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A CD40-targeted peptide controls and reverses type 1 diabetes in NOD mice

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Abstract

Aims/Hypothesis—The CD40–CD154 interaction directs autoimmune inflammation.

Therefore, a longstanding goal in the treatment of autoimmune disease has been to control the formation of that interaction and thereby prevent destructive inflammation. Antibodies blocking CD154 are successful in mouse models of autoimmune disease but, while promising when used in humans, unfortunate thrombotic events have occurred, forcing the termination of those studies.

Methods—To address the clinical problem of thrombotic events caused by anti-CD154 antibody treatment, we created a series of small peptides based on the CD154 domain that interacts with CD40 and tested the ability of these peptides to target CD40 and prevent type 1 diabetes in NOD mice.

Results—We identified a lead candidate, the 15-mer KGYY₁₅ peptide, which specifically targets CD40-positive cells in a size- and sequence-dependent manner. It is highly efficient in preventing hyperglycaemia in NOD mice that spontaneously develop type 1 diabetes. Importantly, KGYY₁₅ can also reverse new-onset hyperglycaemia. KGYY₁₅ is well tolerated and functions to control the cytokine profile of culprit Th40 effector T cells. The KGYY₁₅ peptide is 87% homologous to the human sequence, suggesting that it is an important candidate for translational studies.

Conclusions—Peptide KGYY₁₅ constitutes a viable therapeutic option to antibody therapy in targeting the CD40–CD154 interaction in type 1 diabetes. Given the involvement of CD40 in autoimmunity in general, it will also be important to evaluate KGYY₁₅ in the treatment of other

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Contribution statement

DHW and GMV contributed to the concept and design of the study. DHW, GMV, MHO, DMW and JRC contributed to the acquisition and interpretation of data and critically revised the article. GMV wrote the manuscript. All authors approved the final version to be published. DHW is the guarantor of this work.

Duality of interest statement

DHW holds the intellectual property on the peptide and is cofounder and chief scientific officer of Op-TMune, which utilises the peptide for cell staining. MHO currently is employed at PAREXEL International; however, at the time of these studies he was a student in the laboratory of DHW. No collaboration or other interests exist between the DHW laboratory and PAREXEL.

autoimmune diseases. This alternative therapeutic approach opens new avenues of exploration in targeting receptor–ligand interactions.

Keywords

Autoimmunity; CD40; CD154; Peptide; Therapeutic; Type 1 diabetes mellitus

Introduction

Although the aetiology of autoimmunity is not clear, CD40–CD154 interactions are critical in the development and perpetuation of autoimmune disease [1–11]. CD40 is expressed on antigen-presenting cells (APCs) [12], but it is expressed more ubiquitously, including on T cells [13–15]. We identified a CD4⁺ T cell subset that expresses CD40, known as Th40 cells, which is necessary and sufficient for transferring diabetes in a mouse model of type 1 diabetes [9, 14–17]. CD40 drives the disease process: CD40^{−/−} and CD154^{−/−} mice do not develop autoimmune disease [18, 19].

CD40 is a member of the tumour necrosis factor (TNF) receptor superfamily [20]. The natural ligand for CD40, CD154 [21], occurs in both membrane-bound and soluble forms. CD154 interacts not only with CD40 but also with $\alpha 5\beta 1$ and $\alpha \text{IIb}\beta 3$ integrins, which may be critical to its signalling function [22]. CD154 may serve as a molecular bridge between these receptors: simultaneous CD154 binding to CD40 and $\alpha 5\beta 1$ induced stronger signalling than binding to each receptor separately [22]. The dynamic CD40–CD154 dyad controls many inflammatory events in B cells [23], macrophages [24], monocytes [25], and dendritic cells [26], as well as in T cells [4, 7, 27].

