

Received Date : 11-Feb-2015

Revised Date : 22-Apr-2015

Accepted Date : 07-Jul-2015

Article type : Regular Article

A recombinant fusion protein derived from dog hookworm inhibits autoantibody-induced dermal-epidermal separation *ex vivo*

Title: A recombinant fusion protein derived from dog hookworm inhibits autoantibody-induced dermal-epidermal separation *ex vivo*

Authors: Annette Kemmer,^{1,#} Katja Bieber,^{2,#} Aida Abadpour,¹ Xinhua Yu,³ Nina Mitschker,¹ Sara Roth,⁴ Claudia Kauderer,¹ Ralf J. Ludwig,² Karsten Seeger,⁵ Jörg Köhl,⁶ Detlef Zillikens,^{1,2} Andreas Recke^{1,2,*}

1. Dept. of Dermatology, Allergology and Venereology, University of Lübeck, Lübeck, Germany
2. Institute of Experimental Dermatology, University of Lübeck, Lübeck, Germany
3. Biochemical Immunology, Research Center Borstel, Leibniz-Center for Medicine and Biosciences, Borstel (Sülfeld), Germany
4. Institute for Medical Microbiology and Hygiene, University of Lübeck, Lübeck, Germany
5. Institute of Chemistry, University of Lübeck, Lübeck, Germany
6. Inst. for Systemic Inflammation Research, University of Lübeck, Lübeck, Germany

These authors contributed equally

This article has been accepted for publication and undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process, which may lead to differences between this version and the Version of Record. Please cite this article as doi: 10.1111/exd.12804

This article is protected by copyright. All rights reserved.

* Corresponding author

Corresponding author's contact details:

Dr. Andreas Recke, Lübeck Institute of Experimental Dermatology, University of Lübeck, Lübeck, Germany. Email: Andreas.Recke@uksh.de. Telephone: +49 451 500 2530. Fax: +49 451 500 2981

Funding sources:

This work received funding from the Deutsche Forschungsgemeinschaft RTG 1727/1 (to A.K, A.R. and D.Z.) and infrastructural support from the Deutsche Forschungsgemeinschaft Cluster of Excellence Inflammation at Interfaces (Cluster 306/2).

Conflict of interest declaration:

There are no conflicts of interest to declare.

Notes:

The genetic sequence used in this study has been deposited at GenBank (URL: <http://www.ncbi.nlm.nih.gov>, last accessed 29.12.2014) under accession number KP314277.

Abstract

The proteins secreted by parasitic nematodes are evolutionarily optimized molecules with unique capabilities of suppressing the immune response of the host organism. Neutrophil inhibitory factor (NIF), which is secreted by the dog hookworm *Ancylostoma caninum*, binds to the $\beta 2$ integrin CD11b/CD18, which is expressed on human neutrophils, eosinophils, monocytes and macrophages and inhibits neutrophil-dependent lung injury and neutrophil invasion of ischemic brain tissue. Neutrophils are key players in the pathogenesis of subepidermal autoimmune blistering diseases (sAIBDs), and their pathogenic activities are crucially dependent on $\beta 2$ integrin functionality. Based on the template of single-stranded, dimerizing antibody derivatives, which are already used in cancer treatment, we designed a novel biologic, NIF-IGHE-CH₄, comprising NIF and the dimerizing but otherwise inert constant heavy subdomain 4 (CH₄) of human IgE (IGHE). This molecule was evaluated in a variety of *in vitro* assays, demonstrating its ability to inhibit pathogenically relevant neutrophil functions such as migration, adhesion and spreading, and release of reactive oxygen species (ROS). Finally, we confirmed that NIF-IGHE-CH₄ inhibits blister formation in an *ex vivo* assay of sAIBD. These results suggest that NIF-IGHE-CH₄ is a novel potential anti-inflammatory drug for the treatment of neutrophil-mediated diseases such as sAIBDs. This study promotes the *drugs from bugs* concept and encourages further research and development focused on turning parasite proteins into useful anti-inflammatory biologics.

Key words (3-5)

Autoimmune blistering disease; neutrophils; neutrophil inhibition; parasite proteins; beta-2 integrin

Introduction

During evolution, parasitic organisms developed unique capabilities to evade host defense and interfere with immune responses. However, these parasitic interferences avoid completely inactivating the host's immune system, as this would severely harm the host and indirectly affect the survival of the parasite itself. Moreover, parasitic infections are often accompanied by the suppression of misdirected immune reactions such as allergies and autoimmune diseases (1). The molecules that parasitic organisms secrete to modulate the host immune system are therefore promising candidates for anti-inflammatory and immunomodulatory drugs for treating autoimmune diseases with minimal adverse effects (2–4).

Subepidermal autoimmune blistering diseases (sAIBDs) are characterized by autoantibodies directed against structural proteins of the dermal-epidermal junction such as type XVII collagen in the case of bullous pemphigoid (BP) (5,6) and type VII collagen in the case of epidermolysis bullosa acquisita (EBA) (7–9). A plethora of evidence indicates that the binding of autoantibodies to skin components leads to Fc- and glycosylation-dependent pathogenicity (10–14). The pathogenic events induce the complement cascade (15–18), innate immune cell recruitment and activation (10,11,19,20) and reactive oxygen species (ROS) (21,22) and metalloprotease (23) release by neutrophils, ultimately resulting in severely itchy dermatitis and tense skin blisters. The origin of the irregular production of autoantibodies remains unclear. However, recent studies have demonstrated that induction of a blistering disease resembling EBA or BP in mice is dependent on the s allele of MHC H2(24), on genetic loci outside the MHC regions (25) and on interactions between genes and the microbiome (26).

Because of their key role in the pathogenesis of sAIBDs, neutrophils (neutrophilic granulocytes) are a promising target for novel anti-inflammatory molecules. Molecules secreted from hookworms, such as *Nippostrongylus brasiliensis* (27), *Haemonchus contortus* (28) and *Ancylostoma caninum* (dog hookworm) (29,30), specifically impair the function of neutrophils. The 41-kDa neutrophil inhibitory factor (NIF) from *Ancylostoma caninum* competes with ICAM-1 to bind to the A-domain of CD11b and selectively inhibits CD11/CD18 (CR-3, Mac-1, $\alpha_M\beta_2$ integrin)-dependent neutrophil functions, such as adhesion to vascular endothelial cells and ROS release upon activation (29–31). The A-domain of CD11b is a major recognition site for complement factor iC3b, fibrinogen and factor X in the coagulation cascade (32). NIF also prevents neutrophil-dependent lung injury *ex vivo* and *in vivo* (33,34) and secondary damage caused by neutrophil infiltration of ischemic brain tissues (35). In all of these studies, the administration of NIF either as a recombinant protein or by *in vivo* transfection to a living organism appeared to be a major challenge. The solution to this challenge might be the construction of an artificial protein that retains the abilities of NIF but has optimized pharmacokinetic and pharmacodynamic profiles.

