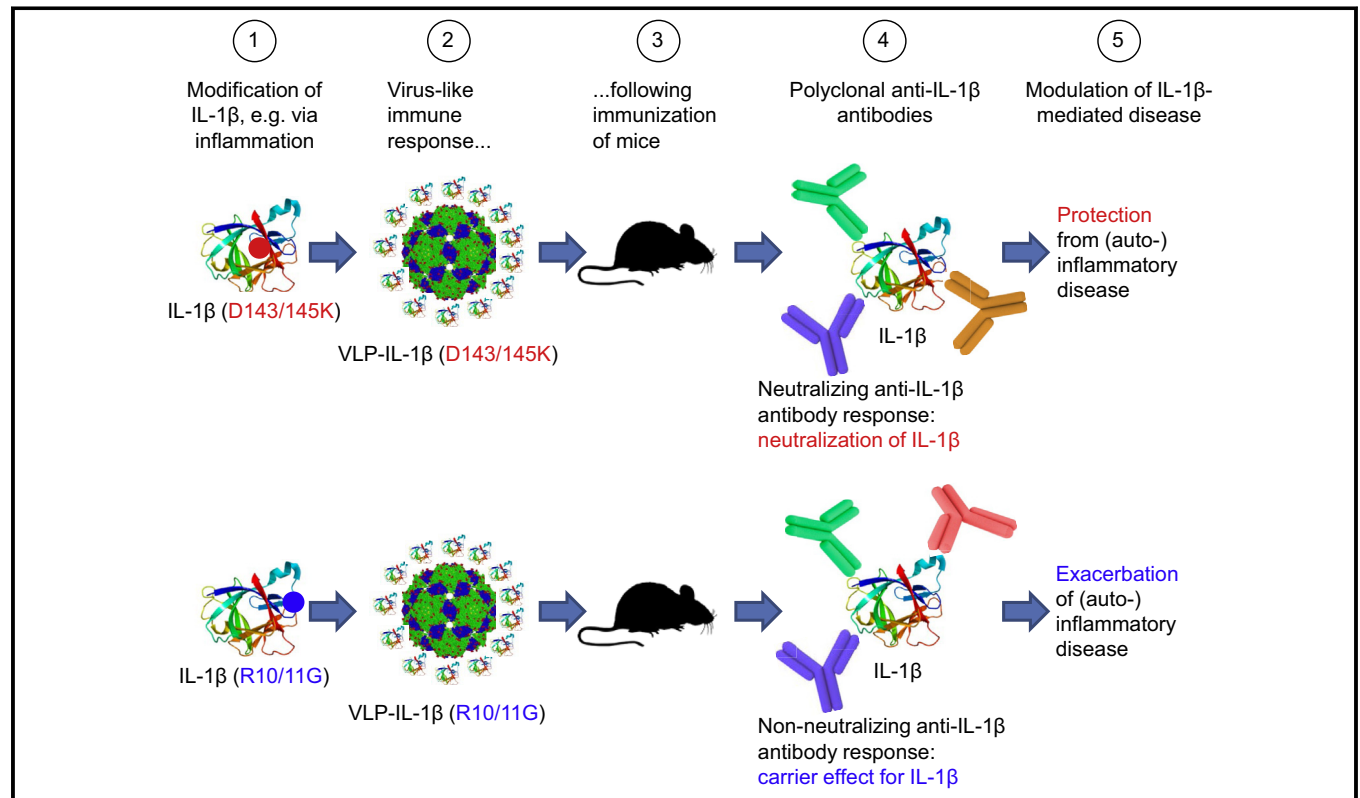


Endogenous polyclonal anti-IL-1 antibody responses potentiate IL-1 activity during pathogenic inflammation

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GRAPHICAL ABSTRACT



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Background: Particular neutralizing mAbs to certain cytokines act as agonists *in vivo* through protection of the cytokine's active site and prolongation of its half-life. Although this principle might be useful for targeted immunotherapy, its role in the pathogenesis of inflammation and autoimmunity is unclear.

Objective: We sought to determine whether slight, structurally nonrelevant modifications of the prototypic proinflammatory cytokine IL-1 β during an immune response could elicit polyclonal anti-IL-1 β antibody responses that modulated IL-1 β 's *in vivo* activity.

Methods: We engineered 2 different IL-1 β variants, thereby mimicking the process of cytokine modification occurring during inflammation, and conjugated them to virus-like particles, followed by immunization of mice. The resulting polyclonal anti-IL-1 β antibody responses were assessed by using *in vitro* and *in vivo* assays, as well as 2 relevant (auto-) inflammatory murine models.

Results: Although antibody responses generated to one variant were potently inhibiting IL-1 β , antibody responses induced by the other variant even potentiated the *in vivo* effects of IL-1 β ; the latter led to enhanced morbidity in 2 different IL-1 β -mediated mouse models, including a model of inflammatory bowel disease and an inflammatory arthritis model.

Conclusion: These data demonstrate that endogenous polyclonal anti-cytokine antibody responses can enhance the cytokine's activity in inflammatory and autoimmune diseases. (J Allergy Clin Immunol 2016;■■■:■■■-■■■.)

Key words: Cytokine, IL-1 β , antibody, autoantibody, inflammation, inflammatory disease, autoinflammatory disease

Autoantibodies to cytokines have been described in various pathologies, including autoimmune diseases with or without susceptibility to infections, chronic inflammatory disorders, and cancer.¹ Neutralizing autoantibodies against GM-CSF have been found in the serum of patients with pulmonary alveolar proteinosis^{2,3} and/or increased susceptibility to cryptococcal meningitis.⁴ Moreover, neutralizing anti-erythropoietin autoantibodies have been associated with pure red cell aplasia, even in subjects who never received recombinant erythropoietin.^{5,6} Neutropenia in patients with rheumatoid arthritis with Felty syndrome has been linked to IgG autoantibodies targeting granulocyte colony-stimulating factor.⁷ Furthermore, patients with rheumatoid arthritis often have neutralizing autoantibodies against TNF- α and IL-1 α .^{8,9} Conditions with increased susceptibility to certain opportunistic pathogens, particularly disseminated nontuberculous mycobacteria, have been shown in adults with circulating anti-IFN- γ autoantibodies.¹⁰⁻¹⁵ Also, chronic mucocutaneous candidiasis in patients with autoimmune polyendocrinopathy syndrome type 1 has been associated with high titers of neutralizing autoantibodies to IL-17 and IL-22, both of which are T_H17-derived cytokines important for immune defense against *Candida* species.^{16,17} Similarly, recurrent staphylococcal cellulitis has been observed in subjects with neutralizing anti-IL-6 autoantibodies.¹⁸ Collectively, most of the autoantibodies associated with the abovementioned pathologies appear to be neutralizing and of the IgG isotype.¹

Healthy subjects can also harbor autoantibodies against cytokines, including GM-CSF, IFN- α , IFN- β , IFN- γ , IL-1 α , IL-2, IL-6, IL-10, and TNF- α .^{1,19-24} However, it is unclear what role anti-cytokine autoantibodies play in apparently healthy subjects and whether and to what extent such autoantibodies predispose to or precipitate certain pathologies. The function of such anti-cytokine antibodies might depend on antibody isotype or IgG subclass, as described for certain disease-related autoantibodies.²⁵ Notably, infections have been reported to lead to considerable titers of anti-cytokine antibodies. Thus some subjects with clinically manifest infection with HIV, a herpesvirus (including EBV, cytomegalovirus, herpes simplex virus, and varicella-zoster virus), measles virus, mumps virus, rubella virus, or an adenovirus were found to harbor high levels of antibodies to IFN- α , IFN- γ , or IL-2.^{22,26,27} Moreover, anti-TNF- α autoantibodies have been shown to arise in human subjects during gram-negative bacterial infections.²⁸ Such pathogen-driven anti-cytokine antibody responses can be of the IgG or IgM isotype and are usually greatest during infection, followed by a decrease, reaching normal levels within a few weeks to months.^{22,28} Interestingly, at least for autoantibodies to IFN- γ , such antibodies

Abbreviations used

DSS:	Dextran sulfate sodium
hIL-1 β :	Human IL-1 β
hIL-1b(D145K):	Human IL-1b carrying D145K point mutation
hIL-1b(R11G):	Human IL-1b carrying R11G point mutation
IL-1b:	IL-1 β with an N-terminal 3-amino-acid extension and a C-terminal Cys-containing linker
IL-1R:	IL-1 receptor
IL-2R:	IL-2 receptor
mIL-1 β :	Mouse IL-1 β
mIL-1b(D143K):	Mouse IL-1b carrying D143K point mutation
mIL-1b(R10G):	Mouse IL-1b carrying R10G point mutation
Q β -hIL-1b(D145K):	Human IL-1b carrying D145K point mutation and conjugated to Q β -VLP
Q β -hIL-1b(R11G):	Human IL-1b carrying R11G point mutation and conjugated to Q β -VLP
Q β -mIL-1b(D143K):	Mouse IL-1b carrying D143K point mutation and conjugated to Q β -VLP
Q β -mIL-1b(R10G):	Mouse IL-1b carrying R10G point mutation and conjugated to Q β -VLP
VLP:	Virus-like particle

were found to be nonneutralizing.²² Altogether, these data suggest that infections can either boost pre-existing clones or lead to *de novo* generation of anti-cytokine autoantibodies, which subsequently can bind and modulate the targeted cytokine's activity. Such phenomena have been shown for different cytokines, including IL-2, IL-4, IL-6, IL-7, and granulocyte colony-stimulating factor, by using a neutralizing mAb against the cytokine, which together form agonistic cytokine/anti-cytokine mAb complexes that potentiate the cytokine's *in vivo* activity.²⁹⁻³⁵