The involvement of CD40 signalling in driving inflammatory events and autoimmunity has led to the CD40–CD154 dyad becoming a primary therapeutic target. By blocking the signal, control of the destructive inflammation becomes possible. However, considering that CD154 interacts with other receptors [22], it is important to tailor the inhibition to avoid undesired consequences. While CD40 antibodies are agonistic in nature, CD154-blocking antibodies are available and can prevent or ameliorate autoimmune disease in several disease models [2, 6, 28], including type 1 diabetes [1, 9]. Anti-CD154 treatment also demonstrated promise in human systemic lupus erythematosus (SLE) [29, 30]. However, unfortunate life-threatening thrombotic events arose, leading to termination of the studies [29, 31]. As a consequence of that result and other antibody-related side-effects, it is desirable to find alternative means of inhibiting the CD40–CD154 interaction. Random peptides that inhibit CD40–CD154 interaction have been tested. However, as a consequence of their random nature, they may inhibit other interactions when employed in vivo [32, 33], and some require to be used at high concentrations [32]. In a more targeted approach, a peptide was designed based on a CD154 domain [34] surrounding one of the amino acids involved in the CD40–CD154 interaction [35, 36]. That peptide demonstrated very low efficacy in blocking experimental autoimmune encephalomyelitis (EAE) [34]. Some small molecule organic compounds inhibited CD40–CD154 interaction but lost their activity in protein-rich medium [37], suggesting further non-specific effects. These compounds are structurally similar to suramin [38], which has toxic side-effects [39].

Peptides are commonly used as human therapeutics and have a good safety record [40–42]. Therefore, based on the potential for peptides to interrupt receptor-ligand interactions we adopted a targeted approach to inhibit the CD40–CD154 interaction. From the CD154 domain critical for interaction with CD40, we designed different-sized peptides spanning several interacting amino acids [35, 36]. This region is 87% identical between humans and mice, suggesting that it forms a functionally important part of CD154. Importantly, this region is not critical for CD154 interaction with other receptors [22]. We tested these peptides in the NOD mouse model of type 1 diabetes by assessing their specificity for CD40 and their ability to prevent and reverse diabetes onset.

Methods

Mice

NOD mice obtained from Taconic (Germantown, NY, USA) were housed at the University of Colorado Denver, an Association for Assessment and Accreditation of Laboratory Animal Care approved facility. Female mice regularly achieved >90% diabetes by 24 weeks of age. All animal experiments were performed under an Institutional Animal Care and Use Committee approved protocol and adhered to the National Institutes of Health Public Health Service Policy on Humane Care and Use of Laboratory Animals.

Peptides and antibodies

Peptides (mouse KGY₁₅, VLQWAKKGY₁₅TMKSN and derivatives; mouse scrambled KGY₁₅, YKNVKQMAYWLTGKS; and human KGY₁₅, VLQWAEKGY₁₅TMSNN) were purchased from New England Peptide (Gardner, MA, USA) and were >98% pure. Anti-CD40 (1C10) and anti-CD4 (GK1.5) antibodies were produced in house. IFN- γ (XMG1.2), TNF- α (MP6-XT22), IL-17A (eBi017B7), IL-6 (MP5–20F3) and IL-2 (JES6-SH4) were obtained from eBioscience (San Diego, CA, USA). Anti-CD40 and anti-hsp70 antibodies, and secondary antibodies used for western blotting (sc-975, sc-33575, sc-2004, sc-2005) were obtained from Santa Cruz Biotechnology (Dallas, TX, USA).

Prevention and reversal of diabetes

For prevention studies, 25 μ g peptide (a dose of 1 mg/kg) in 100 μ l PBS was administered by weekly i.v. injection for 20 weeks, beginning at 5 weeks of age. Blood glucose was monitored weekly until 36 weeks of age. Animals with three consecutive blood glucose measurements >13.9 mmol/l were considered diabetic.

For diabetes reversal studies, starting at 10–12 weeks of age, blood glucose levels were monitored daily until diabetes onset (blood glucose >13.9 mmol/l), and then 100 μ g peptide (a dose of 4 mg/kg) was administered by daily i.v. injection for 6 days or longer.

