2B4 – A potential target in *Staphylococcus aureus* associated allergic inflammation

Pratibha Gaur[1]*, Mansour Seaf[1]*, Nirit Trabelsi[2], Orly Marcu[2], Daria Gafarov[1], Ora Schueler-Furman[2], Ofer Mandelboim[3], Micha Ben-Zimra[1]**, Francesca Levi-Schaffer[1]**

[1]Pharmacology & Experimental Therapeutics Unit, School of Pharmacy, Institute for Drug Research, Faculty of Medicine, The Hebrew University of Jerusalem, Israel

[2]Department of Microbiology & Molecular Genetics, IMRIC, Faculty of Medicine, The Hebrew University of Jerusalem, Israel

[3]The Lautenberg Center for General and Tumor Immunology, The Hebrew University Hadassah Medical School, IMRIC, Jerusalem, Israel

*Equal contribution

** Equal contribution

**Corresponding Author: Prof Francesca Levi-Schaffer, Pharmacology & Experimental Therapeutics Units, School of Pharmacy, Institute for Drug Research, Faculty of Medicine, The Hebrew University of Jerusalem, Israel

Email: francescal@ekmd.huji.ac.il; Tel:+972-2-6757512

Downloaded from https://academic.oup.com/cei/advance-article/doi/10.1093/cei/uxad089/7242641 by Butler University user on 23 August 2023

© The Author(s) 2023. Published by Oxford University Press on behalf of the British Society for Immunology. All rights reserved. For permissions, please e-mail: journals.permissions@oup.com

Abbreviations:

Activating receptor	-	AR
Allergic Effector Unit	-	AEU
Allergic rhinitis	-	AR
Atopic dermatitis	-	AD
Bone marrow derived eosinophils	-	BMEos
Dendritic cells	-	DCs
Enzyme - linked immunosorbent assay	-	ELISA
Eosinophils	-	Eos
Eosinophil peroxidase	-	EPO
Flow cytometry	-	FC
Human 2B4 fusion protein	-	h2B4-Fc
human KIR1-Fc	-	hKIR1-Fc
Inhibiting receptor	-	IR
Immunoreceptor Tyrosine-based Switch Motifs	-	ITSMs
Mast cells	-	MCs
Microscale thermophoresis	-	MST
Mouse BW thymoma cell-line	-	BW
Murine 2B4 fusion protein	-	m2B4-Fc
Natural killer	-	NK
Platelet activating factor	-	PAF
SLAM-associated protein	-	SAP

Downloaded from https://academic.oup.com/cei/advance-article/doi/10.1093/cei/uxad089/7242641 by Butler University user on 23 August 2023

Signaling Lymphocytes Activation Molecules	-	SLAM
Staphylococcus aureus	-	SA
Staphylococcus enterotoxin B	-	SEB
Soluble CD48	-	sCD48

Accepted Manuscript

Downloaded from https://academic.oup.com/cei/advance-article/doi/10.1093/cei/uxad089/7242641 by Butler University user on 23 August 2023

Abstract

Staphylococcus aureus (SA) and its exotoxins activate eosinophils (Eos) and mast cells (MCs) via CD48, a GPI-anchored receptor belonging to the Signaling Lymphocytes Activation Molecules (SLAM) family. 2B4 (CD244), an immuno-regulatory transmembrane receptor also belonging to the SLAM family, is the high affinity ligand for CD48. 2B4 is expressed on several leukocytes including NK cells, T cells, basophils, monocytes, dendritic cells (DCs) and Eos. In the Eos and MCs crosstalk carried out by physical and soluble interactions (named the 'Allergic Effector Unit', AEU), 2B4-CD48 binding plays a central role.

As CD48 and 2B4 share some structural characteristics and SA colonization accompanies most of the allergic diseases, we hypothesized that SA exotoxins (e.g., Staphylococcus enterotoxin B, SEB), can also bind and activate 2B4 and thereby possibly further aggravate inflammation. To check our hypothesis, we employed *in vitro, in- silico* and *in vivo methods*. By Enzyme-linked immunosorbent assay (ELISA), flow cytometry (FC), fluorescence microscopy and microscale thermophoresis (MST), we have shown that SEB can bind specifically to 2B4. By Eos short- and long-term activation assays, we confirmed the functionality of the SEB-2B4 interaction. Using computational modeling, we identified possible SEB-binding sites on human and mouse 2B4. Finally, *in vivo,* in a SEB-induced peritonitis model, 2B4-KO mice showed a significant reduction of inflammatory features compared to WT mice.

Downloaded from https://academic.oup.com/cei/advance-article/doi/10.1093/cei/uxad089/7242641 by Butler University user on 23 August 2023

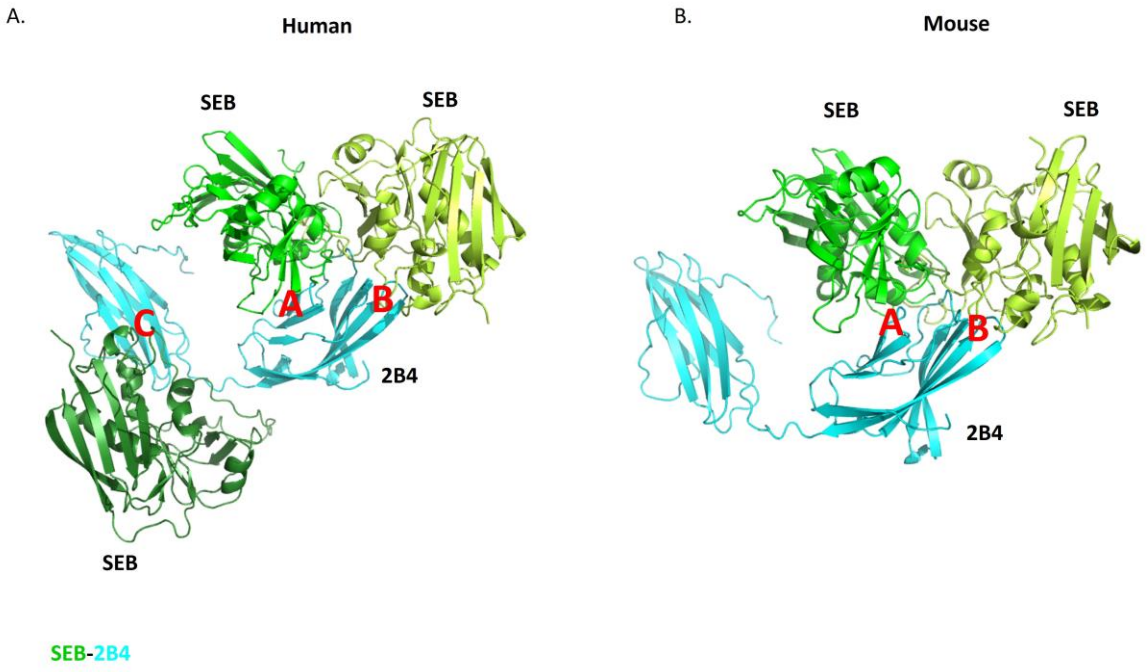
Altogether, the results of this study confirm that 2B4 is an important receptor in SEB-mediated inflammation and therefore a role is suggested for 2B4 in SA associated inflammatory conditions.

Keywords:- Staphylococcus enterotoxin B (SEB), 2B4, sCD48, Eosinophils, SEB induced peritonitis

Accepted Manuscrip

Downloaded from https://academic.oup.com/cei/advance-article/doi/10.1093/cei/uxad089/7242641 by Butler University user on 23 August 2023

Graphical abstract



Accepted Manu

Downloaded from https://academic.oup.com/cei/advance-article/doi/10.1093/cei/uxad089/7242641 by Butler University user on 23 August 2023

Introduction

2B4 (CD244) and CD48, both belong to the SLAM protein family and, as expressed on the membrane of most immune cells, form a high-affinity ligand-receptor couple for each other[1]. 2B4 is a transmembrane receptor composed of an extracellular segment containing two immunoglobulin (Ig)-like domains, a transmembrane region, and a cytoplasmic tail containing four Immunoreceptor Tyrosine-based Switch Motifs (ITSMs). The ITSMs of 2B4 bind and phosphorylate SLAM-associated protein (SAP), which further transduces signaling for cell activation. Nevertheless, the third ITSM can bind cytoplasmic inhibitory phosphatases SHP1, SHP2 and SHIP, hence suggesting 2B4 also as an inhibitory receptor on some cells under specific conditions. Indeed it seems that expression levels of the adaptor molecules, their availability and competitive binding define whether 2B4 will act as an inhibitory or activating receptor (IR or AR)[2].