In this study, we designed and expressed a novel biomolecule that targets and inhibits innate immune cells. The design of this molecule combines the functional properties of *Ancylostoma caninum*-derived NIF with the stability and versatility of small immunoproteins (SIPs) that have already been successfully used in patient therapy (36–38). Moreover, this design allows the

construction of bifunctional heterodimeric molecules, with NIF as an anti-inflammatory agent and an antibody moiety for targeting to the site of inflammation. We show that this novel biomolecule has a low toxicity and effectively inhibits neutrophil migration, adhesion and ROS release *in vitro*, as well as leukocyte-dependent dermal-epidermal separation in an *ex vivo* model of sAIBDs.

Methods

Ethics statement

The ethics committee of the University Hospital of Schleswig-Holstein, Campus Lübeck (No. 04-061, 04-144, and 04-143), approved the studies on serum, skin and leukocytes from patients and healthy volunteers.

Human materials

The BP patient sera used in this study were characterized based on their 1) linear deposition of IgG at the dermal-epidermal junction, as determined by indirect immunofluorescence microscopy of human skin cryosections; 2) reactivity with the epidermal side of 1 M salt-split skin; 3) reactivity with the 180-kDa band formed by keratinocyte extract, as determined by immunoblotting; and 4) positive reactivity with BP180 NC16A, as determined by ELISA. Normal human donor sera were confirmed to be non-reactive in the above-described assays.

Construction and expression of NIF-IGHE-CH₄

NIF (Uniprot ID Q16969, aa 18-274) and IGHE-CH₄ (Uniprot ID P01854, aa 324-428) were synthesized by GeneArt® (Life Technologies GmbH, Darmstadt, Germany). The DNA sequences were codon-optimized by GeneArt® for expression in *Spodoptera frugiperda* Sf21 insect cells and were cloned into the insect cell expression vector pMIBV5/HisC (Life Technologies GmbH, Darmstadt, Germany) using the Hind III and Not I ligation sites.

For polyclonal stable expression of secreted NIF-IGHE-CH₄, Sf21 insect cells cultivated in Insect-XPRESS™ protein-free insect cell medium (Lonza, Basel, Switzerland) were used. The Sf21 cells were transfected using FuGENE HD (Roche, Mannheim, Germany) according to the manufacturer's recommendations. After 4 days, the transfected cells were transferred into Insect-XPRESS™ containing Blasticidin S (Life Technologies GmbH, Darmstadt, Germany) at 40 µg/ml. After 14 days, the polyclonal population of surviving Sf21 cells was further sub-cultivated in Insect-XPRESS™ with 20 µg/ml Blasticidin S.

For high-performance expression, stably transfected Sf21 cells were transferred into Erlenmeyer flasks for shaking culture at an initial density of 5×10⁵ cells/ml. After 5 days, the cell supernatants were harvested.

Purification of NIF-IGHE-CH₄ using Strep-Tactin affinity chromatography

The supernatants from stably transfected Sf21 insect cells were dialyzed against 50 mM Tris-HCl/150 mM NaCl/2.5 mM MgCl₂ at pH 8.0 and treated with Benzonase (Sigma-Aldrich Chemie GmbH, Munich, Germany) and BioLock solution (IBA GmbH, Göttingen, Germany) according to the manufacturers' recommendations. Sterile-filtered supernatants were applied to Strep-Tactin Superflow high-capacity columns (IBA GmbH, Göttingen, Germany) after pre-equilibration with buffer W (100 mM Tris-HCl (pH 8.0)/150 mM NaCl/1 mM EDTA). After washing with buffer W, NIF-IGHE-CH₄ was eluted using buffer W containing 2.5 mM desthiobiotin.

The purified NIF-IGHE-CH₄ was analyzed by SDS-PAGE under both reducing and non-reducing conditions. After electrophoresis, the gels were either stained with colloidal Coomassie brilliant blue (39) or subjected to Western blotting on nitrocellulose membranes. To detect recombinant NIF-IGHE-CH₄ by immunoblotting, a peroxidase conjugate (IBA GmbH, Göttingen, Germany) was used.

FACS analysis of NIF-IGHE-CH₄ binding

Flow cytometric analysis was used to determine NIF-IGHE-CH₄ binding to leukocyte subpopulations. For this purpose, NIF-IGHE-CH₄ was conjugated to DyLight 488 (Thermo Fisher Scientific, p/a Perbio Science Deutschland, Bonn, Germany) according to the manufacturer's instructions. Human whole blood was collected from a healthy volunteer. For erythrocyte lysis, Buffer EL (Qiagen, Venlo, The Netherlands) was added to the whole blood at a 5:1 ratio and incubated for 10 min at room temperature. The leukocytes were washed twice with FACS buffer (PBS/0.5% BSA/0.02% NaN₃). Leukocytes (5×10^5) were stained by incubation with DyLight 488-conjugated or unconjugated NIF-IGHE-CH₄ and with PE-CD3, APC-CD19, APC-CD15 or PE-CD11b on ice for 30 min to discriminate leukocyte subpopulations. All antibodies were purchased from BD Biosciences, San Diego, CA, USA. After washing twice with FACS buffer, the cells were resuspended in 1 ml of PBS and analyzed on a FACSCalibur® (BD Biosciences, San Diego, CA, USA). Data acquisition and analysis were performed with CellQuest Pro (BD Biosciences, San Diego, CA, USA).

Preparation of granulocytes from whole blood

Granulocytes for use in *in vitro* assays were isolated from freshly collected whole human blood using Polymorphprep™ (Axis-Shield GmbH, Heidelberg, Germany) according to the manufacturer's instructions. The typical purity of PMN yielded by this method was about 85% (Supplemental Figure S1).

