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Kv1.3 blockade by ShK186 modulates CD4+ effector memory T-cell activity of patients with Granulomatosis with polyangiitis.

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Abstract

Objectives: Granulomatosis with polyangiitis (GPA) is a chronic relapsing systemic autoimmune vasculitis. Current treatment of GPA is unsatisfactory as it relies on strong immunosuppressive regimens, with either cyclophosphamide or rituximab, that reduce the immunogenicity of several vaccines and are risk factors of severe form of COVID-19. This emphasizes the need to identify new drug target and to develop treatment strategies with less harmful side effects. Since CD4\(^+\) effector memory T cells (T\(_{EM}\)) play a key role in the pathogenesis of GPA, we aimed in this study to modulate CD4\(^+\)T\(_{EM}\) cell activity via Kv1.3 blockade using the specific peptide inhibitor, ShK-186.

Methods: Peripheral blood of 27 GPA-patients in remission and 16 age- and sex-matched healthy controls (HCs) were pre-incubated in vitro in the presence or absence of ShK-186, followed by stimulation with PMA, calcium ionophore and brefeldin-A. The effect of ShK-186 on the cytokine production (IFN\(\gamma\), TNF\(\alpha\), IL-4, IL-17, IL-21) within total and subsets of CD4\(^+\)T\(_H\) cells were assessed using flow cytometry.

Results: ShK-186 reduced the expression level of IFN\(\gamma\), TNF\(\alpha\), IL-4, IL-17, and IL-21 in CD4\(^+\)T\(_H\) cells from GPA-patients in vitro. Further analysis performed on sorted CD4\(^+\) T cell subsets, revealed that ShK-186 predominantly inhibited the cytokine production of CD4\(^+\)T\(_{EM}\) cells. ShK-186 treatment reduced the production of the pro-inflammatory cytokines to the level seen in CD4\(^+\) T\(_H\) cells from HCs.

Conclusions: Modulation of cellular effector function by ShK-186 may constitute a novel treatment strategy for GPA with high specificity and less harmful side effects.
Graphical Abstract

Rheumatology key messages:

- ShK-186 treatment normalized the production of the pro-inflammatory cytokines in CD4⁺T_H cells from GPA-patients.
- ShK-186 predominantly affects the cytokine production of CD4⁺T_EM cells without impairing other CD4⁺T cell subsets.
- Selective targeting of CD4⁺T_EM cells by ShK-186 might have value in the treatment of GPA-patients.
Introduction

Granulomatosis with polyangiitis (GPA) is the prototype of anti-neutrophil cytoplasmic antibody (ANCA) associated vasculitis (AAV). It is a chronic relapsing systemic autoimmune disease characterized by medium to small sized vessel vasculitis predominantly affecting the upper and lower respiratory tract and kidneys which may result in life-threatening complications[1,2]. Current treatment consists of nonspecific immunosuppressive therapy including cyclophosphamide in combination with corticosteroids[3–5]. More recently, B cell depletion therapy with rituximab has been demonstrated to be equally effective as conventional therapy in inducing disease remission[6]. Unfortunately, these immunosuppressive drugs, rituximab in particular, also reduce the immunogenicity of several vaccines and are risk factors of severe form of Covid-19, emphasizing the need to identify novel molecular targets to develop more selective and less harmful treatment strategies[7–9].

It remains unknown how GPA develops but accumulating evidence indicates a key role for CD4\textsuperscript{+} effector memory T cells (T\textsubscript{EM}) and their inflammatory cytokines (such as interleukin (IL)-17, IL-21, and interferon-gamma (IFN\textgamma)) in the induction and progression of GPA[10–15] Therefore, selective targeting of CD4\textsuperscript{+}T\textsubscript{EM} cells without impairing other arms of cellular immunity might have value in the treatment of GPA-patients.

CD4\textsuperscript{+}T\textsubscript{EM} cells preferentially rely on the voltage-gated potassium Kv1.3 channels to maintain their activation and effector function[16],[17]. It has been shown that Kv1.3 channels are highly expressed on CD4\textsuperscript{+}T\textsubscript{EM} cells (~1500 channels per cell), whereas naïve (CD4\textsuperscript{+}T\textsubscript{NAIVE}) and central memory (CD4\textsuperscript{+}T\textsubscript{CM}) CD4\textsuperscript{+}T cells express lower levels of Kv1.3 channels (~250 channels per cell)[17]. CD4\textsuperscript{+}T\textsubscript{EM} cells use the Kv1.3 channels to regulate Ca\textsuperscript{2+} signaling by controlling the membrane potential. During activation, intracellular Ca\textsuperscript{2+} stores are released leading to a depletion of these stores and an influx of extracellular Ca\textsuperscript{2+}. This influx maintained by the release of K\textsuperscript{+} through Kv1.3 channels, which helps sustain the elevated levels of Ca\textsuperscript{2+} required for optimal T\textsubscript{EM} cell activation. Blocking Kv1.3 channels can inhibit this process by preventing the release of K\textsuperscript{+}, leading to a decrease in Ca\textsuperscript{2+} influx and subsequently inhibiting T\textsubscript{EM} cell
Therefore, Kv1.3 channels may serve as an attractive target for specific immunomodulation in $T_{EM}$ cell mediated chronic or autoimmune diseases. Kv1.3 channels can be selectively inhibited by synthetic analogs of a native ShK toxin isolated from the sea anemone *Stichodactyla helianthus*. Previous studies have demonstrated that blocking of Kv1.3 channels by ShK analogs ameliorates disease development in animal models of multiple sclerosis (MS), rheumatoid arthritis (RA), type 1 diabetes mellitus (T1DM), and contact dermatitis without compromising protective immune responses to acute infections[18–20]. In particular, ShK-186, a 37 amino acid synthetic analogue of ShK, has shown promising long-lasting therapeutic potential in animal models of autoimmunity due to its high selectivity and tight binding to and slow release from the Kv1.3 channels on T cells[21]. Accordingly, we hypothesized that selective blocking of Kv1.3 channels on CD4$^+$ $T_{EM}$ cells from GPA-patients, using a highly potent ShK-186 peptide, reduce their pathogenic activity through modulating their pro-inflammatory cytokine production. Selective targeting of CD4$^+$ $T_{EM}$ cells without impairing other arms of cellular immunity might have value in the treatment of GPA.
Martials and methods

