Immunological characterization of Man2 as a novel adjuvant and rFlaA:Betv1 as a therapeutic candidate for allergy treatment

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Summary

Type I hypersensitivity disorders are caused by exaggerated Th2-biased, IgE-mediated inflammatory reactions against harmless environmental allergens. The treatment options for type I allergies are limited, and "allergen vaccination," or allergen-specific immunotherapy (AIT), is currently the only method to re-establish immunological tolerance towards the respective allergens. AIT is performed by repeated administration of allergen extracts shifting allergen-specific Th2 responses towards Th1 responses. However, AIT consists of long treatment schedules, has the risk of inducing anaphylactic side effects, and partially has a low efficacy due to weakly immunogenic allergen molecules. Therefore, novel adjuvants need to be developed for either enhancing or modifying the overall immune responses in AIT.

This thesis aimed to evaluate the immune-modulating capacity of **(1)** β-(1→4)-mannobiose (Man2) as a novel adjuvant on myeloid dendritic cells (mDCs), and the new therapeutic candidate (rFlaA:Betv1, consisting of *Listeria monocytogenes* flagellin A fused to the major birch pollen allergen Bet v 1) on either **(2)** epithelial cells or **(3)** macrophages for type I allergy treatment.

1) The present results have shown that Man2 could induce a mainly "Toll"-like receptor 4 (TLR4)- and partially complement C3a receptor (C3aR)-dependent production of inflammatory cytokines (IL-6, TNF-α, IL-1β, IFN-β, and IL-10) and the upregulation of co-stimulatory molecules (CD40, CD80, and CD86) on mDCs. Besides, Man2 activated mitogen-activated protein kinase (MAPK)-/ nuclear factor kappa-light-chain-enhancer of activated B cells (NFκB)-pathways, and triggered the Warburg Effect in mDCs. Finally, Man2-stimulated mDCs enhanced antigen-specific, T cell-derived IL-2 production, demonstrating that Man2 has potential as a vaccine adjuvant.

2) In our group´s previous studies, rFlaA:Betv1 was shown to induce mDCs activation, but its effects on other cell types remained unknown. The data presented in this thesis demonstrated, that rFlaA:Betv1 could induce a MAPK- and NFκB-dependent, but TLR5-independent secretion of CCL2, CCL20, and IL-6 from lung epithelial cells. Additionally, rFlaA:Betv1 induced a p38-MAPK and cyclooxygenase 2 (COX2)-dependent prostaglandin E2 (PGE_2) production from epithelial cells, which could modulate mDC responses by decreasing their IL-12- and TNF-α-production. Therefore, epithelial cells also contribute to the immune-modulating capacity of rFlaA:Betv1, by modulating the responses of antigen-presenting cells (APCs).

3) Besides DCs, macrophages are another important type of APC. This thesis demonstrated, that macrophages could also be activated by rFlaA:Betv1 stimulation. Here, rFlaA:Betv1 induced a Myeloid differentiation primary-response protein 88 (MyD88)-, Jun N-terminal kinases (JNK)-MAPK-, and partially TLR5-dependent inflammatory cytokine secretion as well as a Warburg Effect from bone marrow-derived macrophages (BMDMs). Furthermore, using RNA-Seq and Western Blot analyses, the upregulation of Janus kinase (JAK)-signal transducer and activator of transcription (STAT)-transcription factor hypoxia-inducible factor 1 α (HIF-1α) signaling was identified, which confirmed results from Seahorse Extracellular Flux technology showing, that rFlaA:Betv1 triggered a pronounced metabolic

shift towards glycolysis in BMDMs. Finally, rFlaA:Betv1-stimulated BMDMs could suppress the secretion of Th2 cytokines from Bet v 1-specific, Th2-biassed $CD4^+$ T cells while increasing both Th1- and anti-inflammatory cytokine secretion.

In summary, the publications presented in this thesis demonstrated that Man2 could be an attractive novel adjuvant, and rFlaA:Betv1 can be an effective therapy for type I allergy treatment. The results in this thesis may contribute to the future development of safe and effective vaccines and therapeutics.

Zusammenfassung

Typ I Allergien zählen zu den Hypersinsitivitätserkrankungen. Sie werden durch überschießende, Th2-basierte und IgE-vermittelte Entzündungsreaktionen verursacht, die sich gegen harmlose Allergene aus unserer Umwelt richten. Die Behandlungsmöglichkeiten für Typ-I-Allergien sind momentan begrenzt, wobei die Allergen-spezifische Immuntherapie (AIT) derzeit die einzige Methode zur Wiederherstellung der immunologischen Toleranz gegenüber den betreffenden Allergenen ist. Während der AIT werden wiederholt Allergenextrakten verabreicht, welche die Allergen-spezifischen Th2-Reaktionen in Richtung Th1-Reaktionen verschieben sollen. Jedoch stellen sich auch bei der AIT einige Herausforderungen: es ergeben sich lange Behandlungszeiten, es besteht das Risiko anaphylaktischer Reaktionen als eine der möglichen Nebenwirkungen und die Wirksamkeit kann aufgrund der schwachen Immunogenizität der Allergene variieren. Um diese Probleme zu lösen müssen neuartige Adjuvantien und Therapeutika entwickelt werden, welche die induzierte Immunantwort bei der AIT entweder verstärken oder modifizieren.

In der vorliegenden Arbeit wurde sowohl die immunmodulierende Wirkung von **(1)** β-(1→4)-Mannobiose (Man2) als neuartiges Adjuvans auf myeloiden dendritischen Zellen (mDCs) getestet als auch, die Wirkung des Fusionsproteins rFlaA:Betv1 (bestehend aus dem *Listeria monocytogenes* Flagellin A, welches mit dem Birkenpollen-Hauptallergen Bet v 1 fusioniert wurde) **(2)** zum einen auf Epithelzellen und **(3)** zum anderen auf Makrophagen untersucht. Dieses soll zur Behandlung von Typ-I-Allergien eingesetzt werden.

1) In der beigefügten Publikation konnte gezeigt werden, dass Man2 die Produktion von Zytokinen (IL-6, TNF-α, IL-1β, IFN-β und IL-10) und die Expression von co-stimulatorischen Molekülen (CD40, CD80 und CD86) auf mDCs induziert. Diese Form der Aktivierung wurde als vorrangig "Toll"-like Rezeptor 4 (TLR4)- und teilweise C3a-Rezeptor (C3aR)-abhängig identifiziert. Zudem aktivierte Man2 in mDCs die mitogen-activated protein kinase (MAPK)-/ nuclear factor kappa-light-chain-enhancer of activated B cells (NFκB)- abhängigen Signaltransduktionswege und induzierte den Warburg-Effekt. Des Weiteren konnten mDCs, welche mit Man2 stimuliert wurden, die Antigen-spezifische IL-2-Produktion von T-Zellen steigern. Hieraus lässt sich schließen, dass Man2 als Adjuvans für Impfstoffe Potential besitzt.

2) In vorhergegangenen Studien unserer Gruppe konnte gezeigt werden, dass rFlaA:Betv1 mDCs aktivieren kann, während die Auswirkungen auf andere Zelltypen bisher unerforscht war. Die Daten dieser Arbeit konnten zeigen, dass rFlaA:Betv1 in Lungenepithelzellen eine MAPK- und NFκB-abhängige, aber TLR5-unabhängige Sekretion der Chemokine CCL2, CCL20 und des Zytokins IL-6 induzieren kann. Die Stimulation mit rFlaA:Betv1 löste in den Epithelzellen eine p38-MAPK- und Cyclooxygenase 2 (COX2)-abhängige Prostaglandin E2 (PGE_2)-Produktion aus, welche die Immunantwort von mDCs veränderte: es wurden geringere Mengen IL-12- und TNF-α ausgeschüttet. Hieraus lässt sich schließen, dass Epithelzellen zur immunmodulierenden Wirkung von rFlaA:Betv1 beitragen, indem sie die Reaktionen Antigen-präsentierender Zellen (APC) modulieren.

3) Neben mDCs stellen auch Makrophagen eine weitere wichtige Art von APCs dar. Innerhalb dieser Arbeit konnte gezeigt werden, dass auch Makrophagen durch rFlaA:Betv1-Stimulation aktiviert werden können. Innerhalb der Makrophagen wird durch rFlaA:Betv1 eine Myeloid differentiation primary-response protein 88 (MyD88)-, Jun N-terminale Kinase (JNK)-MAPK- und teilweise TLR5-abhängige Zytokinsekretion induziert. Zudem konnte die Induktion des Warburg-Effekts in Makrophagen beobachtet werden, die aus dem Knochenmark differenziert wurden (bone marrow derived macrophages, BMDMs). Darüber hinaus wurde mit Hilfe von RNA-Sequenzierung und Western Blot Analysen die Hochregulierung des Janus kinase (JAK)-Signaltransduktionsweges und des signal transductor and activator of transcription (STAT)-Transkriptionsfaktors hypoxia-inducible factor 1 α (HIF-1α) identifiziert. Diese Ergebnisse konnten mithilfe „Seahorse“ Extracellular Flux Assays bestätigt werden. Es zeigte sich, dass rFlaA:Betv1 eine ausgeprägte Verschiebung des Metabolismus in Richtung der Glykolyse in BMDMs verursachte. Abschließend konnte gezeigt werden, dass die Stimulation mit rFlaA:Betv1 in einer Kokultur bestehend aus BMDMs mit Bet v 1-spezifischen, Th2-basierten, $CD4^+$ T-Zellen die Ausschüttung von Th2-Zytokinen unterdrückt, während gleichzeitig die Ausschüttung von Th1- und entzündungshemmenden Zytokinen erhöht wurde.

Die Ergebnisse, welche in Verlauf dieser Arbeit publiziert werden konnten, lassen darauf schließen, dass es sich bei Man2 um ein potentielles Adjuvans handelt, während rFlaA:Betv1 als potentiell neuartiges Therapeutikum für die Behandlung von Typ I Allergien eingesetzt werden könnte. Durch die Ergebnisse dieser Studien konnte ein wichtiger Beitrag für die künftige Entwicklung sicherer und effektiver Impfstoffe und Therapeutika geleistet werden.

This thesis is based on the following publications:

- Ting-Yu Cheng, **Yen-Ju Lin**, Wataru Saburi, Stefan Vieths, Stephan Scheurer, Stefan Schülke, and Masako Toda. β-(1→4)-Mannobiose Acts as an Immunostimulatory Molecule in Murine Dendritic Cells by Binding the TLR4/MD-2 Complex. *Cells.* 10(7): 1774, 2021 *(IF in 2021: 6.6)*

- **Yen-Ju Lin**†, Garibald Papp†, Csaba Miskey, Anna Fiedler, Alexandra Goretzki, Sonja Wolfheimer, Jennifer Zimmermann, Peter Crauwels, Zoltán Ivics, Ger van Zandbergen, Stefan Vieths, Stephan Scheurer, and Stefan Schülke. The Flagellin:Allergen Fusion Protein rFlaA:Betv1 Induces a MyD88− and MAPK-Dependent Activation of Glucose Metabolism in Macrophages. *Cells*. 10(10): 2614, 2021 *(IF in 2021: 6.6)*

- **Yen-Ju Lin**, Adam Flaczyk, Sonja Wolfheimer, Alexandra Goretzki, Annette Jamin, Andrea Wangorsch, Stefan Vieths, Stephan Scheurer, and Stefan Schülke. The Fusion Protein rFlaA:Betv1 Modulates DC Responses by a p38-MAPK and COX2-Dependent Secretion of PGE_2 from Epithelial Cells. *Cells.* 10(12): 3415, 2021 *(IF in 2021: 6.6)*

- Alexandra Goretzki, Jennifer Zimmermann, **Yen-Ju Lin**, and Stefan Schülke. Immune metabolism -An opportunity to better understand allergic pathology and improve treatment of allergic diseases? *Front. Allergy.* 3:825931, 2022

- **Yen-Ju Lin**, Alexandra Goretzki, and Stefan Schülke. Immune Metabolism of IL-4-Activated B Cells and Th2 Cells in the Context of Allergic Diseases. *Front. Immunol.* 12:790658, 2021 *(IF in 2020: 7.561)*

- Alexandra Goretzki†, **Yen-Ju Lin**†, and Stefan Schülke. Immune metabolism in allergies, does it matter? - A review of immune metabolic basics and adaptations associated with the activation of innate immune cells in allergy. *Allergy*. 76(11):3314-3331, 2021 *(IF in 2020: 13.146)*

†Equal contribution

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1 General Introduction

1.1 Pathology and clinical relevance of type I allergies

Allergic diseases are a growing health and economic problem in developed countries with IgE-mediated type I hypersensitivity disorders affecting more than 25% of the population[1,2]. Immunologically, onset and maintenance of type I allergies are caused by exaggerated Th2-mediated immune responses, directed against otherwise harmless environmental antigens.