The role of 2B4 as expressed mostly by NK cells in viral infections is well established. For example, in influenza, it plays an activating role and its increased expression on NK cells results in the stronger killing of influenza virus-infected cells[3]. On the other hand, in chronic HBV infection, 2B4 plays an inhibitory role and its high expression on virus-specific $CD8^+$ T cells results in a decrease in anti-viral immune response[4]. In bacterial infections, to the best of our knowledge, no specific role for 2B4 has been investigated other than two reports showing its high expression on $CD8^+$ T cells in patients infected by mycobacterium-tuberculosis (MTB)[5,6].

The SA is one of the most common human commensal gram-positive bacteria that occasionally becomes an opportunistic pathogen in skin and respiratory tract disorders[7,8]. In allergic inflammation, pathogenic SA is highly common in asthma[9], atopic dermatitis (AD)[10] and allergic rhinitis (AR)[11] where its involvement enhances the severity of the disease[12].

Accepted Manuscript

Downloaded from https://academic.oup.com/cei/advance-article/doi/10.1093/cei/uxad089/7242641 by Butler University user on 23 August 2023

MCs and Eos are the main players in allergic diseases[13,14]. We have studied possible interactions of SA with Eos and MCs, and found that SA and its exotoxin SEB bind and activate MCs[15] and Eos[16] via CD48. Moreover, we have described the importance of CD48 as expressed by human and mouse MCs and of 2B4 as expressed by both human and mouse Eos[17,18] in their pro-inflammatory physical crosstalk, termed by us the 'Allergic Effector Unit' (AEU)[19,20].

As CD48 and 2B4 belong to the same receptor family and share structural characteristics, we hypothesized that SA and/or its exotoxins (e.g., SEB), can also bind and activate 2B4 possibly influencing inflammation. We therefore conducted *in vitro, in-silico* and *in vivo* studies that demonstrate a specific and functional interaction of SEB with 2B4. Our results provide strong evidence for a possible functional role of 2B4 in SA infections.

Material and Methods

Generation of human 2B4-Fc (h2B4-Fc) and mouse 2B4-Fc (m2B4-Fc)

Fusion proteins were generated as previously described[21,3]. In brief, the sequence encoding the extracellular part of human 2B4 was amplified by PCR using the 5' primer CCCACCGGT GCCGCCACC ATG CTG GGG CAA GTG GTC ACC (including AgeI restriction site) and the 3' primer GGATCCGG CCA AAA TCT GAA TTC CTG (including BamHI restriction site). This PCR fragment was cloned into an expression vector containing a mutated Fc portion of human IgG1 (CSI-Ig Puro plasmid). The same protocol was performed for the generation of the mouse 2B4-Ig fusion protein (m2B4-Fc) using the relevant primers for the extracellular part of mouse 2B4 - 5' primer CCC ACCGCT GCCGCCACC ATG TTG GGG CAA GCT GTC CTG (including AgeI restriction site) and 3' primer GGG ATCCGG CAG AAA TCT GAA ATT CGAA (including BamHI restriction

Accepted Manuscript

Downloaded from https://academic.oup.com/cei/advance-article/doi/10.1093/cei/uxad089/7242641 by Butler University user on 23 August 2023

site). Fusion proteins were generated in 293T cells and were purified on a protein G column (HiTrap™ protein G HP, GE Health Care, Rockville, MD, USA).

ELISA assay

ELISA plates were coated with SEB, 10µg/ml in PBS, for overnight (ON) at 4°C. Afterwards, plates were washed 4 times with wash buffer (PBS-Tween 20, 0.05%) and blocked with BSA 1% in PBS for 2hr at room temperature (RT). Then, plates were washed 4 times with wash buffer and h2B4-Fc or hKIR1-Fc were added at different concentrations (0-10µg/ml) for 2hr at RT. Afterwards, plates were washed 4 times with wash buffer and biotinylated anti-human-IgG (Jackson ImmunoResearch, West Grove, PA, USA) was added for 1hr at RT. Plates were then washed (wash buffer X4) and Streptavidin-HRP (Jackson ImmunoResearch, USA) was added for 30 min at RT. Plates were washed (wash buffer X4) and subsequently TMB solution was added. Developing color was measured by a plate reader Cytation/3 (BioTek, Shoreline, USA) at OD of 650nm.

Mouse BW thymoma (BW) cells

BW cells were generously given to us by Prof. O. Mandelboim (Lautenberg Center for General and Tumor Immunology and Cancer Research, Hebrew University of Jerusalem, Israel). BWh2B4 cells were generated and BW/BWh2B4 cell FACS experiments were performed as previously described[22]. For FACS analysis distinct BW cells were blocked (BSA, 5% in PBS) for 15 min on ice and washed once with wash buffer. Labelled SEB (produced by the use of Monolith NT™.115 Protein Labeling kit BLUE-NHS; Nano temper, Munchen, Germany) was added for 40 min on ice, washed and cell fluorescence was then analyzed using Cytoflex (Beckman Coulter, Indianapolis, IN, USA).

Accepted Manuscript

Downloaded from https://academic.oup.com/cei/advance-article/doi/10.1093/cei/uxad089/7242641 by Butler University user on 23 August 2023

BW cell assay

BW cell assay was performed as described previously[22]. ELISA plates were coated with different concentrations of SEB or CD48-Fc/2B4-Fc (1μg/well) (positive control) at 37°C for 2hr, then plates were aspirated to remove the supernatants. BW and BWh2B4/BWhCD48 cells ($5x10^4$/200μl in RPMI medium) were added to the plates for 48hr at 37°C. Cell activation was assessed by measurement of IL-2 secretion using a commercial kit (PeproTech, Modi'in, Israel).

Microscale thermophoresis (MST)

For MST experiments, Monolith NT.115 instrument (Nano temper technologies, Munchen, Germany) was used at the following settings - 20% MST power, 40s and 80% LED power, at 25°C. SEB was labelled using the Monolith NT^{TM}.115 protein labelling kit BLUE (Nano temper technologies, Munchen, Germany) and standard MST capillaries were used. The interaction was assessed by labelled SEB and unlabelled h2B4-Fc or a control human-Ig. Constant concentrations of labelled SEB were maintained during the assay while unlabelled h2B4-Fc or a control human-Ig were added at serial dilutions in PBS. The accuracy of the experimental settings was confirmed by a typical denaturation-sensitivity test.

Preparation of human peripheral blood Eosinophils (Eos)

For purification of human Eos, blood donations of mildly atopic non-treated volunteers (with Eos counts of 5%-15%) were processed as detailed previously[2]. Briefly, samples (150 ml) were left to sediment for 1hr in 6% Dextran (Sigma-Aldrich, Jerusalem, Israel). Leukocytes were centrifuged on Ficoll - Hypaque (700g, 25 min), and neutrophils and lymphocytes in the granulocyte-enriched pellet were tagged using anti-CD16 and anti-CD3 micromagnetic beads, respectively. Neutrophils and lymphocytes were eliminated by negative selection via

Accepted Manuscript

Downloaded from https://academic.oup.com/cei/advance-article/doi/10.1093/cei/uxad089/7242641 by Butler University user on 23 August 2023

magnetic cell sorting, and the resulting cell suspension was 95-100% Eos pure (as determined by Kimura staining) and >98% viable (Trypan blue exclusion). Cells were washed and resuspended in a culture-enriched medium consisting of RPMI 1640 supplemented with heat-inactivated fetal bovine serum (10%), penicillin-streptomycin solution (100 µg/ml) (Biological Industries, Beit Haemek, Israel), and GM-CSF (20 ng/ml).

All human blood samples were collected following ethical approval of the Hadassah-Hebrew University Human Experimentation Helsinki Committee and written informed consent was obtained according to its guidelines.

Eosinophil functional experiments

Blocking 2B4-binding site in SEB: SEB was pre-incubated with or without h2B4-Fc at 5µg/ml in PBS/medium for 30 min at 37°C and thereafter added to $1.5x10^5$ Eos for either 40 min or 18hr and cell activation was assessed by quantifying the supernatants for the released EPO or IL-8, respectively.

Blocking of 2B4 on human Eos: $1.5x10^5$ Eos per sample were incubated with sCD48 in PBS (5µg/ml, 40 min on ice), washed twice with ice-cold PBS after which SEB was added (or not) for cell activation at 10µg/ml in PBS or Eos culture medium. After 45 min in PBS, or 18hr, in Eos medium, supernatants were collected and analyzed by the use of the relevant ELISA kit (PeproTech, Modi'in, Israel), for EPO and IL-8 levels, respectively.