Measurement of granulocyte migration activity

Granulocyte migration activity was measured in 24-well chemotaxis chambers (Transwell Permeable Supports, Corning, Corning B.V. Life Sciences, Amsterdam, The Netherlands) using the transwell method. Granulocytes were freshly prepared as described above, resuspended in RPMI 1640 at 5×10^6 cells/ml together with varying concentrations of NIF-IGHE-CH₄, and pre-incubated for 30 min at 37 °C and 5% CO₂ in a humidified incubator. Next, 100 µl of cell suspension was added to the upper compartment of each transwell chamber. A total of 600 µl of RPMI 1640, together with 10 or 30 nM complement factor C5a, was added to the lower compartment. The plates were

incubated for 1 h in a humidified incubator, as described above. The medium and cells from the lower compartment were mixed with 100 μ l of 1% Triton X-100 for cell lysis. To count the number of migrated cells, glucuronidase activity was measured in the cell lysates using 0.1 M sodium acetate (pH 4.5)/0.01 M p-nitrophenyl-beta-glucuronide and was compared with a cell standard. After incubation for 3-4 h at room temperature, the beta-glucuronidase reaction was stopped by adding 0.4 M glycine-HCl (pH 10.3). Absorbance at 540 nm was read using a VICTOR 3TM reader (PerkinElmer Inc., Waltham, MA, USA).

Measurement of granulocyte adhesion and spreading

Autoimmune complex-induced granulocytes adhesion was monitored and quantified by real-time impedance measurement using the xCELLigence System (Roche, Penzberg, Germany) (40). Freshly isolated granulocytes were added to an E-Plate 96 VIEW coated with or without standardized autoimmune complexes, formed by pre-incubation of plates with 10 μ g/ml recombinant hCOL7EF-GST (12) fragments (Thr³⁹⁴-Ala⁶⁰⁰ of human collagen VII NC1, UNIPROT entry Q02388), blocking with 1% BSA in PBS/0.05% Tween 20 and incubation with recombinant anti-hCOL7 hIgG1 antibodies (12) diluted to 1 μ g/ml in PBST/1% BSA. NIF-IGHE-CH₄ was added at concentrations between 100 ng/ml and 10 μ g/ml. The relative change in the measured electrical impedance represents cell adhesion and is expressed as the arbitrary unit of the cell index. The area under the curve (AUC) of the cell index curve was calculated for a 3-h recording time after the autoimmune complex stimulation.

***In vitro* disease model of immobilized immune-complex-induced ROS release by granulocytes**

ROS release by granulocytes during stimulation with immobilized immune complexes was measured as previously described (12). Briefly, white microtiter plates (Greiner Bio-One, Frickenhausen, Germany) were coated with 10 μ g/ml recombinant hCOL7EF-GST fragments, blocked with 1% BSA in PBS/0.05% Tween 20 and incubated with recombinant anti-hCOL7 hIgG1 antibodies diluted to 1 μ g/ml in PBST/1% BSA. Subsequently, granulocytes isolated from freshly collected human whole blood were resuspended in dye-free RPMI 1640 chemiluminescence medium supplemented with 1% FCS, 10 mg/ml luminol and different concentrations of NIF-IGHE-CH₄, incubated for 30 min on ice, and then placed into the pretreated microtiter plates. The chemiluminescence activity kinetics were measured at 1-min intervals with 60 repetitions using a VICTOR 3TM microplate reader (PerkinElmer Inc., Waltham, MA, USA). FMLP was used at a final concentration of 10 μ M as the positive control.

Determination of cellular toxicity

The induction of apoptosis and necrosis in granulocytes incubated with NIF-IGHE-CH₄ was measured using the Annexin V-FITC Detection Kit (PromoKine, Heidelberg, Germany). Granulocytes were incubated in microtiter plates coated with or without autoimmune complexes at varying concentrations of NIF-IGHE-CH₄, as specified above for the ROS release assay. As controls, cells were treated either with PMA (30 nM) or exposed to UVB light. After 30 min, the cells were washed and stained with annexin V and propidium iodide according to the manufacturer's instructions and analyzed by flow cytometry as specified above.

Measurement of leukocyte-dependent dermal-epidermal separation (DES)

Ex vivo DES induction was performed as previously described (41). Briefly, human neonatal foreskin cryosections were incubated with control sera or sera from BP patients. The size of the cryosections ranged from 1-2 cm. After washing, the slides were incubated for 3 h at 37 °C with $2-2.5 \times 10^7$ leukocytes isolated by dextran sedimentation of freshly collected, heparinized blood from a healthy volunteer and treated with or without NIF-IGHE-CH₄. After fixation in 4% formaldehyde, the slides were stained with hematoxylin and eosin and examined by light microscopy to examine the recruitment of leukocytes to the basement membrane and split formation within the dermal-epidermal junction (DEJ).

Statistics

The open-source software GNU R version 3.15 (42) was used for statistical analysis, together with the packages *clinfun* for the non-parametric Jonckheere-Terpstra k-sample trend test (function *jonckheere.test*) and *coin* for the stratified non-parametric approximative k-sample permutation test (function *oneway_test*) and the stratified exact Wilcoxon Mann-Whitney rank sum test (function *wilcox_test*). The package *boot* was used for non-linear regression model analyses by bootstrapping.

The Bonferroni-Holm method or hierarchical testing was used for multiple test correction in the case of independent or hierarchically ordered hypotheses.

Combined scatter and box plots or combined dot and curve regression plots are used to present the data in *as raw a form as possible*, with each dot representing one separate experiment. Moreover, each experiment was repeated at least three times to ensure reproducibility of results. The dot and curve regression plots contain the fitted curve and a 95% confidence band, represented as a shaded area.

Non-linear sigmoidal regression was performed using *nls* of the GNU R basic *stats* package and the following four-parameter logistic function:

$$y = a + \frac{b - a}{1 + e^{\frac{c - \log(x)}{d}}}$$

with *a* and *b* as the lower and upper asymptotes, *c* as the turning point and *d* as the slope.

For the description of effect sizes, Glass's Δ was used:

$$\Delta = \frac{\bar{x}_1 - \bar{x}_2}{\sigma_1}$$

with $\bar{x}_1$ and $\bar{x}_2$ as the mean values of two groups and σ_1 as the standard deviation of group 1.