Study population

Twenty-seven GPA-patients in remission and 16 age-matched healthy controls (HCs) (5 males and 11 females, mean age of 60 years, range [27 – 77]) were included in this study. The diagnosis of GPA was established according to the definition of the Chapel Hill Consensus Conference and fulfilled the classification of the American College of Rheumatology[22]. Only GPA-patients without clinical signs and symptoms of active disease and considered to have complete remission of their disease, as indicated by a Birmingham Vasculitis Activity Score of 0, were included in this study[23]. All patients were PR3-ANCA positive at disease diagnosis, and 20 patients had biopsy-proven vasculitis. At time of sampling eighteen patients were PR3-ANCA positive as indicated by an ANCA titer ≥1:40. The PR3-ANCA titers were measured by indirect immunofluorescence (IIF) on ethanol-fixed human granulocytes according to the standard procedure as described previously[24]. Twenty-one patients were considered to have generalized disease, and six patients were considered to have localized disease, in which the disease was confined to the upper and lower respiratory tract. None of the patients experienced an infection at the time of sampling as indicated by a median CRP level of 5.8 mg/L. Eight of the twenty-seven GPA-patients were treated with maintenance immunosuppressive therapy at the time of blood withdrawal. One GPA-patient received azathioprine, five GPA-patients received azathioprine in combination with prednisolone, and two GPA-patients were treated with low dose prednisolone. Detailed clinical and laboratory characteristics of the patients are summarized in table 1. All patients and healthy controls provided informed consent and the local medical ethics committee of the University Medical Center Groningen approved the study.

Sample preparation and in vitro peripheral blood stimulation

Lithium-heparinized venous blood was obtained from GPA-patients and HCs. Immediately after blood withdrawal, 2 ml of blood was mixed with 2 ml of RPMI 1640 (Lonza, Basel, Switzerland),
supplemented with 50 µg/ml Gentamicin (GIBCO, Life Technologies, Grand Island, NY, USA) and 10 % fetal calf serum. The diluted blood samples were aliquoted into 5 mL polypropylene tubes (Falcon®, Corning incorporated) at 400 µL per tube. Next, the blood samples were pre-incubated in the presence or absence of ShK-186 (dose range [0.1 nM – 100 nM]; Kineta Inc, Seattle, WA, USA) for 1 hour at 37 °C, followed by stimulation with 50 ng/ml phorbol myristate acetate (PMA; Sigma-Aldrich, St Louis, MO, USA) and 2 mM calcium ionophore (CaI, Sigma-Aldrich). The cultures were incubated for 16 hours at 37 °C with 5% CO₂. As a negative control, one sample was kept without stimulation. To inhibit cytokine release from cells, 10 µg/ml brefeldin A (BFA; Sigma-Aldrich) was added to each sample.

Immunofluorescence staining of peripheral blood

After stimulation of the peripheral blood, erythrocytes were lysed using ammonium chloride and the cells were washed in wash buffer (PBS containing 1% (w/v) bovine serum albumin (BSA)). T cells were stained with Brilliant Violet 605-conjugated anti-CD3 (Biolegend, San Diego, CA, USA), APC-eF780-conjugated anti-CD8 (eBioscience, San Diego, CA, USA), FITC-conjugated CD45RO (BD Pharmingen™, Franklin Lakes, NJ, USA) and PE-Cy7-conjugated CCR7 (BD Pharmingen™) for 15 minutes at room temperature. Cells were fixed with 100 µl fixation reagent A (Fix/Perm medium A, life technologies, Breda, The Netherlands) for 15 minutes. After washing, cells were resuspended in 100 µl permeabilization reagent B (Fix/Perm medium B, life technologies) and labeled with PerCP-Cy5.5-conjugated anti-IL-4 (Biolegend), APC-conjugated anti-IL-17A (eBioscience), PE-conjugated anti-IL-21 (eBioscience), Alexa Fluor®700-conjugated anti-IFNγ (BD Pharmingen™) and Pacific Blue-conjugated anti-TNFα (Biolegend) for 30 minutes at room temperature in the dark. Finally, the samples were washed and analyzed by nine-color flow cytometric analyses on BD™ LSR II flow cytometer. Data were collected for 5 * 10⁵ events for each sample and plotted using Kaluza v1.5a (Beckman Coulter, Brea, CA, USA). Because stimulation reduces the surface expression of CD4 on T cells, CD4⁺T cells were identified indirectly by gating CD3-positive and CD8-negating lymphocytes. Gated CD4⁺T cells were further displayed as density dot plots for the evaluation of intracellular cytokine production. The unstimulated
negative control sample was used to discriminate cytokine producing from non-cytokine producing CD4+ T cell populations.

