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ARTICLE

Preclinical efficacy and safety of an anti-IL-1β vaccine for the treatment of type 2 diabetes

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Neutralization of the inflammatory cytokine interleukin-1β (IL-1β) is a promising new strategy to prevent the β-cell destruction, which leads to type 2 diabetes. Here, we describe the preclinical development of a therapeutic vaccine against IL-1β consisting of a detoxified version of IL-1β chemically cross-linked to virus-like particles of the bacteriophage Qβ. The vaccine was well tolerated and induced robust antibody responses in mice, which neutralized the biological activity of IL-1β, as shown both in cellular assays and in challenge experiments *in vivo*. Antibody titers were long lasting but reversible over time and not associated with the development of potentially harmful T cell responses against IL-1β. Neutralization of IL-1β by vaccine-induced antibodies had no influence on the immune responses of mice to *Listeria monocytogenes* and *Mycobacterium tuberculosis.* In a diet-induced model of type 2 diabetes, immunized mice showed improved glucose tolerance, which was mediated by improved insulin secretion by pancreatic β-cells. Hence, immunization with IL-1β conjugated to virus-like particles has the potential to become a safe, efficacious, and cost-effective therapy for the prevention and long-term treatment of type 2 diabetes.

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INTRODUCTION

Type 2 diabetes is a chronic metabolic disorder characterized by the presence of hyperglycemia due to defective insulin secretion, insulin action, or a combination of both. In the early stage of disease, the predominant abnormality is reduced insulin sensitivity of body cells. Initially, this is counteracted by an increase in circulating insulin through expansion of β-cell mass and secretory function. Later in the disease, both functionality and numbers of pancreatic β-cells decline, causing the development of hyperglycemia and overt diabetes. If not properly controlled by glucose-lowering drugs, type 2 diabetes can lead to serious complications, including diabetic nephropathy, vascular disease, diabetic retinopathy, diabetic neuropathy, and liver damage.

Although the mechanisms of β-cell failure in type 2 diabetes remain a matter of debate, metabolic stress and inflammatory pathways have been implicated. Metabolic stress caused by repetitive glucose excursions, dyslipidemia, and adipokines can induce an inflammatory response, which is characterized by local cytokine secretion, islet immune-cell infiltration, β-cell apoptosis, amyloid deposits, and eventually fibrosis. Furthermore, amyloid deposits in islets have been shown to activate the inflammasome, resulting in IL-1β secretion.[1] In recent years, IL-1β has emerged as a master cytokine, which regulates islet chemokine production and causes impaired insulin production and β-cell death (reviewed in ref. 2). High concentrations of glucose induce β-cell production of IL-1β, which may lead to impaired β-cell function and apoptosis in human pancreatic islets.[3] Blockade of IL-1 signaling by administration of recombinant IL-1 receptor antagonist or neutralizing monoclonal antibodies has been shown to improve glycemic control in animal models of type 2 diabetes.[4–7] Furthermore, treatment of type 2 diabetes patients with recombinant human IL-1 receptor antagonist (Anakinra) resulted in significantly decreased glycated hemoglobin levels (a reliable readout for long-term glycemia) and improved β-cell function.[8,9] Similar results were obtained with different monoclonal antibodies directed against IL-1β, including Canakinumab,[10] Gevokizumab,[11] and LY2189102.[12] Accordingly, IL-1β antagonism is now in phase 3 of clinical development as a treatment for diabetes and its cardiovascular complications (CANTOS study, NCT01327846).

We have previously shown that immunizing mice with recombinant IL-1β chemically cross-linked to virus-like particles (VLPs) of the bacteriophage Qβ induces neutralizing antibodies against IL-1β.[13] A possible hurdle for the direct translation of this potentially cost-effective and convenient therapy to clinical use is the inflammatory activity and systemic toxicity of the IL-1β contained in the vaccine. Clinical trials exploring the hematopoietic effects of IL-1β in cancer patients have shown that s.c. injection of as little as 4 ng/kg can cause fever.[14–16] Intravenous injections of doses ≥ 10 ng/kg caused grade 3 fever and severe hypotension in a majority of patients.[17] An anticipated vaccine dose of 100 µg of IL-1β-Qβ would deliver 20–30 µg (~250–400 ng/kg) of potentially bioactive IL-1β,

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which could cause considerable adverse effects in humans. Other concerns related to the use of self-specific immunization against IL-1β in humans include the potential for induction of irreversible IL-1β-neutralizing antibody responses with possible consequences on the immune response to invading pathogens. In theory, immunization against IL-1β could also lead to IL-1β-specific T cell responses, potentially causing tissue damage and immunopathology.

Here, we describe the design and preclinical characterization of a VLP-based vaccine containing a mutant form of IL-1β with strongly reduced inflammatory activity. This vaccine showed good tolerability in mice and induced long-lasting, but reversible, IL-1β-neutralizing antibody titers. While not causing any measurable immunosuppressive or immunopathological effects, the vaccine improved glucose tolerance and enhanced insulin secretion in a mouse model of diet-induced diabetes.

RESULTS

Production of a genetically detoxified form of human IL-1β as vaccine antigen

To design an anti-IL-1β vaccine suitable for human use, we introduced a series of mutations into wild-type human IL-1β (hIL-1β) and investigated their effects on the inflammatory activity and antigenic profile of the molecule (Spohn *et al*., manuscript in preparation). One of the explored muteins, hIL-1b(D145K), showed a particularly favorable profile in this regard. Compared to wild-type hIL-β, this mutein carries an N-terminal extension of three amino acids (Met-Asp-Ile), a C-terminal hexahistidine tag and cysteine-containing linker, as well as an amino acid substitution from aspartate to lysine at position 145. The hIL-1b(D145K) mutein showed a strongly reduced ability to assemble into the active IL-1β signalling complex, which consists of IL-1β, IL-1 receptor I (IL-1RI) and the IL-1R accessory protein (IL-1RAcP). As compared to wild-type IL-1β, the mutein showed a 3,500-fold reduced affinity to IL-1RI (Figure 1a) and a complete inability to recruit the accessory protein to the preformed IL-1RI-IL-1β(mutein) complex (Figure 1b). In contrast, binding to the nonsignaling decoy receptor IL-1RII was completely preserved, indicating that the overall conformation and antigenic properties of the mutein have been essentially preserved (Figure 1c). This result was further confirmed by far-UV and near-UV CD spectroscopy. No significant differences were detected between hIL-1b(D145K) and wild-type hIL-1β with regard to shape of the spectra or peak intensities (data not shown). Hence, the overall secondary and tertiary structure was conserved between hIL-1β and the mutein hIL-1β(D145K). As a result of the combined defects in hIL-1RI and IL-1RAcP binding, the mutein showed a 12,000-fold reduced ability to induce IL-6 secretion from HeLa cells (Figure 1d) and a 16,000-fold reduced cytopathic effect on A375 cells (Figure 1e). An i.p. injection of 1 µg hIL-1b(D145K) into mice induced 45-fold lower serum concentrations of IL-6 as compared with the same amount of wild-type IL-1β (Figure 1f). Next, hIL-1b(D145K) was conjugated