Generation of murine bone marrow-derived eosinophils (BMEos) from 2B4-KO and WT mice

Bone marrow-derived eosinophils (BMEos) were obtained by culturing bone marrow (BM) cells obtained from femurs of 2B4-KO and WT (C57BL/6) mice as previously described[17].

Accepted Manuscript

Downloaded from https://academic.oup.com/cei/advance-article/doi/10.1093/cei/uxad089/7242641 by Butler University user on 23 August 2023

In brief, to obtain BMEos, BM derived progenitors were placed in RPMI 1640 media supplemented with 20% fetal bovine serum, 100,000 U/ml penicillin, 100 mg/ml streptomycin, 25 mM HEPES, 1 mM sodium pyruvate, 1X nonessential amino acids, 2mM glutamine (Biological Industries, Kibbutz Beit-Haemek, Israel), and 50 mM β-ME (Invitrogen, Modi'in, Israel). The media, replaced every other day, was supplemented by recombinant murine SCF and FLT3-ligand (PeproTech, Modi'in, Israel, 100 ng/ml each) from days 0–4, and recombinant mouse IL-5 (R&D Systems Minneapolis, MN, USA 10 ng/ml) from day 5 and on. Cells were cultured for at least 12 days and were not used beyond day 21. Before all the experiments, cells were assessed for >95% viability (by Trypan Blue exclusion), and FC for the characteristic Eos surface markers Siglec-F+ (clone E50-2440, BD Biosciences, Brunswick, New Jersey, USA) and CCR3 (clone REA122, Miltenyi Biotec, Shoham, Israel).

All mouse cells were harvested according to the ethical approval of the Animal Ethics Committee of The Hebrew University of Jerusalem, Israel.

BMEos activation

For short-term stimulation of WT and 2B4-KO BMEos, $1.5x10^5$/well were seeded and washed twice in ice-cold PBS. Next, cells were re-suspended in BSA 0.1% in PBS, and SEB was added (to final concentration of 10µg/ml) for cell activation (37ºC, 45 min, 5% CO2). For positive control, cells were incubated with PAF at 10µM. EPO release was measured as described above.

For long-term stimulation of BMEos, $1.5x10^5$ cells/well were seeded and washed twice in ice-cold PBS. Next, cells were re-suspended in the BMEos growing media (above) with SEB (10µg/ml) or media alone for 18 hr (37ºC, 5% CO2). Afterwards, sCD48 levels were

Accepted Manuscript

Downloaded from https://academic.oup.com/cei/advance-article/doi/10.1093/cei/uxad089/7242641 by Butler University user on 23 August 2023

quantitated by commercially available mouse CD48 ELISA kit (Icosagen AS, Tartu maakond, Estonia) according to the manufacturer's' instructions.

In-silico modeling of SEB-2B4 interaction

We generated models of the SEB-2B4 interaction using two different strategies: (1) Docking of the monomer structures using ClusPro[23,24], and (2) modeling of the full complex starting from the sequence of the partners using AlphaFold2 (AF2)[25]. All structures were visualized using Pymol 2.5.4 (Schrödinger).

Docking with ClusPro: We docked the solved structure of SEB (PDB ID 3SEB)[26] to the solved structure of 2B4 and CD48, separately and together (based on the solved structure of the 2B4-CD48 complex, PDB ID 2PTT)[23] using the ClusPro 2.0 protein docking server (https://cluspro.bu.edu/login.php)[25,26]. We inspected the top 10 models.

Modeling the interaction using AF2: Structure prediction of the interactions between SEB and 2B4, as well as between SEB and the complex of 2B4 and CD48, were performed with the publicly available AF2 Google Colab (https://colab.research.google.com/github/sokrypton/ColabFold/blob/main/AlphaFold2.ipynb)[27,24], using multimer versions with default parameters and Amber refinement. The input included the query sequence without using any structure templates (see Table 1 for details about the sequences used).

Selection of models: We applied the Rosetta InterfaceAnalyzer[23] to analyze the interfaces of the different structural models generated by AF2. InterfaceAnalyzer calculates a number of features of a given interface, such as predicted binding energy, buried interface surface areas, surface complementarity, packing quality and more.

Accepted M

Downloaded from https://academic.oup.com/cei/advance-article/doi/10.1093/cei/uxad089/7242641 by Butler University user on 23 August 2023

We ran InterfaceAnalyzer with the following flags:

-in:file:l input_list -out:file:score_only score.sc -compute_packstat true -pack_input true -pack_separated true -add_regular_scores_to_scorefile true -use_jobname true -pose_metrics:inter_group_neighbors_cutoff 4

The output scores that were most important when evaluating the results of the analysis were:

dG_cross dG_cross/dSASAx100 dSASA_polar dSASA_hphobic dSASA_int fa_atr fa_rep fa_sol fa_elec hbond_E_fraction hbonds_int delta_unsatHbonds nres_int packstat per_residue_energy_int

Mice

2B4-KO mice, a kind gift from Prof. V. Kumar (University of Chicago, Chicago, IL, USA) were housed and bred in the specific pathogen-free animal facilities of The Hebrew University of Jerusalem. C57BL/6 (WT) mice were purchased from Harlan Laboratories Inc, Jerusalem, Israel. Mice were euthanized by CO_2 inhalation.

All murine experiments were approved by the Animal Experimentation Ethics Committee of the Hebrew University of Jerusalem and performed according to the committee's guidelines.

Staphylococcal enterotoxin B-induced peritonitis in 2B4-KO mice

2B4-KO (8-week-old) and their age-matched C57BL/6 WT mice, were injected i.p. with 200 µl of SEB 5 µg/ml in PBS, or PBS alone. After 48 hr mice were euthanized by CO_2 inhalation followed by cervical dislocation, and peritoneum was then lavaged with cold PBS (3 ml/mouse). Spleens were also collected, and their weight was recorded. Lavages were centrifuged (7 min, 300 g, 4°C) and supernatants were saved in -20°C. Peritoneal cells were

Accepted Manuscript

Downloaded from https://academic.oup.com/cei/advance-article/doi/10.1093/cei/uxad089/7242641 by Butler University user on 23 August 2023

re-suspended in PBS containing 0.1% BSA and 5% goat serum for 15 min on ice for blocking. Cells were then washed and analyzed by FC, using BD FACS Calibur, as follows: First, for total cell numbers, all events in samples were recorded for 30 sec at a medium flow rate. Second, Granulocytes, lymphocytes and monocytes were identified by physical parameters (FSC, SSC) and their absolute numbers were evaluated by specific gating (as described in[28] Fig. S5). Third, Eos were identified by double staining using anti-Siglec-F (clone E50-2440, BD Biosciences, Brunswick, New Jersey, USA) and anti-CCR3 (clone REA122, Miltenyi Biotec, Shoham, Israel) and the relevant isotype matched control Abs. sCD48 levels were quantitated in peritoneal lavages by commercially available mouse CD48 ELISA kit (Icosagen AS, Tartu maakond, Estonia) according to the manufacturer's' instructions.

Data normalization

Where indicated, data were normalized by division, in each experiment, of all results by the average of the not activated (NA) sample results.

Statistical analysis

All experiments were done at least three times unless otherwise stated. Repetition of experiments with human eosinophils was performed with distinct individuals. *In vitro* experiments were done in triplicates (n=3) and *in vivo* experiments (n=2) included 5-6 mice in each treatment group. The data were analyzed using GraphPad Prism 6 software and statistical significance was determined using a two-tail unpaired Student's "t-test" between two groups or ANOVA for more than two groups. "P" value of <0.05 was considered significant. Data are mean ±SD/SEM, *$p<0.05$, **$p<0.01$, ***$p<0.001$.

Accepted Manuscript

Downloaded from https://academic.oup.com/cei/advance-article/doi/10.1093/cei/uxad089/7242641 by Butler University user on 23 August 2023

Results

Staphylococcus enterotoxin B (SEB) binds to 2B4

To check whether SEB can bind 2B4, we first performed an ELISA experiment where increasing concentrations of h2B4-Fc or hKIR1-Fc as a negative control, were added to plate-bound SEB. A strong significant dose-dependent binding of h2B4-Fc to SEB was observed (Fig. 1A) in contrast with a weak binding of the negative control that did not show any dose dependence. Similarly, increasing concentrations of mouse 2B4-Fc (m2B4-Fc) were added to plate-bound SEB and dose-dependent binding was detected (Fig. 1B).