Isolation of CD4+ T<sub>NAIVE</sub> and CD4+ T<sub>EM</sub> cells

PBMCs of 5 HCs were used for cell sorting experiments. Cell suspensions were stained for CD3, CD8, CD45RO, and CCR7. CD4+ T cells were gated negatively as CD3-positive and CD8-negative cells and sorted into: CD4+ T<sub>NAIVE</sub> (CD45RO<sup>-</sup>CCR7<sup>+</sup>), CD4+ T<sub>CM</sub> (CD45RO<sup>+</sup>CCR7<sup>+</sup>), CD4+ T<sub>EM</sub> (CD45RO<sup>+</sup>CCR7<sup>-</sup>), and CD4+ T<sub>T</sub> (CD45RO<sup>-</sup>CCR7<sup>-</sup>) cell fractions on a MoFLO Astrios sorter (Beckman Coulter). The purity of the sorted CD4+ T cell subsets, as determined by a post sort analysis, was > 98% for all sort CD4+ T cell subsets. From each subset, 2.5 * 10<sup>5</sup> cells were incubated in the presence or absence of ShK-186 (dose range [0.1 nM – 100 nM]; Kineta Inc) for 1 hour at 37 °C, followed by stimulation with 50 ng/ml PMA (Sigma-Aldrich) and 2 mM CaI (Sigma-Aldrich) in the presence of BFA. Following incubation for 16 hours at 37 °C with 5% CO<sub>2</sub>, cells were washed, premeabilized, and stained intracellularly for IL-4 (Biolegend), IL-17A, IL-21 (eBioscience), IFNγ (BD Pharmingen™), and TNFα (Biolegend). Finally, the samples were acquired on a BD™ LSR II flow cytometer (BD Biosciences) and data was analyzed using Kaluza 1.5a. Unstimulated samples were used as negative control for setting gates to define cytokine producing cells.

Statistical analysis

Statistical analysis was performed using GraphPad prism (GraphPad Software, San Diego, CA, USA). Data are presented as median values or mean ± SEM, as indicated. Data were analyzed with the D’Agostino & Pearson omnibus normality test for Gaussian distribution. For comparison between GPA-patients and HCs the unpaired t-test was used for data with Gaussian distribution and the Mann-Whitney U test for data without Gaussian distribution. For intra-individual comparison between samples treated with or without ShK-186, the paired t test was used for data with Gaussian distribution and the Wilcoxon
singed rank test for data without Gaussian distribution. Differences were considered statistically significant at 2-sided \( P \)-values equal to or less than 0.05.

**Results**

**T cell subset distribution in peripheral blood of GPA-patients in remission**

We first assessed the distribution of CD4\(^+\)T cell subsets in the peripheral blood of GPA-patients in remission and HCs. CD4\(^+\)T cell subsets were identified based on the surface expression of CD45RO and CCR7 and divided into CD4\(^+\)T\(_{\text{NAIVE}}\) cells (CD45RO\(^-\)CCR7\(^+\)), CD4\(^+\)T\(_{\text{CM}}\) (CD45RO\(^+\)CCR7\(^+\)), CD4\(^+\)T\(_{\text{EM}}\) cells (CD45RO\(^-\)CCR7\(^-\)) and CD4\(^+\) terminal differentiated cells (T\(_{\text{TD}}\), CD45RO\(^-\)CCR7\(^-\)) (figure 1A). We found that the percentage of circulating CD4\(^+\)T\(_{\text{EM}}\) cells from GPA-patients was significantly higher compared to HCs (figure 1B). The percentage of circulating CD4\(^+\)T\(_{\text{NAIVE}}\) cells was significantly lower in GPA-patients compared to HCs, whereas the percentage of CD4\(^+\)T\(_{\text{CM}}\) cells did not differ. In addition, the percentage of circulating CD4\(^+\)T\(_{\text{TD}}\) cells was significantly higher in GPA-patients compared to HCs.

To rule out the possibility that the increased proportion of CD4\(^+\)T\(_{\text{EM}}\) cells was influenced by current treatment, we compared the proportions of CD4\(^+\)T\(_{\text{EM}}\) cells between GPA-patients off treatment and GPA-patients receiving immunosuppressive maintenance therapy. No significant differences were found between the treated and untreated patient group (data not shown).