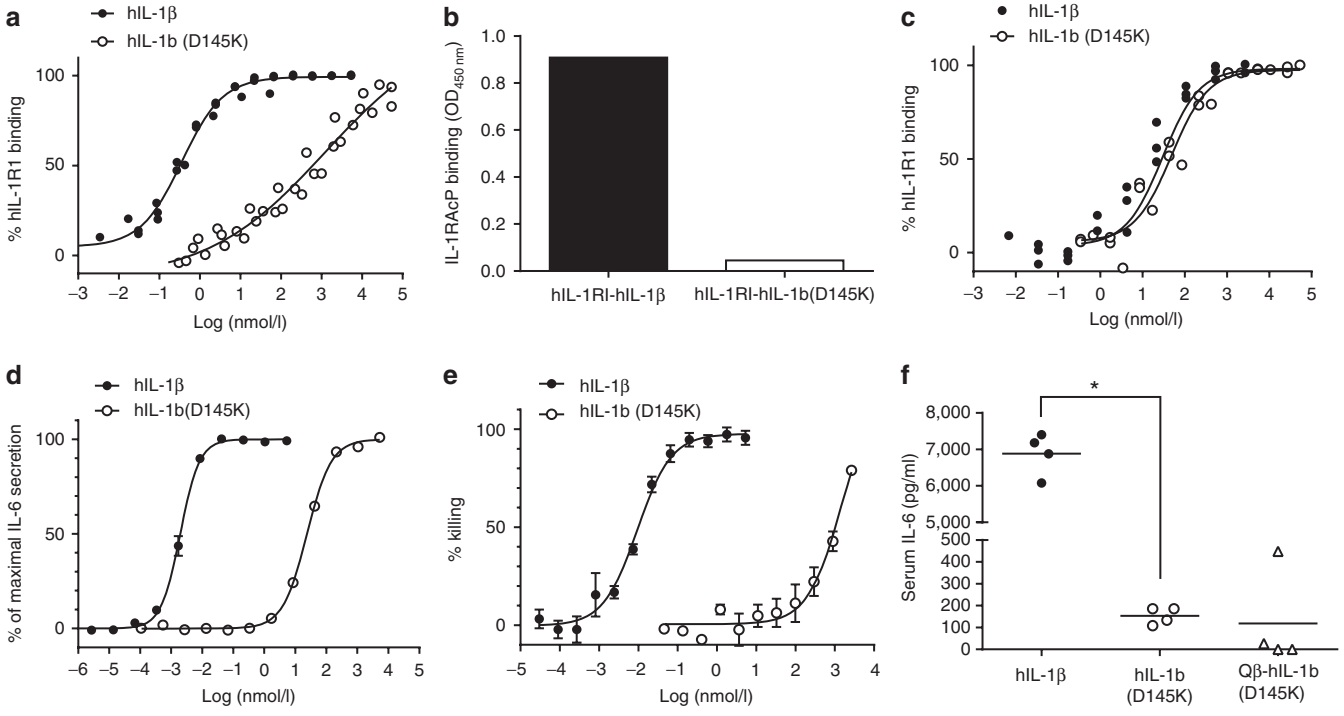

Figure 1 Characterization of the detoxified vaccine antigen hIL-1b(D145K). (**a**) hIL-1RI binding. Serial dilutions of wild-type hIL-1β or hIL-1b(D145K) were mixed with a constant amount of 1 nmol/l biotinylated hIL-1β and applied to ELISA plates that had been coated with hIL-1RI. Obtained OD values were reciprocally transformed to express % receptor binding of wild-type hIL-1β or hIL-1b(D145K), respectively. (**b**) Formation of the ternary hIL-1RI-hIL-1β-hIL-1RAcP signaling complex. Wild-type human IL-1β (0.4 µg/ml) or hIL-1b(D145K) (100 µg/ml) were incubated with hIL-1RAcP (1 µg/ml) and applied to ELISA plates that had been coated with 1 µg/ml of human IL-1RI. Formation of the ternary complex was detected with a hIL-1RAcP-specific antibody. (**c**) hIL-1RII binding. Experimental conditions were as in **a** but with hIL-1RII coated on the ELISA plate. (**d**) IL-6 secretion. HeLa cells were incubated with serial dilutions of wild-type hIL-1β or hIL-1b (D145K). After an incubation of 4 hours, IL-6 was quantified in supernatants by Sandwich ELISA. (**e**) Cytopathic effect. A375 cells were incubated with serial dilutions of either wild-type hIL-1β or hIL-1b(D145K). After 7 days, viable adherent cells were stained with crystal violet and quantified by measuring optical densities at 600 nm. Shown are mean values from triplicate measurements ± SEM. (**f**) *In vivo* inflammatory activity. Groups of female C57BL/6 mice ($n = 4$) were injected i.p. with 1 µg of either wild-type hIL-1β or hIL-1b(D145K) or s.c. with 25 µg of the conjugate vaccine Qβ-hIL-1b(D145K). Three hours after injection, sera were collected, and IL-6 levels were quantified with a Quantikine ELISA kit (*$P < 0.0001$).

to Qβ VLPs, and reactogenicity of the resulting conjugate vaccine was tested in mice. As shown in Figure 1f, s.c. injection of 25 µg Qβ-hIL-1b(D145K) induced barely detectable serum levels of IL-6. Thus, the hIL-1b(D145K) mutein seemed to be sufficiently detoxified to be used as an anti-IL-1β vaccine antigen for human use.

Immunogenicity of the detoxified murine IL-1β VLP vaccine in mice

To enable preclinical testing of the anti-IL-1β vaccine in the context of self-specific immunization, we produced the murine version of hIL-1b(D145K) (=mIL-1b(D143K)) and conjugated it to Qβ VLPs. Figure 2a shows that a single injection of the conjugate vaccine Qβ-mIL-1b(D143K) induced antimurine IL-1β (mIL-1β) IgG titers of 17,600 by day 21, which increased to 33,900 by day 42. An additional injection on day 42 led to a further increase in titers to a maximum of 65,200 by day 70. In the absence of further injections, the titers then declined with an approximate half-life of 2–3 months. Moderate mIL-1β-neutralizing titers of 50 were detected in sera from day 21, which increased to 300 on day 42 and reached their maximum of 1,200 on day 70. Thereafter, they declined in parallel to the enzyme-linked immunosorbent assay (ELISA) titers until the end of the observation period. The ratio of mIL-1β-specific neutralization versus ELISA titers (Figure 2b) increased from day 21 to 70, indicating an augmentation in the average affinity of the produced anti-IL-1β antibodies over time. To assess if the magnitude and quality of the anti-mIL-1β antibody response is affected by the repertoire of IL-1β-specific B cells, we compared wild-type and IL-1β-deficient mice. If the presence of IL-1β in wild-type animals causes a certain degree of B cell tolerance toward IL-1β as an antigen, one would expect that the Qβ-mIL-1b(D143K) vaccine induces stronger IL-1β-specific B cell responses in IL-1β-deficient mice due to the absence of B cell tolerance and thus a larger number and a broader repertoire of IL-1β-specific B cells. Groups of IL-1β-deficient and wild-type control mice were immunized with Qβ-mIL-1b(D143K), and antibody responses against mIL-1β and the Qβ VLP carrier were analyzed by ELISA. As shown in Figure 2c, IgG titers against the foreign antigen Qβ were not significantly reduced in mutant mice, indicating that IL-1β deficiency does not lead to a general suppression of the humoral response. Importantly, both ELISA titers against mIL-1β (Figure 2c) as well as IL-1β-neutralizing titers (Figure 2d) were similar between wild-type and IL-1β-deficient mice, suggesting that no obvious restriction of the IL-1β-specific B cell repertoire is present in wild-type mice.