Next, we analyzed SEB binding to 2B4 in the BW cell reporter system. BW null cells were engineered to express human 2B4 (BWh2B4), as confirmed by FC analysis using anti-2B4 mAb (Fig. S1). Binding of fluorescent-labeled SEB to BWh2B4 or BW null cells as a negative control, was analyzed by FC and specific binding of SEB with 2B4 was demonstrated (Fig. 1C). This result is similar to the one observed with fluorescent-labeled SEB and BWhCD48 cells (our internal positive control, data not shown). Staining of BWh2B4 cells with both fluorescent-labeled SEB and anti-2B4 mAb, showed, by the use of fluorescent confocal microscopy, co-localization of SEB and 2B4 (Fig. 1D). Finally, using microscale thermophoresis (MST) analysis, we confirmed the specific binding of SEB to h2B4-Fc with a Kd of 75+/- 10.2 nM (Fig. 1E) as compared with no binding of SEB to human-IgG used as a negative control (Fig. S2).

Altogether, these results demonstrate a specific binding of SEB to 2B4.

In-silico study predicts possible SEB-2B4 interactions

To better understand the interaction between SEB and 2B4, we generated structural models of SEB-2B4 binding using AlphaFold2 (AF2)[29] and the global docking protocol ClusPro[25,30]. We generated models for binding of SEB to human 2B4 and to mouse 2B4, where the partial structure of the 2B4-CD48 complex that has been already solved[23] was also

Downloaded from https://academic.oup.com/cei/advance-article/doi/10.1093/cei/uxad089/7242641 by Butler University user on 23 August 2023

used for our modeling. The resulting models suggest three possible interfaces between human 2B4 and SEB, hereby termed interactions A, B and C (Fig. 2A). Two of these interactions (A & B) were sampled also for the mouse 2B4-SEB interaction (Fig. 2B). According to Rosetta Interface Analyzer, the most probable conformation is interface A.

SEB activates human and mouse eosinophils through 2B4

To evaluate the functionality of SEB-2B4 binding, we employed freshly isolated primary human peripheral blood Eos known to express 2B4[2] (and CD48[16]) and to be activated by SEB[16,28]. We have activated these Eos with SEB that was pre-incubated (or not) with 2B4-Fc for blocking of its proposed 2B4 binding site/s or with D1-Fc for negative control. Use of the "2B4-blocked" SEB for human Eos activation resulted in a significant decrease in the immediate EPO release (Fig. 3A, left panel). A non-significant trend of decrease was also found in the long-term IL-8 release (Fig. 3A, right panel).

Another way to test the functionality of SEB-2B4 interaction is to block 2B4 expressed on Eos prior to SEB activation. As blocking Abs for 2B4 are not commercially available we used instead the specific ligand for 2B4, CD48, in its soluble form (sCD48) shown previously by us to block specifically 2B4 for anti-2B4 mAb binding and activation[28]. Figure 3B shows that pre-incubation of human Eos with sCD48 resulted in a significant decrease in SEB-induced cell activation as measured by both short- and long-term Eos mediators (EPO and IL-8, 3B left and right panels, respectively). This result indicates that binding of sCD48 to 2B4 competes with 2B4 binding by SEB or at least interfere with its activation by SEB. Overall, these results confirmed that SEB activation of human Eos is mediated, at least in part, by binding and activation of 2B4.

To further establish the activating effect of 2B4 binding by SEB, BMEos from 2B4-KO or WT mice (both confirmed by FC, Fig. S3) were activated with SEB. As expected, WT

Downloaded from https://academic.oup.com/cei/advance-article/doi/10.1093/cei/uxad089/7242641 by Butler University user on 23 August 2023

BMEos reacted to SEB activation with a significant release of the activation markers EPO and sCD48, shown by us as a major marker of eosinophilic inflammation[28, 31]. In contrast, 2B4-KO BMEos responded to SEB activation with a lower release of both inflammatory markers (Fig. 4A & 4B). Activation of BMEos by PAF, was less sensitive to the knock-out of 2B4 (Fig. S4) implying for the specificity of 2B4 activation by SEB.

Reduced inflammation in 2B4-KO mice in SEB-induced peritonitis

Following the *in vitro* and *in-silico* results, we evaluated the consequence of the absence of 2B4 in our well-established mouse model of SEB-induced peritonitis[16] (represented schematically in Fig. 5A). As previously shown, in WT mice, SEB induced a robust peritonitis with a significant increase in total peritoneal cell numbers (compared with PBS control). This increase was significantly reduced in 2B4-KO mice (Fig. 5B). More detailed analyses (demonstrated in Fig. S5) revealed similar decreases in SEB-induced increases in numbers of peritoneal monocytes (Fig. 5C), granulocytes (Fig. 5D), lymphocytes (Fig. 5E), sCD48 levels (Fig. 5F), and in spleen weight (Fig. 5G). Moreover, monocyte, granulocyte, lymphocyte, sCD48 level and spleen weight did not show any significant increase in 2B4-KO mice following SEB injection (compared with mice injected with PBS). No significant difference was observed in total eosinophil numbers between WT and 2B4-KO mice (Fig. 5H).

Discussion

Our first finding in the current study is that SEB, the main exotoxin of SA, binds specifically to human and mouse 2B4, which to the best of our knowledge, is the first evidence for binding of 2B4 with a bacterial toxin. We analyzed the affinity of h2B4-SEB binding by the use of MST, and found a K_D of 75+/- 10.2 nM, indicating a high affinity interaction relative to the published K_D for the typical 2B4-CD48 interaction (8μM for the human and 16-37μM

Accepted Manuscript

Downloaded from https://academic.oup.com/cei/advance-article/doi/10.1093/cei/uxad089/7242641 by Butler University user on 23 August 2023

for the mouse proteins)[23,32]. Our structural models also provide atom-level, plausible suggestions for the SEB-2B4 interaction.

This finding together with our previous data showing that SEB can activate Eos[33] known to express a functional 2B4[2], and the clinical data demonstrating that SA and Eos both play a role in allergic inflammation[28,33], prompted us to test the functionality of the 2B4-SEB interaction. We have shown in human Eos that either blocking the binding site for 2B4 in SEB or blocking 2B4 expressed on the Eos with its ligand sCD48, result in significant inhibition of SEB activation. Moreover, in mouse BMEos, genetic elimination of 2B4 resulted in a robust inhibition of SEB-induced activation. These results confirm that SEB binds 2B4 and thereby activate both human and mouse eosinophils.

Interestingly we have shown, as mentioned above, that SEB binds and activate human eosinophils via CD48, the ligand/activator of 2B4, and its absence in CD48-KO BMEos leads to a significant decrease of SEB-induced activation[16]. The facts that: 1. SEB activation is strongly decreased in both CD48-KO and 2B4-KO BMEos and 2. blocking of either 2B4 (present results) or CD48[16] on human Eos interferes strongly with SEB activation - imply that both 2B4 and CD48 are indispensable for SEB activation of eosinophils. This assumption is also supported by our experiments in BW cells where expression of either human CD48 or 2B4 alone was not enough for SEB activation (Fig. S6). The apparent need for expression of both receptors for SEB activation, raises the hypothesis that on Eos, SEB forms a ternary complex with 2B4 and CD48 and this ternary complex activates the cells. As 2B4-CD48 cis interaction was shown in NK[34] cells and also in T cells[35,36] (where 2B4 binds in cis to CD2 - the SLAM low affinity 2B4 receptor which replaces CD48), it is likely to occur also on Eos and may form a "docking site" for SEB. Nevertheless, our result that sCD48 blocks 2B4 on human Eos for SEB activation, implies a competition between SEB and CD48 on binding of 2B4. This apparent competition raises the alternative hypothesis - that on human Eos SEB

Accepted Manuscript

Downloaded from https://academic.oup.com/cei/advance-article/doi/10.1093/cei/uxad089/7242641 by Butler University user on 23 August 2023

forms separate complexes with either CD48 or 2B4 and both complexes are needed for cell activation.

It is noteworthy that sCD48 that showed competition with SEB, binds 2B4 in trans which differs from the proposed cis interaction between cell-membrane expressed 2B4 and CD48. It might be that while the cis interaction allows the participation of SEB as a third partner, the trans interaction created when sCD48 displaces the membrane bound CD48 precludes it.

To shed additional light on the feasibility of a SEB-2B4-CD48 ternary complex, we added CD48 to the *in silico* modeling. Fig. 6A suggests that CD48 can join as a third component to two of the three human-2B4-SEB predicted structures (interactions A and C, but not B, see in Fig. 2) and also to the two predictions for the SEB-mouse 2B4 interaction (Fig. 6B), suggesting that theoretically, a ternary complex can be formed.