**Increased intracellular pro-inflammatory T cell cytokine production in GPA-patients**

Effector T cells produce pro-inflammatory cytokines (such as IL-4, IL-17, IL-21, TNF\(\alpha\), and IFN\(\gamma\)) that are presumed to be involved in the disease pathogenesis of GPA. Therefore, we next analyzed the pro-inflammatory cytokine profile of CD4\(^+\)T\(_{\text{H}}\) cell from GPA-patients and HCs. In all samples the production of intracellular IL-4, IL-17, IL-21, TNF\(\alpha\), and IFN\(\gamma\) was determined in CD4\(^+\)T\(_{\text{H}}\) cells by flow cytometry (figure 2A). As shown in figure 2, the expression of all pro-inflammatory cytokines within CD4\(^+\)T\(_{\text{H}}\) cells was significantly higher in GPA-patients compared to HCs.
Of note, it has become evident that CD4+ T<sub>H</sub> cells may produce additional cytokines besides their signature cytokine. For example, T<sub>H</sub>1 cells may produce IL-17 in addition to their signature cytokine IFNγ, and T<sub>H</sub>17 cells produce IL-21 in addition to their signature cytokine IL-17[25]. We, therefore, assessed the proportion of CD4+ T<sub>H</sub> cells producing 2 cytokines (TNFα+IFNγ+, IFNγ+IL-17+, and IL-17+IL-21+). As shown in figure 2B, CD4+ T<sub>H</sub> cells from GPA-patients in remission produce significantly higher percentages of TNFα+IFNγ+, IFNγ+IL-17+, and IL-17+IL-21+ cytokines as compared to CD4+ T<sub>H</sub> cells from HCs. Overall, these results demonstrate the pro-inflammatory nature of the CD4+ T<sub>H</sub> cells in patients with GPA.

**ShK-186 reduced the production of cytokines in CD4+ T<sub>H</sub> cells from GPA-patients to the level seen in CD4+ T<sub>H</sub> cells from HCs.**

Next, we questioned whether the pro-inflammatory cytokine production could be regulated by Kv1.3 channel blockade, using the highly potent Kv1.3 peptide blocker ShK-186. To this end, we stimulated peripheral blood samples of GPA-patients in the presence and absence of ShK-186 and analyzed the intracellular cytokine production of IL-4, IL-17, IL-21, TNFα, and IFNγ in CD4+ T<sub>H</sub> cells from GPA-patients (supplementary figure s1, available at Rheumatology online). As shown in figure 3, addition of ShK-186 to stimulated cell cultures significantly reduced the production of IL-17, IL-21, TNFα, and IFNγ in CD4+ T<sub>H</sub> cells from GPA-patients. The effect of ShK-186 on the production of IL-17, IL-21, TNFα and IFNγ was dose dependent (figure 3B). Interestingly, the production of IL-17, IL-21, TNFα and IFNγ in CD4+ T<sub>H</sub> cells was normalized to median cytokine levels detected in HC. Remarkably, the suppressive effect of ShK-186 on IL-4 production was less pronounced.

In addition, the percentage of CD4+ T<sub>H</sub> cells producing TNFα+IFNγ+, IFNγ+IL-17+, and IL-17+IL-21+ was significantly suppressed by ShK-186 in a dose dependent manner (figure 3C).

It is worth mentioning that the reduction in cytokine production upon pretreatment with ShK-186 was not attributed to cell death, as ShK-186 does not affect the viability of CD4+ T cells (Supplementary Figure S2, available at Rheumatology online).
ShK-186 inhibits cytokine production of CD4^{+} T\textsubscript{EM} cells

As described previously, CD4^{+} T\textsubscript{EM} cells express significantly higher numbers of Kv1.3 channels on their plasma membrane compared to CD4^{+} T\textsubscript{NAIVE} cells and CD4^{+} T\textsubscript{CM} cells\cite{17,26}. Therefore CD4^{+} T\textsubscript{EM} cells are the most likely target for ShK-186. To study if Kv1.3 channel blockade by ShK-186 selectively targets cytokine production of CD4^{+} T\textsubscript{EM} cells, we tested the effect of ShK-186 on FACS sorted CD4^{+} T\textsubscript{NAIVE}, CD4^{+} T\textsubscript{CM}, CD4^{+} T\textsubscript{EM}, and CD4^{+} T\textsubscript{TD} cells (figure 4A). First, we observed that the pro-inflammatory cytokine production of IL-4, IL-17, and IFN\gamma after \textit{in vitro} stimulation was significantly increased in CD4^{+} T\textsubscript{EM} cells compared to the other CD4^{+} T cell subsets (CD4^{+} T\textsubscript{NAIVE}, CD4^{+} T\textsubscript{CM}, and CD4^{+} T\textsubscript{TD} cells) (figure 4). IL-21 was significantly increased in CD4^{+} T\textsubscript{EM} cells compared to CD4^{+} T\textsubscript{NAIVE} and CD4^{+} T\textsubscript{TD} cells, whereas no difference was observed compared to CD4^{+} T\textsubscript{CM} cells. TNF\alpha was produced by all CD4^{+} T cell subsets, although the CD4^{+} T\textsubscript{EM} cells showed the highest expression levels of TNF\alpha compared to those of CD4^{+} T\textsubscript{NAIVE}, CD4^{+} T\textsubscript{CM}, and CD4^{+} T\textsubscript{TD} cells. Overall, \textit{in vitro} stimulation with PMA and CaI showed that CD4^{+} T\textsubscript{EM} cells are the major producers of pro-inflammatory cytokines in comparison to other CD4^{+} T cell subsets (figure 4B). Addition of ShK-186 inhibited CD4^{+} T\textsubscript{EM} cells from producing IL-4, IL-17, TNF\alpha, and IFN\gamma in a dose dependent manner, whereas the effect was less pronounced in other CD4^{+} T\textsubscript{H} cell subsets, as their cytokine production remains almost unchanged before and after treatment (figure 4B). It is worth mentioning that the production of IL-4 and IL-17 by isolated CD4^{+} T\textsubscript{H} subsets other than CD4^{+} T\textsubscript{EM} cells were less pronounced, which could potentially impact the ability of ShK-186 to reduce cytokine production in these subsets. In contrast, the production of IL-21 was slightly affected in both CD4^{+} T\textsubscript{CM} and CD4^{+} T\textsubscript{EM} cells, but reached a significant decrease at a dose of 100nM in CD4^{+} T\textsubscript{EM} only. It should be noted that a slight reduction was observed in TNF\alpha and IFN\gamma production in other CD4^{+} T\textsubscript{H} subsets, mainly CD4^{+} T\textsubscript{CM} cells. This might be expected as part of T\textsubscript{CM} cells may develop to T\textsubscript{EM} cells upon stimulation, and thus express higher levels of Kv1.3 channels that can be targeted by ShK-186.
Interestingly, we observed that TNFα+IFNγ+CD4+T\textsubscript{H} cells were predominantly present within the CD4+T\textsubscript{EM} subset. Addition of ShK-186 demonstrated a significant dose dependent inhibition of TNFα+IFNγ+ production by CD4+T\textsubscript{EM} cells compared to the other CD4+T cell subsets (figure 4B).