During allergic sensitization, allergens come in contact with the epithelia barriers (e.g. in the lung or the gastrointestinal system), resulting in disruption of epithelial integrity and allowing allergens to cross the epithelia barriers where they subsequently come in contact and are taken up by antigen presenting cells (APCs) (Figure 1). APCs further process and present the allergen-derived peptides to naïve T cells, promoting the differentiation of naïve T cells into Th2 cells (Figure 1). Additionally, cytokines (e.g. IL-25) secreted by allergen-stimulated epithelial cells, and damage associated molecular patterns (DAMPs, e.g. IL-33) produced by stressed/damaged epithelial cells can expand the population of innate like lymphocytes type II cells (ILC2s) which further produce Th2-promoting cytokines[3–5] (Figure 1). The activated allergen-specific Th2 cells subsequently produce the Th2 cytokines IL-4, IL-5, IL-9, and IL-13 which can in turn promote expansion, maturation, and/or functional activation of inflammatory cells, including mast cells and eosinophils[6] (Figure 1). Moreover, these Th2 cytokines also promote the isotype switching of B cells resulting in the production of allergen-specific IgE antibodies[7] (Figure 1). Allergen-specific IgE antibodies then bind to the high affinity IgE receptor FcεRI on the surface of mast cells and eosinophils (Figure 1). The binding of IgE to FcεRI is characterized by both high specificity and avidity (KDs of up to 10^{-10} M), resulting in the efficient long-term loading of mast cells and eosinophils with the generated IgE antibodies[8].

During the elicitation phase, second contact with the offending allergen results in the crosslinking of surface bound allergen-specific IgE antibodies, triggering both mast cell and eosinophil activation, and the subsequent inflammatory responses caused mast cell- and eosinophil degranulation[9,10] (Figure 1).

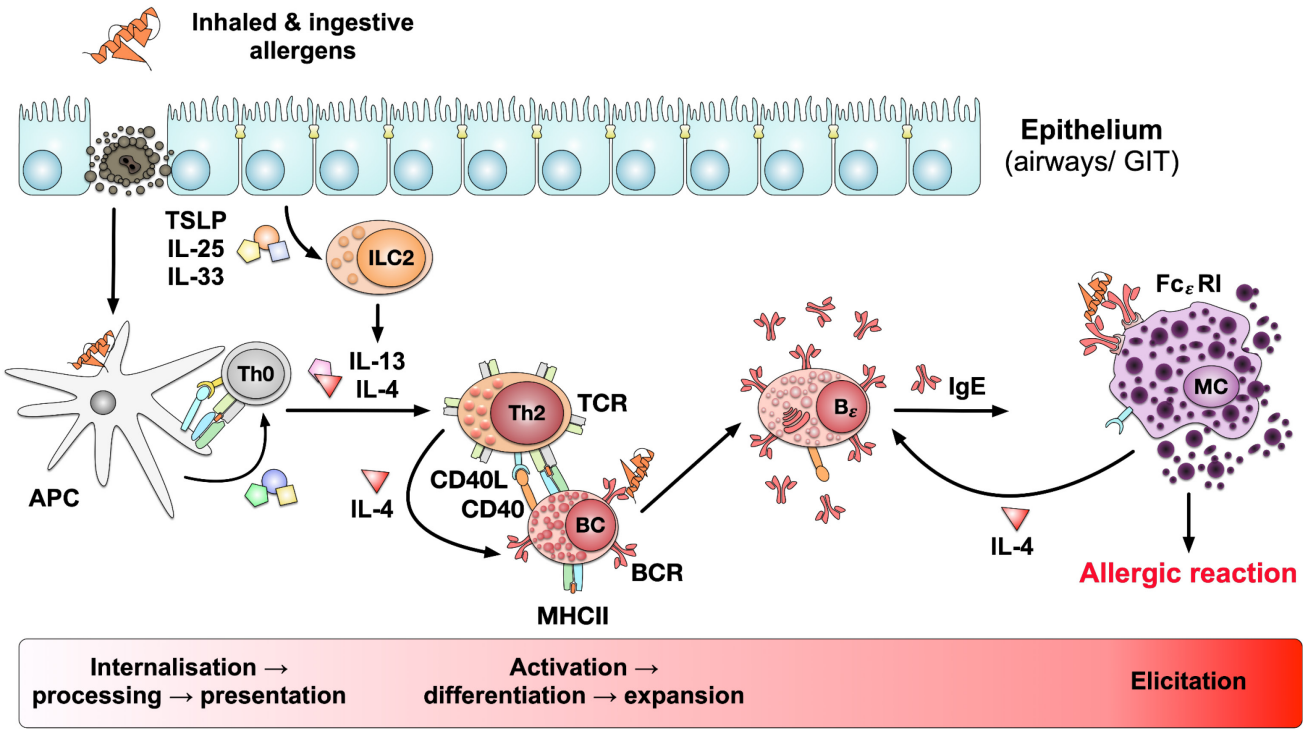

Figure 1: Pathomechanism of type I allergy

Epithelial cells are the first line of contact with allergens. In allergic patients, this contact results in disruption of epithelial integrity, allowing the allergen to get in contact with APCs, which take up, process, and present allergen-derived peptides to naïve T cells. Besides, epithelial cells also secrete inflammatory mediators, resulting in the ILC2-driven differentiation of naïve allergen-specific T cells into Th2 cells. These in turn mediate the activation and differentiation of allergen-specific B cells into IgE-producing plasma cells[3–5,7]. IgE then binds to the high affinity IgE receptor FcεRI on the surface of mast cells (and eosinophils), and this sensitization of mast cells (and eosinophils) with allergen-specific IgE marks the end of the sensitization phase. Upon second contact with the allergen, surface-bound IgE antibodies are cross-linked, resulting in both mast cell- and eosinophil activation, degranulation, and the elicitation of allergic reactions[6,8]. Abbreviations: GIT: gastrointestinal tract, TSLP: thymic stromal lymphopoietin, APC: antigen presenting cell, ILC2: innate like lymphocyte type II, TCR: T-cell receptor, BCR: B-cell receptor, MC: mast cell.

1.2 Current treatment options for type I allergies

The current treatment for type I allergy is limited and can be differentiated into (1) symptomatic treatment to immediately reduce acute clinical symptoms and (2) allergen-specific immunotherapy to achieve long-term desensitization towards the respective allergens.

For acute symptomatic treatment, several available medications have been used for years. For example, corticosteroids suppress Th2 cell-mediated inflammation by unspecifically reducing cell activation, cytokine-, and chemokine production[11]. Moreover, β2 adrenergic receptor agonists such as salbutamol and terbutaline are highly effective in reducing allergic airway symptoms by relaxing smooth muscle tissue[11]. Besides, H1-antihistamines can block the action of the inflammatory mediator histamine through the H1 receptor and improve clinical symptoms[11,12]. Finally, omalizumab, a humanized monoclonal anti-IgE antibody that blocks IgE from binding to both FcεRI (high-affinity IgE receptor) and

FcεRII (low-affinity receptor), was approved for clinical use in 2003[13]. Besides, Mepolizumab (Nucala®) and Reslizumab (Cinquaero® or Cinquair®), antibodies that can neutralize IL-5, and Benralizumab (Fasenra®) that blocks IL-5 receptor subunit α (IL-5Rα) expressed by eosinophils and basophils have also been used to reduce eosinophil-mediated inflammation in asthmatic subjects[14]. However, these pharmacotherapies cannot prevent sensitization to the allergens, and the treatment is costly, has side effects, and must be repeated as long as symptoms persist, which often means a lifetime[15].

In contrast to this, "allergen vaccination", or allergen-specific immunotherapy (AIT) may cure type I allergy. AIT is an immune-modifying therapy with the goal to re-establish immunological tolerance towards the respective allergen(s) and the induction of blocking IgG antibodies by repeated administration of allergen extracts[15–17]. Successful immunotherapy in humans is accompanied by shifting allergen-specific Th2 responses towards Th1 responses (immune deviation), the induction of regulatory T cells (T_{reg}) (immune regulation), and a reduction in mast cell numbers[15–17]. The induced Th1 cells may produce interferon gamma (IFN-γ), in order to stimulate B cells to produce allergen-specific IgG_4 antibodies instead of the allergy-causing IgE antibodies[15]. Moreover, previous studies showed that AIT-activated $CD4^+CD25^+$ T_{regs} can secrete both IL-10 and transforming growth factor-β (TGF-β), which are known to suppress mast cell- and eosinophil activation, Th2 responses, and to induce IgG_4 and IgA antibody production[15,18,19].

Currently, AIT can be either administrated subcutaneously or sublingually, but the drawbacks include long treatment schedules (lasting months to years), and the risk to induce anaphylactic side effects with an incidence of 0.1-5% in treated patients[20,21]. To improve AIT, new administration routes (oral, intralymphatic, or epicutaneous) were studied, but did not demonstrate significant improvements on the above-mentioned drawbacks[22]. Therefore, new treatment strategies for type I allergies are still required.

1.3 Novel strategies for type I allergy treatment

Currently, several strategies to establish new treatments for type I allergy have been proposed in order to reduce side effects and improve immunogenicity of conventional AIT.

1.3.1 Allergoids

Allergoids are chemically modified allergen products obtained by polymerizing native allergen extracts by treating them with either formaldehyde or glutaraldehyde[23]. Allergoids have been shown to display a reduced IgE-binding activity (allergenicity)[24] combined with the induction of reduced local and systemic side effects[25]. Besides, a clinical study for Der p 1 and Der p 2 house dust mite (HDM) allergoids also showed decreased levels of allergen-specific IgE antibodies, in parallel with increasing IgG_4 antibodies in the treated patients[26]. Currently, there is only a limited number of studies available

directly comparing allergoids with unmodified allergen extracts. A study from Henmar *et al.* compared the allergenicity and immunogenicity of four commercially available grass pollen allergoids with three conventional grass pollen AIT products[27]. Their results showed no differences between allergoids and conventional AIT products in the ability to suppress basophil activation *in vitro* when testing blood samples from 20 grass pollen allergic patients[27]. Moreover, two of the four tested allergoids, which demonstrated the lowest basophil-activating capacity, also had significantly lower immunogenicity than conventional AIT products inducing lower levels of allergen-specific IgG antibodies *in vivo*[27]. These data indicated that allergoids may improve safety but so far did not significantly improve efficacy of allergy treatment.