As mentioned above, interaction A is the most probable SEB-2B4 conformation, and it is also the preferred one when modeling the interaction between SEB and the isolated IgG domain of 2B4 (using the crystal structure PDB id 2ptt) by both AF2 and ClusPro. Although interaction A allows theoretically the formation of the ternary complex, it might induce allosteric effects that lead to changes in 2B4 conformation and thereby interfere with sCD48 binding and/or functional cooperation and vice versa. In the human system, interaction B determines a competition between CD48 and SEB binding to 2B4 because it predicts that SEB binds 2B4 exactly in its CD48 binding site.

As yet, more research is needed to determine unequivocally between the two above mentioned hypotheses and to prove or disprove the formation of the ternary complex.

Following the in vitro and in-silico studies, we checked the significance of 2B4 in SEB activation in vivo in our mouse model of SEB-induced peritonitis[16,28], in WT versus 2B4-KO mice. Peritonitis was assessed at 48 hr following induction, a time by which we can reasonably assume the innate immunity is mostly activated and probably adaptive immunity

Downloaded from https://academic.oup.com/cei/advance-article/doi/10.1093/cei/uxad089/7242641 by Butler University user on 23 August 2023

nuscript

also develops due to super-antigen activity of SEB on T cells[37]. In agreement with our previous report[16], in WT mice, SEB injection induced a significant increase in spleen weight, in total peritoneal cell numbers particularly monocytes, granulocytes, lymphocytes and Eos, and in peritoneal sCD48 levels. In contrast, in 2B4-KO mice, SEB injection resulted in a significantly lower increase of all inflammatory markers, including spleen weight, with the notable exception of Eos numbers that increased similar to the WT mice. This general reduction in inflammatory parameters in 2B4-KO mice is in agreement with our *in vitro* results implying a major function for 2B4 in SEB-induced inflammation.

Regarding the normal increase in Eos numbers in 2B4-KO mice, it could have been induced by a strong activation, by SEB, of 2B4-KO MCs. MCs are a main Eos-recruiting cell in Th2-inflammation and indeed 2B4 has been found to function in mouse MCs as an inhibitory receptor[17]. Therefore, it might be that activation of 2B4-KO MC induces an intensive eosinophil recruitment. Nevertheless, the general decrease in inflammatory parameters in the 2B4-KO mice reflects the predominance of the activating function of 2B4 on immune cells other than MCs in the mouse system. Noteworthy, Eos were found to be the major source of sCD48 in SEB-induced peritonitis[28], therefore, the significant decrease in sCD48 levels in 2B4-KO mice implies that in spite of Eos substantial SEB-induced recruitment, their activation is mediated by and therefore inhibited in the absence of 2B4. The present results are in agreement with and bolster our previous results[17] showing the importance of 2B4 in OVA-SEB-induced AD inflammation.

In summary, we have demonstrated a functional binding of SEB to cell surface 2B4 *in vitro*, and *in vivo* and provided *in silico* structural models of their interaction/s. All these results strongly support the conclusion that 2B4 is an important receptor in SEB-mediated inflammation in allergy and beyond and therefore may constitute an interesting therapeutic target.

Accepted Manuscript

Downloaded from https://academic.oup.com/cei/advance-article/doi/10.1093/cei/uxad089/7242641 by Butler University user on 23 August 2023

Figure Legends

Figure 1

Staphylococcus enterotoxin B (SEB) binds to 2B4

SEB was adsorbed to 96W-plate and increasing doses of (A) h2B4-Fc, hKIR1-Fc or (B) m2B4-Fc were added, and binding was analyzed by ELISA. (C) Binding of SEB to cell expressed 2B4 was analyzed by FC, using fluorescent labeled-SEB, BW null (BW) and BWh2B4 cells. (D) Co-localization of SEB and 2B4 was confirmed in BWh2B4 versus BW null cells by confocal microscopy. Arrows indicate 2B4-SEB double positive cells. (E) Affinity of SEB binding with 2B4 was evaluated by MST analysis. A-E – Data, shown as mean ±SEM, are representative of n=3 independent experiments. *p<0.05, **p<0.01, ***p<0.001.

Figure 2

Proposed *in-silico* models for SEB binding to 2B4

In silico study reveals (A) three possible interfaces for interaction (A, B, C) between human 2B4 (cyan) and SEB (A-green, B-lemon and C-forest) and (B) two possible interfaces for interaction (A, B) between mouse 2B4 (cyan) and SEB (A-green and B-lemon).

Figure 3

SEB activates human eosinophils through 2B4

(A) Human Eos were activated with SEB that was pre-incubated with h2B4-Fc or D1-Fc for negative control (both at 5 μg/ml, 30 min, 37˚C) and EPO (left panel) and IL-8 (right panel) release was measured by ELISA. (B) Eos were incubated with or w/o sCD48 followed by activation with SEB. EPO (left panel) and IL-8 (right panel) secretion was measured by commercial ELISA kits. Normalized data are shown as mean ±SEM, n=3. *p<0.05, **p<0.01, ***p<0.001.

Accepted Manuscript

Downloaded from https://academic.oup.com/cei/advance-article/doi/10.1093/cei/uxad089/7242641 by Butler University user on 23 August 2023

Figure 4

SEB activates murine eosinophils through 2B4

BMEos from WT and 2B4-KO mice were activated by SEB and EPO (A) and sCD48 (B) secretion was measured by commercial ELISA kits. Normalized data are shown as mean ±SEM, n=3. **p < 0.01, ***p < 0.001.

Figure 5

Reduced inflammation in 2B4-KO mice in SEB induced peritonitis

SEB was injected i.p. to induce peritonitis in WT versus 2B4-KO mice. (A) A schematic description of the SEB-induced peritonitis mouse model. Peritoneal lavage cells were analyzed by FC (B-G). (B) Total cell number, (C) Monocyte number, (D) Granulocyte number, (E) Lymphocyte number, (F) Peritoneal lavage sCD48 level assessed by ELISA, (G) Spleen weight and (H) Peritoneal lavage Eos number. Data, shown as mean ± SD of each mice group (5-6 mice/group), are representative of n=2 experiments. *p < 0.05, **p < 0.01, ***p < 0.001.

Figure 6

***In-silico* modeling shows possible SEB-2B4-CD48 tertiary complexes**

(A) 2 possible models (A, C) for ternary complexes of human 2B4 (cyan), human CD48 (magenta) and SEB (A-green, and C-forest). (B) 2 possible models (A, B,) for ternary complexes of mouse 2B4 (cyan), mouse CD48 (magenta) and SEB (A-green and B-lemon).

Accepted Manuscript

Downloaded from https://academic.oup.com/cei/advance-article/doi/10.1093/cei/uxad089/7242641 by Butler University user on 23 August 2023

Table 1: Protein sequences used in this study. The sequence range used for the full protein (in parentheses: for the extracellular first IgG domain) is indicated: -

Protein	Uniprot IDs	
2B4	CD244_HUMAN 22-209 (29-130)	CD244_MOUSE 22-215 (22-130)
CD48	CD48_HUMAN 29-212 (29-130)	CD48_MOUSE 29-209 (29-130)
SEB	ETXB_STAAU 28-266	

Accepted Manuscript

Downloaded from https://academic.oup.com/cei/advance-article/doi/10.1093/cei/uxad089/7242641 by Butler University user on 23 August 2023

Acknowledgments

We wish to thank Madelyn Segev for her editorial assistance.

Conflict of interests

The authors declare that they have no conflicts of interest.

Author Contribution Statement

PG and MS performed the experiments, analysed the data, and wrote the manuscript. OSF, OM and NT performed *in-silico* modelling and analysis. DG helped in experiments. OM provided the methodological assistance. FLS and MBZ designed the research and provided overall supervision and edited the final draft. All authors have read and contributed to the final draft.

Funding

This work was supported by grants from the Aimwell Charitable Trust (UK), Emalie Gutterman Memorial Endowed Fund (USA), and the Israel Science Foundation (ISF, grant number : 442/18) to F. Levi- Schaffer

Data Availability

The data generated and/or analyzed during the present study can be made available upon reasonable request to the corresponding authors.