Results

NIF-IGHE-CH₄ is expressed as a dimeric molecule

For the expression of dimeric NIF-IGHE-CH₄ (Fig. 1a), the NIF and human IgE heavy chain constant domain 4 DNA sequences were fused together using a short Gly-Ser-Gly-Gly (GSGG) linker sequence by gene synthesis (sequence available in GenBank, URL: <http://www.ncbi.nlm.nih.gov>, under accession number KP314277). The DNA sequence was re-translated from the amino acid

sequence including codon optimization for high-scale expression in Sf21 insect cell cultures. To stabilize the dimerization, a free cysteine was added to the C-terminus, and a Strep-tag II sequence (WSHPQFEK) was added for purification by affinity chromatography. This sequence was subcloned into the pMIB/V5-HisC insect cell expression vector, to which a honeybee mellitin secretion signal was added to aid later purification from cell culture supernatants. Sf21 insect cells were transfected with this vector, and a polyclonal population of stably transfected cells was selected using Blasticidin S. The intended configuration of the final construct was a dimeric molecule, as shown in Fig. 1b.

The NIF-IGHE-CH₄ protein was purified using Strep-Tactin affinity chromatography. SDS-PAGE followed by Coomassie staining (Fig. 1c) demonstrated that a gross fraction of NIF-IGHE-CH₄ protein was expressed in a completely dimeric form, with only a small amount of non-dimerized molecules. Under non-reducing conditions, monomeric NIF-IGHE-CH₄ appeared to have a smaller molecular weight than under reducing conditions. This was an expected artifact due to the incomplete denaturation under non-reducing conditions.

Dimeric NIF-IGHE-CH₄ preferentially binds to granulocytes and macrophages

To investigate its cellular targets, NIF-IGHE-CH₄ was conjugated to the fluorescent label DyLight 488, and its binding to freshly prepared human peripheral blood leukocytes was assessed by flow cytometry (Fig. 2a-d).

The binding properties of NIF-IGHE-CH₄ were specifically measured against T cells, B cells, granulocytes and monocytes. NIF-IGHE-CH₄ bound most strongly to granulocytes (Glass's $\Delta = 58.18$) and monocytes (Glass's $\Delta = 67.86$), correlating with the presence of CD11b/CD18 on the surface of these cell populations. In contrast, NIF-IGHE-CH₄ bound to T and B cells to a much lesser extent, as reflected by small effect estimator values (Glass's $\Delta = 2.22$ and 5.6 , respectively).

Dimeric NIF-IGHE-CH₄ inhibits granulocyte migration, adhesion and ROS release

Next, the functional effects of NIF-IGHE-CH₄ were determined using a variety of neutrophil function assays (Fig. 3a-c). The transwell method was used to investigate whether NIF-IGHE-CH₄ inhibits the C₅a-induced granulocyte migration. The dose-response curve (Fig. 3a) showed a significant drop ($p=1.34 \times 10^{-7}$ at $10 \text{ nM C}_5\text{a}$ and $p=0.0278$ at $30 \text{ nM C}_5\text{a}$) in the granulocyte migratory activity, with an EC₅₀ between 0.01 and $0.2 \text{ }\mu\text{g/ml}$.

The effect of NIF-IGHE-CH₄ on granulocyte adhesion was measured using the xCELLigence System (Fig. 3b), with or without pre-coating of microtiter plates with immobilized autoimmune complexes. Spreading activity was enhanced approximately 4- to 5-fold by the immobilized immune complexes but significantly dropped to pre-stimulation levels following the addition of approximately $10 \text{ }\mu\text{g/ml}$ NIF-IGHE-CH₄ (Jonckheere-Terpstra test, $p = 0.00018$, EC₅₀ $\approx 1.6 \text{ }\mu\text{g/ml}$ ($0.16-17$)). NIF-IGHE-CH₄ also inhibited spontaneous spreading activity (Jonckheere-Terpstra test, $p = 0.005$, EC₅₀ $\approx 0.78 \text{ }\mu\text{g/ml}$ ($0.15-4$)).

To investigate whether NIF-IGHE-CH₄ also inhibits ROS release by granulocytes stimulated with immobilized autoimmune complexes, we incubated freshly prepared granulocytes with standardized autoimmune complexes at saturating concentrations (Fig. 3c). NIF-IGHE-CH₄ was

added at varying concentrations to the cells, and luminol-enhanced chemiluminescence was recorded. Normalized AUC values were calculated from the ratio of the chemiluminescence kinetics of respective wells to those of negative control wells. The dose-response regression of the normalized AUC values versus NIF-IGHE-CH₄ at different concentrations showed a significant ($p = 0.00132$) drop, with an EC₅₀ between 0.58-5.4 $\mu\text{g/ml}$.

Dimeric NIF-IGHE-CH₄ is not toxic to granulocytes

To exclude the possibility that the inhibition of granulocyte function was caused by toxic effects of NIF-IGHE-CH₄, freshly prepared granulocytes were incubated in microtiter plates with varying concentrations of NIF-IGHE-CH₄, with or without pre-coating of plates with autoimmune complexes (Fig. 3d). Apoptosis and necrosis were assessed by flow cytometry after staining with annexin V-FITC and propidium iodide, respectively. The annexin V-FITC and propidium iodide-negative cell fraction represented vital cells. Interestingly, no significant toxic effect for NIF-IGHE-CH₄ up to a concentration of 50 $\mu\text{g/ml}$ was observed compared to no treatment; this was found with both unstimulated cells and cells co-incubated with immobilized autoimmune complexes (Fig. 3d). In contrast, NIF-IGHE-CH₄ seems to protect granulocytes from both apoptosis and necrosis.

Dimeric NIF-IGHE-CH₄ abolishes leukocyte-dependent DES

sAIBD pathogenesis depends on neutrophil migration, adhesion and ROS release. All of these functions are inhibited by NIF-IGHE-CH₄. Therefore, we tested the ability of NIF-IGHE-CH₄ to inhibit autoantibody-induced blister induction, i.e., leukocyte-induced DES in human skin cryosections. For this purpose, neonatal foreskin cryosections were incubated with a BP patient serum or normal healthy control sera (NHS) diluted 1:3 in PBS (Fig. 4a and b). Subsequently, the cryosections were covered with a layer of freshly isolated leukocytes, together with NIF-IGHE-CH₄ at a concentration of 0, 25 or 50 $\mu\text{g/ml}$ (Fig. 4b-d). After 2.5 h, the cryosections were washed to remove the leukocytes and then stained with hematoxylin and eosin. To evaluate the extent of DES, the length of the DEJ with separation was measured relative to the total length. This experiment was repeated a minimum of 5 times for each serum sample, using a different healthy blood donor for each leukocyte preparation. For data quality control, only experimental runs with greater than 50% DES for the positive controls were analyzed further.