Cell purification and flow cytometry

Spleens were homogenised in red blood cell lysing buffer (Sigma-Aldrich, St Louis, MO, USA), washed, and then the cells were either stained immediately or sorted into Th40 and APC populations, as previously described [8], and then stained. Lymph node lymphocytes were isolated using Lympholyte-M (Cedarlane Labs, Burlington, NC, USA). Intracellular

staining was performed on cells that were fixed immediately after isolation, without ex vivo stimulation, using permeabilisation buffer (eBioscience). Flow cytometry was performed using a MACSQuant Analyzer (Miltenyi Biotec, Auburn, CA, USA) and analysis was performed using FlowJo software (Ashland, OR, USA).

Peptide pull down and western blotting

Splenic CD4^{hi}, CD4^{lo} (Th40), CD8 and MHCII (APCs) cells were sorted as previously described [8, 43]. Cells were lysed in 20 mmol/l Tris-HCl pH 7.5, 2 mmol/l EDTA, 137 mmol/l NaCl, 0.1% Triton X-100, 1 µg/ml each leupeptin and aprotinin, 0.2 mmol/l phenylmethanesulfonyl fluoride, and 0.4 mmol/l sodium orthovanadate. KGY₁₅ or scrambled peptide conjugated to Tosyl-activated magnetic beads (Invitrogen, Grand Island, NY, USA) was immediately added to cell lysates and incubated for 20–30 min. Beads were washed three times with lysis buffer before elution of bound material. Western blots were performed as previously described [8].

Serum antibody and T cell antigen recall tests

Serum and splenic cells were collected from mice that had been injected weekly for 7 weeks with 1 mg/kg KGY₁₅ peptide. For western blotting of the KGY₁₅ peptide and recombinant soluble CD154 (200 ng each per lane), membranes were blocked in 6% milk in Tris-buffered saline/Tween-20 (milk-TNT) then incubated in a 1:670 dilution of serum in milk-TNT followed by a 1:5000 dilution of anti-mouse IgG antibody. Splenic cells were MHCII depleted, and the remaining T cells were labelled with carboxyfluorescein succinimidyl ester (CFSE) and washed. MHCII⁺ cells (4×10^5) were loaded by incubation with 0.5 µg KGY₁₅ peptide for 2 h, washed and combined with labelled T cells (4×10^5). After incubation for 5 days, CFSE dilution (indicating cell proliferation) was measured by flow cytometry.

Histology

Fixed and sectioned pancreases were stained with haematoxylin and eosin (H&E) or aldehyde fuchsin. Scoring for insulitis and insulin granules was conducted as indicated in the figure legends. More than 100 islets were scored in each group.

Data analysis

Data analysis was performed using GraphPad Prism (GraphPad Software, La Jolla, CA, USA) using tests indicated in the text and figure legends.

Results

Peptides spanning several amino acids critical to CD40–CD154 interaction

Given the plethora of clinical problems associated with antibodies for immune intervention, it is essential to employ alternative means to inhibit inflammatory interactions. We designed peptides based on a domain of the CD154 protein sequence known to be critical for binding to CD40 (Fig. 1a). The peptides span three of the five amino acids in human CD154 required for interaction with CD40: K143, Y145 and Y146 (Fig. 1a; amino acids in bold;

K142, Y144 and Y145 in the mouse sequence) [35, 36]. A 15 amino acid stretch of this region is 87% identical between mouse and human sequences (Fig. 1a; boxed, analysed by BLASTp at the National Center for Biotechnology Information; <http://blast.ncbi.nlm.nih.gov/>), indicating that it is a functionally important part of the protein. In contrast, a previously studied peptide that demonstrated very low efficacy in inhibiting EAE (Fig. 1a; highlighted in blue) [34] has only 64% homology to the human CD154 sequence.