**Discussion**

In the present study, we show that pro-inflammatory cytokine producing CD4+T\textsubscript{H} cells are proportional increased in the circulation of GPA-patients in remission compared to HCs. We found that *in vitro* pharmacological blockade of Kv1.3 channels using ShK-186 decreased the production of pro-inflammatory cytokines including IL-17, IL-21, TNFα, and IFNγ of CD4+T\textsubscript{H} cells from GPA-patients. Importantly, ShK-186 treatment did not completely inhibit cytokine production but rather reduced the production of these pro-inflammatory cytokines to the level seen in CD4+T\textsubscript{H} cells from HCs. Furthermore, addition of ShK-186 predominantly affected cytokine production of CD4+T\textsubscript{EM} cells with minimal effects on cytokine production of other CD4+T\textsubscript{H} cell subsets (i.e. CD4+T\textsubscript{NAIVE}, CD4+T\textsubscript{CM}, and CD4+T\textsubscript{TD} cells).

Our observation that CD4+T\textsubscript{H} cells from GPA-patients display an increased pro-inflammatory cytokine profile compared to cells from HCs is consistent with previous reports demonstrating increased production of IFNγ and TNFα by PBMCs and CD4+T\textsubscript{H} cells of GPA-patients[11,27,28]. In addition, we and others have demonstrated that circulating IL-17 and IL-21 producing CD4+T\textsubscript{H} cells are significantly increased in GPA-patients even in remission[13,14,29].

Next, we demonstrated that the increase in pro-inflammatory cytokine production in CD4+T\textsubscript{H} cells from GPA-patients can be prevented by ShK-186 treatment. These data are in line with previous reports showing that ShK-186 preferentially suppresses production of IL-2, IFNγ, TNFα from synovial T-cells (mainly consisting of CD4+T\textsubscript{EM} cells) of RA patients[20]. In addition, Chi et al. have demonstrated that ShK-186 suppresses cytokine production in human T cells from whole blood[30]. Similar to our observations, these authors reported that Shk-186 was most effective in suppressing the production of IL-
2 followed by IFNγ and IL-17 but had a minor effect only on IL-4 production. Interestingly, it has been shown that TCR induced Ca\(^{2+}\) signaling is lower in T\(_{H}2\) cells than in T\(_{H}1\), T\(_{H}17\) or naïve T cells suggesting that Kv1.3 mediated T cell activation is differently regulated not only in T cell subsets (i.e. T\(_{NAIVE}\), T\(_{CM}\) and T\(_{EM}\)) but also between different T cell phenotypes [31,32]. This could explain the fact that blocking Kv1.3 channels using ShK-186 has a more pronounced effect on the pro-inflammatory cytokines IFNγ and IL-17 compared to IL-4.

Beside its effect on T cells, we have previously explored the anti-inflammatory effect of ShK-186 on B cells. We found that ShK-186 modulates the effector functions of B cells of GPA-patients in vitro by reducing the production of ANCA-s and pro-inflammatory cytokines [33]. Thus, utilizing a Kv1.3-based therapy in GPA would represent a significant improvement over existing therapies. In addition, ShK-186 may also be beneficial in the treatment of COVID-19, as it has the potential to mitigate the production of pro-inflammatory cytokines and thus suppresses the cytokine storms occurring in COVID-19 patients [34].