The extent of DES with the BP patient serum was significantly higher than that with the NHS (Wilcoxon Mann-Whitney test, $p=0.014$, stratified by leukocyte donor). The effect of different concentrations of NIF-IGHE-CH₄ was evaluated using a 1-sided, non-parametric, one-way test (Fig. 4e), which revealed significant dose-dependent inhibition of autoantibody-induced DES ($p=0.0056$, stratified by leukocyte donor).

Discussion

In the search for treatment options with less iatrogenic morbidity and mortality for patients with autoimmune diseases such as subepidermal blistering diseases, molecules derived from parasites and other infectious microorganisms are promising novel drugs that have been optimized by evolution to be highly effective and to have minimal side effects. Parasitic hookworms secrete

proteins that potently inhibit the functions of the neutrophils of the host organism (27–30). One such protein is NIF, which binds to CD11b/CD18 and inhibits neutrophil functions such as neutrophil adhesion to vascular endothelial cells and ROS release upon neutrophil activation (29–31). Importantly, these secreted proteins do not seem to lose their function, e.g. due to an immune response, as parasites can survive in their hosts for decades (1). In this study, we generated a recombinant form of NIF that inhibited granulocyte adhesion, migration and activation *in vitro*, as well as neutrophil-dependent dermal-epidermal separation *ex vivo*.

The combination of proteins with an antibody scaffold often improves their stability and pharmacokinetics and aids purification by standard methods (43). The combination of single-chain variable fragments (scFvs) with the dimerizing domain of immunoglobulins, also termed SIPs (36–38), has been used to successfully construct single-chain antibodies. The ability of SIPs to activate immune cells or complement is stripped by using only part of the Fc portion, i.e. the CH₄ subdomain. In the current study, we therefore linked NIF with the dimerizing CH₄ subdomain of human IgE (IGHE-CH₄) and added a cysteine to stabilize the dimer. This molecule was cloned into a vector that allows expression in a high-density culture of stably transfected insect cells.

NIF-IGHE-CH₄ purified from insect cell supernatants forms stable dimers that specifically bind to granulocytes and monocytes/macrophages but not to CD3- or CD19-positive lymphocytes, as determined by flow cytometry. As expected, this finding corresponds to CD11b/CD18 (CR-3, Mac-1, $\alpha_M\beta_2$ integrin) expression in these cells (44,45). Therefore, the dimeric fusion molecule exhibited the same binding behavior as NIF.

Complement activation is one of the first events that occurs following the binding of autoantibodies to the DEJ (15,17,19,46), leading to the generation of chemoattractant complement fragments such as C5a, which recruit innate immune cells into the skin. Our results show that NIF-IGHE-CH₄ specifically inhibits the granulocyte migration triggered by complement factor C5a and fMLP (not shown). Similar effects have been achieved by knocking out CD11b or by injecting neutralizing antibodies against CD11b into a neonatal mouse model of BP (47). In both cases, the mice were completely resistant to experimental BP induction by the passive transfer of rabbit antibodies against mouse type XVII collagen. This resistance was associated with an impaired accumulation of neutrophils in the skin, whereas complement deposition and mast cell and neutrophil degranulation were equivalent to those in both wild-type and untreated mice.

In a later phase of the pathogenesis of experimental BP and EBA, autoantibodies bound to the DEJ directly activate the invading neutrophils via Fc γ receptors (22,41,48–50). Although the specific contribution of eosinophils has not been elucidated, they might be important to mediate pathogenic effects by IgE-type autoantibodies (51,52). Our results show that NIF-IGHE-CH₄ inhibits adhesion, migration and ROS release of granulocytes upon activation by immobilized autoimmune complexes in a dose-dependent manner. Therefore, NIF-IGHE-CH₄ has a dual anti-inflammatory effect in the pathogenesis of sAIBDs; NIF-IGHE-CH₄ inhibits the recruitment as well as the activation of innate immune cells by autoantibodies.

Using an *ex vivo* model of subepidermal blistering diseases, we demonstrated the potential of NIF-IGHE-CH₄ as a potential novel anti-inflammatory agent. NIF-IGHE-CH₄ abolished leukocyte-dependent blister induction, i.e., dermal-epidermal separation, in cryosections of human skin incubated with BP patient sera in a dose-dependent manner.

These results demonstrate that dog hookworm-derived NIF-IGHE-CH₄ and other NIF-derived biologics have the potential to be used as anti-inflammatory drugs in patients with autoimmune diseases. The successful dimerization is a prerequisite for the construction of bifunctional heterodimers; e.g. NIF combined with an antibody moiety for targeting of the molecule. Moreover, NIF-derived biologics might be useful as inhibitors of neutrophils and other innate immune cells for the treatment of stroke (35) or the prevention of atrial fibrillation (53). Further studies are required to optimize and enhance the NIF-IGHE-CH₄ biomolecule for use in animal studies of sAIBD. The molecular design of dimeric NIF-IGHE-CH₄ provides a basis for further modifications, e.g. the expression in a heterodimeric format with dual functionality. Already promising by itself, NIF-IGHE-CH₄ is only one example of how parasite proteins can be adapted into novel biologics with potential healthcare applications.

Acknowledgments

We thank Ingeborg Atefi, Marina Kongsbak-Reim, Vanessa Krull and Tanja Sörensen for help with the patient sera and Christian Karsten for advice on the microscopic imaging and Anja Kerstein for advice on neutrophil staining. We furthermore thank Dr. Mohammed Almasry and Dr. Andrea Ortfeld (University of Lübeck) for providing human neonatal foreskin samples; and we also thank all of the volunteers who donated blood for the experiments performed in this study.

This manuscript has been edited for English language usage, grammar, spelling and punctuation by native English-speaking editors at NPG Language Editing (certificate oA03-1832-91C4-D52A-B6FD).

Contributions

A. K., K. B., A. A., N. M., S. R., C. K., X. Y. and A. R. performed experiments; A. K., K. B., A. A., X. Y. and A. R. analyzed the data; A. K., K. B., A. A., N. M., S. R., X. Y., K. S., J. K., R. J. L., D. Z. and A. R. contributed to writing of the manuscript. A. R., D. Z., K. S., K. B., J. K. designed research and discussed results.