Accepted Manuscript

Downloaded from https://academic.oup.com/cei/advance-article/doi/10.1093/cei/uxad089/7242641 by Butler University user on 23 August 2023

References

1. Georgoudaki AM, Khodabandeh S, Puiac S, et al. CD244 is expressed on dendritic cells and regulates their functions. *Immunol Cell Biol*. 2015;93(6). doi:10.1038/icb.2014.124
2. Munitz A, Bachelet I, Fraenkel S, et al. 2B4 (CD244) Is Expressed and Functional on Human Eosinophils. *J Immunol*. 2005;174(1). doi:10.4049/jimmunol.174.1.110
3. Duev-Cohen A, Bar-On Y, Glasner A, et al. The human 2B4 and NTB-A receptors bind the influenza viral hemagglutinin and co-stimulate NK cell cytotoxicity. *Oncotarget*. Published online 2016. doi:10.18632/oncotarget.7597
4. Raziorrouh B, Schraut W, Gerlach T, et al. The immunoregulatory role of CD244 in chronic hepatitis B infection and its inhibitory potential on virus-specific CD8+ T-cell function. *Hepatology*. 2010;52(6). doi:10.1002/hep.23936
5. Wang Y, Zhong H, Xie X, et al. Long noncoding RNA derived from CD244 signaling epigenetically controls CD8+ T-cell immune responses in tuberculosis infection. *Proc Natl Acad Sci U S A*. 2015;112(29). doi:10.1073/pnas.1501662112
6. Yang B, Wang X, Jiang J, Zhai F, Cheng X. Identification of CD244-expressing myeloid-derived suppressor cells in patients with active tuberculosis. *Immunol Lett*. 2014;158(1-2). doi:10.1016/j.imlet.2013.12.003
7. Martina F, Perrotta F, Nicolai A, et al. Staphylococcus Aureus in chronic airway diseases: An overview. *Respir Med*. 2019;155. doi:10.1016/j.rmed.2019.07.008
8. Jinnestål CL, Belfrage E, Bäck O, Schmidtchen A, Sonesson A. Skin barrier impairment correlates with cutaneous Staphylococcus aureus colonization and sensitization to skin-associated microbial antigens in adult patients with atopic dermatitis. *Int J Dermatol*. 2014;53(1). doi:10.1111/ijd.12198

Accepted Manuscript

Downloaded from https://academic.oup.com/cei/advance-article/doi/10.1093/cei/uxad089/7242641 by Butler University user on 23 August 2023

9. Huvenne W, Hellings PW, Bachert C. Role of staphylococcal superantigens in airway disease. *Int Arch Allergy Immunol*. 2013;161(4). doi:10.1159/000350329

10. Fyhrquist N, Muirhead G, Prast-Nielsen S, et al. Microbe-host interplay in atopic dermatitis and psoriasis. *Nat Commun*. 2019;10(1). doi:10.1038/s41467-019-12253-y

11. Suvarnsit K, Kiratisin P, Bunnag C, Tantilipikorn P. Prevalence of nasal carriage of Staphylococcus aureus in allergic rhinitis patients and healthy controls in Thailand. *Asian Pacific J Allergy Immunol*. 2021;39(3). doi:10.12932/AP-080719-0598

12. Higaki S, Morohashi M, Yamagishi T, Hasegawa Y. Comparative study of staphylococci from the skin of atopic dermatitis patients and from healthy subjects. *Int J Dermatol*. 1999;38(4). doi:10.1046/j.1365-4362.1999.00686.x

13. Metcalfe DD, Pawankar R, Ackerman SJ, et al. Biomarkers of the involvement of mast cells, basophils and eosinophils in asthma and allergic diseases. *World Allergy Organ J*. 2016;9(1). doi:10.1186/s40413-016-0094-3

14. Gaur P, Zaffran I, George T, Rahimli Alekberli F, Ben-Zimra M, Levi-Schaffer F. The regulatory role of eosinophils in viral, bacterial, and fungal infections. *Clin Exp Immunol*. 2022;209(1):72-82. doi:10.1093/cei/uxac038

15. Rocha-de-Souza CM, Berent-Maoz B, Mankuta D, Moses AE, Levi-Schaffer F. Human mast cell activation by Staphylococcus aureus: Interleukin-8 and tumor necrosis factor alpha release and the role of toll-like receptor 2 and CD48 molecules. *Infect Immun*. 2008;76(10). doi:10.1128/IAI.00270-08

16. Minai-Fleminger Y, Gangwar RS, Migalovich-Sheikhet H, et al. The CD48 receptor mediates Staphylococcus aureus human and murine eosinophil activation. *Clin Exp Allergy*. Published online 2014:1335-1346. doi:10.1111/cea.12422

17. Elishmereni M, Fyhrquist N, Singh Gangwar R, Lehtimäki S, Alenius H, Levi-Schaffer F. Complex 2B4 Regulation of Mast Cells and Eosinophils in Murine

Accepted Manuscript

Downloaded from https://academic.oup.com/cei/advance-article/doi/10.1093/cei/uxad089/7242641 by Butler University user on 23 August 2023

Allergic Inflammation. *J Invest Dermatol*. Published online 2014. doi:10.1038/jid.2014.280

18. Elishmereni M, Bachelet I, Ben-Efraim AHN, Mankuta D, Levi-Schaffer F. Interacting mast cells and eosinophils acquire an enhanced activation state in vitro. *Allergy Eur J Allergy Clin Immunol*. 2013;68(2). doi:10.1111/all.12059
19. Minai-Fleminger Y, Elishmereni M, Vita F, et al. Ultrastructural evidence for human mast cell-eosinophil interactions in vitro. *Cell Tissue Res*. 2010;341(3). doi:10.1007/s00441-010-1010-8
20. Gangwar RS, Pahima H, Puzzovio PG, Levi-Schaffer F. Update on Eosinophil Interaction with Mast Cells: The Allergic Effector Unit. In: *Methods in Molecular Biology*. Vol 2241. ; 2021. doi:10.1007/978-1-0716-1095-4_18
21. Lankry D, Gazit R, Mandelboim O. Methods to identify and characterize different NK cell receptors and their ligands. *Methods Mol Biol*. 2010;612. doi:10.1007/978-1-60761-362-6_17
22. Elias S, Kahlon S, Duev-Cohen A, Mandelboim O. A BW reporter system for studying receptor-ligand interactions. *J Vis Exp*. 2019;2019(143). doi:10.3791/58685
23. Velikovsky CA, Deng L, Chlewicki LK, Fernández MM, Kumar V, Mariuzza RA. Structure of Natural Killer Receptor 2B4 Bound to CD48 Reveals Basis for Heterophilic Recognition in Signaling Lymphocyte Activation Molecule Family. *Immunity*. 2007;27(4). doi:10.1016/j.immuni.2007.08.019
24. Mirdita M, Schütze K, Moriwaki Y, Heo L, Ovchinnikov S, Steinegger M. ColabFold: making protein folding accessible to all. *Nat Methods*. 2022;19(6). doi:10.1038/s41592-022-01488-1
25. Kozakov D, Hall DR, Xia B, et al. The ClusPro web server for protein-protein docking. *Nat Protoc*. 2017;12(2). doi:10.1038/nprot.2016.169

Accepted Manuscript

Downloaded from https://academic.oup.com/cei/advance-article/doi/10.1093/cei/uxad089/7242641 by Butler University user on 23 August 2023

26. Benjamin Stranges P, Kuhlman B. A comparison of successful and failed protein interface designs highlights the challenges of designing buried hydrogen bonds. *Protein Sci*. 2013;22(1). doi:10.1002/pro.2187

27. Papageorgiou AC, Tranter HS, Acharya KR. Crystal structure of microbial superantigen staphylococcal enterotoxin B at 1.5 Å resolution: Implications for superantigen recognition by MHC class II molecules and T-cell receptors. *J Mol Biol*. 1998;277(1). doi:10.1006/jmbi.1997.1577

28. Gangwar RS, Levi-Schaffer F. SCD48 is anti-inflammatory in Staphylococcus aureus Enterotoxin B-induced eosinophilic inflammation. *Allergy Eur J Allergy Clin Immunol*. Published online 2016:829-839. doi:10.1111/all.12851

29. Jumper J, Evans R, Pritzel A, et al. Highly accurate protein structure prediction with AlphaFold. *Nature*. 2021;596(7873). doi:10.1038/s41586-021-03819-2

30. Kozakov D, Beglov D, Bohnuud T, et al. How good is automated protein docking? *Proteins Struct Funct Bioinforma*. 2013;81(12). doi:10.1002/prot.24403

31. Pahima H, Puzzovio PG, Levi-Schaffer F. A novel mast cell-dependent allergic peritonitis model. *Clin Exp Immunol*. 2021;205(3). doi:10.1111/cei.13619

32. Schuhmachers G, Ariizumi K, Mathew PA, Bennett M, Kumar V, Takashima A. 2B4, a new member of the immunoglobulin gene superfamily, is expressed on murine dendritic epidermal T cells and plays a functional role in their killing of skin tumors. *J Invest Dermatol*. 1995;105(4). doi:10.1111/1523-1747.ep12323533

33. Minai-Fleminger Y, Gangwar RS, Migalovich-Sheikhet H, et al. The CD48 receptor mediates *Staphylococcus aureus* human and murine eosinophil activation. *Clin Exp Allergy*. 2014;44(11):1335-1346. doi:10.1111/cea.12422

34. Claus M, Wingert S, Watzl C. Modulation of natural killer cell functions by interactions between 2B4 and CD48 in cis and in trans. *Open Biol*. 2016;6(5).