1.3.2 Recombinant allergens

Recombinant DNA technology opened another window for improving allergy immunotherapy. Here, highly pure recombinant allergens can be produced with low batch-to-batch variation in order to replace the more heterogeneous natural allergen extracts[28]. Moreover, recombinant allergens also allow for the modification of allergens such as mutating IgE-binding epitopes, producing short amino acid segments, or generating allergen dimers or trimers that retain antigenicity but suppress allergenicity in order to prevent side effects[23]. For example, Vrtala and colleagues showed that injection of recombinant rBet v 1 trimers consisting of either amino acids 1-74 or 75-160 could induce higher levels of IgG_1 antibodies in mice, and these IgG antibodies also inhibited the binding of birch pollen allergic patients' IgE to wild type Bet v 1[29]. Another study from Wai *et al.* identified six major immunodominant T cell epitopes of tropomyosin, the major shrimp allergen of *Metapenaeus ensis* (Met e 1). Oral administration of recombinant rMet e 1-T cell epitope peptides resulted in increased levels of IgG_{2a} antibodies as well as lower levels of the Th2 cytokines IL-4 and IL-5 in a shrimp-allergic mouse model[30]. Although most of the available *in vivo* and *in vitro* studies showed promising results on recombinant modified allergens to improve allergy treatment, their effects in humans are still unknown. A phase I clinical trial demonstrated, that treatment with mutated IgE-binding epitopes of the recombinant peanut allergens Ara h 1, Ara h 2, and Ara h 3 resulted in unexpected significant adverse events in 5 out of 10 tested peanut-allergic patients[31], indicating that more pre-clinical and clinical research is still needed to safely apply these molecules.

1.4 Adjuvants

The other promising strategy to improve type I allergy treatment is adding adjuvants to the low-immunogenic allergens to either enhance or modify the overall induced immune responses. Currently, adjuvant research is a hot topic not only for improving allergy treatment but also for the development of other vaccines. Here, I briefly summarize the knowledge on the different adjuvants currently under investigation for improving AIT (see also Figure 2).

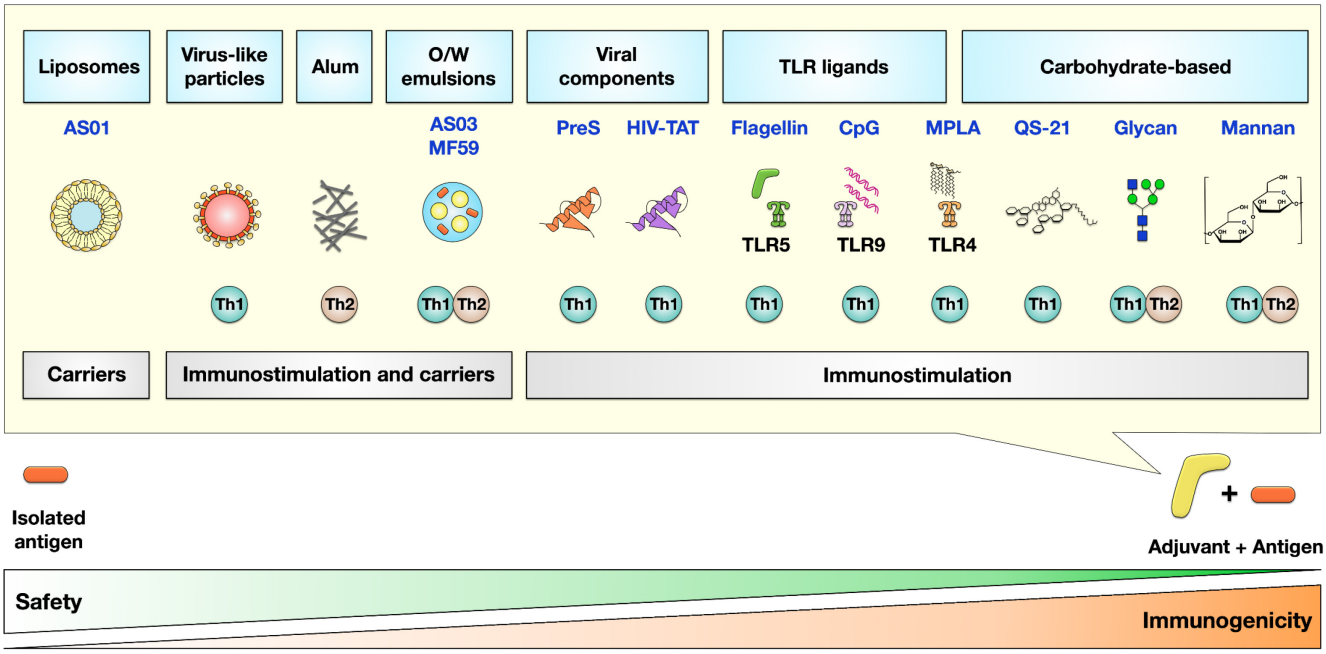

Figure 2: Adjuvants that are currently studied to improve AIT

The types of adjuvants can be classified based on their carrier or immunostimulatory capabilities[32]. Here, liposomes mainly serve as carriers, while virus-like particles, alum, and O/W emulsions have both carrier and immune stimulating characteristics. Viral components (PreS or HIV-TAT), TLR ligands (flagellin, CpG, or MPLA), and carbohydrate-based adjuvants (QS-21, glycan, or mannan) have immunomodulatory properties to trigger different immune responses. The known capacity of these adjuvants to induce Th1 or Th2 responses is also indicated. For more information, see text. Abbreviations: AS: adjuvant system, Alum: aluminum hydroxide, O/W: oil-in-water, PreS: HBV-derived T-cell epitope, HIV-TAT: HIV type 1 trans-activating regulatory protein, TLR: "Toll"-like receptor, MPLA: monophosphoryl lipid A.

Adjuvants can be categorized according to their carrier- or immunostimulatory properties (Figure 2). As carriers, liposomes have been considered attractive adjuvants because of their low toxicity and high effectiveness in packaging and delivering water-soluble antigens to target cells[33]. However, the disadvantage for liposomes is their lack of immune-activating capacity, which can be overcome by combining liposomes with additional adjuvants. For this, one liposome-based formulation, the commercial adjuvant system 01 (AS01), which also contains the "Toll"-like receptor 4 (TLR4)-ligand monophosphoryl lipid A (MPLA) and the natural saponin product QS-21 has already been approved for use in malaria- (Mosquirix) and shingles (Shingrix) vaccines[34]. The potential of liposomes to carry different allergen extracts has been tested *in vivo*[35], and results showed that liposome-encapsulated allergen extracts could induce higher levels of allergen-specific IgG antibodies paralleled with lower IgE production[35]. Compared to liposomes, virus-like particles (VLPs) have the advantage to serve as effective delivery vehicles while also offering strong immune-modulating capacities due to their highly repetitive and ordered structure[36]. Up to now, several studies applying VLPs showed their potential to improve allergic responses in allergic animal models *in vivo*[36]. However, so far no human clinical study investigating VLPs for allergy treatment has been finished and reported. A phase I clinical trial announced by the United States Food & Drug Administration (FDA) recently started in January 2022

and will analyze VLPs that display the major peanut allergen Ara h 2[37] for treating peanut-allergic patients. Hopefully, this trial will give us some insights in the potential of VLPs for allergy treatment.

Besides VLPs, aluminum hydroxide (Alum) and oil in water (O/W) emulsions also have both carrier and immune-stimulating characteristics (Figure 2). Since Glenny *et al.* reported, that Alum could be used as an adjuvant in 1926[38], Alum has nowadays become the most widely used adjuvant for different licensed human vaccines against infectious diseases[39]. Alum has also been used in most subcutaneous allergy immunotherapy (SAIT) products[40] because of its ability to adsorb proteins and prolong the exposure of these antigens to immune cells at the injection site to increase immune responses[39]. In addition, Alum-stimulated dendritic cells (DCs) could further promote $CD4^+$ T cell differentiation, especially towards Th2 cells[39]. Therefore, since Alum is well known to induce Th2-biased immune responses, for allergy immunotherapy, Alum adjuvant was reported to correlate with observed side effects such as urticaria[41]. Based on these findings, the improved application of Alum as an adjuvant for type I allergy treatment is still needed in the future.

Currently, two O/W emulsion adjuvants AS03 and MF59, have already been licensed for clinical use, especially for various Influenza vaccines[42]. Mechanistically, O/W emulsions have the advantage of gradually releasing the antigen at the injection site, which stimulates the activation of plasma cells producing antigen-specific antibodies and generates mixed Th1/Th2 responses[43,44]. A recent study from O'Konek and Baker, Jr. used nanoscale O/W emulsions formulated with peanut allergens (PN-NE) to suppress allergic responses after either oral or systemic peanut allergen challenge in mice[45]. Their results showed decreased production of Th2 cytokines, as well as higher Th1- and anti-inflammatory cytokine production in PN-NE-treated peanut-allergic mice[45], indicating the potential for using O/W emulsions in allergy treatment.

The next category of adjuvants have strong immunostimulatory capabilities but no carrier characteristics (Figure 2). These can be further sub-classified into viral components, TLR ligands, and carbohydrate-based adjuvants (Figure 2). Several viral components have been proposed as adjuvants for allergy treatment[36]. For example, the HBV-derived T-cell epitope PreS peptide fused with different allergens has been tested both *in vivo* (mice and rabbits)[46,47] and in human clinical trials[48,49]. Moreover, HIV type 1 trans-activating regulatory protein (HIV-TAT) fused with pollen allergen also demonstrated the induction of higher allergen-specific IgG antibody and Th1 cytokine IFN-γ production after immunization in mice[50], indicating the potential of using viral proteins as adjuvants for generating allergy vaccines.

In this thesis, I will test the TLR5 agonist flagellin and the mannooligosaccharide Man2 as adjuvants for assessing their potential on improving allergy treatment. Therefore, in the following section, I will explain carbohydrate-based adjuvants and TLR ligands more in detail, including their current application for developing allergy vaccines.

1.4.1 Carbohydrate-based adjuvants

Carbohydrates are the most common type of biomolecule found in nature. They play an important role in modulating both innate and adaptive immunity[51]. Their high biocompatibility and low toxicity make carbohydrates interesting candidates for novel adjuvants. Currently, two carbohydrate-based adjuvants, QS-21 and MPLA, have been licensed for clinical use[34,52] (Figure 2). Although presently no study tested QS-21 as an adjuvant for allergy treatment, QS-21 was shown to induce Th1-biased immune responses with high titers of IgG_{2a} and IgG_{2b} antibodies[34], making it an attractive candidate for future research.

Besides QS-21 and MPLA, several different other compounds are also under investigation. For example, glucans, the polysaccharides derived from plants or microorganisms composed of D-glucose units linked by different glycosidic bonds[53]. The mechanisms by which glucans, which can vary in structure, type of linkage, and length of glucans, activate immune cells are not fully clear. A clinical study demonstrated that orally-administrated superfine dispersed β-(1,3)-glucan (particle-size: 0.08 μm), instead of non-dispersed shiitake extract (particle-size: 288 μm), could alleviate patient symptoms like rhinorrhea, sneezing, nasal congestion, and itchy watery eyes induced by Japanese cedar pollen[54]. Besides clinical symptoms, the superfine dispersed β-(1,3)-glucan treatment group also displayed lower pollen-specific IgE antibody profiles[54]. These results showed that (1) glucans could be an attractive adjuvant for improving AIT, and (2) that the size of the carbohydrate has a strong influence on the induced immune responses.