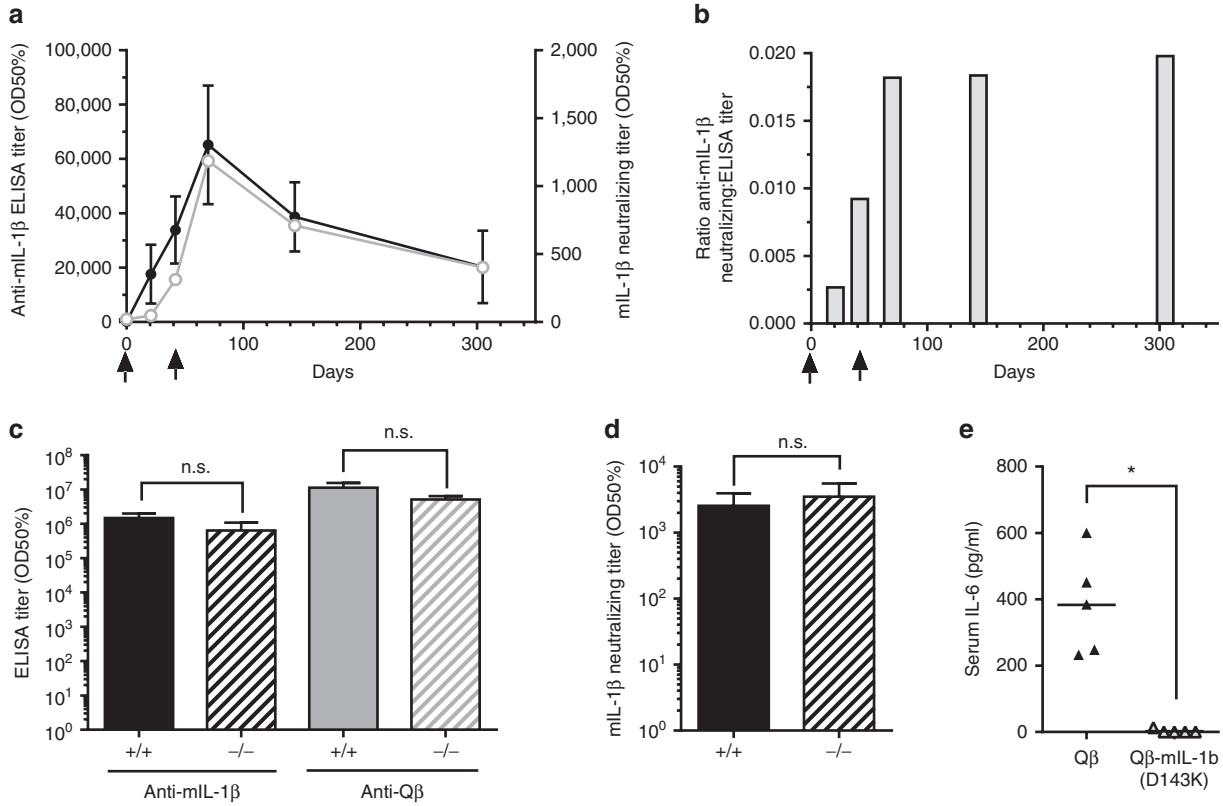

Figure 2 Immunogenicity of Qβ-mIL-1b(D143K) in mice. (**a**) Induction of anti-mIL-1β antibody titers. Female C57BL/6 mice (n = 5) were immunized twice (days 0 and 42, arrows) with 10 µg of Qβ-mIL-1b(D143K) in the presence of Alum. At the indicated time points, mouse IL-1β (wild type)-specific IgG antibody titers were measured by ELISA (filled black circles). At the same time points IL-1β neutralizing titers were determined *ex vivo* in the HeLa IL-6 secretion assay as described in *Materials and Methods* (open gray circles). Shown are mean titers ± SEM. (**b**) Ratio of IL-1β neutralizing versus ELISA titers over time. The ratios of IL-1β- neutralizing versus IL-1β-specific IgG ELISA titers were calculated for the group means of each time point of the experiment described in **a**. Vaccine injections are indicated by arrows. (**c**) Antibody responses in IL-1β-deficient mice. Groups (n = 4) of female C57BL/6 IL-1β-deficient mice (-/-) or C57BL/6 control mice (+/+) were immunized on days 0, 14, and 28 with 10 µg of Qβ-mIL-1b(D143K) in the presence of Alum. On day 42, mouse IL-1β (wild type)- as well as Qβ-specific IgG antibody titers were measured by ELISA. Shown are mean titers ± SEM. (**d**) Induction of IL-1β-neutralizing titers in IL-1β-deficient mice. Mouse IL-1β (wild type)-neutralizing titers were determined in sera from day 42 of the experiment described in **c**. Shown are mean titers ± SEM. (**e**) *In vivo* neutralization of IL-1β in Qβ-mIL-1b(D143K)-immunized mice. Groups of female C57BL/6 mice (n = 5) were immunized s.c. on days 0, 14, and 28 with 1 µg of Qβ-mIL-1b(D143K) or Qβ VLPs as control. On day 42, mice were challenged with an i.p. injection of 1 µg wild-type mouse IL-1β. Three hours after challenge, sera were collected and IL-6 levels were quantified with a Quantikine ELISA kit. Shown are data from individual mice and group means (*P < 0.001; n.s., not significant).

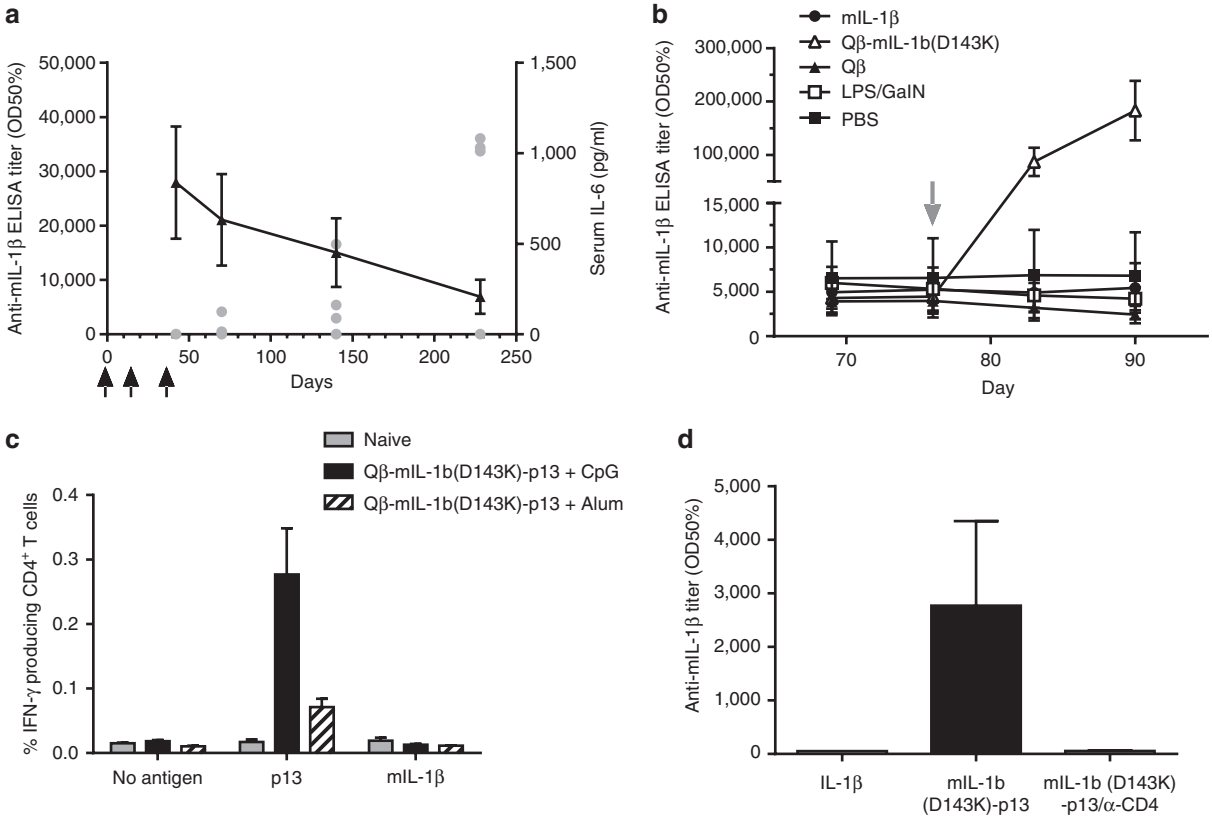

Figure 3 Safety assessment of the Qβ-mIL-1b(D143K) vaccine in mice. (**a**) Time course of anti-IL-1β antibody responses. Female C57BL/6 mice ($n = 4$) were immunized s.c. on days 0, 14, and 35 (arrows) with 1 µg of Qβ-mIL-1b(D143K). On days 42, 70, 140, and 228, mice were challenged with i.p. injections of 1 µg wild-type mouse IL-1β. Three hours after challenge, sera were collected, and IL-6 levels were quantified by ELISA. Single data points (filled gray circles) represent individual animals. Antimouse IL-1β (wild type) IgG antibody titers were determined at the same time points by ELISA and are represented as group means ± SEM (black triangles). (**b**) Effect of increased IL-1β levels on antimouse IL-1β IgG antibody titers. Groups of female C57BL/6 mice ($n = 5$) were immunized s.c. on day 0 with 50 µg Qβ-mIL-1b(D143K). On day 76, mice received either an i.v. injection of 100 ng wild-type mouse IL-1β (in 100 µl PBS), an i.p. injection of a mixture of 1 ng *Escherichia coli* lipopolysaccharide, and 20 mg N-galactosamine (Sigma-Aldrich), or a s.c. injection of 50 µg Qβ-mIL-1b(D143K). Control groups received either an i.v. injection of 100 µl PBS or a s.c. injection of 50 µg Qβ VLPs. Mice were bled on days 69, 76, 83, and 90 and mouse IL-1β (wild type)-specific IgG antibody titers were determined by ELISA. Shown are group means ± SEM. (**c**) Measurement of IL-1β-specific T cell responses after vaccination with Qβ-mIL-1b(D143K). Groups of mice were immunized with Qβ-mIL-1b(D143K)-p13 in the presence of CpG or Alum, respectively, as described in *Materials and Methods*. One group of female C57BL/6 mice was kept naive. After immunization, splenocytes were isolated from all mice and stimulated with BMDC that had been loaded either with synthetic p13 peptide or with wild-type mouse IL-1β. Nonspecific IFNγ-release from $CD4^+$ T cells was determined by incubation of splenocytes with mock-pulsed BMDC (no antigen). Antigen-specific IFNγ-producing $CD4^+$ T cells were determined by fluorescence-activated cell sorting. Shown are group means ± SEM. (**d**) Th cell dependence of anti-IL-1β antibody induction. Groups of mice received s.c. injections of either wild-type mouse IL-1β or mIL-1b(D143K)-p13, each in the presence of incomplete Freund's adjuvant. A subgroup of mIL-1b(D143K)-p13-immunized mice were additionally injected with a depleting anti-CD4 antibody. Mouse IL-1β (wild type)-specific IgG antibody titers were analyzed by ELISA. Shown are group means ± SEM.