Accepted Manuscript

Downloaded from https://academic.oup.com/cei/advance-article/doi/10.1093/cei/uxad089/7242641 by Butler University user on 23 August 2023

doi:10.1098/rsob.160010

35. Lissina A, Ambrozak DR, Boswell KL, et al. Fine-tuning of CD8+ T-cell effector functions by targeting the 2B4-CD48 interaction. *Immunol Cell Biol*. 2016;94(6). doi:10.1038/icb.2016.17

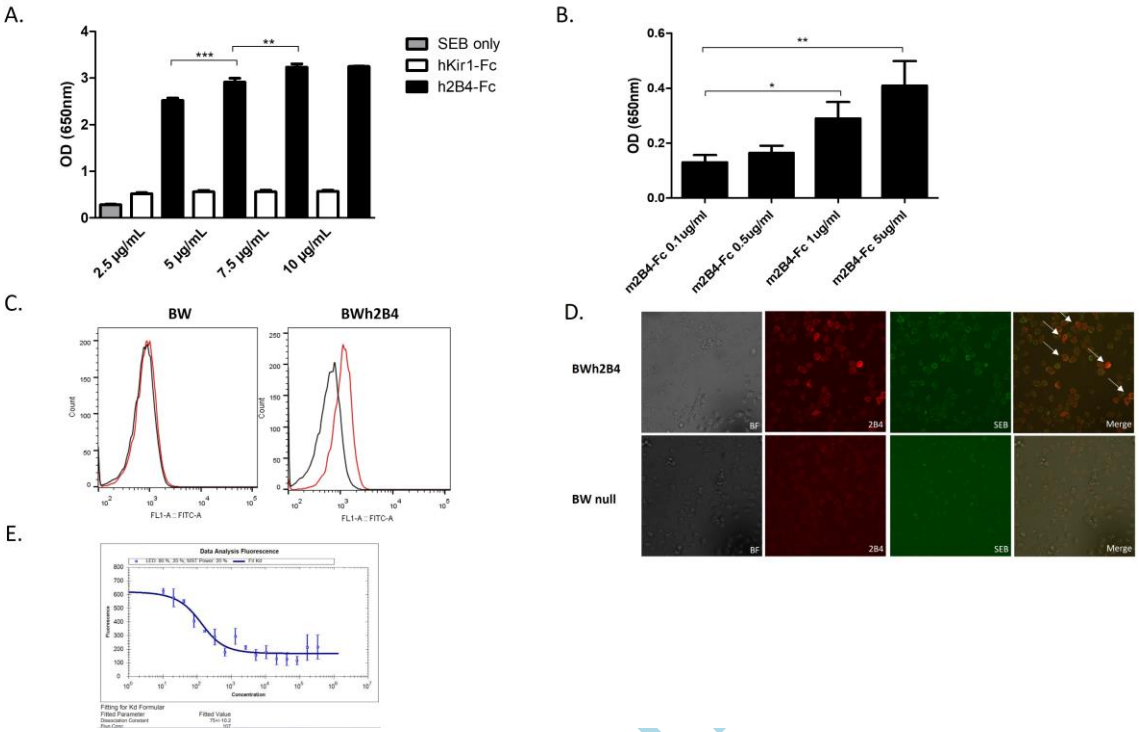
36. Li B, Lu Y, Zhong MC, et al. Cis interactions between CD2 and its ligands on T cells are required for T cell activation. *Sci Immunol*. 2022;7(74). doi:10.1126/sciimmunol.abn6373

37. D'Orazio JA, Stein-Streilein J. Human natural killer (NK) cells present staphylococcal enterotoxin B (SEB) to T lymphocytes. *Clin Exp Immunol*. 1996;104(2). doi:10.1046/j.1365-2249.1996.08698.x

Accepted Manuscript

Downloaded from https://academic.oup.com/cei/advance-article/doi/10.1093/cei/uxad089/7242641 by Butler University user on 23 August 2023

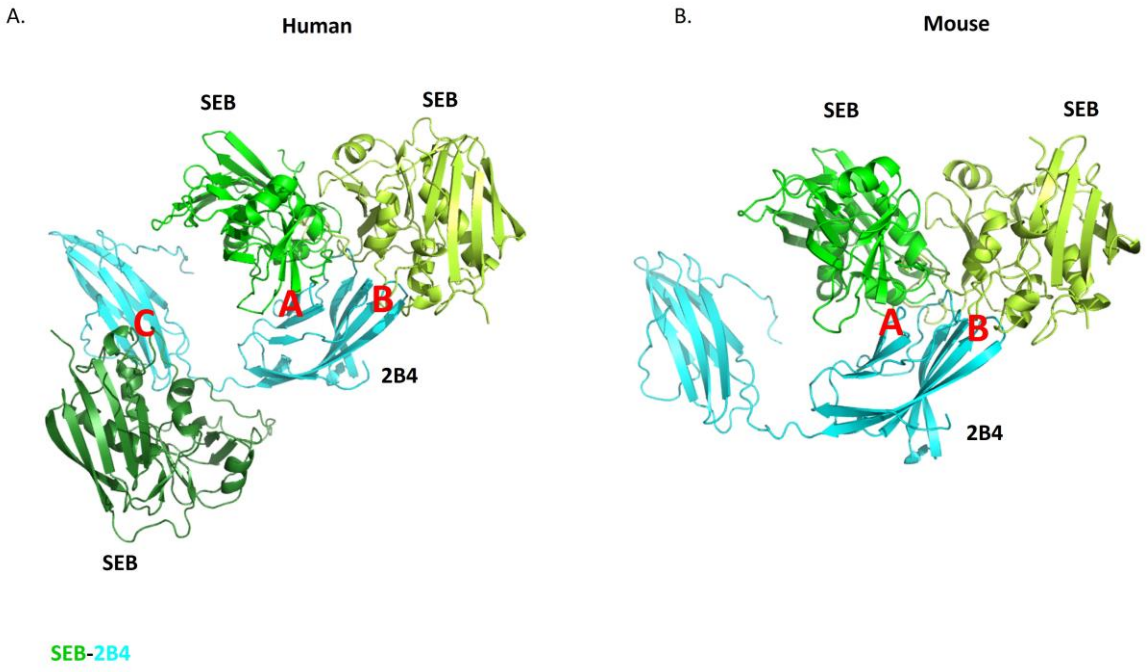
Figure 1A-E



Accepted Manuscript

Downloaded from https://academic.oup.com/cei/advance-article/doi/10.1093/cei/uxad089/7242641 by Butler University user on 23 August 2023