1.4.1.1 Mannan

Mannan is a β-(1,4)-mannose polysaccharide which can be found in the cell walls of plant cells or fungi[34], is also been proposed as a novel adjuvant for allergy treatment. Mannan can modulate immune responses by binding to both mannan- and C-type lectin receptors, which leads to the activation of complement pathways[53]. Moreover, these two receptors are also expressed by APCs[53]. Therefore, mannan was shown to activate DCs and macrophages, including increased phagocytosis, NOD-, LRR- and pyrin domain-containing protein 3 (NLRP3) inflammasome activation, and cytokine secretion[34,53]. The first study that used mannan as an adjuvant for vaccine development was reported in 1992. Okawa *et al.* conjugated mannan with hepatitis B virus (HBV) 139-147 peptide[55]. This conjugate was shown to induce higher IgG titers compared to HBV peptide-dextran conjugates[55]. In the following years, the native, oxidized, or reduced form of mannan conjugated with different antigens has been tested as an adjuvant for targeting APCs[34], showing higher antigen uptake and presentation with distinct Th1- or Th2-responses[34].

The currently available studies using mannan as an adjuvant for improving allergen immunotherapy are limited. Weinberger and colleagues oxidized mannan from the yeast *Saccharomyces cerevisiae* to

generate reactive aldehyde groups for covalent attachment of egg allergen ovalbumin (OVA) via amine-containing residues (MN–OVA)[56]. Their results showed, that MN–OVA was taken up more strongly by DCs both *in vitro* and *in vivo* compared to OVA alone[56]. Moreover, MN–OVA also induced higher IgG antibody production at the injection site[56]. Another approach was to conjugate defatted grass pollen grain-based allergoids from *Phleum pratense* with the native form of mannan extracted from *Saccharomyces cerevisae* (PM)[57–59]. Here, data demonstrated that PM induced higher inflammatory cytokines (IL-6 and IL-10) with lower production of the Th2 cytokine IL-4, promoted mammalian target of rapamycin (mTOR)-dependent glycolysis, and could be more efficiently taken up by human monocyte-derived DCs (hmoDCs) *in vitro* when compared to either pollen extracts or pollen allergoids[57–59]. From *in vivo* analysis, PM was shown to stimulate the differentiation of $CD4^+CD25^{high}FOXP3^+$ T_{reg} cells, paralleled by increased pollen-specific IgG_{2a}/IgE ratios[58]. These studies demonstrated the potential of mannan as an adjuvant to improve AIT.

Research showed that differences in size, structure, and purification method of carbohydrates from natural sources might lead to distinct immune responses[54,60]. However, the influence of mannan with different sizes and linkages on immune cell activation is still unknown. Therefore, in this thesis, I tested the effects of different sizes and linkages of mannooligosaccharides on dendritic cell activation in order to investigate their adjuvant potential for improving AIT.

1.4.2 Toll-like receptors agonists

Innate immune cells express pattern recognition receptors (PRRs) (e.g. TLRs and NOD-like receptors (NLRs)) to directly recognize pathogen-associated molecular patterns (PAMPs) that are part of invading pathogens[61]. After PAMPs bind to PRRs, innate immune cells become activated and further initiate and regulate adaptive immunity[61]. Besides, different PRR-ligand can induce different levels of Th1-, Th2-, or Th17-immune responses[61]. Due to this intrinsic immune activating potential, PRR-ligands are highly interesting adjuvant candidates for vaccine development.

For example, the synthetic, immunostimulatory oligodeoxynucleotide containing unmethylated CpG motifs (CpG-ODN), that can bind to TLR9, have been tested as vaccine adjuvants[62] (Figure 2). The proposed advantages of using CpG-ODN for allergen-specific immunotherapy include: (1) CpG-ODN can activate DCs, especially plasmacytoid DCs (pDCs), to produce IL-10 and TGF-β that in turn drive differentiation and activation of T_{reg} responses[62]. (2) Since B cells express high levels of TLR9, CpG-ODN can directly activate B cells. (3) CpG-ODN was reported to induce systemic Th1-responses[63]. These mechanisms and *in vivo* studies demonstrated the potential of CpG-ODN as an adjuvant for advanced AIT treatment. However, so far there is no human clinical study to connect these preclinical findings to a clinical application.

Besides, the immunological effects of MPLA, a carbohydrate-based adjuvant which can bind to TLR4[64] have also been well studied. It is known, that MPLA can induce Th1-biased immune responses and promote both IgG_1- and IgG_4-dominated humoral immune responses without boosting IgE production[65,66], which makes MPLA an attractive adjuvant for improving AIT. Pollinex® Quattro (PQ) is an allergen therapeutic containing glutaraldehyde-modified pollen allergoids adsorbed to L-tyrosine and further adjuvanted with MPLA[67]. It is currently the only advanced AIT combined with adjuvant investigated for clinical use in allergology. PQ was launched on the market in 1999 for allergic rhinitis treatment against grass-, tree-, olive-, and weed allergens[67]. Compared to traditional AIT that normally requires months to years of treatment, PQ is based on a "short-term specific immunotherapy (ST-AIT)" consisting of only four pre-seasonal injections of increasing dosages[68]. Clinical studies showed, that PQ treatment significantly reduced nasal and ocular clinical symptoms, as well as shifting immune responses from allergic Th2- toward Th1-responses characterized by higher grass-pollen-specific IgG antibody production[68]. Moreover, *in vivo* rat and dog pre-clinical studies showed no toxicological findings as well as no significant local and systemic adverse events in human clinical trials[68,69]. Taken together, PQ is a successful example of combining allergens with adjuvant, enabling the improvement on the major disadvantages of traditional AIT regarding long treatment periods and high occurrence of side effects.

1.4.2.1 Flagellin

Vaccines adjuvanted with the TLR5-ligand flagellin[70], a bacterial motility protein forming the main body of the bacterial flagellum, were reported to be both safe and well-tolerated in clinical trials[71,72]. Here, flagellin was demonstrated to be an effective mucosal adjuvant triggering Th1-biased, protective immune responses[73–75]. One of the major advantages of flagellin as an adjuvant is its proteinaceous nature, allowing for the efficient generation of fusion proteins consisting of flagellin and the antigen of choice by recombinant DNA technology. Flagellin-containing fusion proteins are attractive vaccines combining the adjuvant activity of the TLR5-ligand and the cargo antigen into a single molecule, allowing for the efficient targeting of antigens to, and simultaneous activation of TLR5-expressing APCs[76–80]. Several studies described *Salmonella typhimurium* flagellin type C (FliC) conjugated with different target antigens to boost protective neutralizing antibody responses[81,82]. Hence, the intrinsic adjuvant activity of flagellin is currently being leveraged in experimental vaccine candidates to induce protective immunity against viral infections (influenza, poxvirus, and West Nile Virus) and bacterial pathogens (*Clostridium tetani*, *Pseudomonas*) (reviewed in[83]). Recently, recombinant FliC fusion proteins incorporating influenza virus antigens have been shown to be safe in phase I clinical trials[71,72,84–87]. These studies demonstrated that flagellin could be a promising adjuvant for future human application.

Flagellin has also been analyzed as an adjuvant for improving traditional AIT. Here, approaches utilizing flagellin were divided into either (1) mixtures of flagellin and different allergens or (2) flagellin fused to

allergens as part of fusion proteins. The first study using flagellin and allergen as a mixture was performed by Lee and Kim *et al.* where they tested the intranasal or intralymphatic injection of *Vibrio vulnificus* FlaB mixed with OVA in an OVA-based mouse model of airway inflammation[88,89]. Their results showed, that the OVA/FlaB mixture could reduce OVA-induced airway hyper-responsiveness, inflammatory cell infiltration in lung tissue, as well as both systemic (IL-4, IL-5, IL-6, IL-17, and IFN-γ) and local (IL-4 and IL-5) cytokine production; while increasing the levels of OVA-specific IgA in serum[88,89]. A follow-up study by the same group investigated the underlying mechanisms in more detail, and found the ability of the OVA/FlaB mixture to suppress allergic responses to be dependent on TLR5[90]. Moreover, FlaB induced the activation of regulatory dendritic cells (rDCs) producing both IL-10 and TGF-β, which suppressed both Th1/Th2 responses and enhanced the activation of T_{reg} cells[90]. Besides DCs and T cells, a recent study also demonstrated the capability of flagellin to stabilize OVA-induced eosinophil activation (releasing major basic protein (MBP) and peroxidase (EPX)) *in vitro* and *in vivo*[91]. Interestingly, the authors also found that higher oxidative stress in OVA-sensitized eosinophils could both be reduced by flagellin treatment and was correlated with decreased allergic inflammation[91]. Additionally, Zeng *et al.* also found that flagellin could diminish allergen-induced oxidative stress in B_{reg} from both a food allergy (FA) mouse model and patients' blood samples[92]. Mixing flagellin as an adjuvant with OVA could improve traditional AIT in OVA-induced FA *in vivo*[92]. These results showed, that as an adjuvant for improving AIT, flagellin can activate different cell types, changing their cytokine secretion and metabolic status that correlates with disease outcome.

On the other hand, flagellin:allergen fusion proteins showed even more promising potential for allergy treatment than the mixture of flagellin and allergen. Tan *et al.* generated a recombinant fusion protein containing *Vibrio vulnificus* FlaB fused to the C-terminus of the HDM allergen Der p 2 (rDerp2:FlaB)[93]. Compared to the non-fused Derp2/FlaB mixture, rDerp2:FlaB more efficiently suppressed airway hyper-responsiveness, serum IgE levels, and bronchoalveolar lavage fluid Th2 cytokines in a HDM-induced mouse asthma model[93]. Moreover, Kitzmüller and colleagues also demonstrated that FliC (lacking the middle hypervariable domain) fused to either the N- or C-terminus of Bet v 1 (rFliC:Betv1 or rBetv1:FliC, respectively) could activate monocyte-derived DCs from pollen-allergic patients[94]. Besides, immunization with both rFliC:Betv1 or rBetv1:FliC fusion proteins also induced lower Bet v 1-specific IgE production paralleled by higher production of IgG antibodies from mice[94], suggesting these fusion proteins to be promising novel treatment options for pollen allergy. Apart from the above-mentioned publications, we are the only group currently focusing on flagellin:allergen fusion protein research. Moreover, so far no other groups has analyzed the molecular mechanisms by which flagellin:antigen fusion proteins activate the immune system in greater detail. In the following sections, I will briefly summarize our previous findings, followed by the open questions and the aim of this thesis.

1.5 Own previous work on flagellin:allergen fusion proteins

In the last 15 years, our group successfully generated recombinant fusion proteins containing *Listeria monocytogenes* flagellin A (FlaA) fused with either the OVA protein fron hens egg (rFlaA:OVA), wild type or hypoallergenic mugwort allergen Art v 1 (rFlaA:Artv1), or the major birch pollen allergen Bet v 1 (rFlaA:Betv1) and analyzed their immune-modulating capacity both *in vitro* and *in vivo*[77–80].

1.5.1 Flagellin:allergen fusion proteins can suppress allergen-induced clinical symptoms *in vivo*

First, we demonstrated that prophylactic vaccination with rFlaA:OVA, but not the mixture of both proteins, could prevent clinical symptoms such as softness of feces, body weight loss, and drop in core body temperature in an OVA-induced intestinal allergy mouse model[79,80]. Moreover, vaccination with all generated rFlaA:allergen fusion proteins was shown to induce higher allergen-specific IgG_{2a} antibodies paralleled with decreased levels of both specific IgE and Th2 cytokines (IL-5 and IL-13) from mouse serum, when compared with the flagellin/allergen mixture[77–80].