Using sorted CD4\(^{+}\)T cell subsets, we observed that cytokine production is most effectively suppressed by ShK-186 in CD4\(^{+}\)T\(_{EM}\) cells. This can be explained by the fact that activation of T-cells has differential effects on the expression of potassium channels in different T-cells subsets. CD4\(^{+}\)T\(_{NAIVE}\) and CD4\(^{+}\)T\(_{CM}\) cells preferentially up-regulate the Ca\(^{2+}\)-activated potassium KCa3.1 channel while CD4\(^{+}\)T\(_{EM}\) cells preferentially increase their Kv1.3 expression [17]. This switch in channel expression significantly affects responsiveness of T-cell subsets to Kv1.3 and KCa3.1 blockers, CD4\(^{+}\)T\(_{EM}\) cells being highly sensitive to Kv1.3 channel blockers and CD4\(^{+}\)T\(_{NAIVE}/T_{CM}\) cells being more sensitive to KCa3.1 channel blockers.

In addition, ShK analogs have shown similar effects on rat T cells in various immune-mediated inflammatory disease models. In these studies, ShK analogs showed efficacy in preventing and ameliorating acute experimental autoimmune encephalomyelitis (EAE, a model of multiple sclerosis) and pristine-induced arthritis in rats [20,35]. Moreover, in a rat model of anti-glomerular basement membrane (GBM) glomerulonephritis, the majority of CD4\(^{+}\)T cells infiltrating the kidney were Kv1.3\(^{high}\) T\(_{EM}\) cells [36]. Rats treated with a Kv1.3 blocker developed less proteinuria and had fewer crescentic glomeruli.
than rats treated with placebo. ShK-186 may therefore be useful in the treatment of autoimmune kidney
disease like GPA.

In addition to the ShK-186, several other types of Kv1.3 blockers have been developed and studied. For
instance, ShK-related peptides derived from parasitic worms, such as AcK1 and BmK1, have been shown
to block Kv1.3 channels and suppress $T_{EM}$ cell responses in vitro and in vivo[37]. Other scorpion venom-
derived peptides, including HsTX1 and Imk, have been also identified as potent Kv1.3 channel blockers,
and demonstrated effectiveness in controlling arthritis and reducing the severity of experimental
autoimmune encephalomyelitis, respectively[38,39]. Additionally, Vm24, a peptide derived from the
scorpion venom, has shown high selectivity and potency in blocking Kv1.3 channels and impairing the
synthesis and secretion of Th-cell cytokines in response to TCR engagement[40]. These Kv1.3 blockers
are currently being investigated as potential therapeutic agents for autoimmune diseases.

Compared to other Kv1.3 blockers, ShK-186 has several advantages. One of the key advantages of ShK-
186 over other Kv1.3 blockers is its high selectivity. It has been shown to be more than 100-fold selective
for Kv1.3 compared to other potassium channels[19]. Another unique aspect of ShK-186 is its tight
binding to the Kv1.3 channels and long duration of action, which may allow for less frequent dosing. The
unique pharmacokinetic profile of ShK-186 may make it a promising therapeutic option for the treatment
of autoimmune diseases.

It is worth noting that in addition to CD4$^+$T$^{EM}$ cells, Kv1.3 channels are also expressed in various tissues
in the body including the kidney, liver and the central nervous system. Therefore, one may argue that
toxic side effects are a potential concern when using Kv1.3 channel blockers. However, Kv1.3 blockers
-especially the ShK analogs) have been shown to have an excellent safety profile in animal
models[19,20,41]. ShK-186 was reported to exhibit no perceptible in vitro toxicity, was negative in the
amses test, and had no effect on cardiac parameters[19]. Furthermore, repeated subcutaneous
administration of ShK-186 in rats did not cause clinical toxicity as evidenced by normal blood cell counts
and serum chemistry parameters, and no signs of histopathological changes in various tissues[19,20].

Moreover, in vivo studies have demonstrated that the efficacy of ShK186 can be achieved without general immunosuppression[41]. In rats, administration of ShK-186 did not compromise the protective immune response to acute viral (Influenza) or bacterial (Chlamydia) infections at pharmacological doses that did ameliorate autoimmune diseases[41]. Importantly, ShK-186 has completed phase 1b trial in psoriasis patients showing the blocker is well tolerated and improve psoriatic skin lesions by inhibiting T cell mediators of inflammation[42].

Therapies targeting CD4+TEM cells via blocking Kv1.3 channels may have an advantage over current therapies in GPA because CD4+TNAIVE and CD4+TCM would escape the inhibition by ShK-186. Leaving TNAIVE and TCM CD4+T cells unimpaired. GPA-patients treated with ShK-186 would therefore be able to preserve protective immune responses against most pathogenic challenges. On the other hand, a potential disadvantage of Kv1.3 blockade is that it likely suppresses all CD4+TEM cells thereby affecting immune responses against chronic infections. However, as demonstrated here, it may be possible to titrate ShK-186 to a dose at which it normalizes, but does not completely suppress, CD4+TEM cell responses.

Moreover, the Kv1.3 blocker is reversible and therapy could be paused in the event of an acute infection, unlike current treatments in GPA (i.e. cyclophosphamide, high dose corticosteroids and rituximab) which take several months to subside.