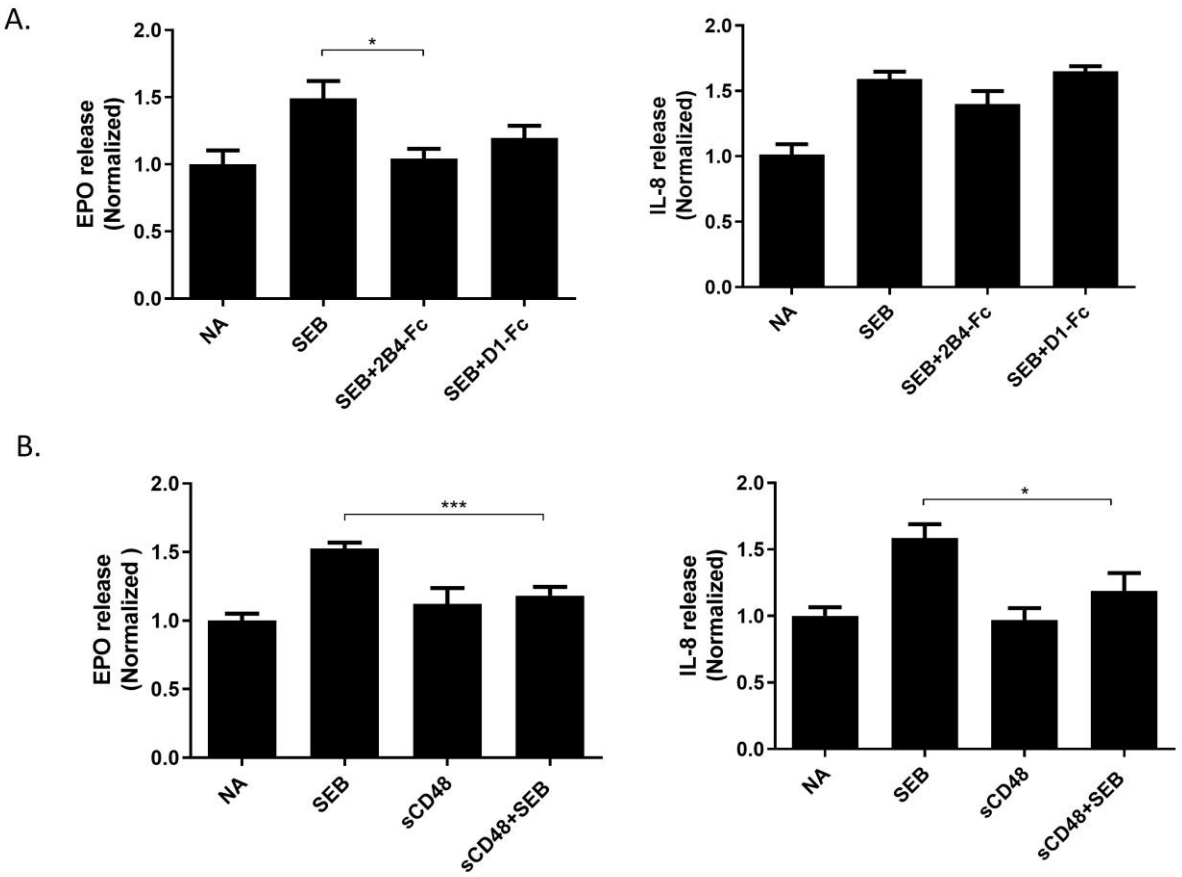
Figure 2A-B



Accepted Manuscript

Downloaded from https://academic.oup.com/cei/advance-article/doi/10.1093/cei/uxad089/7242641 by Butler University user on 23 August 2023

Figure 3A-B

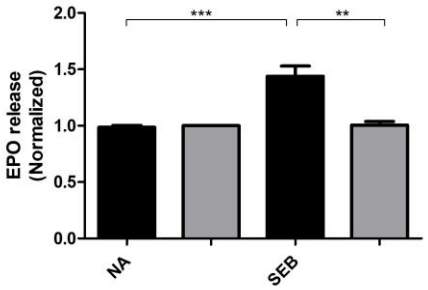


Accepted

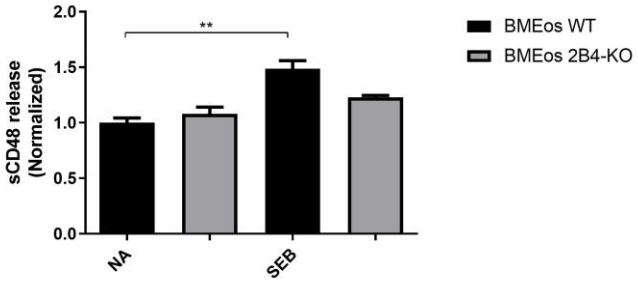
Downloaded from https://academic.oup.com/cei/advance-article/doi/10.1093/cei/uxad089/7242641 by Butler University user on 23 August 2023

Figure 4A-B

A.



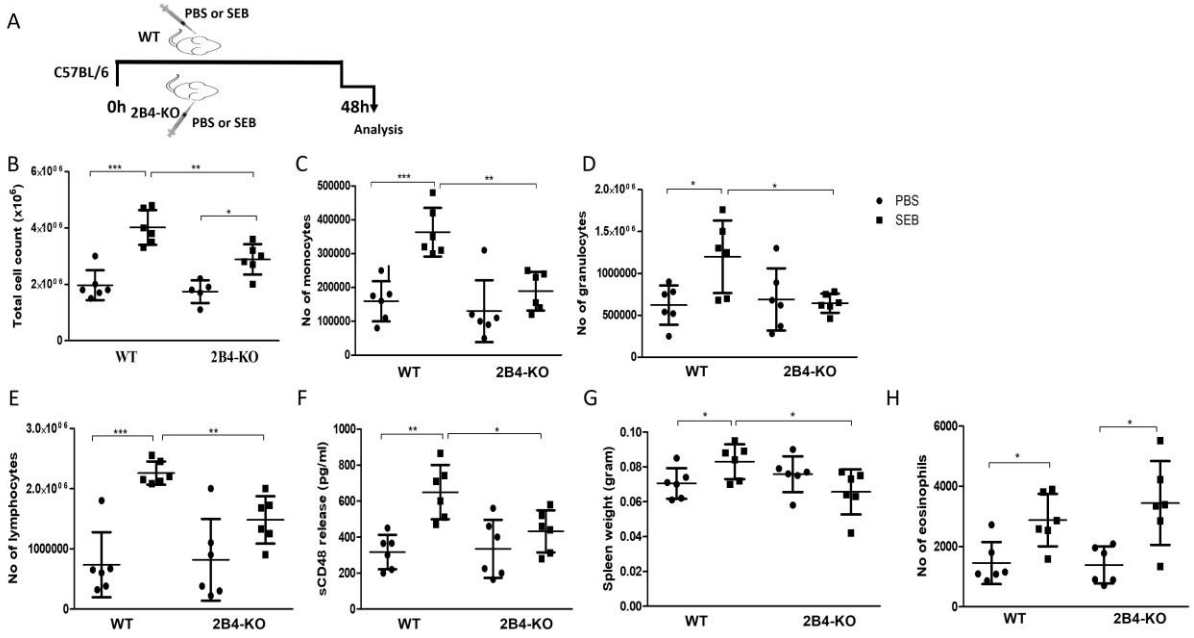
B.



Accepted Manuscript

Downloaded from https://academic.oup.com/cei/advance-article/doi/10.1093/cei/uxad089/7242641 by Butler University user on 23 August 2023

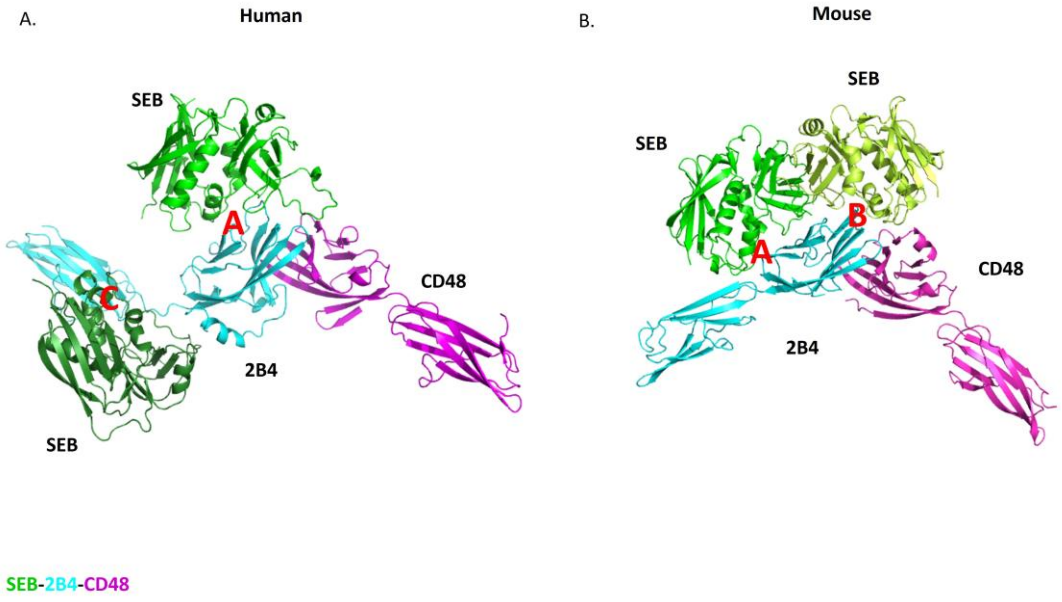
Figure 5A-H



Accepted Manuscript

Downloaded from https://academic.oup.com/cei/advance-article/doi/10.1093/cei/uxad089/7242641 by Butler University user on 23 August 2023

Figure 6A-B



SEB-2B4-CD48

Accepted Manus

Downloaded from https://academic.oup.com/cei/advance-article/doi/10.1093/cei/uxad089/7242641 by Butler University user on 23 August 2023