1.5.2 Flagellin:allergen fusion proteins activate mDCs which subsequently suppress Th2 responses

Next, we checked which cell types might contribute to the observed immune modulating effects of the flagellin:allergen fusion proteins *in vivo*. Here, we first analyzed one of the most important target cells for vaccination, dendritic cells. Our results showed, that rFlaA:allergen-stimulated myeloid dendritic cells (mDCs) could modulate allergen-specific T cells responses, including suppressing Th2 cytokine (IL-4, IL-5, and IL-13) production[77,78,80]. Interestingly, rFlaA:OVA was shown to also suppress Th1 cytokine IFN-γ[80], while both rFlaA:Artv1 and rFlaA:Betv1 could induce higher IFN-γ secretion in mDC:$CD4^+$ T cell (TC) co-cultures *in vitro*[77,78], indicating that different conjugates might result in slightly different overall responses. We also checked the cytokines secreted from mDCs after stimulation with the flagellin-containing fusion proteins, and could show that, compared to rFlaA/allergen mixtures, fusion proteins induced both higher pro- (IL-6, IL-1β, TNF-α, and IL-12) and anti-inflammatory (IL-10) cytokine production[76–79,95]. Moreover, the suppressive effect on allergen-specific Th2 cytokine production was demonstrated to depend on the induced anti-inflammatory IL-10 secretion from rFlaA:allergen-stimulated mDCs[79]. Besides cytokines, rFlaA:allergen-treated mDCs also express higher surface levels of the maturation markers CD40, CD69, CD80, and CD86[76,77]. Next, to further better understand how rFlaA:allergen fusion proteins mediate dendritic cell responses, we moved on to dissect the underlying intracellular signaling pathways.

1.5.3 Flagellin:allergen fusion proteins induce a MyD88- and MAPK-dependent, but TLR5-independent activation of mDCs

In nature, flagellin can bind to cell-surface expressed TLR5, which further recruits downstream signaling molecule myeloid differentiation primary-response protein 88 (MyD88). This leads to the activation of interleukin-1 receptor-associated kinase (IRAK)1/4 and TNF-receptor-associated factor 6 (TRAF6)[96,97] (Figure 3). TRAF6 and IRAK1 in turn activate TGF-β-activated kinase 1 (TAK1), resulting in the phosphorylation of IκB kinase (IKK) complex and activation of mitogen-activated protein kinases (MAPK)[96,97] (Figure 3). The phosphorylation of IKK leads to the degradation of IκB and the activation of the nuclear factor kappa-light-chain-enhancer of activated B cells (NFκB) pathway[96,97] (Figure 3). Both NFκB- and MAPK-activation can trigger the activation of many different genes (Figure 3), which finally induce immune cells to express co-stimulatory markers, migrate to the draining lymph nodes, and secrete cytokines and chemokines involved in inflammatory responses[96,97]. Therefore, we also checked if rFlaA:allergen fusion protein-mediated mDC activation depended on either TLR5- or MyD88-signaling pathways. Interestingly, while rFlaA:OVA induced a TLR5-dependent IL-10 production, all cytokines secreted after stimulation of mDCs with rFlaA:Bev1 were shown to be TLR5-independent[78,79]. Moreover, in MyD88-deficient mDCs, both rFlaA:OVA- and rFlaA:Bev1-induced mDC activation and cytokine secretion was abrogated[78,79]. Furthermore, in our most recent publication, we demonstrated higher phosphorylation of all investigated MAPK (Jun N-terminal kinases (JNK)-, extracellular signal-regulated kinase 1/2 (ERK)- and p38-MAPK), as well as decreased expression of IκB with higher levels of phosphorylated NFκB molecules after rFlaA:Bev1 stimulation in mDCs compared to the equimolar mixture of rFlaA and rBet v 1[95]. Using different inhibitors we could show, that the induced intracellular pathways differentially contributed to rFlaA:Bev1-induced inflammatory cytokine secretion[95]. These results showed that, instead of via the activation of the surface TLR5, the induced mDC activation was mediated by intracellular MyD88-, MAPK-, and NFκB-pathways. This strong activation of intracellular signaling pathways by rFlaA:allergen fusion proteins is likely due to their tendency to form high molecular aggregates. In line with this speculation, we also showed that rFlaA:OVA, rFlaA:Artv1, and rFlaA:Betv1 were taken up more strongly by mDCs than the respective mixtures of both proteins[77–79]. The higher uptake of rFlaA:Betv1 by mDCs also contributed to the observed cytokine production[78].

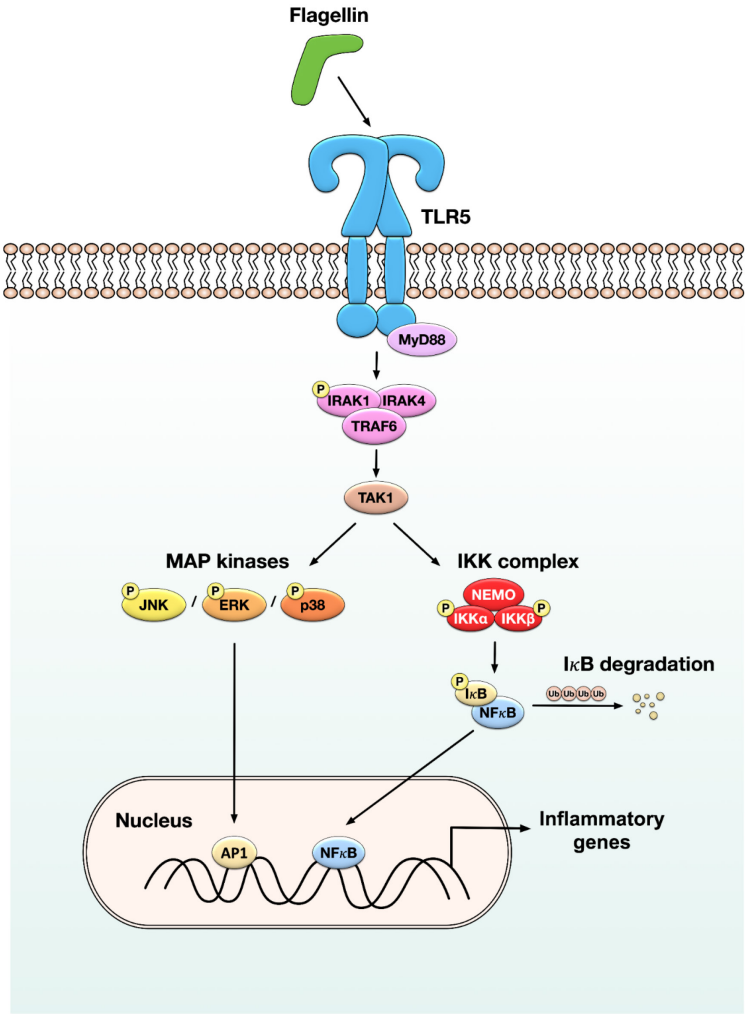

Figure 3: Intracellular signaling events triggered by the activation of TLR5

The binding of flagellin to TLR5 recruits the adaptor protein MyD88, and further triggers its association with IRAK4, IRAK1, and TRAF6 as a complex[96,97]. IRAK4 in turn induces the phosphorylation of IRAK1 and induces the activation of TAK1. TAK1 then phosphorylates both MAPK and IKKα & IKKβ that belong to the IKK complex[96,97]. The IKK complex can phosphorylate IκB, leading to the ubiquitylation and degradation of protein, allowing the transcription factor NFκB to translocate to the nucleus[96,97]. NFκB- and MAPK-activated transcription factor AP1 both can regulate inflammatory gene expression and activate immune responses[96,97]. Abbreviations: TLR5: Toll-like receptor 5, MyD88: myeloid differentiation primary-response protein 88, IRAK: interleukin-1 receptor-associated kinase, TRAF: TNF-receptor-associated factor, TAK1: transforming-growth-factor-β-activated kinase, MAPK: mitogen-activated protein kinase, JNK: Jun N-terminal kinases, ERK: extracellular signal-regulated kinase 1/2, IKK: IκB kinase, NFκB: nuclear factor kappa-light-chain-enhancer of activated B cells, AP1: activator protein 1.

1.5.4 Flagellin:allergen fusion proteins induce a MyD88-, MAPK-, and mTOR-dependent, but TLR5-independent Warburg Effect in mDCs

When analyzing the effects of flagellin:allergen fusion proteins on mDCs, we also observed a striking yellow-coloring of cell culture medium[77,78,95]. This phenomenon, which is called the "Warburg Effect", was first described by Otto Warburg, who discovered it under aerobic conditions in cancer cells[98]. The phenomenon is due to cells changing their glucose metabolism from complete oxidation of glucose in

oxidative phosphorylation (OXPHOS) towards preferentially generating lactate in glycolysis (Figure 4). Under normal metabolic conditions, cells take up glucose from their surrounding and process it into pyruvate[99]. The pyruvate is then imported into the mitochondrion, where it is metabolized in a multistep process called the Krebs cycle, resulting in the generation of CO_2 exported from the cell and energy in the form of the reduction equivalents NADH and $FADH_2$[100] (Figure 4A). These reduction equivalents are then used in OXPHOS at the inner membrane of the mitochondrion. Here, oxygen molecules are used as terminal electron acceptors to form a proton gradient over the inner mitochondrial membrane that drives the generation of large amounts of ATP by the ATP synthase complex[100] (Figure 4A). However, under certain conditions, the cell can predominantly produce lactate from glucose instead of using oxygen to completely metabolize glucose into CO_2[101]. The accumulation of lactate leads to the export of protons that turns the red color of the phenol red pH indicator dye in the cell culture medium to yellow (Figure 4B). As we have learned in the last years, this switch towards glycolysis (also called the Warburg Effect), not only allows cancer cells to survive under oxygen-deprived conditions but is also used to fulfill the rapid energy demand of activated immune cells, especially APCs like dendritic cells and M1 macrophages[102–106]. Monitoring the metabolic changes occurring in immune cells can also give us hints on either disease progression or treatment outcome. During allergic inflammation, the involved immune cells also change their metabolic phenotypes. These changes and their functional implications were discussed in detail in several reviews published by us[107–109].