Many inflammatory and autoimmune disorders have been linked to pathogens. Therefore we hypothesized that anti-cytokine antibodies arising during infections might affect the susceptibility toward or the disease course of such pathologies. To study this hypothesis without having to account for confounding effects because of infection, we used replication-deficient virus-like particles (VLPs) of the bacteriophage Q β chemically linked to engineered versions of IL-1 β . We chose IL-1 β for its known properties as a proinflammatory cytokine³⁶ and generated IL-1 β variants to mimic cytokine modification, such as increased or reduced glycosylation, which can occur during inflammation.³⁷ Immunization of mice with Q β displaying IL-1 β variants induced high titers of polyclonal anti-IL-1 β antibodies. However, one of the 2 prototypic variants induced neutralizing antibodies, whereas the other variant led to production of polyclonal antibody responses that enhanced the *in vivo* activity of IL-1 β and exacerbated pathology in mouse models of inflammatory disorders.

METHODS

Construction, expression, and purification of IL-1 β variants

The DNA sequence encoding mature human IL-1 β (hIL-1 β) was amplified by means of PCR from human liver cDNA and cloned into expression vector pET42T.³⁸ For the murine version, the DNA sequence encoding mature mouse IL-1 β (mIL-1 β) was amplified by means of PCR from cDNA of TNF- α -activated murine macrophages and cloned into pET42T. As a consequence of the cloning procedure, the Ala and Val residues at the N-termini of IL-1 β were substituted by a short N-terminal extension (Met-Asp-Ile), and the

C-termini were fused to a hexahistidine tag and cysteine containing linker; the resulting protein was termed IL-1b. The Quik-Change Site-directed Mutagenesis Kit (Stratagene, La Jolla, Calif) was used to introduce site-specific mutations into these constructs to yield expression vectors for the hIL-1 β variants D145K and R11G and their mouse counterparts D143K and R10G. *Escherichia coli* BL21 cells harboring these expression plasmids were used to express the recombinant IL-1 β variants, followed by purification from soluble fraction by using affinity chromatography.

Production of cytokine conjugates

Q β -VLPs were produced, as previously described.³⁹ IL-1 β variants were incubated for 30 minutes at room temperature with a 5-fold molar excess of tri(2-carboxyethyl)phosphine hydrochloride to reduce the cysteine residue of the linker. The reduced proteins were then mixed with the derivatized Q β -VLPs at a molar ratio of Q β monomer to IL-1b protein of 1:1 (2:1 for human IL-1 β carrying the R11G point mutation [hIL-1b[R11G]] and mouse IL-1 β carrying the R10G point mutation [mIL-1b[R10G]]), and incubated for 1 hour at room temperature to allow cross-linking. Free IL-1b protein was removed by means of extensive dialysis against PBS (pH 7.4) by using cellulose ester membranes with a cutoff of 300 kDa (Spectrum Laboratories, Rancho Dominguez, Calif).

Production of gevokizumab

The light chain and γ 2 version of the heavy chain of gevokizumab were cloned into the expression vector pCB15. The resulting plasmid was transfected into HEK-293T cells by using Lipofectamin Plus (Invitrogen, Carlsbad, Calif), and antibody-expressing cells were enriched by means of selection in the presence of puromycin. Gevokizumab was purified from cell-culture supernatants by means of affinity chromatography on protein G Sepharose columns.

Inhibition of IL-1 β -induced IL-6 secretion

HeLa cells were seeded in 96-well cell-culture plates at a density of 4.5 to 6 $\times$ 10⁴ cells/well. Serial dilutions of immune sera were preincubated in complete MEM medium containing 10% FBS for 1 hour at room temperature with a constant amount of 200 pg/mL (approximately 10 pmol/L) wild-type hIL-1 β (R&D Systems, Minneapolis, Minn) and then incubated for 4 hours with the seeded cells. IL-6 concentrations in cell-culture supernatants were then determined, as previously described.⁴⁰ Neutralizing titers of sera were expressed as the reciprocal of those serum dilutions that led to half-maximal inhibition of hIL-1 β activity.

Immunization and analysis of antibody responses

All VLP-IL-1b conjugates were diluted in PBS (pH 7.4) to 200 μ L and injected subcutaneously at 2 ventral sites (100 μ L each) without additional adjuvants. Sera were obtained at the indicated time points, serially diluted in PBS containing 0.05% Tween and 2% BSA, and assessed by using ELISA plates coated with 1 μ g/mL wild-type hIL-1 β (PeproTech, Rocky Hills, NJ). For determination of antibody specificities, sera were applied either to hIL-1 β -His-C captured on nickel-coated plates (His-Grab; Pierce, Rockford, Ill)⁴⁰ or wild-type hIL-1 β captured on ELISA plates coated with 1 μ g/mL gevokizumab. Antibodies were detected by means of incubation with horseradish peroxidase-conjugated goat anti-mouse IgG antibody, as previously described.⁴¹ ODs were determined by using an ELISA reader (Bio-Rad Laboratories, Hercules, Calif). Titers were expressed as the reciprocal of those serum dilutions that lead to half-maximal OD at 450 nm.

Challenge and measurement of IL-6 and hIL-1 β levels

Wild-type hIL-1 β was injected intraperitoneally, and serum IL-6 levels were measured 3 and 6 hours after injection by using a mouse IL-6 Quantikine ELISA kit (R&D Systems). For determination of hIL-1 β concentrations, sera were serially diluted in PBS/0.05% Tween-20/2% BSA and applied to ELISA plates that had been coated with a mouse anti-hIL-1 β IgG₁ mAb (R&D Systems). Bound hIL-1 β was detected with biotinylated polyclonal goat anti-hIL-1 β IgG (R&D Systems). Recombinant hIL-1 β (PeproTech) served as a standard.

Collagen-induced arthritis model

Groups of male 8-week-old DBA/1 mice (n = 8) were immunized subcutaneously 3 times at biweekly intervals with 50 μ g of the indicated Q β -VLP-mIL-1b conjugates or Q β -VLPs as a control. One week after the last immunization (day 35), all mice were injected intradermally with 200 μ g of collagen in complete Freund adjuvant, followed by a second injection of the same amount of collagen in incomplete Freund adjuvant 3 weeks later (day 56). Thereafter, body weights were recorded, mice were examined daily in a blind and randomized fashion for signs of arthritis, and a clinical score was assigned to each limb, as previously described.⁴²

Colitis model

Groups of 6- to 8-week old C57BL/6 mice (n = 5) were immunized subcutaneously 3 times at biweekly intervals with 10 to 25 μ g of the different Q β -VLP-mIL-1b conjugates or Q β -VLPs as a control. One week after the last immunization, mice received 3% dextran sulfate sodium (DSS) in their drinking water for 7 days. To follow colitis development, DSS-treated and control mice were weighed every other day from day 0 to day 5 and then daily from day 5 to day 9 in a blinded and randomized fashion. For assessment of the severity of colitis, histologic slides were prepared, as previously established,⁴³ and average histologic scores in the proximal, middle, and distal portions of hematoxylin and eosin-stained colons of mice on day 9 of DSS treatment were determined in a blinded and randomized fashion, as published.⁴⁴

Statistics

P values were calculated as indicated by using either 2-way ANOVA with the Bonferroni multiple comparison test or 1-way ANOVA with the Tukey multiple comparison test. The expected variance was similar between the groups that were compared.

RESULTS

Generation of polyclonal anti-IL-1 β antibody responses in mice

To produce a form of IL-1 β suitable for generation of polyclonal antibody responses in mice, we modified wild-type hIL-1 β to comprise both an N-terminal extension of 3 amino acids and a C-terminal Cys-containing linker and designated the resulting cytokine hIL-1b. The N-terminal extension of hIL-1b reduced the overall inflammatory activity of the cytokine, thereby allowing the administration of immunogenic doses in the microgram range without inducing strong systemic inflammatory responses (G.S., unpublished data). The C-terminal Cys-containing linker was introduced to facilitate chemical conjugation to VLPs of the bacteriophage Q β .