References

- 1 Wilson M S, Maizels R M. Regulation of allergy and autoimmunity in helminth infection. *Clin Rev Allergy Immunol* 2004; **26**: 35–50.
- 2 Hewitson J P, Grainger J R, Maizels R M. Helminth immunoregulation: the role of parasite secreted proteins in modulating host immunity. *Mol Biochem Parasitol* 2009; **167**: 1–11.
- 3 Lightowlers M W, Rickard M D. Excretory-secretory products of helminth parasites: effects on host immune responses. *Parasitology* 1988; **96 Suppl**: S123–166.
- 4 Proudfoot L. Parasitic helminths tip the balance: potential anti-inflammatory therapies. *Immunology* 2004; **113**: 438–440.
- 5 Giudice G J, Emery D J, Diaz L A. Cloning and primary structural analysis of the bullous pemphigoid autoantigen BP180. *J Invest Dermatol* 1992; **99**: 243–250.
- 6 Giudice G J, Emery D J, Zelickson B D *et al.* Bullous pemphigoid and herpes gestationis autoantibodies recognize a common non-collagenous site on the BP180 ectodomain. *J Immunol Baltim Md* 1950 1993; **151**: 5742–5750.

- 7 Woodley D T, Chang C, Saadat P *et al.* Evidence that anti-type VII collagen antibodies are pathogenic and responsible for the clinical, histological, and immunological features of epidermolysis bullosa acquisita. *J Invest Dermatol* 2005; **124**: 958–964.
- 8 Woodley D T, Briggaman R A, O’Keefe E J *et al.* Identification of the skin basement-membrane autoantigen in epidermolysis bullosa acquisita. *N Engl J Med* 1984; **310**: 1007–1013.
- 9 Gammon W R, Murrell D F, Jenison M W *et al.* Autoantibodies to type VII collagen recognize epitopes in a fibronectin-like region of the noncollagenous (NC1) domain. *J Invest Dermatol* 1993; **100**: 618–622.
- 10 Liu Z, Sui W, Zhao M *et al.* Subepidermal blistering induced by human autoantibodies to BP180 requires innate immune players in a humanized bullous pemphigoid mouse model. *J Autoimmun* 2008; **31**: 331–338.
- 11 Sitaru C, Mihai S, Otto C *et al.* Induction of dermal-epidermal separation in mice by passive transfer of antibodies specific to type VII collagen. *J Clin Invest* 2005; **115**: 870–878.
- 12 Recke A, Trog L M, Pas H H *et al.* Recombinant human IgA1 and IgA2 autoantibodies to type VII collagen induce subepidermal blistering ex vivo. *J Immunol Baltim Md 1950* 2014; **193**: 1600–1608.
- 13 Recke A, Sitaru C, Vidarsson G *et al.* Pathogenicity of IgG subclass autoantibodies to type VII collagen: induction of dermal-epidermal separation. *J Autoimmun* 2010; **34**: 435–444.
- 14 Collin M, Ehlers M. The carbohydrate switch between pathogenic and immunosuppressive antigen-specific antibodies. *Exp Dermatol* 2013; **22**: 511–514.
- 15 Mihai S, Chiriac M T, Takahashi K *et al.* The alternative pathway of complement activation is critical for blister induction in experimental epidermolysis bullosa acquisita. *J Immunol Baltim Md 1950* 2007; **178**: 6514–6521.[cited 2012 Aug 26]
- 16 Natsuga K, Nishie W, Shinkuma S *et al.* Antibodies to pathogenic epitopes on type XVII collagen cause skin fragility in a complement-dependent and -independent manner. *J Immunol Baltim Md 1950* 2012; **188**: 5792–5799.
- 17 Liu Z, Giudice G J, Swartz S J *et al.* The role of complement in experimental bullous pemphigoid. *J Clin Invest* 1995; **95**: 1539–1544.[cited 2012 Aug 26]
- 18 Iwata H, Kitajima Y. Bullous pemphigoid: role of complement and mechanisms for blister formation within the lamina lucida. *Exp Dermatol* 2013; **22**: 381–385.
- 19 Sitaru C, Chiriac M T, Mihai S *et al.* Induction of complement-fixing autoantibodies against type VII collagen results in subepidermal blistering in mice. *J Immunol* 2006; **177**: 3461–3468.
- 20 Woodley D T, Ram R, Doostan A *et al.* Induction of epidermolysis bullosa acquisita in mice by passive transfer of autoantibodies from patients. *J Invest Dermatol* 2006; **126**: 1323–1330.
- 21 Chiriac M T, Roesler J, Sindrilaru A *et al.* NADPH oxidase is required for neutrophil-dependent autoantibody-induced tissue damage. *J Pathol* 2007; **212**: 56–65.
- 22 Yu X, Holdorf K, Kasper B *et al.* FcγRIIA and FcγRIIIB are required for autoantibody-induced tissue damage in experimental human models of bullous pemphigoid. *J Invest Dermatol* 2010; **130**: 2841–2844.
- 23 Shimanovich I, Mihai S, Oostingh G J *et al.* Granulocyte-derived elastase and gelatinase B are required for dermal-epidermal separation induced by autoantibodies from patients with epidermolysis bullosa acquisita and bullous pemphigoid. *J Pathol* 2004; **204**: 519–527.
- 24 Ludwig R J, Recke A, Bieber K *et al.* Generation of antibodies of distinct subclasses and specificity is

linked to H2s in an active mouse model of epidermolysis bullosa acquisita. *J Invest Dermatol* 2011; **131**: 167–176.