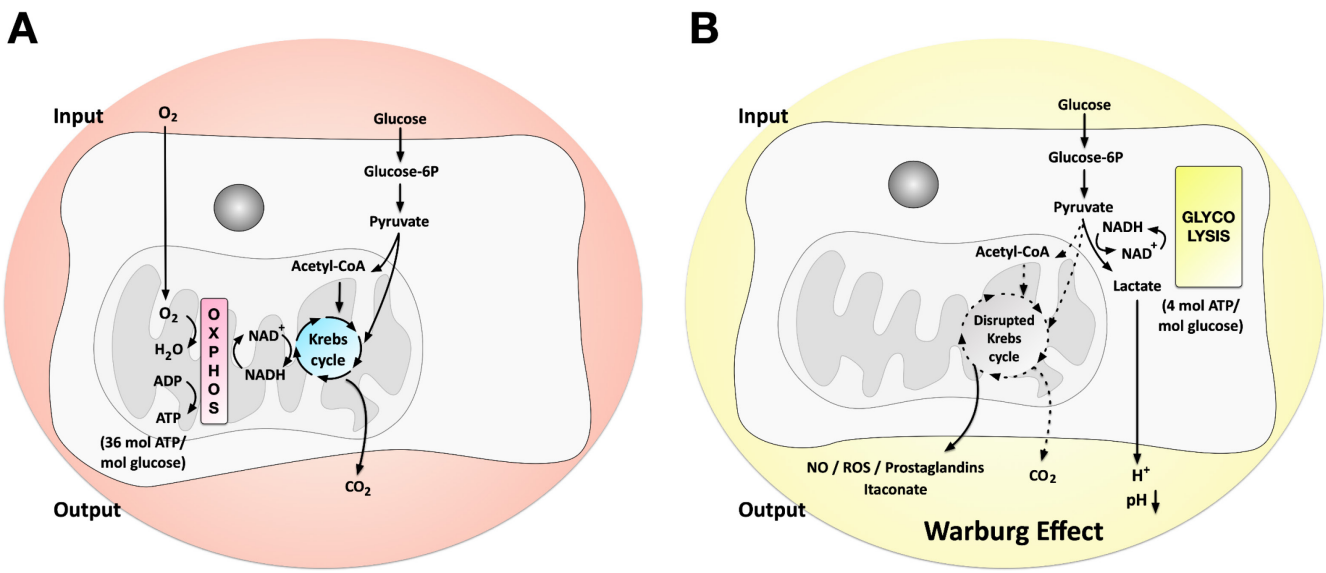

Figure 4: Steady-state glucose metabolism compared to the Warburg Effect observed in either cancer cells or activated immune cells

Under normal conditions, cells take up glucose from the medium and convert it into pyruvate[99] (A). Pyruvate is then transported into the mitochondrion and metabolized to Acetyl-CoA that is used in the Krebs cycle to generate CO_2 and the reduction equivalents NADH and $FADH_2$ for the next step of cellular respiration that takes place at the inner mitochondrial membrane[100]. This step, known as OXPHOS, requires oxygen as terminal electron acceptor. Finally, 36 mol of ATP/mol glucose are generated after OXPHOS, which is the main energy source of eukaryotic cells[100] (A). Under certain conditions (for example in cancer cells, strongly proliferating cells, and activated M1-macropahges or dendritic cells), cells prefer to

convert pyruvate into lactate rather than metabolizing pyruvate via the Krebs cycle[101] (B). Although this process only generates 2 mol of ATP/mol glucose, the rate of generating ATP is faster than the complete oxidation of glucose in the mitochondria[110]. Besides, this lack of pyruvate in the mitochondrion results in a "disrupted Krebs cycle," leading to the accumulation of biosynthetic intermediates (e.g. NO, ROS, prostaglandins, and itaconate) needed for certain immune cell effector functions[109]. Moreover, the predominant production of lactate leads to a decrease in extracellular pH that turns the red color of the phenol red dye in the cell culture medium to yellow, known as the "Warburg Effect" (B). Abbreviations: OXPHOS: oxidative phosphorylation, NO: nitric oxide, ROS: reactive oxygen species.

Therefore, we further analyzed which signaling pathways contributed to the rFlaA:allergen fusion protein-induced Warburg Effect from mDCs, and found that it was again MyD88-dependent but TLR5-independent[78]. Interestingly, we identified a JNK-MAPK-dependent activation of the mTOR pathway to mediate the rFlaA:Betv1-induced Warburg Effect in mDCs[78,95]. Furthermore, blocking of either mTOR- or JNK-MAPK-activation with either rapamycin or SP600125 suppressed the rFlaA:allergen-induced Warburg Effect from mDCs[77,78,95]. mTOR is a conserved serine/threonine protein kinase that belongs to the phosphatidylinositol 3-kinase-(PI3K) family. mTOR can recognize and integrate various nutritional and environmental stimuli, including levels of growth factors, cellular energy reserves, and stress levels[111]. Recent research has revealed that mTOR is a master regulator of cellular metabolism that also affects innate and adaptive immune responses[111]. Based on these functions of mTOR, we also demonstrated that blocking the mTOR pathway could suppress both rFlaA:Artv1- and rFlaA:Betv1-induced anti-inflammatory IL-10 secretion[77,78], showing mTOR to connect metabolic changes and dendritic cell functions after rFlaA:allergen fusion protein stimulation.

1.6 Summary of our previous work and open questions

Taken together, we made a detailed *in vivo* and *in vitro* analysis of the immune modulating properties of flagellin:allergen fusion proteins and the underlying signaling pathways and molecular mechanisms. So far our studies focused on the activation of dendritic cells by the generated rFlaA:allergen fusion proteins. We found that rFlaA:allergen fusion proteins could activate DCs which were able to suppress allergen-specific Th2 responses, supporting our original concept, that rFlaA:allergen fusion proteins can potentially be a novel treatment for type I allergies (our findings are summarized in Figure 5). However, dendritic cells are likely not the only cell type that contribute to the overall immune responses induced by flagellin:allergen fusion proteins. So far, the effects of rFlaA:allergen fusion protein on other cell types remains largely unknown. Besides, additional studies to find and characterize novel adjuvants and dissecting their mechanisms of action are still essential for future (allergy) vaccine development. Therefore in this thesis, I analyzed (1) the potential of a carbohydrate-based mannooligosaccharides (Man) with different sizes and linkages as a novel adjuvant, and also investigated the effects of rFlaA:Betv1 on either (2) lung epithelial cells or (3) macrophages.

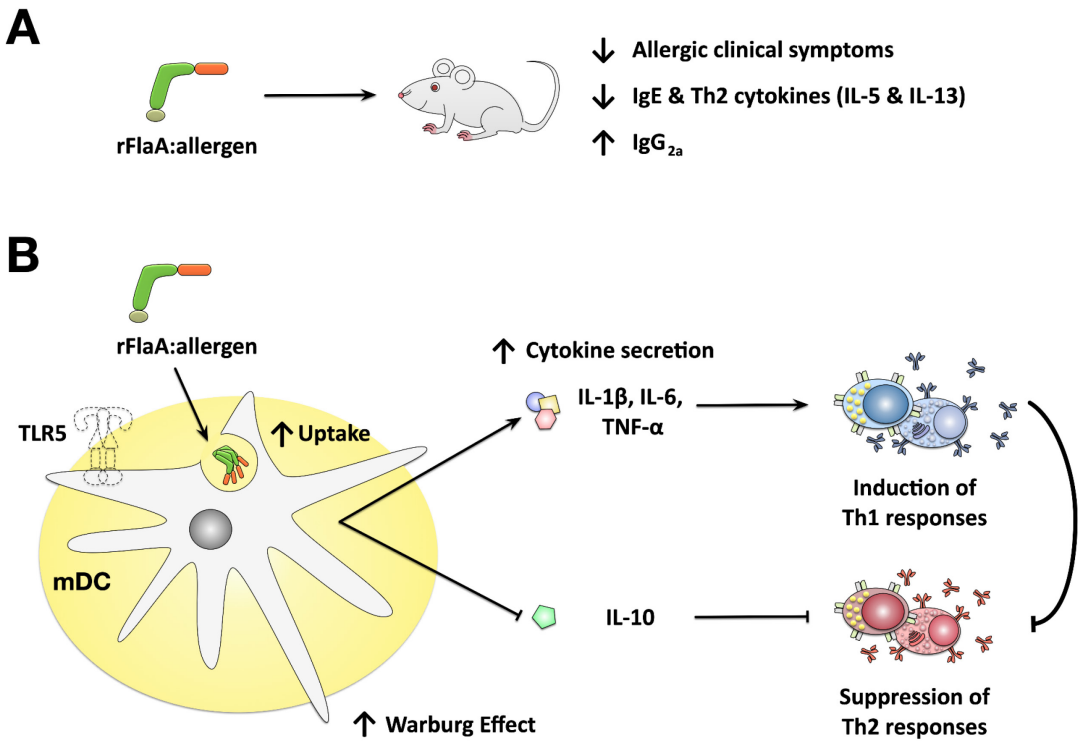

Figure 5: Currently published *in vivo* and *in vitro* mechanisms of rFlaA:allergen fusion proteins

Prophylactic vaccination with rFlaA:OVA could prevent OVA-induced allergic clinical symptoms *in vivo*[79,80], and all investigated rFlaA:allergen fusion proteins were shown to induce allergen-specific IgG_{2a} antibodies, with decreased levels of both IgE and Th2 cytokines from mouse serum after injection[77–80] (A). The effects of rFlaA:allergen fusion proteins on mDC activation were also analyzed *in vitro*[76–79,95] (B). Results showed that rFlaA:allergen fusion proteins could form high molecular aggregates, which were taken up more strongly by mDCs than the respective mixtures of both proteins[77–79]. Moreover, rFlaA:allergen fusion proteins could induce a TLR5-independent secretion of pro- and anti-inflammatory cytokines[78,79] that was shown to promote allergen-specific Th1 responses while suppressing Th2 responses in mDC:$CD4^+$ T cell co-cultures *in vitro*[77–80]. Finally, rFlaA:allergen fusion proteins were shown to induce metabolic changes in mDCs, resulting in the yellow-coloring of cell culture medium, which is called the "Warburg Effect"[77,78,95] (B). For more information, see text. Abbreviations: rFlaA:allergen: fusion protein consisting of *Listeria monocytogenes* flagellin fused to allergen, TLR: "Toll"-like receptor, mDC: myeloid dendritic cell.

2 Aims of this thesis

The main purpose of this thesis was to analyze the immune-modulating effects of mannooligosaccharides (Man) as novel adjuvants and the vaccine candidate (rFlaA:Betv1, consisting of *Listeria monocytogenes* flagellin fused to the major birch pollen allergen Bet v 1) for type I allergy treatment using different immune cell types.

The specific questions included:

Investigation of mannooligosaccharides (see also **Figure 6** ① & chapter 1):

- Could different size and linkage of Man activate mDCs by stimulating cytokine production, changing their metabolic state, and further affect T cell responses?
- Which signaling pathways contribute to the activation of mDCs by Man?

Investigation of the immune modulating effects of rFlaA:Betv1 on

Epithelial cells (see also **Figure 6** ② & chapter 2):

- Could epithelial cells be activated by rFlaA:Betv1?
- What is the detailed mechanism of rFlaA:Betv1-mediated epithelial cells activation?
- How do rFlaA:Betv1-stimulated epithelial cells modulate mDC responses?

Macrophages (see also **Figure 6** ③ & chapter 3):

- Could rFlaA:Betv1 activate bone marrow-derived macrophages (BMDMs)?
- Which signaling pathways contribute to the observed rFlaA:Betv1-induced BMDM cytokine production and glycolytic phenotype?
- Could rFlaA:Betv1-stimulated BMDMs modulate allergen-specific T cell responses?