In this study, blood samples from GPA-patients in remission were evaluated for the effect of ShK-186, and not in those with active disease. We have previously shown that during active disease CD4+TEM cells appear to migrate towards inflamed tissues[12]. Analysis of ShK-186 effect on circulating CD4+TH cells in GPA-patients with active disease will exclude cells migrated to inflamed tissue which are, probably, the most relevant cells. Therefore, studying samples from patients in remission seems more relevant for this analysis. However, it should be noted that our findings may not fully reflect the effect of ShK-186 on TEM cells during disease progression, and therefore future studies should aim to include samples from patients with active disease to better understand the potency of ShK-186 on CD4+ TEM cells in different
stages of the disease. Future *in vivo* studies should also consider the impact of ShK-186 on PR3-specific T cells and its impact on protective immunity.

In conclusion, the data presented here demonstrate that the Kv1.3 blocker ShK-186 suppresses pro-inflammatory cytokine production in CD4$^+$ T$_H$ cells from GPA-patients, and predominantly affects the cytokine production of CD4$^+$ T$_{EM}$ cells. Importantly, ShK-186 treatment reduced the production of the pro-inflammatory cytokines to the level seen in CD4$^+$ T$_H$ cells from HCs. These findings support the potential of selective Kv1.3 blockade as a therapeutic strategy for GPA-patients.

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Author Contributions:

All authors contributed to the concept and design. LL performed the experiments, statistical analysis, drafted the manuscript, and contributed to interpretation of the data. WA and PH contributed to interpretation of the data and critically revised the manuscript. AR and CS contributed to inclusion of patients with GPA, and assessed and participated in the interpretation of clinical data, and critical revision of the manuscript. EM-E, ET, and SI critically revised the manuscript. All authors read and approved the final manuscript.

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The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Data availability statement:
The data that support the findings of this study are available from the corresponding author on request.

Table

| Table 1: Clinical and laboratory characteristics of the GPA-patients at the time of blood sampling |
| GPA |
| Subjects, n (% male) | 27 (44%) |
| Age, mean (range) | 61 (34 – 79) |
| PR3-ANCA positive\(^1\), n (% positive) | 18 (67%) |
| Localized / generalized disease, n (% generalized) | 6 / 21 (78%) |
| CRP (mg/L), median (range) | 5.8 (<0.3 – 11) |
| eGFR\(^2\) ml/min/1.73m\(^2\), median (range) | 64 (15 – 91) |
| Disease duration in years, median (range) | 9.5 (1.3 – 30.8) |
| Number of previous relapses, median (range) | 1 (0 – 6) |
| Non / maintenance immunosuppressive therapy\(^3\), n | 19 / 8 |

\(^1\)ANCA-positive titer ≥1:40, ANCA-negative ≤1:20

\(^2\)Estimated Glomerular Filtration Rate (eGFR) was calculated using the Chronic Kidney Disease Epidemiology Collaboration (CKD-EPI) equation.

\(^3\)Immunosuppressive maintenance therapy: azathioprine, azathioprine + prednisolone, or prednisolone.
Figures

Figure 1: Increased percentage of circulating CD4+ T EM cells in GPA-patients. A) Representative flow cytometry dot plots of CD45RO and CCR7 expression to identify four CD4+ T cell subsets in the peripheral blood of a GPA-patient in remission (right plot) and a HC (left plot). B) Percentages of CD45RO−CCR7− (T NAIVE), CD45RO−CCR7+ (T CM), CD45RO+CCR7− (T EM) and CD45RO+CCR7− (T TD) subsets within the CD4+ T cell population in peripheral blood of GPA-patient in remission (filled squares; n=27) and HCs (open circles; n=16). Horizontal bar represent median percentage. **p<0.01, ***p<0.001 versus HCs.

Figure 2: Higher percentage of intracellular cytokine production in circulating CD4+T H cells from GPA-patients. Peripheral blood of GPA-patients and HCs was stimulated with PMA and CaI and analyzed with flow cytometry for intracellular IL-4, IL-17, IL-21, TNFα, and IFNγ cytokine expression. A) Percentages of IL-4+, IL-17+ IL-21+, TNFα+, and IFNγ+ CD4+T H cells from GPA-patient in remission (filled squares; n=27) and HCs (open circles; n=16). B) Percentages of TNFα+IFNγ+, IFNγ+IL-17+, and IL-17+IL-21+ within CD4+T H cells from GPA-patient in remission (filled squares; n=27) and HCs (open circles; n=16). Horizontal bar represent median percentage. *p<0.05, **p<0.01, ***p<0.001 versus HCs.

Figure 3: Dose dependent suppression of pro-inflammatory cytokines by ShK-186 in CD4+T H cells from GPA-patients. Peripheral blood of GPA-patients and HCs was stimulated with PMA and CaI with and without increasing concentrations of ShK-186. Intracellular IL-4, IL-17, IL-21, TNFα, and IFNγ cytokine production in CD4+T H cells was analyzed using flow cytometry. A) Representative flow cytometry dot plots of cytokine expression within CD4+ T H cells after stimulation in the presence (lower panels) and absence (upper panels) of ShK-186 from a GPA-patient in remission. B) Percentages of cytokine producing CD4+ T H cells after stimulation in the presence and absence of ShK-186 from GPA-patients in remission (grey box and whiskers; n=27). C) Percentages of TNFα+IFNγ+, IFNγ+IL-17+, and IL-17+IL-21+ within CD4+T H cells after stimulation in the presence and absence of ShK-186 from GPA-
patients in remission (grey box and whiskers; \(n=27\)). Box and whiskers plots (tukey), boxes represent median values and interquartile range. Red horizontal dashed line represent median percentage of cytokine production by \(\text{CD}^4\text{T}_{\text{H}}\) cells from HCs. *\(p<0.05\), **\(p<0.01\), ***\(p<0.001\) versus stimulated \(\text{CD}^4\text{T}_{\text{H}}\) cells without ShK-186.