A 15 amino acid sequence is the optimal size for diabetes prevention

In order to determine whether peptides from the region described above are efficacious in preventing diabetes, 5-week-old NOD mice were treated with 8-mer, 10-mer, 13-mer, 15-mer and 24-mer peptides spanning the KxYY motif (Fig. 1a; shown in bold). The 15-mer peptide was highly efficacious in preventing diabetes, with only 10% of treated mice becoming diabetic, while the 13-mer peptide demonstrated some ability in preventing diabetes, with only 30% of treated mice becoming diabetic (Fig. 1b). Additionally, both peptides significantly ($p<0.0001$) delayed diabetes onset in mice that did become diabetic, with the 15-mer peptide outperforming the 13-mer peptide ($p<0.0001$). The smaller peptides were unable to prevent diabetes, although they could delay the onset (Fig. 1b; $p<0.0001$). The 24-mer peptide showed no efficacy at all, resulting in a diabetes incidence rate identical to that of the untreated group (Fig. 1b). The fluctuation in diabetes incidence at weeks 30 and 31 in the 15-mer peptide-treated group was the result of a single mouse that became diabetic despite the treatment but reverted to euglycaemia with continued treatment.

Diabetes prevention by the KGY_Y₁₅ 15-mer peptide is sequence specific

As shown in Fig. 1b, the 15-mer peptide (referred to as KGY_Y₁₅) was the most efficacious in diabetes prevention. A scrambled version of KGY_Y₁₅ completely lacked the ability to prevent diabetes (Fig. 2a; sequences noted above graph). Additionally, a peptide spanning the RGD region of CD154, which is known to interact with α IIb β 3 [22], did not prevent diabetes (Fig. 2a; sequence noted above graph).

Additional amino acids are critical for the interventional efficacy of KGY_Y₁₅

Given that the size of the peptide, as well as its amino acid sequence, was shown to be critical for its function, we performed a structure–activity relationship (SAR) study by creating a peptide series in which single amino acids were replaced with glycine. This substitution strategy revealed that L137, Y144, T146, M147 and N150 are critical for the *in vivo* intervention function (Fig. 2b; no other single substitutions altered efficacy). Curiously, substitution of T146 for glycine increased diabetes incidence to 100% (Fig. 2b). When Y145, shown to be critical for CD154 interaction with CD40 [35, 36], was exchanged for a glycine residue, there was no reduction in the interventional efficacy of the peptide. Importantly, all of the amino acids shown to be critical for peptide efficacy are conserved between the mouse and human sequences (Fig. 2b; compare with Fig. 1a).

KGY_Y₁₅ ameliorates insulinitis

Although the specific mechanism is unknown, blocking CD154 prevents diabetes [1] and also insulinitis [9], which is defined as T cell and other immune cell infiltrates into beta islets.

We determined the extent of insulinitis by comparing KGY₁₅-treated pancreases to diabetic NOD mouse pancreases (Fig. 3a, b). KGY₁₅ treatment did not prevent immune cell infiltration into the pancreas itself but, importantly, prevented entry into beta islets, as demonstrated by the peri-insulitis pattern in H&E stained sections (Fig. 3a). Scoring the infiltration levels of the islets revealed that, while 39% of the diabetic pancreatic islets demonstrated a score of 3 or 4, only 13% of the KGY₁₅-treated islets showed a score at that level, and the majority (72%) scored 0 or 1 (Fig. 3c).

KGY₁₅ reverses overt hyperglycaemia

While the ability of KGY₁₅ to prevent type 1 diabetes onset in mice is evident, it is not easy to predict the development of type 1 diabetes in humans and it is not feasible to treat everybody with the goal of preventing disease in a subset of the population. Therefore, it is highly desirable to be able to effectively treat the disease once it is diagnosed. With this goal in mind, we allowed NOD mice to progress to diabetes (blood glucose >13.9 mmol/l), and then new-onset diabetic mice received a 4 mg/kg daily dose of KGY₁₅. Using this strategy, 56% of diabetic animals returned to euglycaemia after 1–2 days (Fig. 4a and b). Additionally, KGY₁₅ treatment initiated after hyperglycaemia onset significantly improved insulinitis (Fig. 4c), as well as insulin granule (Fig. 4d), scores.