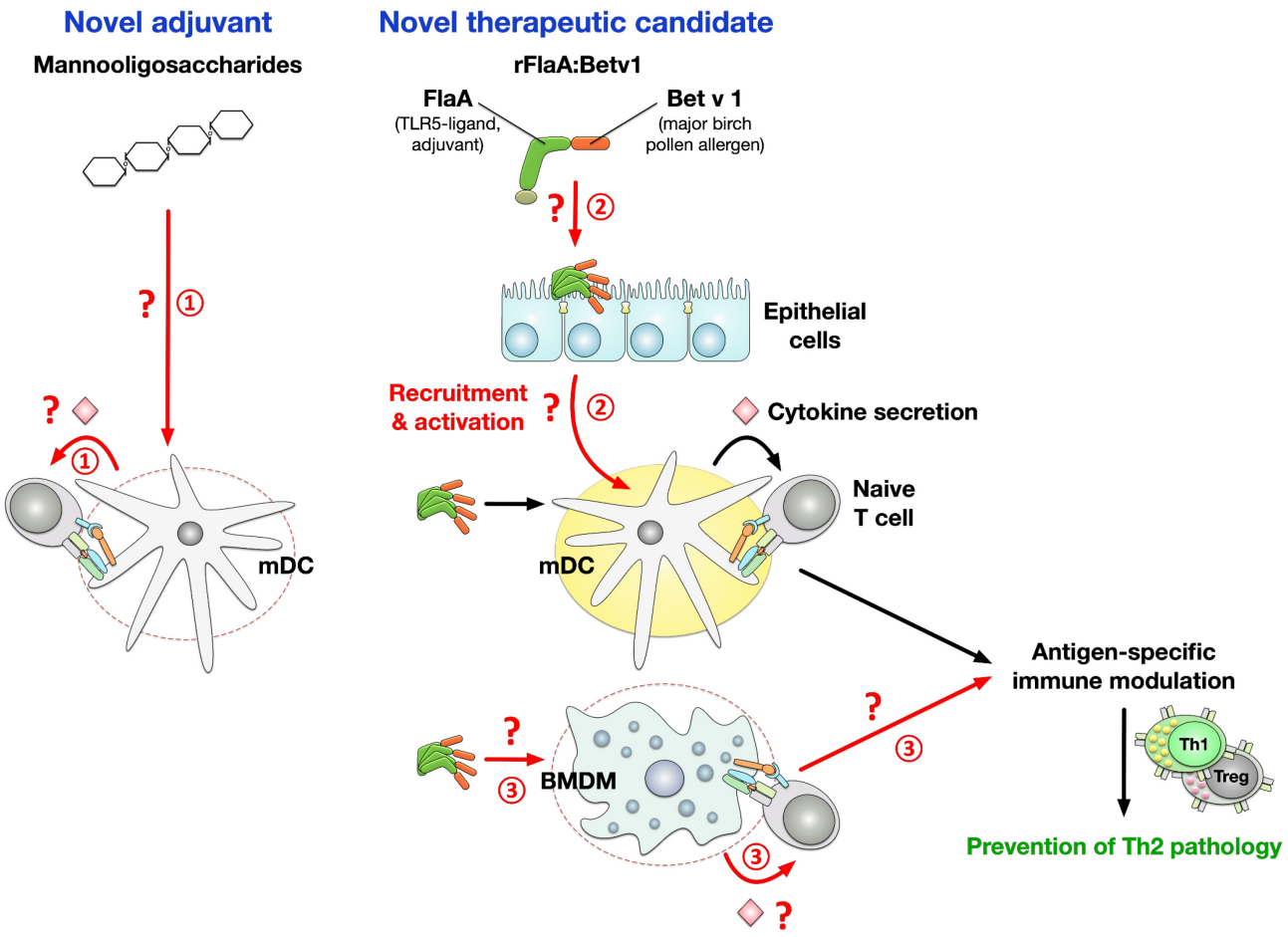

Figure 6: Graphical overview of the thesis' aims

Depicted are the three subprojects analyzing the potential of mannooligosaccharides as novel adjuvants ①, and the investigation of different cell types (epithelial cells ② & macrophages ③) which contribute to the immune modulation by the flagellin:allergen fusion protein rFlaA:Betv1. Abbreviations: mDCs: myeloid dendritic cells, BMDMs: bone marrow-derived macrophages.

3 Chapter 1

β-(1→4)-Mannobiose Acts as an Immunostimulatory Molecule in Murine Dendritic Cells by Binding the TLR4/MD-2 Complex

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- Resource: W.S.
- Data curation: M.T.
- Data interpretation: S.V., S.S. (Stephan Scheurer), S.S. (Stefan Schülke), M.T.
- Writing—original draft preparation: M.T. and T.-Y.C.
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Figures contributed by Yen-Ju Lin:

- Figure 6A
- Figure 7A, B, C (cooperation with Stefan Schülke)

cells

MDPI

Article

β-(1→4)-Mannobiose Acts as an Immunostimulatory Molecule in Murine Dendritic Cells by Binding the TLR4/MD-2 Complex

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Abstract: Some β-mannans, including those in coffee bean and soy, contain a mannose backbone with β-(1→4) bonds. Such mannooligosaccharides could have immunological functions involving direct interaction with immune cells, in addition to acting as prebiotics. This study aimed at assessing the immunological function of mannooligosaccharides with β-(1→4) bond, and elucidating their mechanism of action using bone marrow-derived murine dendritic cells (BMDCs). When BMDCs were stimulated with the mannooligosaccharides, only β-Man-(1→4)-Man significantly induced production of cytokines that included IL-6, IL-10, TNF-α, and IFN-β, and enhanced $CD4^+$ T-cell stimulatory capacity. Use of putative receptor inhibitors revealed the binding of β-Man-(1→4)-Man to TLR4/MD2 complex and involvement with the complement C3a receptor (C3aR) for BMDC activation. Interestingly, β-Man-(1→4)-Man prolonged the production of pro-inflammatory cytokines (IL-6 and TNF-α), but not of the IL-10 anti-inflammatory cytokine during extended culture of BMDCs, associated with high glucose consumption. The results suggest that β-Man-(1→4)-Man is an immunostimulatory molecule, and that the promotion of glycolysis could be involved in the production of pro-inflammatory cytokine in β-Man-(1→4)-Man-stimulated BMDCs. This study could contribute to development of immune-boosting functional foods and a novel vaccine adjuvant.

Keywords: mannobiose; dendritic cells; immunostimulation

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1. Introduction

Mannans are D-mannose-based polysaccharides that comprise the outermost layer of the cell wall in plants, fungi, and yeast [1]. Mannans range from pure mannans containing only mannose to glucomannans and galactomannans comprised of mannosyl residues linked by various glucosidic linkages such as α-(1→2)-, α-(1→3)-, α-(1→6)-, β-(1→2)-, and/or β-(1→4)-links bonds [2,3]. The immunological properties of mannans are diverse, depending on their structures and source. For instance, N-linked and O-linked mannans derived from *Candida albicans* induce anti-fungal protective immunity [4]. Mannans are also significant virulence factors associated with the severity and pathogenesis of *Candida* infections [2–4]. In contrast, α-mannan from the cell wall of the yeast *Saccharomyces cerevisiae* induces anti-inflammatory responses by inducing the anti-inflammatory cytokine IL-10 and the co-inhibitory molecule programmed death-ligand 1 in dendritic cells (DCs) [5]. β-Galactomannans from soy also appears to induce such anti-inflammatory properties [6]. These observations indicate that structures of mannosyl residues are an important factor determining the immunological properties of mannan.

Mannans are a valuable source of mannooligosaccharides [3]. Physical, chemical, and mechanical methods that include enzymatic hydrolysis, acid hydrolysis, and ultrasonic

degradation of mannans have been developed to prepare mannooligosaccharides in food technology [1,3]. In general, non-digestible oligosaccharides are expected to improve gastrointestinal health by inducing both the growth of beneficial bacteria and the production of short-chain fatty acids (SCFAs) by bacteria [7,8]. In addition, a previous study showed that crude mannooligosaccharides or β-Man-(1→4)-Man (β-(1→4)-mannobiose: Man2) stimulates cytokine production in RAW264.7 macrophages, and exerts anti-inflammatory effects in murine models [9,10]. However, the oligomer size of β-(1→4)-mannooligosaccharides that induces a potent immunological response is unknown.

DCs play a central role in immune responses; they are essential as a bridge between innate and acquired immunity [11,12]. DCs are equipped with a diverse array of specialized receptors, including co-stimulatory molecules and pattern-recognition receptors to specifically recognize "non-self" components [13]. This study assessed the function of several mannooligosaccharides with β-(1→4) bonds on bone marrow-derived murine DCs (BMDCs) and their underlying mechanisms. Among the tested mannooligosaccharides, only Man2 was capable to activate BMDCs. Man2 induced production of cytokines including type I interferon with anti-viral and immune-modulating functions in DCs via Toll like receptor (TLR)-4 and complement C3a receptor (C3aR) engagement. In addition, Man2 enhanced the T-cell stimulatory capacity of DCs. The results suggest that Man2 has potential as a vaccine adjuvant and an immunostimulatory component for functional foods.

2. Materials and Methods

2.1. Preparation of β-Mannooligosaccharides

β-Man-(1→4)-Man (Man2), β-Man-(1→4)-Man2 (Man3), β-Man-(1→4)-Man3 (Man4), β-Man-(1→4)-Man4 (Man5), β-Man-(1→4)-Glc (Manβ-4Glc), and β-Glc-(1→4)-Man (Glcβ-4Man), were prepared as described previously [14,15]. Preparation of β-Man-(1→4)-GlcNAc (Manβ-4 GlcNAc) is described in the online repository.

2.2. Animals

Female C57BL/6J or BALB/c mice were purchased from either Japan SLC, Inc., or Jackson Laboratories (Bar Harbor, ME, USA). OT-II mice were provided by Prof. Ishi Naoto (Tohoku University). The mice were housed under pathogen free conditions. All animal experiments were performed in accordance with either the rules on animal experiments at Tohoku University (2019AgA-026) or the German animal protection law (granting authority: RP Darmstadt).

2.3. Cell Culture

Raw264.7 cells were obtained from Riken BioResource Research Center, and maintained in Minimum Essential Media (MEM: Sigma-Aldrich, St. Louis, MO, USA) containing 10% fetal bovine serum (FBS: Sigma-Aldrich, cat. 273012, St. Louis, MO, USA), 0.1 mM non-essential amino acids (NEAA: FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan), 100 units/mL penicillin, and 100 μg/mL streptomycin (Gibco). The cells were passaged using 0.25% trypsin + 0.02% EDTA solution (FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan) to adjust cell density at 3×10^5 cells/mL twice a week. BMDCs were generated by culturing BM cells from mice in RPMI 1640 medium (Sigma-Aldrich, St. Louis, MO, USA) containing 10% FBS, 1 mM sodium pyruvate (Sigma-Aldrich, St. Louis, MO, USA), 10 mM HEPES (Sigma-Aldrich, St. Louis, MO, USA), 0.1 mM 2-mercaptoethanol (Sigma-Aldrich, St. Louis, MO, USA), 100 units/mL penicillin, 100 μg/mL streptomycin, and 20 to 50 ng/mL of rGM-CSF (Peprotech, Rocky Hill, NJ, USA) for 8 days [16]. Approximately 2.0 to 3.0 $\times 10^7$ of BM cells were collected from one mouse (C57BL/6 or BALB/c mice, female, age of three to four months), and 4.0 to 6.0 $\times 10^7$ of BMDCs were generated.

2.4. Cell Stimulation Assay

Raw264.7 cells or BMDCs (1×10^6 cells/mL) were stimulated with the indicated concentrations of β-mannooligosaccharide or either 5.0 ng/mL or 1.0 μg/mL of lipopolysac-

charide from *Salmonella enterica* serotype abortus equi (LPS: Sigma-Aldrich, L5886, St. Louis, MO, USA) in 12-well plates (total medium amount: 1 mL) or 24-well plates (total medium amount: 0.5 mL). To identify receptors on BMDCs binding Man2, the cells were treated for 30 min before the addition of 50 µM Man2 with following inhibitors: 100 nM TAK-242 (Merck Millipore, Burlington, MA, USA), 50 µM C34 (Sigma-Aldrich, St. Louis, MO, USA), 100 µM SB290157 (C3 receptor inhibitor, Sigma-Aldrich, St. Louis, MO, USA), 100 µg/mL of WGP® Soluble (InVivogen, San Diego, CA, USA), or 200 µg/mL of mannan (Sigma-Aldrich, St. Louis, MO, USA). In addition, Cell Counting Kit-8 (CCK-8) assay using WST-8 (2-(2-methoxy-4-nitrophen yl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt) (Dojindo Laboratories) was performed to assess the influence of receptor inhibitors on viability of BMDCs.