- 25 Ludwig R J, Müller S, Marques A d C *et al.* Identification of quantitative trait loci in experimental epidermolysis bullosa acquisita. *J Invest Dermatol* 2012; **132**: 1409–1415.
- 26 Srinivas G, Möller S, Wang J *et al.* Genome-wide mapping of gene-microbiota interactions in susceptibility to autoimmune skin blistering. *Nat Commun* 2013; **4**: 2462.
- 27 Keir P A, Brown D M, Clouter-Baker A *et al.* Inhibition of neutrophil recruitment by ES of *Nippostrongylus brasiliensis*. *Parasite Immunol* 2004; **26**: 137–139.
- 28 Anbu K A, Joshi P. Identification of a 55 kDa *Haemonchus contortus* excretory/secretory glycoprotein as a neutrophil inhibitory factor. *Parasite Immunol* 2008; **30**: 23–30.
- 29 Moyle M, Foster D L, McGrath D E *et al.* A hookworm glycoprotein that inhibits neutrophil function is a ligand of the integrin CD11b/CD18. *J Biol Chem* 1994; **269**: 10008–10015.
- 30 Muchowski P J, Zhang L, Chang E R *et al.* Functional interaction between the integrin antagonist neutrophil inhibitory factor and the I domain of CD11b/CD18. *J Biol Chem* 1994; **269**: 26419–26423.
- 31 Rieu P, Ueda T, Haruta I *et al.* The A-domain of beta 2 integrin CR3 (CD11b/CD18) is a receptor for the hookworm-derived neutrophil adhesion inhibitor NIF. *J Cell Biol* 1994; **127**: 2081–2091.
- 32 Diamond M S, Springer T A. A subpopulation of Mac-1 (CD11b/CD18) molecules mediates neutrophil adhesion to ICAM-1 and fibrinogen. *J Cell Biol* 1993; **120**: 545–556.
- 33 Barnard J W, Biro M G, Lo S K *et al.* Neutrophil inhibitory factor prevents neutrophil-dependent lung injury. *J Immunol Baltim Md 1950* 1995; **155**: 4876–4881.
- 34 Zhou M Y, Lo S K, Bergenfeldt M *et al.* In vivo expression of neutrophil inhibitory factor via gene transfer prevents lipopolysaccharide-induced lung neutrophil infiltration and injury by a beta2 integrin-dependent mechanism. *J Clin Invest* 1998; **101**: 2427–2437.
- 35 Jiang N, Moyle M, Soule H R *et al.* Neutrophil inhibitory factor is neuroprotective after focal ischemia in rats. *Ann Neurol* 1995; **38**: 935–942.
- 36 Wyss M T, Spaeth N, Biollaz G *et al.* Uptake of 18F-Fluorocholine, 18F-FET, and 18F-FDG in C6 gliomas and correlation with 131I-SIP(L19), a marker of angiogenesis. *J Nucl Med Off Publ Soc Nucl Med* 2007; **48**: 608–614.
- 37 Perrino E, Steiner M, Krall N *et al.* Curative properties of noninternalizing antibody-drug conjugates based on maytansinoids. *Cancer Res* 2014; **74**: 2569–2578.
- 38 Spaeth N, Wyss M T, Pahnke J *et al.* Radioimmunotherapy targeting the extra domain B of fibronectin in C6 rat gliomas: a preliminary study about the therapeutic efficacy of iodine-131-labeled SIP(L19). *Nucl Med Biol* 2006; **33**: 661–666.
- 39 Kang D, Gho Y S, Suh M *et al.* Highly Sensitive and Fast Protein Detection with Coomassie Brilliant Blue in Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis. *Bull Korean Chem Soc* 2002; **23**: 1511–1512.
- 40 Scrace S, O'Neill E, Hammond E M *et al.* Use of the xCELLigence system for real-time analysis of changes in cellular motility and adhesion in physiological conditions. *Methods Mol Biol Clifton NJ* 2013; **1046**: 295–306.
- 41 Sitaru C, Kromminga A, Hashimoto T *et al.* Autoantibodies to type VII collagen mediate Fcgamma-

dependent neutrophil activation and induce dermal-epidermal separation in cryosections of human skin. *Am J Pathol* 2002; **161**: 301–311.

- 42 Team R D C. R: A Language and Environment for Statistical Computing [Internet]. Vienna, Austria: 2012. DOI: <http://www.R-project.org/>
- 43 Rezaee S A R, Gracie J A, McInnes I B *et al.* Inhibition of neutrophil function by the Kaposi's sarcoma-associated herpesvirus vOX2 protein. *AIDS Lond Engl* 2005; **19**: 1907–1910.
- 44 Hu X, Wohler J E, Dugger K J *et al.* beta2-integrins in demyelinating disease: not adhering to the paradigm. *J Leukoc Biol* 2010; **87**: 397–403.
- 45 Solovjov D A, Pluskota E, Plow E F. Distinct roles for the alpha and beta subunits in the functions of integrin alphaMbeta2. *J Biol Chem* 2005; **280**: 1336–1345.
- 46 Karsten C M, Pandey M K, Figge J *et al.* Anti-inflammatory activity of IgG1 mediated by Fc galactosylation and association of FcγRIIB and dectin-1. *Nat Med* 2012; **18**: 1401–1406.
- 47 Liu Z, Zhao M, Li N *et al.* Differential roles for beta2 integrins in experimental autoimmune bullous pemphigoid. *Blood* 2006; **107**: 1063–1069.
- 48 Ludwig R J, Kalies K, Köhl J *et al.* Emerging treatments for pemphigoid diseases. *Trends Mol Med* 2013; **19**: 501–512.
- 49 Kasperkiewicz M, Nimmerjahn F, Wende S *et al.* Genetic identification and functional validation of FcγRIV as key molecule in autoantibody-induced tissue injury. *J Pathol* 2012; **228**: 8–19.
- 50 Zhao M, Trimbeger M E, Li N *et al.* Role of FcRs in animal model of autoimmune bullous pemphigoid. *J Immunol Baltim Md 1950* 2006; **177**: 3398–3405.[cited 2012 Aug 26]
- 51 Messingham K N, Holahan H M, Frydman A S *et al.* Human eosinophils express the high affinity IgE receptor, FcεRI, in bullous pemphigoid. *PloS One* 2014; **9**: e107725.
- 52 Messingham K a. N, Holahan H M, Fairley J A. Unraveling the significance of IgE autoantibodies in organ-specific autoimmunity: lessons learned from bullous pemphigoid. *Immunol Res* 2014; **59**: 273–278.
- 53 Friedrichs K, Adam M, Remane L *et al.* Induction of atrial fibrillation by neutrophils critically depends on CD11b/CD18 integrins. *PloS One* 2014; **9**: e89307.

Figure legends.

Figure 1 – The single-stranded NIF-IGHE-CH₄ fusion protein is expressed as a dimeric molecule.