To investigate if the vaccine-induced antibodies were able to neutralize the inflammatory activity of IL-1β *in vivo*, we immunized mice three times with either Qβ-mIL-1b(D143K) or Qβ VLPs as control and challenged them by an i.v. injection of wild-type mIL-1β protein. Figure 2e shows that the challenge induced average IL-6 serum levels of ~400 pg/ml in Qβ VLP-immunized mice, whereas no IL-6 could be detected in serum of Qβ-mIL-1b(D143K)-immunized mice. Thus, the inflammatory activity of mIL-1β was completely neutralized in the latter group of mice. We conclude that Qβ-mIL-1b(D143K) is highly immunogenic in mice and induces mIL-1β-specific IgG antibodies, which efficiently neutralize the activity of IL-1β *in vitro* and *in vivo*.

Cross-reactivity of Qβ-mIL-1b(D143K)-induced antibodies with mIL-1α was not directly assessed, but we had previously found that antibodies induced with a very similar mIL-1β vaccine showed minimal to no cross-reactivity.[13] To further support this notion, mice were immunized with the Qβ-mIL-1β vaccine as described in ref. 13 and challenged with both IL-1α and IL-1β. While the inflammatory activity of IL-1β was fully neutralized in Qβ-mIL-1β-immunized mice, the activity of IL-1α was unaffected (Supplementary Figure S1), indicating that Qβ-mIL-1β-induced antibodies are not able to cross-neutralize IL-1α.

Neutralization of mIL-1β by Qβ-mIL-1b(D143K) is reversible and not boosted by endogenous IL-1β

A possible safety concern for the use of a vaccine against IL-1β in humans is uncontrolled, irreversible neutralization of the cytokine through the induction of long-lasting or even steadily increasing titers of IL-1β-specific antibodies. Such persisting anti-IL-β antibody titers could have long-term consequences for immunity against infectious diseases. To test for this possibility in a preclinical setting, we first immunized mice three times with Qβ-mIL-1b(D143K) to induce a robust mIL-1β-neutralizing antibody response. Starting

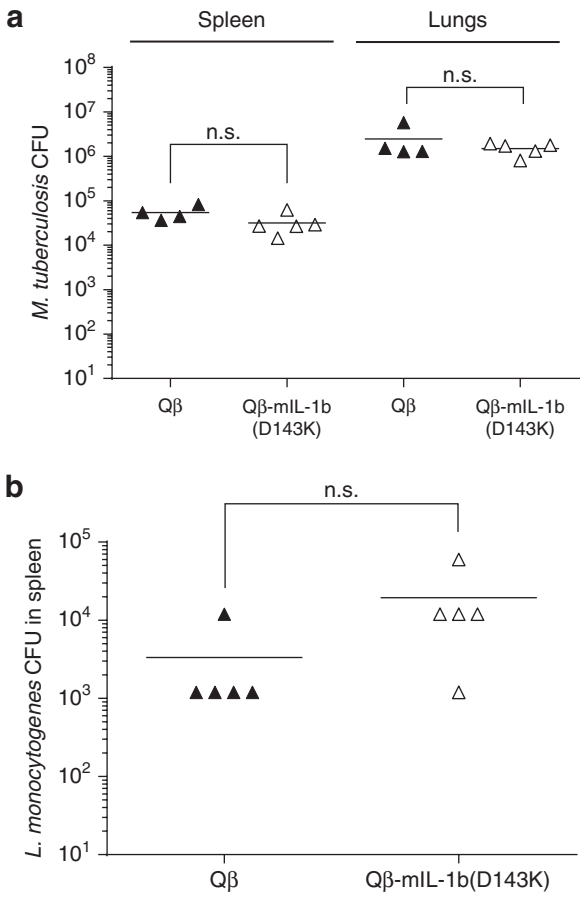

Figure 4 Effect of immunization with Qβ-mIL-1b(D143K) on susceptibility to infection with intracellular bacteria. (**a**) *Mycobacterium tuberculosis*. Groups of mice ($n = 5$) were immunized s.c. with either Qβ-mIL-1b(D143K) or Qβ VLPs and infected with *Mycobacterium tuberculosis* via the aerosol route. At 36 weeks postinfection, bacterial load was determined in lungs and spleens of infected mice as described in *Materials and Methods*. (**b**) *Listeria monocytogenes*. Groups of mice ($n = 5$) were immunized s.c. with either Qβ-mIL-1b(D143K) or Qβ VLPs and infected i.p. with *L. monocytogenes*. Bacterial load was determined after 3 days in spleens of infected mice as described in *Materials and Methods* (n.s., not significant).

from the peak of the antibody response, we then followed the mIL-1β-specific antibody titers over an extended time period while measuring the neutralizing activity of the antibodies *in vivo* by repeated challenges with a constant dose of recombinant wild-type mIL-1β. On day 42, at the peak of the antibody response, anti-mIL-1β IgG titers of ~27,900 were measured, and an i.p. challenge with 1 µg of mIL-1β did not induce any elevation of serum IL-6 levels, indicating complete neutralization of the applied dose of mIL-1β by vaccine-induced antibodies (Figure 3a). During the follow-up phase, antibody titers continuously declined and responsiveness to the challenge was gradually regained. On day 228, three out of four mice produced robust amounts of IL-6 in response to injection with mIL-1β. Importantly, the IL-1β-specific antibody response was not boosted by the repeated administration of recombinant wild-type IL-1β, indicating that nonconjugated IL-1β is unable to activate IL-1β-specific B cells induced by vaccination.

IL-1β is produced as part of the innate immune response to infectious agents or other conditions of inflammation. In order to determine whether a sudden and natural rise in endogenous IL-1β levels has the ability to boost anti-IL-1β antibody titers induced by vaccination, mice were immunized once with Qβ-mIL-1b(D143K) and subsequently injected with lipopolysaccharide and N-galactosamine in order to induce an endogenous increase in IL-1β.[18] Control groups received phosphate-buffered saline (PBS) or additional injections of Qβ-mIL-1b(D143K) or Qβ VLPs alone. Mouse IL-1β-specific antibodies were measured before injection and 1 and 2 weeks thereafter. Results presented in Figure 3b show that lipopolysaccharide/N-galactosamine injection had no influence on anti-IL-1β antibody titers, indicating that increased endogenous levels of IL-1β do not boost the existing antibody response induced by vaccination with Qβ-mIL-1b(D143K). As expected, only an additional injection of Qβ-mIL-1b(D143K) was able to boost the mIL-1β-specific antibody response.

Absence of IL-1β-specific CD4+ T cell induction after immunization with Qβ-mIL-1b(D143K)

A potential concern regarding vaccination against IL-1β is the induction of T cells specific for IL-1β, which could have the potential to cause immunopathology. In general, induction of such self-reactive T cells is not expected, since T cell tolerance toward self-antigens is generally very strict due to the clonal deletion of autoreactive T cells during development. We nevertheless investigated whether IL-1β-specific T cells are induced by immunization with Qβ-mIL-1b(D143K). As we did not expect strong IL-1β-specific T cell responses, it was important to have a reliable internal positive control. To this end, a fusion protein consisting of mIL-1b(D143K) and a strong CD4+ T cell epitope derived from the glycoprotein of mouse lymphocytic choriomeningitis virus (p13)[19] was produced (mIL-1b(D143K)-p13) and coupled to Qβ VLPs. One group of mice was immunized with the vaccine Qβ-mIL-1b(D143K)-p13 mixed with CpG. CpGs are known as strong inducers of T cell responses and were chosen to maximize the likelihood of inducing an antigen-specific CD4+ T cell response. A second group of mice was immunized three times with Qβ-mIL-1b(D143K) in aluminum hydroxide (Alum), thus mimicking the intended use in humans. One week after the last immunization, splenocytes from all mice were isolated and stimulated with bone marrow-derived dendritic cells that were loaded with p13 peptide or recombinant wild-type mouse IL-1β, respectively. Figure 3c shows that Qβ-mIL-1b(D143K)-p13-immunized animals exhibited a sizeable population of p13-specific Th cells as determined by intracellular cytokine staining. Animals immunized in the presence of CpG had higher frequencies of specific CD4+ T cells compared to the group receiving the vaccine in Alum (0.28 versus 0.07%, respectively). Only a low nonspecific p13 response was observed in naive animals (0.02%). Importantly, IL-1β-reactive CD4+ T cells could not be detected in any of the groups. Hence, immunization with Qβ-mIL-1b(D143K)-p13, while capable of inducing a strong CD4+ response to the foreign CD4+ T cell epitope p13, did not lead to the induction/expansion of IL-1β-specific CD4+ T cells, even if very strong adjuvants such as CpG were used.