We then generated different variants of hIL-1b by introducing site-specific mutations to mimic modifications occurring during inflammatory responses and possibly leading to alterations or masking of certain cytokine sites.³⁷ For generating these hIL-1b variants, we selected amino acid substitutions that have been described in the literature to result in reduced IL-1 β bioactivity without affecting the overall tertiary structure (and thus antigenicity) of the cytokine.⁴⁵⁻⁴⁸ Interestingly, of these hIL-1b variants, hIL-1b carrying R11G point mutation (hIL-1b[R11G]) and hIL-1b(D145K) (Fig 1, A) elicited antibody responses with opposing *in vitro* and *in vivo* effects, leading us to further investigate the antibody responses generated on immunization with these 2 IL-1 β muteins. Receptor-binding assays confirmed that the mutations R11G and D145K had no marked effect on binding to either IL-1 receptor (IL-1R) I or IL-1RII (see Fig E1, A and B, in this article's Online Repository at www.jacionline.org). As

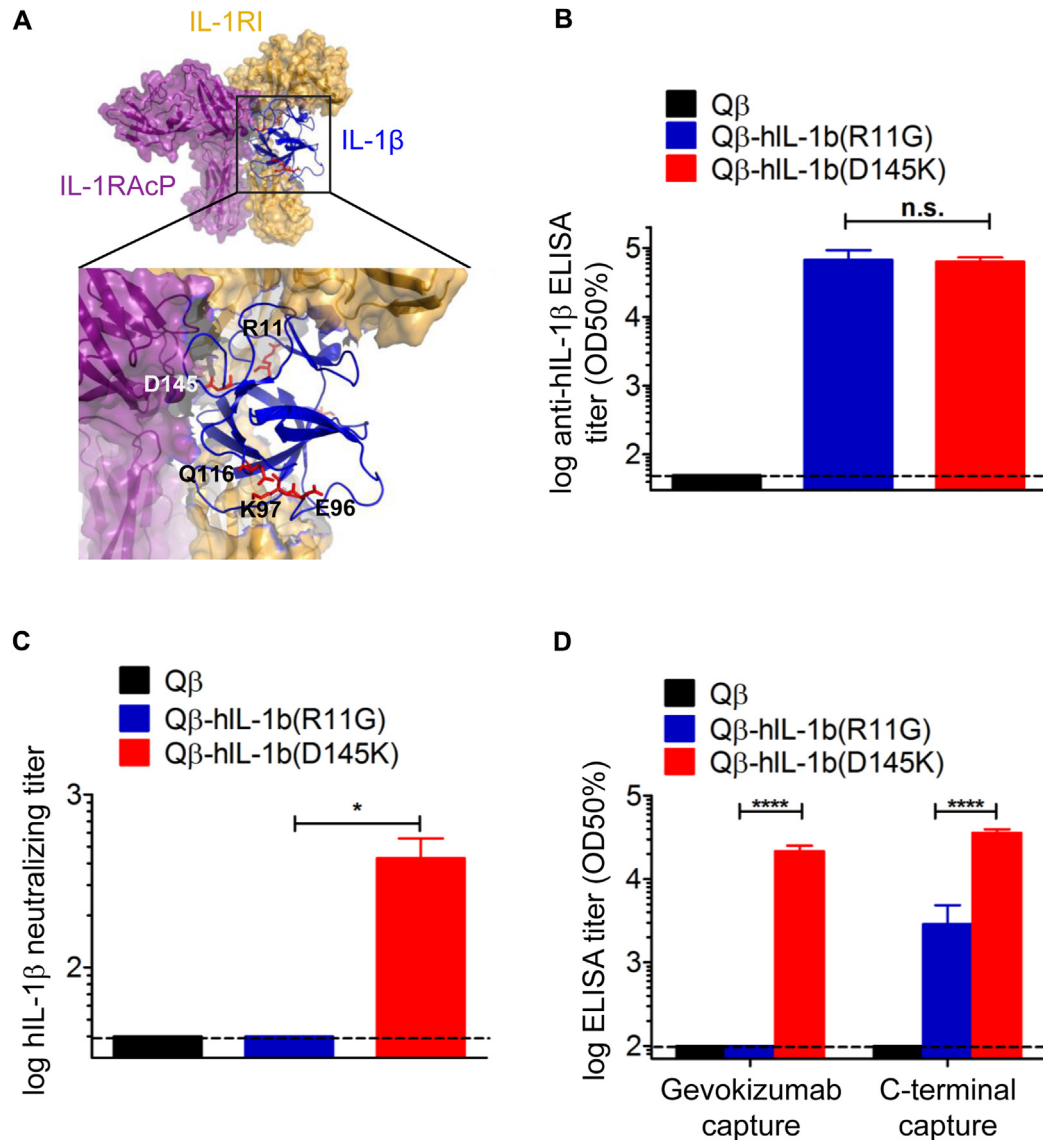


FIG 1. Generation of different endogenous polyclonal anti-IL-1 β antibody responses. **A**, Three-dimensional structure of the IL-1 β -IL-1RI complex (accession code 3O4O),⁴⁸ showing the different IL-1R interaction sites of IL-1 β . **B**, Groups of C57BL/6 mice ($n = 4$) were immunized on days 0, 14, and 28 with 25 μ g of Q β -hIL-1b(R11G), Q β -hIL-1b(D145K), or Q β -VLPs as a control. Wild-type (WT) hIL-1 β -specific total IgG ELISA titers were measured in sera collected 25 days after the last immunization. **C**, Neutralizing antibody titers were determined by incubating HeLa cells with serial dilutions of the same sera as in Fig 1, B, which had been premixed with a constant amount of WT hIL-1 β . **D**, Serial dilutions of the same sera as in Fig 1, B, were applied to hIL-1 β -His-C captured on Ni²⁺-coated plates (C-terminal capture) or to WT hIL-1 β captured on gevokizumab-coated ELISA plates. Shown are group means $\pm$ SEMs from one of 2 experiments with similar results. Dashed lines indicate detection limits. P values were determined by using 1-way ANOVA with the Tukey multiple comparison test. n.s., Not significant. * $P < .05$ and **** $P < .0001$.

expected, the inflammatory activity of hIL-1b was strongly reduced by the introduced mutations, indicating that functionally important sites of hIL-1b had been mutated (see Fig E1, C and D).

We conjugated each hIL-1b variant to Q β -VLPs and subcutaneously immunized C57BL/6 mice, leading to high titers of IgG antibodies recognizing wild-type hIL-1 β independently of the introduced mutation (Fig 1, B). However, when assessing the *in vitro* neutralizing capacity of the induced antibodies using inhibition of hIL-1 β -induced release of IL-6 from HeLa cells, we observed a striking difference between the 2 polyclonal antibody responses. Whereas immunization with hIL-1 β

carrying the D145K point mutation and conjugated to Q β -VLP (Q β -hIL-1b[D145K]) led to antibodies with strong neutralizing activities, Q β -hIL-1b(R11G) did not elicit any such response (Fig 1, C).

We investigated whether alterations in the specificities of the induced antibodies could account for the different neutralizing capacities. We performed sandwich ELISAs in which hIL-1 β was captured either through its C-terminus or through the mAb gevokizumab, which binds with very high affinity to an epitope comprising the amino acids E96, K97, and Q116.⁴⁹ In the first case the region opposite of the C-terminus of hIL-1 β is

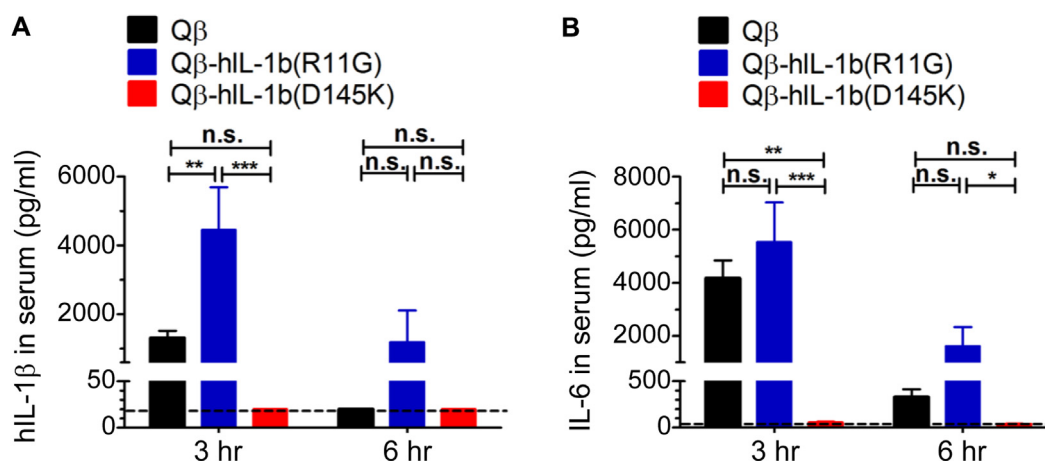


FIG 2. Modulation of hIL-1 β activity by Q β -IL-1 β mutein-induced antibodies. Groups of C57BL/6 mice ($n = 4$) were immunized as in Fig 1, followed by an intraperitoneal injection of 1 μ g of WT hIL-1 β . Serum levels of human IL-1 β (A) and mouse IL-6 (B) were measured 3 and 6 hours after the challenge. Shown are group means $\pm$ SEMs of pooled data from 2 experiments performed under the same conditions. Dashed lines indicate detection limits of IL-1 β (20 pg/mL) and IL-6 (40 pg/mL) quantification assays. P values were determined by using 1-way ANOVA with the Tukey multiple comparison test. n.s., Not significant. * $P < .05$, ** $P < .01$, and *** $P < .001$.