KGY₁₅ binds to Th40 cells

To test KGY₁₅ peptide binding to CD40 on the surface of T cells, sorted NOD splenic Th40 cells were costained with FITC-conjugated KGY₁₅ peptide and anti-CD40 antibody. Seventy-eight per cent of Th40 cells showed double positive staining for CD40 and KGY₁₅ (Fig. 5a). APCs are known to express CD40. Therefore, as a comparison, we stained splenic MHCII⁺ cells that had been depleted of CD4 and CD8, and found that 64% of APCs showed double positive staining for KGY₁₅ and CD40 (Fig. 5b). Several distinct subsets of cells stained positively for CD40 and KGY₁₅ in both cell populations. Although not tested here, those cells may express different CD40 receptor constellations, as we have demonstrated for Th40 cells [44]. Moreover, in the APC population, it is likely that different subpopulations of MHCII⁺ cells (macrophages, B cells, dendritic cells) are differentially stained.

KGY₁₅ pulls down CD40

Many cell types express CD40 and, interestingly, Th40 cells from autoimmune NOD mice express differentially glycosylated CD40 protein [44, 45]. Peptide pull-downs were performed to further investigate the interaction of KGY₁₅ with CD40. KGY₁₅ readily pulled down a CD40 band of approximately 45 kDa from splenic CD4^{hi} cells, CD8 cells and APCs (Fig. 5c). CD40 was also readily pulled down from Th40 cells, but two smaller and one larger band were present (Fig. 5c), which may represent differentially glycosylated CD40 protein, as we have demonstrated before for this cell type [44, 45]. A scrambled version of KGY₁₅ did not pull down any CD40 protein bands, thus demonstrating the specificity of the interaction between KGY₁₅ and the CD40 protein (Fig. 5c). Hsp70, which is known to non-specifically bind peptides, was assayed as a control. The scrambled peptide was as efficient as KGY₁₅ in pulling down Hsp70 protein, demonstrating that the scrambled peptide is functional and further indicating the specificity of KGY₁₅ (Fig. 5c).

KGY₁₅ does not appear to elicit peptide-specific antibody production or T cell antigen recall

An important aspect to consider in the peptide treatment of disease is whether the peptide itself elicits an antigen response. We could not detect serum antibodies against the peptide or recombinant soluble CD154 in mice that had been treated with KGY₁₅ for 7 weeks [electronic supplementary material (ESM) Fig. 1a]. Similarly, when T cells from treated mice were exposed to autologous APCs loaded with peptide, we could not detect proliferation (ESM Fig. 1b). In addition, mice did not demonstrate any discomfort during administration of the treatment. Therefore, we conclude that the peptide does not elicit a discernible immune response.

KGY₁₅ alters cytokine production by Th40 cells

In addressing the mechanism of KGY₁₅ action, we examined its impact on cytokine production by Th40 cells. KGY₁₅ treatment significantly reduced the production of several inflammatory cytokines by Th40 cells, as measured in cells from peripheral and pancreatic lymph nodes immediately after their removal (Fig. 6). IFN- γ production, which is strongly associated with diabetes progression, was significantly reduced, along with TNF- α and IL-6, in peripheral lymph nodes, while IL-2 was not affected. TNF- α , IL-6 and IL-2 levels were significantly reduced in pancreatic lymph node Th40 cells. In splenic Th40 cells, there was a reduction in several cytokines, although this was not statistically significant.

Discussion

A long-term goal in the treatment of autoimmune disease has been to control the CD40–CD154 interaction. Initial studies utilising an antibody against CD154 were highly successful in animal models of autoimmune diseases [1, 6, 9, 28]. However, when translational studies were performed in SLE patients, life-threatening thrombotic events occurred and the studies were halted. Ironically, though, the CD40–CD154 block was successful [29, 31]. This fact and other inherent problems in the therapeutic use of antibodies, including the induction of antibody production directed against the treatment antibody and Fc-receptor binding, have intensified the hunt for an alternative means of inhibiting the CD40–CD154 interaction. Here, we demonstrate that through analysing a series of small peptides targeting CD40, a lead candidate for drug development has emerged. A 15 amino acid sequence, KGY₁₅, based on the CD154 domain critical for interacting with CD40, specifically binds CD40⁺ T cells and APCs from autoimmune NOD mice. These results suggest that KGY₁₅ can affect several compartments of the adaptive immune system to stem inflammatory responses.