(a) Single-stranded design of the NIF-IGHE-CH₄ fusion protein expression construct. *Signal*, honeybee mellitin secretion signal sequence; *NIF*, *Ancylostoma caninum* neutrophil inhibitory factor (Uniprot ID Q16969, aa 18-274); *Linker*, GSGG linker sequence; *IGHE_HUMAN*, human immunoglobulin E heavy-chain constant domain 4 (Uniprot ID P01854, aa 324-428) for dimerization induction; *Cys*, additional free cysteine for disulfide bridge formation for dimer stabilization; *Strep-tag II*, fusion tag (sequence NWSHPQFEK) for detection and purification. (b) Schematic of the molecular conformation of the dimers. (c) Coomassie staining of NIF-IGHE-CH₄ separated by 7.5% SDS-PAGE under both reducing (*red.*) and non-reducing (*non-red.*) conditions.

Figure 2 – The NIF-IGHE-CH₄ fusion protein preferentially binds to granulocytes and macrophages.

FACS analysis of human peripheral blood leukocytes using a DyLight 488-conjugated NIF-IGHE-CH₄ fusion protein (**DyLight 488-conjugated NIF**) compared with unconjugated NIF-IGHE-CH₄ (**NIF**). Leukocyte subpopulations were gated (1) by FSC/SSC or (2) by SSC/PE-CD₃, APC-CD₁₉, APD-CD₁₅ or PE-CD_{11b}. The fluorescence signal of DyLight 488-conjugated NIF-IGHE-CH₄ was then (3) compared with that of unconjugated NIF-IGHE-CH₄ in a histogram plot using Glass's Δ as an effect estimator to evaluate the difference between conjugated and non-conjugated NIF-IGHE-CH₄. **(a)** The binding of NIF-IGHE-CH₄ to CD₃-positive T cells (Glass's Δ = 2.22), **(b)** to CD₁₉-positive B cells (Glass's Δ = 5.6), **(c)** to CD₁₅-positive granulocytes (Glass's Δ = 58.18) and **(d)** to CD_{11b}-positive monocytes (Glass's Δ = 67.86).

Figure 3 – The NIF-IGHE-CH₄ fusion protein inhibits granulocyte migration, immune-complex-induced spreading and ROS release.

(a) The inhibition of complement factor C_{5a}-induced neutrophil migration by NIF-IGHE-CH₄ was assessed in 3 separate experiments using the Boyden chamber transwell method. C_{5a} was used at 10 nM or 30 nM, and NIF-IGHE-CH₄ was added to the upper compartment of Boyden chambers at concentrations ranging from 50 pg/ml to 5 μ g/ml. The combined scatter and curve regression plots show the fitted 4-parameter sigmoidal regression curve, including a 95% confidence band. Dose dependency was evaluated using a 1-sided Jonckheere-Terpstra trend test ($p = 1.34 \times 10^{-7}$ at 10 nM C_{5a} and $p = 0.0278$ at 30 nM C_{5a}); EC₅₀ = 0.01-0.2 μ g/ml. **(b)** Autoimmune complex-induced neutrophil adhesion and spreading were assessed using the xCELLigence System with microtiter plates with or without pre-coating with autoimmune complexes of recombinant hCOL7EF autoantigen and recombinant anti-hCOL7 hIgG1 autoantibody. Spreading activity (cell index) was calculated as the AUC value of the spreading kinetics recorded for 3 h. The combined scatter and curve regression plots show the fitted 4-parameter sigmoidal regression curve, including a 95% confidence band. The logarithmic scale is broken to include the values at a concentration of 0 μ g/ml. The inhibition of both spontaneous (**w/o IC**, $p=0.005$, EC₅₀ $\approx$ 0.78 μ g/ml (0.15-4)) and autoimmune complex-induced (**w/ IC**, $p=0.00019$ EC₅₀ $\approx$ 1.6 μ g/ml (0.16-17)) neutrophil spreading by NIF-IGHE-CH₄ was evaluated using a 1-sided Jonckheere-Terpstra trend test. AU, arbitrary units. **(c)** The inhibition of ROS release by autoimmune-complex activated neutrophils. Neutrophils were pre-incubated for 30 min on ice with varying concentrations of NIF-IGHE-CH₄ and then added to microtiter plates pre-incubated with recombinant hCOL7EF autoantigen and recombinant anti-hCOL7 hIgG1 autoantibody immune complexes at pre-optimized concentrations. Luminol-enhanced chemiluminescence due to ROS release was recorded each minute for 60 min. The combined scatter and curve regression plots of the chemiluminescence AUC values show the fitted 4-parameter sigmoidal regression curve, including a 95% confidence band. Dose dependency was evaluated by a 1-sided Jonckheere-Terpstra trend test ($p = 0.00132$); EC₅₀ = 0.58-5.4 μ g/ml. Horizontal dashed line, cell control without autoimmune complexes. **(d)** The toxicity of NIF-IGHE-CH₄ was assessed by flow cytometry after staining with annexin V and propidium iodide. Neutrophils were incubated for 60 min on microtiter plates with (**w/ IC**) or without (**w/o IC**) immobilized autoimmune complexes, as described above, with concentrations of NIF-IGHE-CH₄ ranging from 0-50 μ g/ml. PMA (30 nM) and UVB exposure were used as controls. Combined scatter and box plots for the vital cell fraction (negative for both annexin V and propidium iodide) are shown.

Figure 4 – NIF-IGHE-CH₄ inhibits antibody-induced DES by leukocytes in cryosections.

Cryosections of human neonatal foreskin were incubated first with previously optimized dilutions of BP patient sera or normal healthy control sera (NHS) and subsequently with freshly prepared leukocytes from healthy volunteers. NIF-IGHE-CH₄ was mixed with the leukocyte suspensions immediately before addition to the slides. After hematoxyling and eosin staining, the degree of DES was evaluated microscopically by a blinded observer. Representative microscopic images show the extent of DES (arrows) for (a) NHS without NIF-IGHE-CH₄, (b) BP patient serum without NIF-IGHE-CH₄, (c) BP patient serum with 25 µg/ml NIF-IGHE-CH₄ and (d) BP patient serum with 50 µg/ml NIF-IGHE-CH₄. All scale bars on the lower right correspond to 100 µm. (e) Combined scatter and box plot representations of the results obtained for NHS and BP patient serum samples at varying concentrations of NIF-IGHE-CH₄ (NIF). Each dot corresponds to a single experiment, with a total of 4 different leukocyte donors. DES was significantly higher with BP patient sera than with NHS (Wilcoxon Mann-Whitney rank sum test, $p=0.014$, stratified by leukocyte donors). Approximative k-sample permutation testing, stratified by leukocyte donor, revealed significant dose dependency in the inhibition of DES by NIF-IGHE-CH₄ ($p = 0.0056$).