preferentially exposed, whereas in the second case the area surrounding the C-terminus and the nearby N-terminus is accessible. Serum antibodies elicited by Q β -hIL-1b(R11G) and Q β -hIL-1b(D145K) were able to recognize hIL-1 β when captured through its C-terminus (Fig 1, D), confirming the results of the direct ELISA (Fig 1, B) and demonstrating that both muteins induced antibodies that associated with hIL-1 β in its native conformation. Interestingly, antibodies induced by Q β -hIL-1b(D145K) were able to bind to gevokizumab-captured hIL-1 β , whereas no such binding was observed for Q β -hIL-1b(R11G)-induced antibodies. These results suggest that antibodies induced by Q β -hIL-1b(D145K) are able to bind to epitopes outside gevokizumab's epitope, whereas antibodies elicited by Q β -hIL-1b(R11G) preferentially recognize epitopes that overlap with gevokizumab's binding site; the latter was further supported by a competitive binding assay in which gevokizumab was able to compete with Q β -hIL-1b(R11G)-induced antibodies but not with Q β -hIL-1b(D145K)-induced antibodies (see Fig E2 in this article's Online Repository at www.jacionline.org). Thus we consider the alternative possibility of gevokizumab influencing allosterically the binding sites of the antibodies elicited by Q β -hIL-1b(R11G) less likely, although we cannot entirely rule out this possibility without structural data at hand.

While the N-terminus of hIL-1 β is strongly involved in interactions with the IL-1Rs, the epitope recognized by gevokizumab locates to a region of hIL-1 β that is not directly involved in interaction with either IL-1R or the accessory protein IL-1RAcP (Fig 1, A).^{48,49} Therefore the specificity of the antibodies induced by Q β -hIL-1b(R11G) for this region might explain the lack of neutralizing ability.

***In vivo* properties of anti-hIL-1 β antibodies**

To explore the *in vivo* properties of the 2 types of polyclonal anti-hIL-1 β antibody responses, we investigated their influence on the pharmacokinetic properties of IL-1 β *in vivo*. To this end, mice were immunized with the 2 Q β -VLP-conjugated hIL-1b

variants, followed by injection of 1 μ g of wild-type hIL-1 β and quantification of hIL-1 β in serum of these mice 3 and 6 hours later. In Q β -VLP-immunized control mice approximately 1100 pg/mL hIL-1 β was detected 3 hours after injection, whereas 6 hours after injection, hIL-1 β levels were less than the detection limit of 20 pg/mL (Fig 2, A). In Q β -hIL-1b(D145K)-immunized mice hIL-1 β levels were less than the detection limit at both time points, indicating efficient neutralization of the cytokine by the induced anti-hIL-1 β antibody response. Conversely, Q β -hIL-1b(R11G)-immunized mice showed increased hIL-1 β concentrations at both time points, which were significantly higher at 3 hours compared with those in control animals (Fig 2, A). The extended bioavailability of hIL-1 β in these mice translated into increased hIL-1 β activity, as evidenced by a tendency toward enhanced serum IL-6 levels in Q β -hIL-1b(R11G)-immunized mice (Fig 2, B). This effect was not due to an antibody-mediated increase in the cytokine's signal strength at the receptor level but merely the result of its increased systemic exposure. Accordingly, the addition of serum from Q β -hIL-1b(R11G)-immunized mice did not increase the IL-1 β -induced secretion of IL-6 by HeLa cells, nor did it neutralize the effect of IL-1 β (Fig 3, A). Serum from Q β -hIL-1b(D145K)-immunized mice, on the other hand, potently inhibited IL-1 β -mediated IL-6 secretion by HeLa cells, confirming the neutralizing ability of the antibodies induced by Q β -hIL-1b(D145K) (Fig 3, A). Thus hIL-1b(D145K) elicits an *in vivo* neutralizing antibody response ("neutralizer"), whereas antibodies generated after immunization with hIL-1b(R11G) appear to act as carriers of hIL-1 β , thereby prolonging the cytokine's half-life and bioavailability ("enhancer").

Modulation of endogenous IL-1 β by specific antibodies

To investigate the functional consequence of the different endogenous polyclonal anti-IL-1 β antibody responses, we generated the murine analogs of hIL-1b(D145K) and hIL-1b(R11G) termed mouse IL-1b carrying D143K point

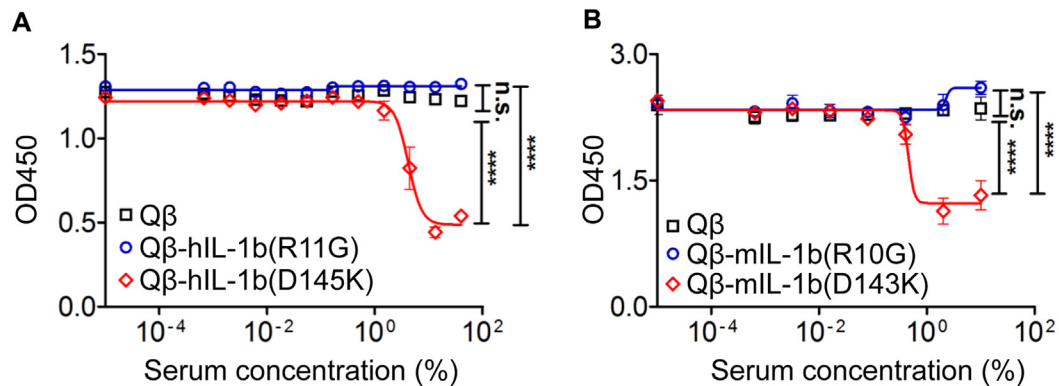


FIG 3. Q β -IL-1 β mutein-induced antibodies show different IL-1 β neutralizing abilities. Groups of C57BL/6 mice ($n = 2$ -3) were immunized on days 0, 14, and 28 with 10 to 15 μ g of the indicated Q β -hIL-1 β mutein conjugates (**A**) or Q β -mIL-1 β mutein conjugates (**B**) or Q β -VLPs as a control. Neutralizing activities of sera from immunized mice were determined by their ability to inhibit hIL-1 β (Fig 3, A) or mIL-1 β (Fig 3, B)-stimulated IL-6 release by HeLa cells. Shown are group means $\pm$ SEMs of 3 independent experiments. P values were determined by using 2-way ANOVA with Bonferroni posttest correction. n.s., Not significant. **** $P < .0001$.

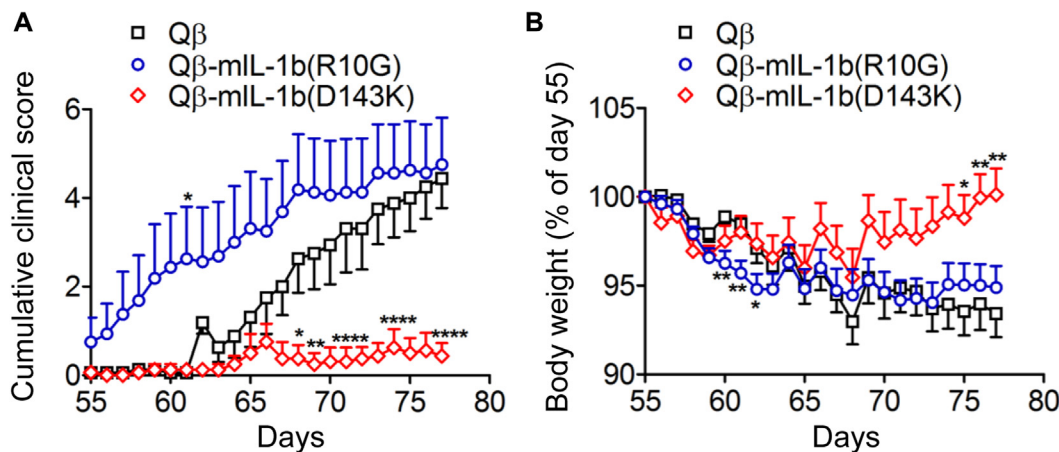


FIG 4. Endogenous polyclonal anti-IL-1 β antibodies exacerbate murine inflammatory arthritis. Groups of mice ($n = 8$) were immunized with the indicated Q β -mIL-1 β mutein conjugates or Q β -VLPs as a control and injected with collagen in adjuvant to induce joint inflammation. Mice were examined daily, clinical scores ranging from 0 to 3 were assigned to each limb (**A**), and body weights were recorded (**B**). Shown are group means $\pm$ SEMs of one of 2 experiments with similar results. P values were determined by using 2-way ANOVA with Bonferroni posttest correction. P values were less than .0001 (Q β -mIL-1b[D143K] vs Q β ; days 71-77), less than .1 (Q β -mIL-1b[R10G] vs Q β ; days 55-61), .29 (Q β -mIL-1b[D143K] vs Q β ; days 55-77), or .03 (Q β -mIL-1b[R10G] vs Q β ; days 55-61). Time points at which mean clinical scores and body weight significantly differed from the Q β control group are indicated. * $P < .05$, ** $P < .01$, and **** $P < .0001$.

mutation (mIL-1b[D143K]) and mIL-1b(R10G), respectively. We first assessed the neutralizing capacity of the antibodies generated *in vivo* after immunization with mouse IL-1b carrying D143K point mutation and conjugated to Q β -VLP (Q β -mIL-1b [D143K]) or Q β -mIL-1b(R10G) using IL-6 production by HeLa cells, as described above. As expected, HeLa cell activation was decreased by Q β -mIL-1b(D143K)-induced antibodies, whereas Q β -mIL-1b(R10G)-induced antibodies did not affect HeLa cell activation (Fig 3, B).