An additional reliable and sensitive readout for the induction of CD4+ T cell responses is the detection of antigen-specific IgG antibodies. Immunization with isolated self antigens does not induce specific IgG antibodies due to the lack of T cell epitopes in the antigen required for T cell help-dependent class switching. The strong IgG response to IL-1β observed after immunization with Qβ-mIL-1b(D143K) is due to the activation of T helper cells specific for epitopes contained in the Qβ VLP carrier. Figure 3d shows that immunization of C57BL/6 mice with recombinant wild-type mouse IL-1β in incomplete Freund's adjuvant (IFA) did not induce any IL-1β-specific IgG antibodies, showing that IL-1β-specific T helper cells have not

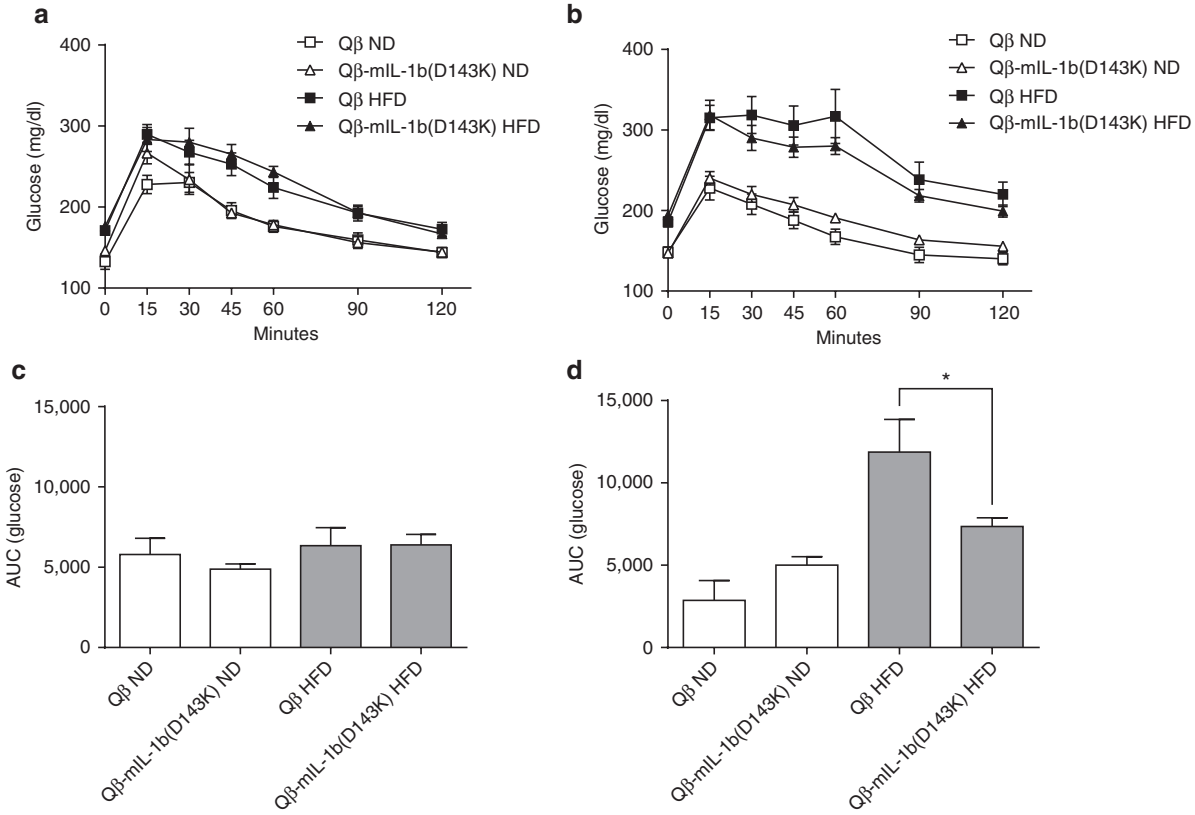

Figure 5 Effect of Qβ-mIL-1b(D143K) immunization on glycemic control in the diet-induced diabetes model. Groups of mice ($n = 8$) were kept on normal diet (ND) or high-fat diet (HFD) and immunized with either Qβ-mIL-1b(D143K) or Qβ VLPs as described in *Materials and Methods*. (**a**) Oral glucose tolerance test before the onset of diabetes (day 57). A solution of glucose in water was applied intragastrically to fasted mice, and glucose concentrations were determined at the indicated time points in blood samples drawn from the tail vein. Shown are group means ± SEM. (**b**) Oral glucose tolerance test in diabetic mice (day 244). The experiment was performed as outlined in **a**. (**c,d**) The areas under the curves (AUC) obtained from the oral glucose tolerance test performed on day 57 (**c**) and day 244 (**d**) were calculated. Shown are group means ± SEM (*$P < 0.05$).

been induced under these conditions. In contrast, immunization with the mIL-1b(D143K)-p13 fusion protein in IFA induced a robust IgG antibody response against IL-1β. This indicates that IL-1β-specific B cells are present in wild-type mice and that the failure to generate IL-1β-specific IgG upon immunization with IL-1β alone is due to the absence of specific T cell help. In fact, ablation of T helper cells by repeated administration of an anti-CD4 antibody completely abolished the IgG response to IL-1β after mIL-1b(D143K)-p13 immunization (Figure 3d). Taken together, these data show that immunization of mice with the self antigen IL-1β alone or in the context of the vaccine Qβ-mIL-1b(D143K) does not result in the induction of IL-1β-specific CD4+ T cells.

Immunization with Qβ-mIL-1b(D143K) does not increase susceptibility of mice to bacterial infection

The IL-1 system is a central element of the innate and adaptive immune response to intracellular bacterial pathogens. Mice genetically deficient for IL-1α and IL-1β[20] or for the receptor IL-1RI[21–23] show increased susceptibility to *Mycobacterium tuberculosis*. Similarly, blocking IL-1RI signaling by administration of monoclonal antibodies[24] or transgenic overexpression of IL-1 receptor antagonist[25,26] causes increased bacterial growth after infection with *Listeria monocytogenes.* We have previously shown that immunization of mice with a Qβ VLP-based vaccine displaying mIL-1β had no influence on the host response to either *M. tuberculosis* or *L. monocytogenes.*[22] To investigate whether the same is true for Qβ-mIL-1b(D143K), we immunized groups of mice with the vaccine or Qβ VLPs as control and infected them with either pathogen. Figure 4a shows that 36 weeks after infection with *M. tuberculosis*, similar bacterial loads were detected in lungs and spleens between Qβ-mIL-1b(D143K)-immunized and control-immunized mice. Mice in both groups gained weight normally, and no significant difference in the average body weights was detected between the groups up to week 36 (data not shown). Similarly, infection with *L. monocytogenes* did not result in significantly increased bacterial burden in spleens of Qβ-mIL-1b(D143K)-immunized mice as compared with Qβ VLP-immunized mice (Figure 4b).

We also investigated whether immunization with Qβ-mIL-1b(D143K) can lead to a reduction in peripheral neutrophil counts, similar to what has been observed clinically in a subgroup of patients receiving IL-1-blocking agents.[8,27–31] Repeated injection of mice with high doses of Qβ-mIL-1b(D143K) resulted in a trend toward reduced neutrophil numbers when compared with Qβ-immunized control mice, without however reaching statistical significance (Supplementary Figure S2).