KGY₁₅ has the advantages of being both small and derived from an endogenous protein that is expressed under normal conditions. Therefore, the likelihood of host tolerance to KGY₁₅ is high. Indeed, we could not detect the presence of autoantibodies to KGY₁₅ or CD154, nor could we elicit T cell antigen recall. The KGY₁₅ peptide was well tolerated by NOD mice injected weekly for 20 weeks and, importantly, prevented diabetes onset. In addition, in NOD mice that were allowed to progress to overt diabetes, albeit new onset, before any treatment was initiated, hyperglycaemia was reversed by administration of

KGYY₁₅. This indicates that KGYY₁₅ can afford some level of control after disease onset and highlights a question that to date remains unanswered: are beta islets actually destroyed by inflammation or is their insulin production simply ‘turned off’ or downregulated? From our data it appears, at least in the early stages of overt diabetes, that beta cells can reinitiate insulin production when pathological conditions, i.e. the inflammatory environment, are controlled. This suggests that the inflammatory milieu dictates whether beta islet cells can induce insulin expression in response to increased glucose levels. A pattern of peri-insulitis was observed instead of complete beta islet infiltration and cytokine production was altered, demonstrating that KGYY₁₅, while not completely able to prevent trafficking to the pancreas, can block entry to the beta islets themselves, thus preserving beta cell integrity.

Peptide size and amino acid sequence are crucial to the interventional activity of KGYY₁₅. SAR data revealed critical amino acid positions in the peptide that had not been detected in earlier CD40–CD154 interaction studies [35, 36]. Clearly, our in vivo functional study differs from previous in vitro mutagenesis studies, and it is also important to note that a peptide (KGYY₁₅) may interact differently than a larger protein (i.e. CD154). Surprisingly, a methionine residue (M147; position 12 in the 15-mer) is required for in vivo function of the peptide. Methionine is oxidation susceptible and is often replaced in therapeutic peptides, yet both this data and stability determination models show that the current 15-amino-acid configuration is extremely stable (100 h, as predicted by ExPASy ProtParam).

The thrombotic events that occurred in anti-CD154 antibody clinical trials were caused by platelet activation by the antibody. The KGYY₁₅ peptide does not have the inherent problems that antibodies carry as therapeutics. It is also not directed against CD154, but rather binds to CD40, which makes a thrombotic problem less likely.

Our data have important implications for new treatment strategies and drug development in autoimmune inflammation. As stated previously, controlling the CD40–CD154 dyad is a critical element in controlling autoimmune inflammation. Previously, the problem has been how to do this safely. KGYY₁₅ treatment represents a major step in that direction, and the fact that this protein sequence is highly conserved (87%) suggests that it would be an excellent candidate for translational studies. Important future studies will include determining the optimal KGYY₁₅ dose and treatment interval, and whether starting treatment later in the process that eventually leads to overt hyperglycaemia is efficacious. This research also opens other avenues for investigation: the development of peptides to directly target known receptors, for example cytokine and chemokine receptors.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations

APC	Antigen-presenting cell
EAE	Experimental autoimmune encephalomyelitis
H&E	Haematoxylin and eosin
SAR	Structure–activity relationship
SLE	Systemic lupus erythematosus

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Fig. 1.