Subsequently, we tested these mIL-1b variants in a murine inflammatory arthritis model induced by collagen in adjuvant. Animals were immunized with nonconjugated Q β -VLPs (control) or with Q β -VLPs linked to either mIL-1b(D143K) or mIL-1b(R10G). Control mice had severe arthritis, peaking at an average score of 4.4 on day 77 after initiation (Fig 4, A), showing

that the immunization itself did not affect the disease severity obtained in this model. Notably, animals receiving the “enhancer” mIL-1b variant R10G started to have disease approximately 1 week earlier, namely on day 55 instead of day 62 for control mice, and showed accelerated weight loss compared with control animals (Fig 4). Contrarily, mice immunized with the “neutralizer” mIL-1b variant D143K showed only minimal signs of disease and no weight loss over the whole observation period (Fig 4).

Similar results were obtained by using another model of inflammation, namely DSS-induced colitis. Mice were immunized with either nonconjugated Q β -VLPs (control) or Q β -VLPs linked to either mIL-1b(D143K) or mIL-1b(R10G). Once animals had generated high antibody titers, they received DSS in their drinking water to induce acute intestinal inflammation. Although

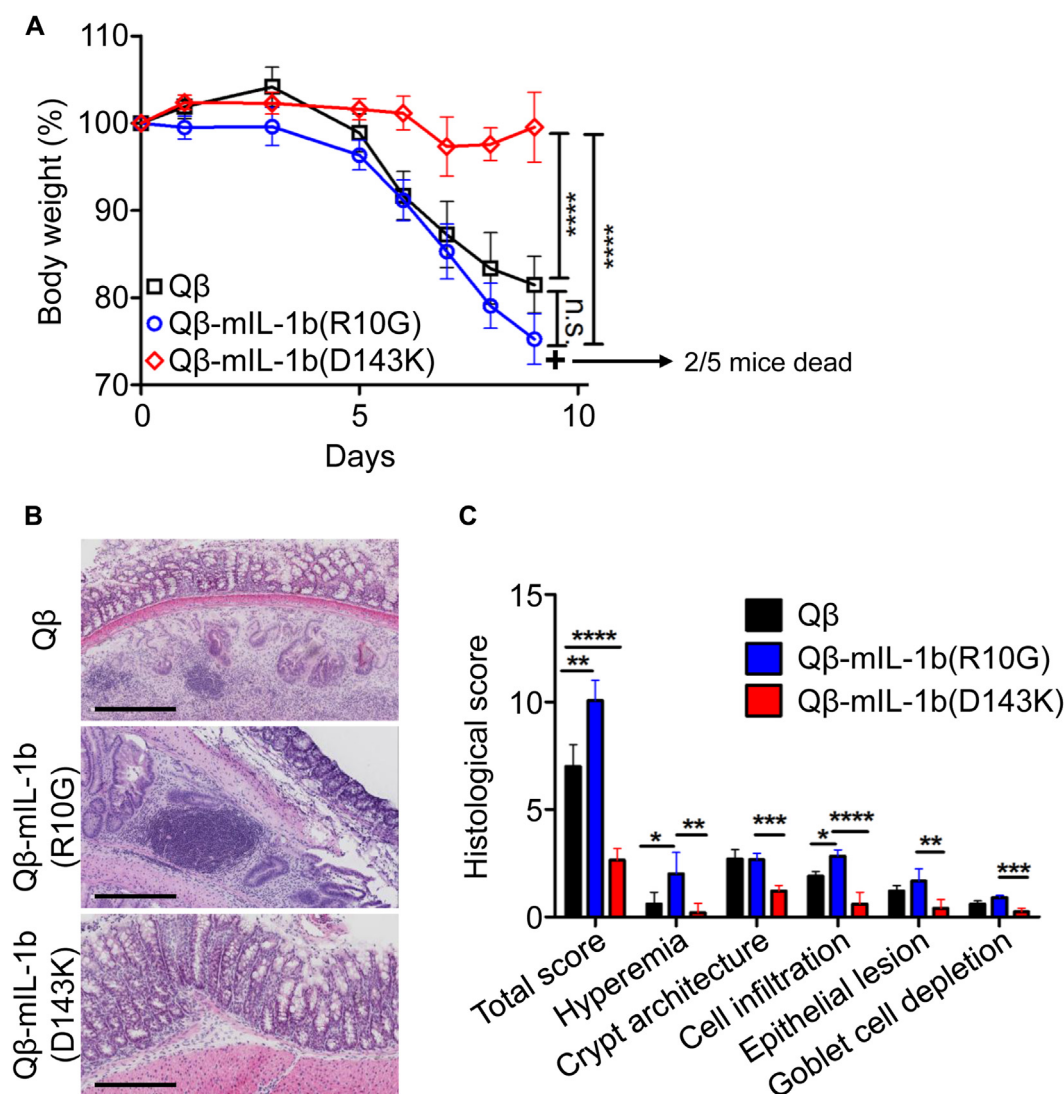


FIG 5. Endogenous polyclonal anti-IL-1 β antibody responses influence inflammatory bowel disease *in vivo*. **A-C**, C57BL/6 mice ($n = 5$) were immunized with Q β -VLPs, Q β -mIL-1b(R10G), or Q β -mIL-1b(D143K), as in Fig 4, followed by induction of colitis with DSS and measurement of body weight (Fig 5, A). Two mice immunized with Q β -mIL-1b(R10G) were found dead on day 9, as indicated. Representative hematoxylin and eosin staining of the colon (Fig 5, B), with scale bars representing 200 μ m, and calculation of histologic scores (Fig 5, C) was performed on day 9 of DSS administration. Shown are groups means $\pm$ SEMs of pooled data of 2 experiments with similar results. *P* values were determined by using 1-way ANOVA with Bonferroni posttest correction. *n.s.*, Not significant. **P* < .05, ***P* < .01, ****P* < .001, and *****P* < .0001.

their body weight did not significantly differ compared with that of control animals, mice immunized with Q β -mIL-1b(R10G) showed signs of increased disease severity, such as rectal bleeding and even death in some instances (Fig 5, A). This was further confirmed by means of histologic analysis of the colon, showing more severe hyperemia and cell infiltration in Q β -mIL-1b(R10G)-immunized mice and resulting in a significantly worse total disease score (Fig 5, B and C). Conversely, animals receiving the Q β -mIL-1b(D143K) conjugate showed barely any signs of disease (Fig 5, A-C).

DISCUSSION

The presence of endogenous anti-cytokine autoantibodies is not unusual. However, the emergence of polyclonal anti-cytokine antibody responses able to boost the cytokine's biological activity

by acting as carriers *in vivo*, as described in this study, is rather unexpected and offers room for speculation on the possible involvement of anti-cytokine autoantibodies in the regulation and exacerbation of inflammatory disorders. The key question is what elements are necessary for the generation of such agonistic polyclonal anti-cytokine antibodies.

As mentioned earlier, infection and inflammation can trigger the generation of anti-cytokine autoantibodies.^{1,8,22,26-28} The emergence of anti-cytokine autoantibodies might be due in part to similarities of a cytokine and a microbial epitope, as shown for anti-IL-2 antibodies during HIV infection, where a hexapeptide homology between the HIV envelope and IL-2 was proposed to lead to anti-IL-2 antibodies through molecular mimicry.²⁶ However, molecular mimicry is unlikely to serve as an explanation for many of the transitory anti-cytokine autoantibody responses

accompanying infections. Rather, these phenomena might be linked to how B-cell tolerance is enforced in the bone marrow, including clonal deletion and receptor editing, with current evidence suggesting that receptor editing is the predominant mechanism of the central tolerance of B cells.^{50,51}

B-cell receptor editing can give rise to B cells that continue to be weakly self-reactive. This phenomenon, called allelic inclusion, leads to B cells carrying 2 or more different B-cell receptor light chains, thus being polyreactive and potentially still self-reactive.^{51,52} Thus it is conceivable that a fraction of pathogen-specific B cells retain reactivity toward the cytokine concerned, the latter of which *per se* is insufficient to cause B-cell stimulation because of very low-avidity interaction. However, once these B cells are appropriately stimulated in the context of infection by their specific pathogen, the cells would also start producing anti-cytokine antibodies. Although most of these latter responses might cease with elimination of the pathogen concerned, continued infection or inflammation, thus providing B-cell survival factors, such as B cell-activating factor, might cause persistence of the anti-cytokine autoantibody response.