Qβ-mIL-1b(D143K) immunization protects from diet-induced diabetes

The therapeutic potential of Qβ-mIL-1b(D143K) immunization in type 2 diabetes was tested in the murine diet-induced model of diabetes.[32] Groups of mice were immunized with Qβ-mIL-1b(D143K)

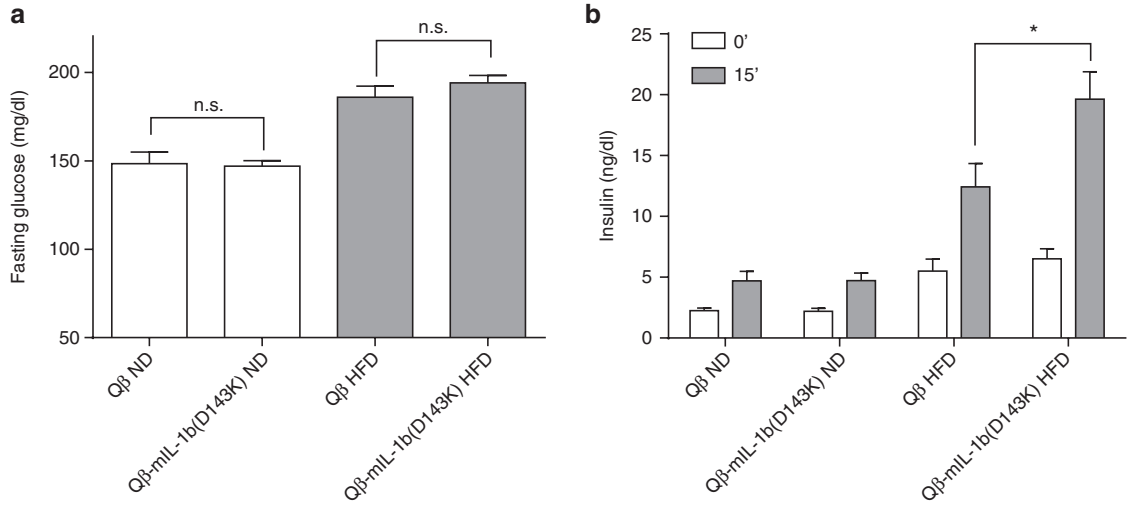

Figure 6 Fasting glucose levels and glucose-induced insulin secretion. (**a**) Fasting glucose levels. Blood glucose levels were determined on day 244 in mice that had been fasted for 5 hours. Shown are group means ±SEM. (**b**) Glucose-induced insulin secretion. On day 253, a solution of glucose in water was applied intragastrically by oral gavage to mice that had been fasted for 5 hours. Before and 15 minutes after gavage, insulin levels were determined in blood samples drawn from the tail vein. Shown are group means ± SEM (*$P < 0.05$, n.s., not significant).

or Qβ VLPs as control and fed a high-fat diet to induce an obese and early diabetic phenotype. As controls, Qβ-mIL-1b(D143K)- or Qβ-immunized mice were kept on normal diet. Qβ-mIL-1b(D143K) immunization resulted in slightly increased weight gain as compared with Qβ-immunized control animals, irrespective of the underlying diet. The difference was, however, mild and did not reach statistical significance at any time point (Supplementary Figure S3). The antidiabetic effect of anti-mIL-1β immmunization was tested in oral glucose tolerance tests at different time points. A fixed dose of glucose was administered intragastrically by oral gavage to fasted mice, and blood glucose levels were monitored for 2 hours thereafter to determine clearance kinetics. Figure 5a shows that before the onset of diabetes (day 57), Qβ-mIL-1b(D143K)- and Qβ-immunized mice showed similar profiles of glucose elimination with a sharp peak in blood glucose levels 15 minutes after administration, followed by a sustained decline and a return to baseline levels after 2 hours. This is reflected in the areas under the curves, which are similar between Qβ-mIL-1b(D143K)-immunized and Qβ control mice and not yet affected by the underlying diet (Figure 5c). Eight months after the first immunization (day 244), a new glucose challenge of Qβ-immunized mice resulted in peak levels of ~320 mg/dl at 15 minutes, but in contrast to the first challenge, these levels did not significantly decline during the next 45 minutes (Figure 5b); only thereafter, blood glucose levels started to decrease, without however returning to baseline levels within the 2-hour observation period. This severe impairment in glucose clearance indicates that Qβ-immunized control mice had developed a strong diabetic phenotype. Qβ-mIL-1b(D143K)-immunized mice challenged at the same time point also showed an initial increase in blood glucose levels to ~320 mg/dl, which was immediately followed by a sustained decline, resulting in glucose levels that were consistently lower than that in Qβ-immunized control mice. Two hours after challenge, blood glucose levels had returned to prechallenge levels in these mice. When plotting the area under the curves resulting from the oral glucose tolerance test on day 244 (Figure 5d), it becomes evident that the high-fat diet had led to a strong glucose intolerance in Qβ-immunized mice, whereas clearance kinetics were more similar to control mice in Qβ-mIL-1b(D143K)-immunized mice.

We next investigated whether the improved glucose clearance in Qβ-mIL-1b(D143K)-immunized mice was due to increased insulin responsiveness or increased insulin secretion by β-cells. Intraperitoenal insulin tolerance tests were performed at different time points (days 61, 88, and 274) but failed to reveal an improved insulin sensitivity in Qβ-mIL-1b(D143K)-immunized mice (data not shown). In addition, there was no effect of Qβ-mIL-1b(D143K)-immunization on fasting glucose (Figure 6a) or fasting insulin levels (Figure 6b, 0'). However, when measuring the insulin response 15 minutes after an oral glucose challenge, we noted significantly increased blood insulin concentrations in Qβ-mIL-1b(D143K)-immunized mice on high-fat diet when compared with Qβ-immunized animals on the same diet (Figure 6b, 15'). Taken together, these data indicate that the vaccine improves glucose clearance not by decreasing insulin resistance but rather by increasing the insulin secretion by β-cells in response to high glucose concentrations.

DISCUSSION

Specific blockade of the key proinflammatory cytokine IL-1β is emerging as a therapeutic option for the prevention and treatment of type 2 diabetes. The experiments and clinical trials leading to this notion were based on interventions with IL-1β-targeted therapy using monoclonal antibodies or small antagonists. Here, we demonstrate in a preclinical mouse model that active immunization against IL-1β may become an additional safe and effective strategy for treatment of this disorder.

In a first step toward the development of an anti-IL-1β vaccine for human use, we designed an IL-1β derivative as vaccine antigen with strongly reduced inflammatory activity. This was warranted because of the strong pyrogenic and hypotensive activity of IL-1β that might otherwise lead to fever and hypotension in a large proportion of vaccinated patients. We introduced a series of specific mutations which strongly reduced the bioactivity of IL-1β, as measured both in cell culture and in challenge experiments in mice. Compared with wild-type human IL-1β, the mutant protein induced 45-fold less IL-6 *in vivo*. Furthermore, the Qβ-hIL-1b(D145K) conjugate vaccine induced barely detectable levels of IL-6. Importantly, the antigenic properties of IL-1β were not

affected by the introduced mutations, as illustrated by the preserved affinity of the mutant protein hIL-1b(D145K) for the decoy receptor IL-1RII, and its unaltered near and far UV spectra. The loss in bioactivity of hIL-1b(D145K) is most likely a consequence of a reduced ability to form the active IL-1β/IL-1RI/IL-1RAcP signaling complex, as indicated by its reduced affinity for the primary receptor IL-1RI and its inability to recruit IL-1RAcP to the preformed IL-1b(D145K)-IL-1RI complex. The reduction in IL-1RI affinity is most likely mediated by the short amino acid extension at the N-terminus of hIL-1b(D145K). Structural data indicate that in wild-type human IL-1β, the N-terminal alanine residue specifically interacts with two amino acids on IL-1RI, while no such interaction could be detected with IL-1RII.[33,34] The reduced IL-1RI binding and preserved IL-1RII binding of hIL-1b(D145K) is therefore consistent with a specific loss of IL-1RI interactions at this amino acid residue. The reduced IL-1RAcP binding is likely a consequence of the D145K mutation, which disrupts a hydrophilic interaction between the aspartate at position 145 in IL-1β and a serine residue at position 185 in IL-1RAcP.[34]

The murine version of the Qβ-hIL-1b(D145K) conjugate vaccine, Qβ-mIL-1b(D143K), induced high titers of IgG autoantibodies against wild-type mouse IL-1β after injection into mice, which could be boosted by additional vaccine injections. These antibodies were neutralizing, as shown by their ability to inhibit the IL-1β-induced secretion of IL-6 from HeLa cells *in vitro* and their ability to neutralize the inflammatory activity of IL-1β *in vivo*. Interestingly, neutralizing titers appeared with a certain delay with respect to the total anti-IL-1β IgG titers, and the ratio between neutralizing and total anti-IL-1β IgG titers increased over time, suggesting the occurrence of affinity maturation. Neutralizing titers were long lasting, but reversible, gradually restoring IL-1β responsiveness in the absence of further vaccine injections. Taken together, these data show that conjugation to Qβ VLPs provides strong foreign Th epitopes to the self antigen IL-1β, which help IL-1β-specific B cells to mount a strong neutralizing IgG antibody response. That the induction of IL-1β-specific IgG antibodies depends on foreign T cell help is clearly shown by the fact that neutralizing titers were not boosted by exogenously administered or endogenously produced IL-1β, but only by additional injections of conjugate vaccine. This notion is further supported by the observation that IL-1β-specific IgG antibodies were induced when a strong Th epitope derived from the glycoprotein of lymphocytic choriomeningitis virus was linked to IL-1β, but not when no such Th epitope was provided. Whereas the conjugate vaccine induced high titers of IL-1β-specific IgG antibodies, it did not induce IL-1β-specific $CD4^+$ T cells, even when a strong adjuvant such as CpG was used. This is an important safety prerequisite for the use of the vaccine in humans, where such T cell responses need to be avoided as they have the potential to cause immunopathology.