**Figure 4: ShK-186 inhibits the pro-inflammatory cytokine production of \(\text{CD}^4\text{T}_{\text{EM}}\) cells.** \(\text{CD}^4\text{T}\) cells subsets (i.e. \(\text{CD}^4\text{T}_{\text{NAIVE}}\), \(\text{CD}^4\text{T}_{\text{CM}}\), \(\text{CD}^4\text{T}_{\text{EM}}\), and \(\text{CD}^4\text{T}_{\text{TD}}\) cells) were isolated from PBMCs of HCs followed by stimulation with PMA and CaI with and without increasing concentrations of ShK-186. Intracellular IL-4, IL-17, IL-21, TNF\(\alpha\), and IFN\(\gamma\) cytokine production in the \(\text{CD}^4\text{T}\) cell subsets was analyzed using flow cytometry. 

**A)** Representative flow cytometry dot plots of \(\text{CD}^4\text{T}\) cells subsets based on surface expression of CD45RO and CCR7 (center dot plot), and the cytokine expression within \(\text{CD}^4\text{T}_{\text{NAIVE}}\) cells (upper left, red), \(\text{CD}^4\text{T}_{\text{CM}}\) cells (upper right, green), \(\text{CD}^4\text{T}_{\text{TD}}\) cells (lower left, purple), and \(\text{CD}^4\text{T}_{\text{EM}}\) cells (lower right, blue) after stimulation in the presence and absence of ShK-186 from a HC.  

**B)** Percentages of intracellular cytokine production in \(\text{CD}^4\text{T}_{\text{NAIVE}}\) cells (red symbol & line), \(\text{CD}^4\text{T}_{\text{CM}}\) cells (green symbol & line), \(\text{CD}^4\text{T}_{\text{TD}}\) (purple symbol & line), and \(\text{CD}^4\text{T}_{\text{EM}}\) cells (blue symbol & line) after stimulation in the presence and absence of ShK-186 from HCs \((n=5)\). Data represent mean values \(\pm\) SEM. ***\(p<0.01\), ****\(p<0.001\) indicate \(\text{CD}^4\text{T}_{\text{EM}}\) cells versus \(\text{CD}^4\text{T}_{\text{NAIVE}}\), \(\text{CD}^4\text{T}_{\text{CM}}\), and \(\text{CD}^4\text{T}_{\text{TD}}\) cells. *\(p<0.05\), **\(p<0.01\), ***\(p<0.001\) indicate \(\text{CD}^4\text{T}_{\text{EM}}\) cells with vs without ShK-186.  

IL-21 production in \(\text{CD}^4\text{T}_{\text{EM}}\) cells is only significant different compared to \(\text{CD}^4\text{T}_{\text{NAIVE}}\) and \(\text{CD}^4\text{T}_{\text{TD}}\) cells and not significant different compared to \(\text{CD}^4\text{T}_{\text{CM}}\) cells.
Figure 1

A

CD4+ T cells

HC

GPA

T_{NAIVE} 54%  T_{CM} 28%
T_{TD} 2%
T_{EM} 16%

CD45RO

B

HC  GPA  HC  GPA  HC  GPA  HC  GPA

% cells (within CD4+ T cells)

CD45RO - + + -

CCR7 + + - -

HC GPA  HC GPA  HC GPA  HC GPA

T_{Naive}  T_{CM}  T_{EM}  T_{TD}

***  **  ***  **
Figure 2

A

IL-4

IL-17

% IL-4 CD4+ T cells

% IL-17 CD4+ T cells

HC GPA

HC GPA

IL-21

TNFα

IFNγ

% IL-21 CD4+ T cells

% TNFα CD4+ T cells

% IFNγ CD4+ T cells

HC GPA

HC GPA

HC GPA

B

TNFα*IFNγ*

IFNγ*IL-17*

IL-17*IL-21*

% TNFα*IFNγ* CD4+ T cells

% IFNγ*IL-17* CD4+ T cells

% IL-17*IL-21* CD4+ T cells

HC GPA

HC GPA

HC GPA
Figure 3

A

CD4+ T cells

B

IL-4

IL-17

IL-21

TNFα

IFNγ

C

TNFα*IFNγ*

IFNγ*IL-17*

IL-17*IL-21*

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Figure 4

A

B

IL-4

IL-17

IL-21

TNFα

IFNγ

ShK-186 [nM]

% CD4+ T cells

% IL-4+ T cells

% IL-17+ T cells

% IL-21+ T cells

% TNFα+ T cells

% IFNγ+ T cells

% TNFα+IFNγ+ T cells

0

0.1

1

10

100