Moreover, infection and inflammation might create a micro-environment causing (1) increased immunogenicity of the cytokine concerned, including an acute physiologic increase in its concentration and modification; (2) enhanced reactivity toward the cytokine through release of pathogen- and danger-associated molecular patterns; (3) suppression of regulatory immune cells, including regulatory T and B cells; and (4) possibly increased antibody affinity to the cytokine by favoring somatic hypermutation.⁵¹ Also, pathogen-specific (follicular) T helper cells likely contribute to this process by providing costimulatory signals through cytokines and CD40–CD40 ligand interaction. The sum of these factors might lead to the generation of a high-avidity anti-cytokine IgG antibody response.

What determines whether such antibody responses result in agonistic or truly neutralizing antibodies? This question is more difficult to answer. We know that agonistic anti-cytokine mAbs usually target one of the receptor-binding epitopes of the cytokine concerned. Thus, for IL-2, the anti-IL-2 mAb S4B6 is thought to cover up the IL-2 receptor (IL-2R) α (CD25)-binding epitope of IL-2, whereas the anti-IL-2 mAb JES6-1 likely interferes with IL-2 binding to IL-2R β (CD122) and IL-2R γ (γ c).^{33,53,54} Interestingly, upon injection into mice, S4B6 binds endogenous IL-2 and thereby redirects IL-2 to memory phenotype CD8⁺ T cells and natural killer cells; conversely, for CD25⁺ regulatory T cells, S4B6 prevents stimulation of these cells by bound IL-2.^{33,55} Likewise, injection of anti-IL-7 mAb M25 to mice is able to neutralize endogenous IL-7 for pre-B and T cells,⁵⁶ whereas M25 serves as an agonist for these cells when administered together with recombinant IL-7.^{32,34} What is common to these and other agonistic anti-cytokine mAbs is that they are neutralizing *in vitro*,⁵⁴ indicating that they interfere with the cytokine binding to one of its receptors.

This was different for the herein described polyclonal agonistic anti-IL-1 β antibodies after stimulation with Q β -mIL-1b(R10G) and Q β -hIL-1b(R11G). Molecular modeling analyses of the hIL-1b(R11G) mutant suggest that the introduced mutation leads to small but significant conformational changes, which are not confined to the mutated sites.⁴⁷ The exchange of Arg to Gly at position 11 leads to a disruption of the interaction between the C γ of R11 and the carbonyl oxygen of K16 and is predicted to

shift the orientation of the amino-terminal and carboxy-terminal β -sheets. As a consequence, subtle changes might be introduced in adjacent regions, such as in the cleft formed by residues in the β 1- β 2, β 8- β 9, β 10- β 11, and β 11- β 12 loops, which contain important receptor-binding sites.⁴⁷ Thus the introduction of the R11G mutation leads to slight structural changes in and around the antiparallel N- and C-terminal β -sheets, which have only minor effects on receptor binding but on immunization lead to a significant change in the specificity of the induced antibodies. Antibodies raised against R11G were nonneutralizing *in vitro*, likely because the part of the antibody response directed against “neutralizing” epitopes in the structurally modified portion of the mutein did not recognize this region in wild-type hIL-1 β , whereas the remainder of the antibody response recognizing “nonneutralizing” epitopes was able to recognize the same epitopes on wild-type hIL-1 β . *In vivo* the binding of hIL-1b(R11G)-raised antibodies to IL-1 β caused a carrier effect, which resulted in an increased bioavailability of IL-1 β , increased biological activity, and, consequently, an exacerbation of inflammation in models of autoimmune disease. It is worth mentioning that our data do not formally exclude a contribution of the Q β -VLP immunization to the herein described IL-1 β -specific effects. However, immunization with Q β -VLP alone did not elicit any IL-1 β -related effects, as shown here, thus arguing that the polyclonal anti-IL-1 β antibodies rather than the Q β -VLP-IL-1 β immunization as a whole exerted the observed effects.

Collectively, we found that particular endogenous polyclonal antibody responses targeting IL-1 β can enhance the cytokine's *in vivo* activity rather than neutralizing it and thus exacerbate pathology in different models of (auto-) inflammation. Similar phenomena could be operational in chronic inflammatory and possibly autoimmune disorders in human subjects.

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Key messages

- During an immune response, anti-IL-1 β autoantibody responses occurred with different *in vitro* and *in vivo* qualities.
- Certain polyclonal anti-IL-1 β autoantibody responses increased IL-1 β 's half-life and biological activity.
- Polyclonal anti-IL-1 β autoantibody responses exacerbated disease in murine models of IL-1 β -driven (auto-) inflammatory pathologies.

REFERENCES

1. Browne SK, Holland SM. Anticytokine autoantibodies in infectious diseases: pathogenesis and mechanisms. *Lancet Infect Dis* 2010;10:875-85.
2. Kitamura T, Tanaka N, Watanabe J, Uchida K, Kanegasaki S, Yamada Y, et al. Idiopathic pulmonary alveolar proteinosis as an autoimmune disease with neutralizing antibody against granulocyte/macrophage colony-stimulating factor. *J Exp Med* 1999;190:875-80.
3. Kitamura T, Uchida K, Tanaka N, Tsuchiya T, Watanabe J, Yamada Y, et al. Serological diagnosis of idiopathic pulmonary alveolar proteinosis. *Am J Respir Crit Care Med* 2000;162:658-62.