Other potential safety concerns for the use of the vaccine in humans are related to the long-term neutralization of IL-1β in humans, which might expose vaccinated individuals to an increased infection risk. Here, we show that vaccination against IL-1β did not cause increased bacterial growth of the intracellular bacterial pathogens *L. monocytogenes* and *M. tuberculosis*. Although it had been demonstrated that the immune response to infection with these pathogens is dependent on IL-1RI signaling, it only recently emerged that IL-1α rather than IL-1β seems to be the main mediator of this response. We have recently shown that neutralization of IL-1α enhanced bacterial growth after infection of mice with *L. monocytogenes* and *M. tuberculosis*, while no such effect could be detected after neutralization of IL-1β.[22]. Our observations are therefore in line with these reports and suggest that the use of the vaccine in humans should not lead to a significant increase in infection rates with these bacteria.

Immunization against IL-1β clearly improved glucose tolerance in a mouse model of diet-induced type 2 diabetes. The improved clearance kinetics in vaccinated animals was due to increased insulin production rather than to improved insulin responsiveness. This indicates that IL-1β mainly affects the pancreas and its ability to produce insulin rather than the ability of the peripheral organs to respond to it. These data are compatible with a recent report using the anti-IL-1β antibody XOMA052, which also detected a major influence on insulin production and only a minor role for improved insulin responsiveness.[6] It has been hypothesized that cytokines other than IL-1β, e.g. TNFα, are stronger drivers of insulin hyporesponsiveness in peripheral tissue.[35] Possibly combined inhibition of IL-1β and TNF may further improve the therapeutic effect in type 2 diabetes, likely however at the cost of increased susceptibility to infection.

Type 2 diabetes is an important disease which is developing into a worldwide pandemic. While the disease was originally largely restricted to western countries, it is now a major problem also in countries not previously adapted to western-style diets. By way of example, type 2 diabetes is currently more prevalent in Mexico than in the United States. These epidemiological facts call for new and affordable medical interventions. As cost of goods for vaccines are a small fraction of those of other biologics, such as monoclonal antibodies, vaccination may be a valuable proposition for affordable treatment of chronic diseases including type 2 diabetes. The here-described vaccination approach may therefore deserve further exploration provided the currently tested monoclonal antibodies deliver solid proof-of-concept.

MATERIALS AND METHODS

Cloning, expression, and purification of hIL-1b(D145K) and mIL-1b(D143K)

For production of the mutein hIL-1b(D145K) the DNA sequence encoding mature human IL-1β was amplified by PCR from human liver cDNA using oligonucleotides 5′-ATATATGATATCCCTGTACGATCACTGAACTGCACG-3′ and 5′-ATATATCTCGAGGGAAGACACAAATTGCATGGTGAAG-3′ (underlined nucleotides indicate *Eco*RV and *Xho*I restriction sites, respectively) and cloned into the expression vector pET42T.[36] For production of mIL-1b(D143K), the DNA sequence encoding mature mouse IL-1β was amplified by PCR from cDNA of TNFα-activated murine macrophages using oligonucleotides 5′-ATATATGATATCCCCATTAGACAGCTGCACTACAGG-3′ and 5′-ATATATCTCGAGGGAAGACACAGATTCCATGGTGAAG-3′ and cloned into the same vector. The resulting plasmids encode the mature forms of human and mouse IL-1β, respectively, fused to a hexahistidine tag and a cysteine containing linker at the C-termini. Due to the introduction of the *Eco*RV restriction site, the Ala and Val residues at the N-termini of mature human and mouse IL-1β, respectively, are substituted by a short N-terminal extension (Met-Asp-Ile) in both fusion proteins. The mutations encoding the D145K and D143K substitutions were finally introduced into the sequences of the human and mouse proteins, respectively, with the Quik-Change Site-directed mutagenesis kit (Agilent Technologies, Basel, Switzerland). *Escherichia coli* BL21 cells were transformed with the resulting plasmids pET42T-hIL-1b(D143K) and pET42T-mIL-1b(D143K), grown at 37 °C to an OD_{600nm} of 0.7–1.0, and protein expression was induced by addition of isopropyl-β-D-thiogalactopyranoside to a final concentration of 1 mmol/l. Growth was continued overnight, bacteria were harvested by centrifugation, and resuspended in buffer A (50 mmol/l NaH_2PO_4, 300 mmol/l NaCl, 20 mmol/l imidazol, and 0.05% Tween-20, pH 8.0). Cells were disrupted by sonication, cellular debris were removed by centrifugation, and the soluble fractions were applied to Ni^{2+}-nitriloacetate agarose columns (Qiagen, Hilden, Germany). After extensive washing with buffer A, bound IL-1b muteins were eluted with buffer B (50 mmol/l NaH_2PO_4, 300 mmol/l NaCl, 250 mmol/l imidazol, and 0.05% Tween-20, pH 8.0). Pooled fractions containing the purified IL-1b muteins were dialysed extensively against PBS pH 7.4.

Determination of hIL-1RI, hIL-1RII, and hIL-1RAcP binding

A recombinant version of wild type human IL-1β carrying a C-terminal hexahistidine tag and additional cysteine residue (hIL-1β-His-C) was site specifically biotinylated at its free cysteine residue using an EZ-link maleimide-PEG_{11}-biotinylation kit (Thermo Fisher Scientific, Lausanne, Switzerland). Biotinylated hIL-1β-His-C bound with the same affinity as wild-type human hIL-1β (R&D Systems, Abingdon, UK) to immobilized recombinant human IL-1RI and IL-RII Fc chimeras (R&D Systems), as shown by Sandwich ELISA using polyclonal goat antihuman IL-1β IgG (R&D Systems) for detection (data not shown). For determination of binding affinities, serial dilutions of nonlabeled wild-type human IL-1β or hIL-1b(D145K) were premixed with a constant amount of 20 ng/ml (~1 nmol/l) of biotinylated hIL-1β-His-C and then transferred to ELISA plates (Nunc Maxisorp, eBioscience, Vienna, Austria) that had been coated with 1 µg/ml of human IL-1RI or human IL-RII Fc chimeras (R&D Systems). Bound biotinylated hIL-1β-His-C was detected with horseradish peroxidase–conjugated streptavidin. Binding curves were fitted by four-parameter logistic equations using GraphPad Prism (GraphPad Software, La Jolla, CA), and inhibition data were reciprocally transformed to express % receptor binding of the respective nonbiotinylated protein. For determination of hIL-1RAcP binding, ELISA plates were coated with 1 µg/ml of human IL-1RI and incubated with 0.4 µg/ml wild-type human IL-1β or 100 µg/ml hIL-1b(D145K) that had each been premixed with 1 µg/ml of hIL-1RAcP-human Fc chimera (R&D Systems). The concentrations of wild-type human IL-1β and hIL-1b(D145K) were chosen because based on the results of the IL-1RI binding assay, they were predicted to lead to saturation of all available hIL-1RI binding sites. Formation of the ternary complex consisting of hIL-1RI, hIL-1β wild type or hIL-1b(D145K), and hIL-1RAcP was detected by a polyclonal goat anti-hIL-1RAcP antibody and a polyclonal horseradish peroxidase-conjugated rabbit antigoat IgG antibody.

IL-1β-induced IL-6 secretion by HeLa cells

HeLa cells (in complete minimal essential medium containing 10% fetal bovine serum) were seeded on 96-well cell culture plates at a density of 6×10^4 cells per well and incubated with serial dilutions of human wild-type IL-1β or hIL-1b(D145K) for 4 hours at 37 °C. For neutralization assays, serial dilutions of immune sera were preincubated in complete minimal essential medium containing 10% fetal bovine serum for 1 hour at room temperature with a constant amount of 10 ng/ml (~0.6 nmol/l) of wild-type mouse IL-1β (Peprotech, London, UK) and then incubated for 4 hours with the seeded cells. For determination of IL-6 concentrations, cell culture supernatants were diluted 1:1 in PBS/0.05% Tween-20/2% bovine serum albumin and transferred to ELISA plates that had been coated with a mouse anti-human IL-6 capture mAb (R&D Systems) at a concentration of 1 µg/ml. Bound human IL-6 was detected with a biotinylated goat anti-human IL-6 mAb (R&D Systems) and horseradish peroxidase-conjugated streptavidin. Recombinant human IL-6 (R&D Systems) was used as a standard. Curves were fitted with four-parameter logistic equations using GraphPad Prism. Biological activities of wild-type human IL-1β and hIL-1b(D145K) were expressed as ED_{50} and neutralizing titers of sera as the reciprocal of those serum dilutions that lead to half maximal inhibition of hIL-1β activity.