A 15-mer CD154-derived peptide prevents diabetes. **(a)** The amino acid sequence of mouse CD154 is depicted, with amino acids known to interact with CD40 shown in bold. A 15 amino acid sequence is highlighted in grey and aligned with the same motif in human CD154. A previously studied peptide [41] is highlighted in blue. **(b)** NOD mice were treated with different sizes of peptides, as indicated (8-mer, black open triangle; 10-mer, black square; 13-mer, black filled triangle; 15-mer, green circle; 24-mer, black diamond; untreated, purple circle; $n=10$ in all groups). Mice were monitored for diabetes onset (blood glucose >13.9 mmol/l). Differences were significant by two-way ANOVA; $p<0.0001$.

Fig. 2.

KGY₁₅ diabetes prevention is sequence dependent, and several amino acid positions are critical for its function. **(a)** KGY₁₅-treated mice (black circle) were compared with mice treated with a scrambled peptide (black square) or a peptide targeting the RGD region (black triangle; sequences indicated above the graph). **(b)** KGY₁₅-treated mice (green circle) were compared with mice treated with a 15-mer peptide series in which single amino acids, as indicated, were substituted for glycine. L137, black filled square; Y144, open black triangle; T146, purple triangle; M147, black diamond; N150, open black circle; $n=10$ in all groups shown in **(a)** and **(b)**, except for L137, where $n=9$. Differences were significant by two-way ANOVA; $p<0.0001$

Fig. 3. KGYG₁₅ ameliorates insulinitis in NOD mice. **(a, b)** When control mice became diabetic (UN diabetic), KGYG₁₅-treated and control mice were killed and histological analysis was performed on their pancreases. Representative images of at least ten H&E stained pancreases are shown. **(c)** Islet insulinitis scores: 0, no infiltration (black); 1, 25% infiltration (striped); 2, 50% infiltration (dark grey); 3, 75% infiltration (light grey); 4, 100% infiltration (white)

Fig. 4.

KGY15 reverses overt hyperglycaemia (**a**, **b**) Starting at 10 to 12 weeks of age (day 0), blood glucose was monitored in NOD mice. Immediately after diabetes onset (arrows; blood glucose >13.9 mmol/l; $n=9$), mice were treated daily with KGY15 and blood glucose was monitored. (**a**) Graph depicts each mouse that underwent hyperglycaemia reversal; (**b**) graph depicts those that did not. (**c**) Islet insulinitis scores in reversed and non-reversed mice: 0, no infiltration (black); 1, 25% infiltration (striped); 2, 50% infiltration (dark grey); 3, 75% infiltration (light grey); 4, 100% infiltration (white). (**d**) Islet insulin granulation scores: 0, less than 25% granulation; 1, 25–50% granulation; 2, 50–75% granulation; 4, 75–100% granulated. Data is represented as the mean $\pm$ SEM; **** $p < 0.0001$ by two-tailed unpaired t test

Fig. 5. KGGY₁₅ specifically interacts with CD40. **(a, b)** Representative dot plots of purified NOD splenic Th40 cells **(a)** and APCs **(b; MHCII⁺ CD4⁻ CD8⁻)** costained with an anti-CD40 antibody (ab) and FITC-conjugated KGGY₁₅ (grey). Cell debris was excluded and gates were set based on isotype stained/unstained cells (black; numbers represent percentages). Cells from three different mice were stained. **(c)** KGGY₁₅ or a scrambled peptide was utilised to pull down cellular proteins from sorted NOD splenic CD4^{hi}, CD8 and Th40 cells, and APCs (MHCII⁺). Western blots are shown for CD40 (top panels) and hsp70 (bottom panels). The experiment was performed three separate times

Fig. 6.

KGY₁₅ controls the production of inflammatory cytokines by Th40 cells. Immediately ex vivo lymphocytes from NOD mice treated with KGY₁₅ (grey) and control mice (black) were stained for cytokines. Data for lymphocytes from (a) peripheral lymph node; (b) pancreatic lymph node; and (c) spleen. Bars show cytokine-positive cells within gated CD4⁺CD40⁺ cell populations (Th40), represented as a percentage. Data are from three individual mice from each group and are represented as the mean $\pm$ SEM. * $p < 0.05$, ** $p < 0.01$ by two-tailed unpaired t test