4. Rosen LB, Freeman AF, Yang LM, Jutivorakool K, Olivier KN, Angkasekwinai N, et al. Anti-GM-CSF autoantibodies in patients with cryptococcal meningitis. *J Immunol* 2013;190:3959-66.
5. Casadevall N, Dupuy E, Molho-Sabatier P, Tobelem G, Varet B, Mayeux P. Autoantibodies against erythropoietin in a patient with pure red-cell aplasia. *N Engl J Med* 1996;334:630-3.
6. Casadevall N, Nataf J, Viron B, Kolta A, Kiladjian JJ, Martin-Dupont P, et al. Pure red-cell aplasia and antierythropoietin antibodies in patients treated with recombinant erythropoietin. *N Engl J Med* 2002;346:469-75.
7. Hellmich B, Csernok E, Schatz H, Gross WL, Schnabel A. Autoantibodies against granulocyte colony-stimulating factor in Felty's syndrome and neutropenic systemic lupus erythematosus. *Arthritis Rheum* 2002;46:2384-91.
8. Wildbaum G, Nahir MA, Karin N. Beneficial autoimmunity to proinflammatory mediators restrains the consequences of self-destructive immunity. *Immunity* 2003;19:679-88.
9. Jouvenne P, Fossiez F, Banchereau J, Miossec P. High levels of neutralizing autoantibodies against IL-1 alpha are associated with a better prognosis in chronic polyarthritis: a follow-up study. *Scand J Immunol* 1997;46:413-8.
10. Doffinger R, Helbert MR, Barcenas-Morales G, Yang K, Dupuis S, Ceron-Gutierrez L, et al. Autoantibodies to interferon-gamma in a patient with selective susceptibility to mycobacterial infection and organ-specific autoimmunity. *Clin Infect Dis* 2004;38:e10-4.
11. Patel SY, Ding L, Brown MR, Lantz L, Gay T, Cohen S, et al. Anti-IFN-gamma autoantibodies in disseminated nontuberculous mycobacterial infections. *J Immunol* 2005;175:4769-76.
12. Kampmann B, Hemingway C, Stephens A, Davidson R, Goodsall A, Anderson S, et al. Acquired predisposition to mycobacterial disease due to autoantibodies to IFN-gamma. *J Clin Invest* 2005;115:2480-8.
13. Hoflich C, Sabat R, Rousseau S, Temmesfeld B, Slevogt H, Docke WD, et al. Naturally occurring anti-IFN-gamma autoantibody and severe infections with *Mycobacterium chelonae* and *Burkholderia coccovenans*. *Blood* 2004;103:673-5.
14. Browne SK, Burbelo PD, Chetchotisakd P, Suputtamongkol Y, Kiertiburanakul S, Shaw PA, et al. Adult-onset immunodeficiency in Thailand and Taiwan. *N Engl J Med* 2012;367:725-34.
15. Browne SK. Anticytokine autoantibody-associated immunodeficiency. *Annu Rev Immunol* 2014;32:635-57.
16. Puel A, Doffinger R, Natividad A, Chrabieh M, Barcenas-Morales G, Picard C, et al. Autoantibodies against IL-17A, IL-17F, and IL-22 in patients with chronic mucocutaneous candidiasis and autoimmune polyendocrine syndrome type I. *J Exp Med* 2010;207:291-7.
17. Kisand K, Boe Wolff AS, Podkrajsek KT, Tserel L, Link M, Kisand KV, et al. Chronic mucocutaneous candidiasis in APECED or thymoma patients correlates with autoimmunity to Th17-associated cytokines. *J Exp Med* 2010;207:299-308.
18. Puel A, Picard C, Lorrot M, Pons C, Chrabieh M, Lorenzo L, et al. Recurrent staphylococcal cellulitis and subcutaneous abscesses in a child with autoantibodies against IL-6. *J Immunol* 2008;180:647-54.
19. Bendtzen K, Hansen MB, Ross C, Svenson M. High-avidity autoantibodies to cytokines. *Immunol Today* 1998;19:209-11.
20. Uchida K, Nakata K, Suzuki T, Luisetti M, Watanabe M, Koch DE, et al. Granulocyte/macrophage-colony-stimulating factor autoantibodies and myeloid cell immune functions in healthy subjects. *Blood* 2009;113:2547-56.
21. Ross C, Hansen MB, Schyberg T, Berg K. Autoantibodies to crude human leucocyte interferon (IFN), native human IFN, recombinant human IFN-alpha 2b and human IFN-gamma in healthy blood donors. *Clin Exp Immunol* 1990;82:57-62.
22. Caruso A, Bonfanti C, Colombrita D, De Francesco M, De Rango C, Foresti I, et al. Natural antibodies to IFN-gamma in man and their increase during viral infection. *J Immunol* 1990;144:685-90.
23. Tiberio L, Caruso A, Pozzi A, Rivoltini L, Morelli D, Monti E, et al. The detection and biological activity of human antibodies to IL-2 in normal donors. *Scand J Immunol* 1993;38:472-6.
24. Hansen MB, Svenson M, Diamant M, Bendtzen K. Anti-interleukin-6 antibodies in normal human serum. *Scand J Immunol* 1991;33:777-81.
25. Bhol K, Mohimen A, Ahmed AR. Correlation of subclasses of IgG with disease activity in pemphigus vulgaris. *Dermatology* 1994;189(suppl 1):85-9.
26. Bost KL, Hahn BH, Saag MS, Shaw GM, Weigent DA, Blalock JE. Individuals infected with HIV possess antibodies against IL-2. *Immunology* 1988;65:611-5.
27. Pozzetto B, Mogensen KE, Tovey MG, Gresser I. Characteristics of autoantibodies to human interferon in a patient with varicella-zoster disease. *J Infect Dis* 1984;150:707-13.
28. Fomsgaard A, Svenson M, Bendtzen K. Auto-antibodies to tumour necrosis factor alpha in healthy humans and patients with inflammatory diseases and gram-negative bacterial infections. *Scand J Immunol* 1989;30:219-23.
29. Klein B, Brailly H. Cytokine-binding proteins: stimulating antagonists. *Immunol Today* 1995;16:216-20.
30. Sato TA, Widmer MB, Finkelman FD, Madani H, Jacobs CA, Grabstein KH, et al. Recombinant soluble murine IL-4 receptor can inhibit or enhance IgE responses in vivo. *J Immunol* 1993;150:2717-23.
31. May LT, Neta R, Moldawer LL, Kenney JS, Patel K, Sehgal PB. Antibodies chaperone circulating IL-6. Paradoxical effects of anti-IL-6 "neutralizing" antibodies in vivo. *J Immunol* 1993;151:3225-36.
32. Finkelman FD, Madden KB, Morris SC, Holmes JM, Boiani N, Katona IM, et al. Anti-cytokine antibodies as carrier proteins. Prolongation of in vivo effects of exogenous cytokines by injection of cytokine-anti-cytokine antibody complexes. *J Immunol* 1993;151:1235-44.
33. Boyman O, Kovar M, Rubinstein MP, Surh CD, Sprent J. Selective stimulation of T cell subsets with antibody-cytokine immune complexes. *Science* 2006;311:1924-7.
34. Boyman O, Ramsey C, Kim DM, Sprent J, Surh CD. IL-7/anti-IL-7 mAb complexes restore T cell development and induce homeostatic T cell expansion without lymphopenia. *J Immunol* 2008;180:7265-75.
35. Woytschak J, Keller N, Krieg C, Impellizzeri D, Thompson RW, Wynn TA, et al. Type 2 interleukin-4 receptor signaling in neutrophils antagonizes their expansion and migration during infection and inflammation. *Immunity* 2016;45:172-84.
36. Dinarello CA. Interleukin-1 in the pathogenesis and treatment of inflammatory diseases. *Blood* 2011;117:3720-32.
37. Van den Steen P, Rudd PM, Dwek RA, Van Damme J, Opdenakker G. Cytokine and protease glycosylation as a regulatory mechanism in inflammation and autoimmunity. *Adv Exp Med Biol* 1998;435:133-43.
38. Spohn G, Jennings GT, Martina BE, Keller I, Beck M, Pumpens P, et al. A VLP-based vaccine targeting domain III of the West Nile virus E protein protects from lethal infection in mice. *Virol J* 2010;7:146.
39. Jegerlehner A, Tissot A, Lechner F, Sebbel P, Erdmann I, Kundig T, et al. A molecular assembly system that renders antigens of choice highly repetitive for induction of protective B cell responses. *Vaccine* 2002;20:3104-12.
40. Spohn G, Schori C, Keller I, Sladko K, Sina C, Guler R, et al. Preclinical efficacy and safety of an anti-IL-1beta vaccine for the treatment of type 2 diabetes. *Mol Ther Methods Clin Dev* 2014;1:14048.
41. Spohn G, Keller I, Beck M, Grest P, Jennings GT, Bachmann MF. Active immunization with IL-1 displayed on virus-like particles protects from autoimmune arthritis. *Eur J Immunol* 2008;38:877-87.
42. Spohn G, Guler R, Johansen P, Keller I, Jacobs M, Beck M, et al. A virus-like particle-based vaccine selectively targeting soluble TNF-alpha protects from arthritis without inducing reactivation of latent tuberculosis. *J Immunol* 2007;178:7450-7.
43. Bouchaud G, Gehrke S, Krieg C, Kolios A, Hafner J, Navarini AA, et al. Epidermal IL-15Ralpha acts as an endogenous antagonist of psoriasisform inflammation in mouse and man. *J Exp Med* 2013;210:2105-17.
44. Kim JJ, Shajib MS, Manocha MM, Khan WI. Investigating intestinal inflammation in DSS-induced model of IBD. *J Vis Exp* 2012;(60); <http://dx.doi.org/10.3791/3678>.
45. Gehrke L, Jobling SA, Paik LS, McDonald B, Rosenwasser LJ, Auron PE. A point mutation uncouples human interleukin-1 beta biological activity and receptor binding. *J Biol Chem* 1990;265:5922-5.
46. Ju G, Labriola-Tompkins E, Campen CA, Benjamin WR, Karas J, Plocinski J, et al. Conversion of the interleukin 1 receptor antagonist into an agonist by site-specific mutagenesis. *Proc Natl Acad Sci U S A* 1991;88:2658-62.
47. Auron PE, Quigley GJ, Rosenwasser LJ, Gehrke L. Multiple amino acid substitutions suggest a structural basis for the separation of biological activity and receptor binding in a mutant interleukin-1 beta protein. *Biochemistry* 1992;31:6632-8.
48. Wang D, Zhang S, Li L, Liu X, Mei K, Wang X. Structural insights into the assembly and activation of IL-1beta with its receptors. *Nat Immunol* 2010;11:905-11.
49. Owyang AM, Issafras H, Corbin J, Ahluwalia K, Larsen P, Pongo E, et al. XOMA 052, a potent, high-affinity monoclonal antibody for the treatment of IL-1beta-mediated diseases. *MAbs* 2011;3:49-60.
50. Halverson R, Torres RM, Pelanda R. Receptor editing is the main mechanism of B cell tolerance toward membrane antigens. *Nat Immunol* 2004;5:645-50.
51. Basten A, Silveira PA. B-cell tolerance: mechanisms and implications. *Curr Opin Immunol* 2010;22:566-74.
52. Liu S, Velez MG, Humann J, Rowland S, Conrad FJ, Halverson R, et al. Receptor editing can lead to allelic inclusion and development of B cells that retain antibodies reacting with high avidity autoantigens. *J Immunol* 2005;175:5067-76.
53. Letourneau S, van Leeuwen EM, Krieg C, Martin C, Pantaleo G, Sprent J, et al. IL-2/anti-IL-2 antibody complexes show strong biological activity by avoiding interaction with IL-2 receptor α subunit CD25. *Proc Natl Acad Sci U S A* 2010;107:2171-6.
54. Arenas-Ramirez N, Woytschak J, Boyman O. Interleukin-2: biology, design and application. *Trends Immunol* 2015;36:763-77.
55. Boyman O, Sprent J. The role of interleukin-2 during homeostasis and activation of the immune system. *Nat Rev Immunol* 2012;12:180-90.
56. Grabstein KH, Waldschmidt TJ, Finkelman FD, Hess BW, Alpert AR, Boiani NE, et al. Inhibition of murine B and T lymphopoiesis in vivo by an anti-interleukin 7 monoclonal antibody. *J Exp Med* 1993;178:257-64.