IL-1β-induced cytopathic effect on A375 cells

A375 cells (in complete minimal essential medium containing 10% fetal calf serum) were seeded on 96-well cell culture plates (2,000 per well) and incubated with titrating amounts of either wild-type human IL-1β or hIL-1b(D145K) for 7 days. Plates were washed with PBS, and viable adherent cells were stained with a solution of 0.5% crystal violet in 20% methanol. After 30 minutes, cells were resuspended in 100% ethanol, and cell densities were quantified by measuring optical densities at 600 nm. Curves were fitted with four-parameter logistic equations, and activities of proteins were expressed as ED_{50}.

Vaccine production

Qβ VLPs were produced as described[37] and reacted with a 2.5-fold molar excess of the heterobifunctional cross-linker succinimidyl-6-(β-maleimidopropionamido)hexanoate (Thermo Fisher Scientific). Unreacted cross-linker was removed by passage over a PD-10 desalting column (GE Healthcare, Glattbrugg, Switzerland). The mIL-1b(D143K) and hIL-1b(D145K) muteins were incubated for 30 minutes at room temperature with a five-fold molar excess of tri(2-carboxyethyl)phosphine hydrochloride to reduce the cysteine residue contained in the linker. The reduced proteins were then mixed with the derivatized Qβ VLPs at a molar ratio of Qβ monomer to IL-1β mutein of 1:1 and incubated for 1 hour at room temperature to allow cross-linking. Unreacted maleimide groups in the cross-linker were blocked by the addition of a 10-fold molar excess of L-cysteine. Free IL-1β muteins were removed by extensive dialysis against PBS, pH 7.4, using cellulose ester membranes with a cutoff of 300 kDa (SpectrumLabs, Breda, The Netherlands).

Immunizations and analysis of antibody induction

Qβ-mIL-1b(D143K) and Qβ control VLPs were diluted in PBS, pH 7.4, to 200 µl and injected s.c. at two ventral sites (100 µl each). Sera were withdrawn at the indicated time points, serially diluted in PBS containing 0.05% Tween and 2% bovine serum albumin, and applied to ELISA plates that had been coated with 1 µg/ml wild-type mouse IL-1β (Peprotech) or 1 µg/ml Qβ VLPs. Bound antibodies were detected by incubation with a horseradish peroxidase-conjugated goat anti-mouse IgG secondary antibody. Optical densities were determined using an ELISA reader (Biorad, Cressier, Switzerland), and titers were expressed as the reciprocal of those serum dilutions that lead to half-maximal OD at 450 nm (OD50%). For challenge experiments, mice were injected i.p. with the indicated proteins, and serum IL-6 was measured 3 hours after injection with a mouse IL-6 Quantikine ELISA Kit (R&D Systems).

Assessment of IL-1β-specific T cell responses

For cloning of the mIL-1b(D143K)-p13 fusion protein, two complementary oligonucleotides (5′-TCGAGTACGGTCTTAACGGTCCTGACATCTACAAAGGTG TTTACCAGTTCAAATCTGTTGAATTCGACC-3′ and 5′-TCGAGGTCGAATTCAAC AGATTTGAACTGGTAAACACCTTTGTAGATGTCAGGACCGTTAAGACCGT AC-3′) encoding the gp61-80 (p13) peptide derived from the glycoprotein of lymphocytic choriomeningitis virus (GLNGPDIYKGVYQFKSVEFD) were annealed and cloned into the *Xho*I site in pET42T-hIL-1b(D143K) located between the mutated IL-1β coding sequence and sequence encoding the hexahistidine tag. The mIL-1b(D143K)-p13 fusion protein was expressed, purified, and conjugated to Qβ VLPs as described above. In a first experiment, two groups of C57BL/6 mice (n = 3 each) were immunized s.c. on days 0, 7, and 13 with 300 µg of Qβ-mIL-1b(D143K)-p13 mixed with either 20 µg CpG or Alum. A third group of C57BL/6 mice (n = 3) was kept untreated. On day 20, splenocytes from all mice were isolated and stimulated with bone marrow derived dendritic cells loaded with 5 µmol/l synthetic p13 peptide (Eurogentec, Hestal, Belgium) or 2 µg/ml recombinant wild-type mouse IL-1β, respectively. Nonspecific IFN-γ release from $CD4^+$ T cells was determined by incubation of splenocytes with mock-pulsed bone marrow derived dendritic cells. Antigen-specific IFNγ-producing $CD4^+$ T cells were determined by fluorescence-activated cell sorting. In a second experiment, groups of female C57BL/6 mice were immunized s.c. on days 0 and 14 with 2.5 µg of wild-type mouse IL-1β mixed with IFA (n = 4) or 2.5 µg of the fusion protein mIL-1b(D143K)-p13 mixed with IFA (n = 8). One subgroup of the mice that had been injected with mIL-1b(D143K)-p13 (n = 4) received repeated injections of a depleting anti-CD4 monoclonal antibody (200 µg/mouse on days −3 and −1 and 100 µg/mouse on days 3, 6, 10, 13, and 17). Mouse IL-1β-specific IgG antibody titers were analyzed on day 32 by ELISA.

M. tuberculosis infection model

Groups of C57BL/6 male mice (n = 5) were immunized s.c. with either Qβ-mIL-1b(D143K) or Qβ VLPs three times before (weeks −5, −3, and −1) and once after (week 10) aerosol infection with 100 colony-forming units/lung of *M. tuberculosis* H37Rv (week 0). Body weights of infected mice were measured weekly over a period of 36 weeks. At the end of the experiment, lungs and spleens of infected mice were removed and homogenized in 0.04% Tween 80. Ten-fold dilutions of the homogenized tissues were plated in duplicate onto Difco Middlebrook 7H10 Agar (BD Biosciences) plates supplemented with 10% Dubos oleic albumin complex medium (BD Biosciences) and 0.5% glycerol and incubated at 37 °C. After 21 days, bacterial colonies were counted.

Listeria monocytogenes infection model

Groups of female C57BL/6 mice (n = 5) were immunized s.c. on days 1 and 25 with 50 µg of Qβ-mIL-1b(D143K) or Qβ and infected on day 47 by

an i.p. injection with 10,000 colony-forming units of *L. monocytogenes*. Three days later, mice were sacrificed, and bacterial load was determined by plating serial dilutions of spleen homogenates on tryptic soy agar plates and counting the bacterial colonies after 24 hours of incubation at 37 °C.

Diet-induced diabetes model

Groups of male C57/BL6 mice ($n = 8$) were fed a high-fat diet (#2127, Provimi Kliba, Kaiseraugst, Switzerland) and immunized five times (days 0, 14, 28, 42, and 147) with 50 µg of either Qβ-mIL-1b(D143K) or Qβ VLPs. As controls, one Qβ-mIL-1b(D143K)- and one Qβ-immunized group ($n = 8$ each) were maintained on normal chow. Individual body weights were recorded three times a week over the entire observation period. Oral glucose tolerance tests were performed at different time points by applying a solution of 0.2 g glucose in water intragastrically by oral gavage to mice that had been fasted for 5 hours and determining glucose concentrations in blood samples drawn from the tail vein before and 15, 30, 45, 60, 90, and 120 minutes after application using AccuChek (Roche Diagnostics, Rotkreuz, Switzerland). Glucose-induced insulin secretion was measured in blood samples drawn before and 15 minutes after glucose application using an ELISA kit (Mercodia, Uppsala, Sweden). For insulin tolerance tests, mice were fasted for 5 hours, and 0.5 mU/g insulin (from porcine pancreas, Sigma-Aldrich, Buchs, Switzerland) was injected i.p., followed by blood glucose concentration measurements as above.

Statistical Analysis

Differences between groups were evaluated by parametric Student's *t*-test. Group means were considered to be statistically different when two-tailed *P* values were <0.05.

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CONFLICT OF INTEREST

G.S., C.S., I.K., K.S., C.S., K.S., G.T.J., and M.F.B. are former employees and hold stock or stock options from Cytos Biotechnology. The other authors declare no conflict of interest.

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