are representative of three experiments. *C*, PBMC were incubated overnight with PBS, *L. casei*, or *S. thermophilus* and challenged with the indicated concentrations of RAHE F(ab')₂. *D*, Supernatants from PBMC incubated overnight with PBS, *L. casei*, or *S. thermophilus*, the responses of which are shown in *C*, were harvested and added to aliquots of PBMC incubated overnight with PBS. One hour later, these were challenged with RAHE F(ab')₂. *E*, PBMC were incubated overnight with PBS or indicated numbers of *L. casei*, washed, and challenged with RAHE F(ab')₂ at the indicated periods. CD203c upregulation was monitored by flow cytometry in FcεRI⁺ CD203c⁺ cells. Results are representative of three experiments. Results are the mean of duplicates (±SD) and are representative of at least three donors.



unravel a novel mechanism by which probiotics can protect from allergy and autoimmunity.

L. casei inhibited IgE-dependent mouse mast cell and human basophil activation when cells were incubated overnight with bacteria before challenge. Nonpathogenic *E. coli* strains were reported to inhibit mast cell activation in vitro (3, 50). Not all nonpathogenic bacteria have this property. Indeed, a strain of *S. thermophilus* inhibited neither mouse mast cell nor human basophil activation. Inhibition could be induced by live but also by irradiated *L. casei*, excluding a role of secreted metabolites. Inhibition was indeed abrogated when mouse mast cells were separated from bacteria by a semipermeable membrane. No inhibition of human basophil activation was observed either when cells were exposed to supernatant of PBMC incubated overnight with bacteria. *L. casei* therefore needs to establish contacts with cells for inhibition to occur.

We failed to identify mouse mast cell receptor(s) that possibly mediate contacts with *L. casei*, but we could exclude all known TLR (51), as well as Nod1/2 (52), that mediate interactions between cells and microorganisms. Inhibition indeed occurred in MyD88^{-/-}TRIF^{-/-} and in Nod1^{-/-}Nod2^{-/-} mast cells. Other receptors and signaling molecules were also dispensable (not shown). These include FcγRIIB, which binds viral (53) and fungal (54) molecules and negatively regulates cell activation, FcγRIIA, which binds *E. coli* and can generate inhibitory signals (55), and DNAX-activation protein 12, which mediates the inhibition of FcR and TLR signaling by triggering receptor expressed on myeloid cells 2 (56).

Inhibition was not due to “desensitization” of mast cells, possibly resulting from prior activation during the incubation with bacteria. Indeed, *L. casei* induced no detectable release of β-hexosaminidase after a 20-min or an overnight incubation, and no secretion of cytokines or chemokines after a 3-h or an overnight

incubation with mast cells; that is, not only when these responses were maximum on stimulation with IgE and Ag, but also over the whole period during which cells were exposed to bacteria. In accordance with this observation, total β-hexosaminidase content was identical in mast cells after an overnight incubation with *L. casei* or without. Noticeably, *L. casei* induced no detectable secretion of IL-10 by mast cells. We did not examine other inhibitory cytokines such as TGF-β. Inhibition was not due to cytotoxicity as judged by mast cell staining with Topro 3 and as confirmed by the reversibility of inhibition observed in both mouse mast cells and human basophils. Inhibition could not be either ascribed to the decreased FcεRI expression and reduced sensitization induced by *L. casei*. Of note, a similar effect on FcεRI expression was observed on human basophils that were already sensitized by endogenous human IgE before they were exposed to *L. casei*. In accordance with these observations, *L. casei* selectively inhibited the Lyn/Syk/LAT pathway, leaving the Fyn/Gab2/PI3K pathway unaffected in mast cells. It affected both proximal events, such as Syk, LAT, and PLCγ phosphorylation, and distal events, such as Erk1/2, JNK, and p38 phosphorylation, as well as the Ca²⁺ response.

As a consequence, *L. casei* inhibited mast cell degranulation and the secretion of IL-5, IL-6, IL-13, TNF-α, MCP-1, and MIP-1α, the four cytokines and the two chemokines induced on FcεRI aggregation. Importantly, *L. casei* also inhibited the release of β-hexosaminidase induced by IgG immune complexes made with GPI and K/BxN serum. *L. casei* can, therefore, inhibit responses that depend on receptors other than FcεRI, in mast cells other than BMMC. PCMC activation by IgG immune complexes indeed depends on FcγRIIA (41, 57). PCMC are mature serosal-type mast cells (39) that may better account for allergic and autoimmune inflammation than BMMC.

Importantly, inhibition of mast cell activation induced in vitro by *L. casei* could be observed in vivo. To this end, we devised

a model of adoptive systemic anaphylaxis. Anaphylaxis, induced on Ag challenge in mice injected with IgE-sensitized BMMC, was abrogated in mice injected with BMMC incubated with *L. casei* prior sensitization with IgE. Inhibition therefore affects all mast cell mediators involved in anaphylaxis. IgE-dependent adoptive anaphylaxis provides a good model of IgE-PSA because mast cell activation through FcεRI engagement is sufficient to account for IgE-PSA.

That *L. casei* could also inhibit IgE-PSA is, however, not readily explained. Whether bacteria could reach mast cells is indeed puzzling if a contact between cells and bacteria is also required in vivo. Mast cells are present in every tissue, but which mast cells are involved in IgE-PSA is unknown. Whether *L. casei* could exit the peritoneal cavity when injected i.p. is not known and, more generally, whether bacteria can get close to mast cells and/or inflammatory cells under physiological or pathological conditions is also unclear. Small numbers of bacteria can be taken up by dendritic (58) or M cells (59) and cross the gut epithelium. Bacteria are transiently found in blood after meals. Whatever the route by which they reach bacteria, mast cells can efficiently protect against a wide spectrum of skin, gut, lung, or peritoneal infections (17). Finally, cells other than mast cells could be affected by *L. casei*. *L. casei* indeed inhibited IgE-dependent activation of human basophils in vitro. Differing from IgE-PSA, K/BxN-arthritis involves many effector cells other than mast cells. K/BxN-arthritis was nevertheless profoundly inhibited by *L. casei*, when administered i.p. Which cells were inhibited and how these cells could meet bacteria in this model needs to be investigated.

IgE-PSA and K/BxN arthritis are models of allergic and auto-immune diseases that bypass the induction phase of the immune response. Pathogenic Abs are indeed passively administered to nonimmunized mice. That both in vivo reactions were inhibited by *L. casei* indicates that this probiotic can inhibit the effector phase of immune inflammation. This finding has fundamental and practical implications. Usually, pathogens promote, whereas probiotics dampen and/or modulate dendritic cell-dependent Ag presentation (19). Likewise, pathogens induce (14–16), whereas *L. casei* inhibits mast cell-dependent inflammation. Microbes therefore control both the induction and the effector phase of immune responses (Supplemental Fig. 4B). The combined effects of pathogens and probiotics may enable physiologic inflammation but prevent pathologic inflammation. The hygiene hypothesis (60) explains the increased incidence of allergies by a decreased exposure to bacteria associated with the Western way of life, resulting in a biased Th1/Th2 polarization (24, 31) in favor of Th2 (61), at the initiation of adaptive immune responses. Not only Th2 diseases, such as allergies (62), but also Th1 diseases, such as type 1 diabetes, Crohn's disease, and multiple sclerosis, however, increased in parallel (63). Our results provide a possible explanation if bacteria also control the effector phase of adaptive immune responses that is shared by the two types of diseases. Based on this conclusion, one can extrapolate that some probiotics may prevent symptoms in allergic or autoimmune patients who have already produced IgE anti-allergen or IgG autoantibodies.

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