METHODS

Determination of binding affinities by means of competitive ELISA

Wild-type human IL-1 β carrying a His-tag and a C-terminal free cysteine residue (hIL-1 β -His-C) was expressed and purified as described for the IL-1 β muteins and then biotinylated at the free cysteine residue by using an EZ-link maleimide-PEG₁₁-biotinylation kit (Pierce). Biotinylated hIL-1 β -His-C bound with the same affinity as wild-type hIL-1 β to immobilized recombinant human IL-1RI and IL-1RII (R&D Systems), as detected in a sandwich ELISA

using polyclonal goat anti-hIL-1 β IgG (R&D Systems) for detection. For determination of binding affinities, serial dilutions of the different hIL-1 β muteins were premixed with a constant amount of 20 ng/mL (corresponding to approximately 1 nmol/L) of biotinylated hIL-1 β -His-C and then transferred to ELISA plates that had been coated with hIL-1RI or hIL-1RII. Bound biotinylated hIL-1 β -His-C was detected with horseradish peroxidase-conjugated streptavidin. Binding curves were fitted by using 4-parameter logistic equations with GraphPad Prism software (GraphPad Software, La Jolla, Calif).

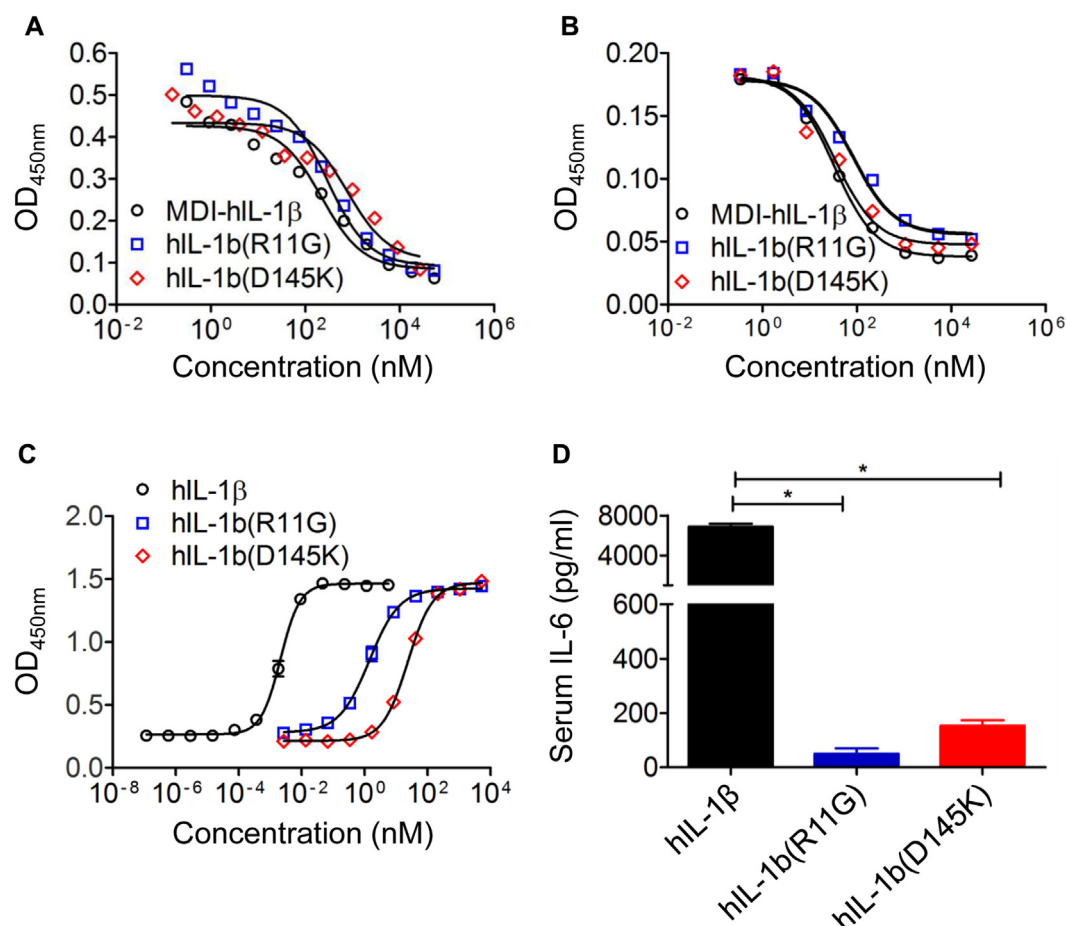


FIG E1. IL-1R binding and IL-1 β bioactivity of the hIL-1 β mutants hIL-1b(R11G) and hIL-1b(D145K). **A** and **B**, ELISA plates were precoated with either IL-1RI (Fig E1, A) or IL-1RII (Fig E1, B), followed by incubation with titrated amounts of either hIL-1b(R11G), hIL-1b(D145K), or hIL-1 β carrying the same N-terminus as the 2 mutants (MDI-hIL-1 β); the hIL-1 β constructs had been premixed with a constant amount of C-terminally biotinylated hIL-1 β -His-C. Shown is receptor-bound biotinylated hIL-1 β -His-C detected by using streptavidin-conjugated horseradish peroxidase. **C**, *In vitro* bioactivity of hIL-1 β constructs was determined by incubating HeLa cells with titrated amounts of wild-type IL-1 β or the indicated hIL-1 β mutants and measurement of IL-6 release by HeLa cells in the supernatant by means of ELISA, with OD_{450nm} values corresponding to the amount of IL-6. **D**, *In vivo* activity of hIL-1 β constructs was assessed by injecting C57BL/6 mice ($n = 4$ per group) intraperitoneally with 1 μ g of wild-type hIL-1 β , hIL-1b(R11G), or hIL-1b(D145K), followed by measuring serum IL-6 levels 3 hours later by using a sandwich ELISA (detection limit, 20 pg/mL). Shown are group means $\pm$ SEMs. Statistical significance of differences was assessed by using 1-way ANOVA and the Tukey multiple comparison test. * $P < .0001$.

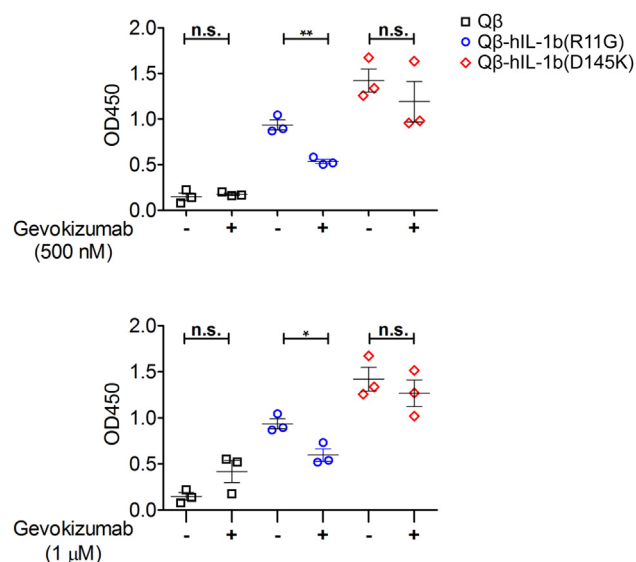


FIG E2. Gevokizumab competes for hIL-1 β binding with antibodies induced by hIL-1b(R11G) but not with antibodies induced by hIL-1b(D145K). Nickel plates were coated with His-tagged hIL-1 β (5 μ g/mL), followed by incubation with PBS (indicated by a minus symbol [-]) or gevokizumab (at 500 nmol/L [top] or 1 μ mol/L [bottom]; indicated by a plus symbol [+]) and serum of mice immunized with either Q β (black squares), Q β -hIL-1b(R11G) (blue circles), or Q β -hIL-1b(D145K) (red diamonds), as indicated. Mouse IgG was detected by using anti-mouse IgG antibody, indicating the presence of His-tagged hIL-1 β -bound anti-hIL-1 β antibodies. Detection of mouse IgG was at background levels in serum of mice immunized with Q β . Data are presented as means $\pm$ SEMs ($n = 3$ sera of different mice of 2 independent experiments [ie, one using 500 nmol/L and another using 1 μ mol/L gevokizumab, both showing comparable results]). P values were calculated with the unpaired Student t test. *n.s.*, Not significant. * $P < .05$ and ** $P < .01$.