2.5. Fluorescence-Activated Cell Sorting (FACS) Analysis

To detect the expression levels of co-stimulatory molecules on the cell surface of RAW-267.4 cells and BMDCs, fluorescein isothiocyanate (FITC)-conjugated rat anti-mouse CD40 (clone 3/23, Biolegend, San Diego, CA, USA), CD80 (clone 16-10A1, Biolegend, San Diego, CA, USA), CD86 (clone GL-1, Biolegend, San Diego, CA, USA), or MHC class II (I-A/I-E) mAbs (clone M5/114.15.2, Biolegend, San Diego, CA, USA) were used. In all stainings, IgG receptors on the cell surface were blocked using either monoclonal antibody (mAb) against CD16/CD32 (clone 2.4G2, Thermo Fisher Scientific, Waltham, MA, USA), or normal murine IgG antibodies (Santa Cruz Biotechnology, Dallas, TX, USA). BMDCs were additionally stained with allophycocyanin (APC)-conjugated anti-mouse CD11c mAb (clone N418, Biolegend, San Diego, CA, USA) to gate the DC population. Fluorescence intensity of stained cells (10,000 cell detection) was measured with flow cytometry using a FACSLyric (BD Bioscience, Franklin Lakes, NJ, USA). Data were analyzed using FlowJo V. 7 (BD Bioscience, Franklin Lakes, NJ, USA).

2.6. Cytokine ELISA

Cytokine concentrations in cell culture supernatants were measured by ELISA, as previously described [16]. For more detailed information, see a Supplemental Information File.

2.7. SDS-PAGE and Western Blotting

BMDCs (1×10^6 cells/mL: total 1 mL) were stimulated with 1.0 to 50 µM Man2 or 10 µg/mL of LPS for 30 min and lysed in lysis buffer (62.5 mM Tris-HCl (pH 6.8 at 25 °C), 2% *w*/*v* SDS, 10% glycerol, 50 mM DTT, and 0.01% *w*/*v* bromophenol blue) for 10 min on ice. Target proteins in lysates were separated by 15% Tris-glycine SDS-polyacrylamide gels prepared with 30% acrylamide/bis-solution (Roth, Karlsruhe, Germany), and transferred to nitrocellulose membranes (GE Healthcare Life Sciences, Marlborough, MA, USA) in transfer buffer (25 mM Tris base, 0.2 M glycine, 20% methanol) at 350 mA for 2 h. After blocking with 5% non-fat milk (Roth), the resolved proteins were detected by the following antibodies obtained from Cell Signaling Technologies: nuclear factor-kappa B (NF-κB) pathway sampler kit, phospho-mitogen-activated protein kinase (MAPK) family antibody sampler kit, mammalian target of rapamycin (mTOR) substrates antibody sampler kit, and loading control anti-histone H3 antibody. Detection was performed with the provided secondary antibodies using ACE Glow substrate (VWR International GmbH, Darmstadt, Germany). Images were captured with iBright™ CL1500 system (Thermo Fischer Scientific, Waltham, MA, USA) and the intensities of protein bands were quantitated by ImageJ software v1.52a (www.imagej.nih.gov, accessed on 1 August 2020, National Institutes of Health, Bethesda, MD, USA).

2.8. Analysis of Metabolic State of BMDCs

The Warburg effect in stimulated BMDC cultures was determined photometrically 72 h post stimulation with the indicated concentrations of 1.0 to 50 µM Man2 and 10 µg/mL of LPS by quantifying the optical density (OD) of culture supernatants at 570 nm by

SpectraMAX340PC (Molecular Devices, San Jose, CA, USA) and calculating the Warburg effect as 1/OD570 normalized to unstimulated controls. Glucose concentrations in culture supernatants were determined 72 h post-stimulation using the Glucose (GO) Assay Kit (Sigma-Aldrich, St. Louis, MO, USA). The metabolic rate was derived mathematically from the measured glucose concentrations by calculating the glucose consumption in % in relation to medium without BMDCs (glucose conc. in RPMI1640 = 2 mg/mL).

2.9. Assessment of T-Cell Stimulatory Capacity of Man2-Stimulated BMDCs

Splenic $CD4^+$ T-cells were isolated from OT-II mice using CD4 (L3T4) MicroBeads (Miltenyi Biotec., Bergisch Gladbach, Germany). To evaluate T-cell activation, $CD4^+$ T-cells (8.0×10^5 cells/mL) and BMDCs (1.6×10^5 cells/mL) were seeded in 96-well plates (total medium amount: 0.2 mL), and stimulated with Man2 and LPS-free ovalbumin (OVA: Seikagaku Cooperation, Chiyoda-Ku, Japan). The supernatants were harvested after 24 h for IL-2 determination by ELISA.

2.10. Statistical Analysis

Differences between mean values were assessed by Dunnett's test or two-way ANOVA tests with confidence intervals adjusted for multiple comparisons according to either Bonferroni or Tukey. Statistical analysis was performed with GraphPad Prism v6 to v8 (GraphPad Software, San Diego, CU, USA) and Bell Curve for Excel (Social Survey Research Information Co., Ltd., Shinjuku-ku, Japan). A p value of <0.05 was considered significant.

3. Results

3.1. Man2 Induces Expression of Co-Stimulatory Molecule and Production of Cytokine in RAW264.7 Cells

To assess the effect of mannooligosaccharides with β-(1→4)-linkage on antigen presenting cells, RAW264.7 cells, a murine macrophage cell culture model, were stimulated with the oligosaccharides. Upon stimulation, expression levels of a co-stimulatory molecule (CD40, an activation marker) and cytokine production in the cells were measured. The chemical formulas of the tested mannooligosaccharides are indicated in Figure 1.

FACS analysis showed that, in addition to LPS (used as positive control), 50 μM as well as 200 μM Man2 remarkably enhanced CD40 expression on the cell surface (Figure 2A and Figure S1). Both Glcβ-4Man and Manβ-4Glc only marginally induced CD40 expression at a concentration of 200 μM, whereas neither Manβ-4GlcNAc, Man3, Man4, nor Man5 did induce detectable levels of CD40 expression (Figure 2A). Man2, but not other mannooligosaccharides, induced production of both the pro-inflammatory cytokine IL-6 and the anti-inflammatory cytokine IL-10 from RAW264.7 cells (Figure 2B). These results indicate that, among the tested mannooligosaccharides, only Man2 was capable to stimulating RAW264.7 cells.

3.2. Man2 Enhances T-Cell Stimulatory Capacity of BMDCs

To assess the effect of Man2 on DC activation, the expression levels of co-stimulatory and MHC class II molecules and cytokine production in BMDCs, primary cultured DCs, were measured 24 h after stimulation with Man2. As well as LPS, Man2 remarkably enhanced the expression of CD40, CD80, and CD86 on the BMDC surface, although this effect was not observed for the expression levels of MHC class II molecules (I-A/I-E) (Figure 3A, Figures S2 and S3). In addition, Man2 induced the production of pro-inflammatory cytokines IL-6 (Figure 3B and Figure S3), TNF-α and IL-1β (Figure S4), anti-inflammatory cytokine IL-10 (Figure 3B and Figure S3), and the anti-viral defense cytokine IFN-β (Figure 3B) in BMDCs. The threshold concentration of Man2 for the induction of detectable levels of CD40 expression and cytokine production in BMDCs, was 1.0 to 5.0 μM (34.43 to 171.2 ng/mL) (Figure 3B, Figures S4 and S5). Other mannooligosaccharides did neither induce the expression of co-stimulatory molecules nor production of cytokines in BMDCs (Figures S2 and S3).

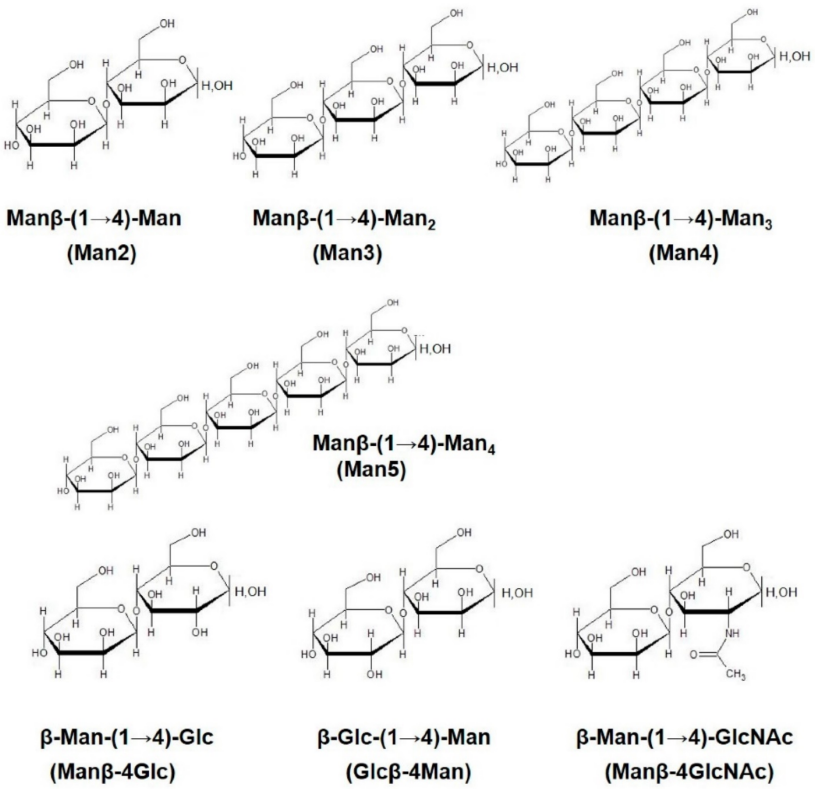

Figure 1. The structure of the tested mannooligosaccharides. Tested samples include β-Man-(1→4)-Man (Man2), β-Man-(1→4)-Man2 (Man3), β-Man-(1→4)-Man3 (Man4), β-Man-(1→4)-Man4 (Man5), β-Man-(1→4)-Glc (Manβ-4Glc), β-Glc-(1→4)-Man (Glcβ-4Man), β-Man-(1→4)-GlcNAc (Manβ-4-GlcNAc).

Next, the effect of Man2 on the T-cell stimulatory capacity of BMDCs was assessed in the co-culture system with splenic OT-II cells, i.e., transgenic $CD4^+$ T-cells expressing monoclonal OVA-specific T-cell receptors on the C57BL/6 background. Here, stimulation with BMDCs with 5.0 or 10 μM Man2 significantly enhanced IL-2 production by OT-II cells upon stimulation with 100 μg/mL of OVA (Figure 3C). A higher concentration of Man2 (50 μM) and OVA (1000 μg/mL) reduced IL-2 production by OT-II cells. This reduction of IL-2 production is likely due to overstimulation of $CD4^+$ T-cells. It is consistent with our previous study showing, that overstimulation of OT-II cells with modified OVA reduced T-cell activation in co-culture with BMDCs [17]. Taken together, these results suggest, that Man2 can enhance the $CD4^+$ T-cell stimulatory capacity of BMDCs.

3.3. Man2 Activates BMDCs via TLR4 and C3aR Engagement

To elucidate the mechanism of Man2-mediated BMDC activation, we attempted to identify receptor(s) that bound to Man2. BMDCs were pre-treated with different inhibitors of putative receptors, which are known to binds carbohydrates, before Man2 stimulation (Figure 4). TLR-4 inhibition by TAK-242 remarkably suppressed the stimulatory effect of Man2 on CD40, and CD80, CD86 expression in BMDCs (Figure 4 and Figure S6A), although the effect was not observed for I-A/I-E expression. In addition to TAK-242, another TLR4 inhibitor C34, C3aR inhibitor SB290157, Dectin-1 inhibitor WGP® Soluble (whole glucan particle), and the mannose receptor (MR, CD206) inhibitor α-mannan all resulted in statistically significant reduction of CD40 expression in Man2-stimulated BMDCs (Figure S6B), but the changes in the expression were low.

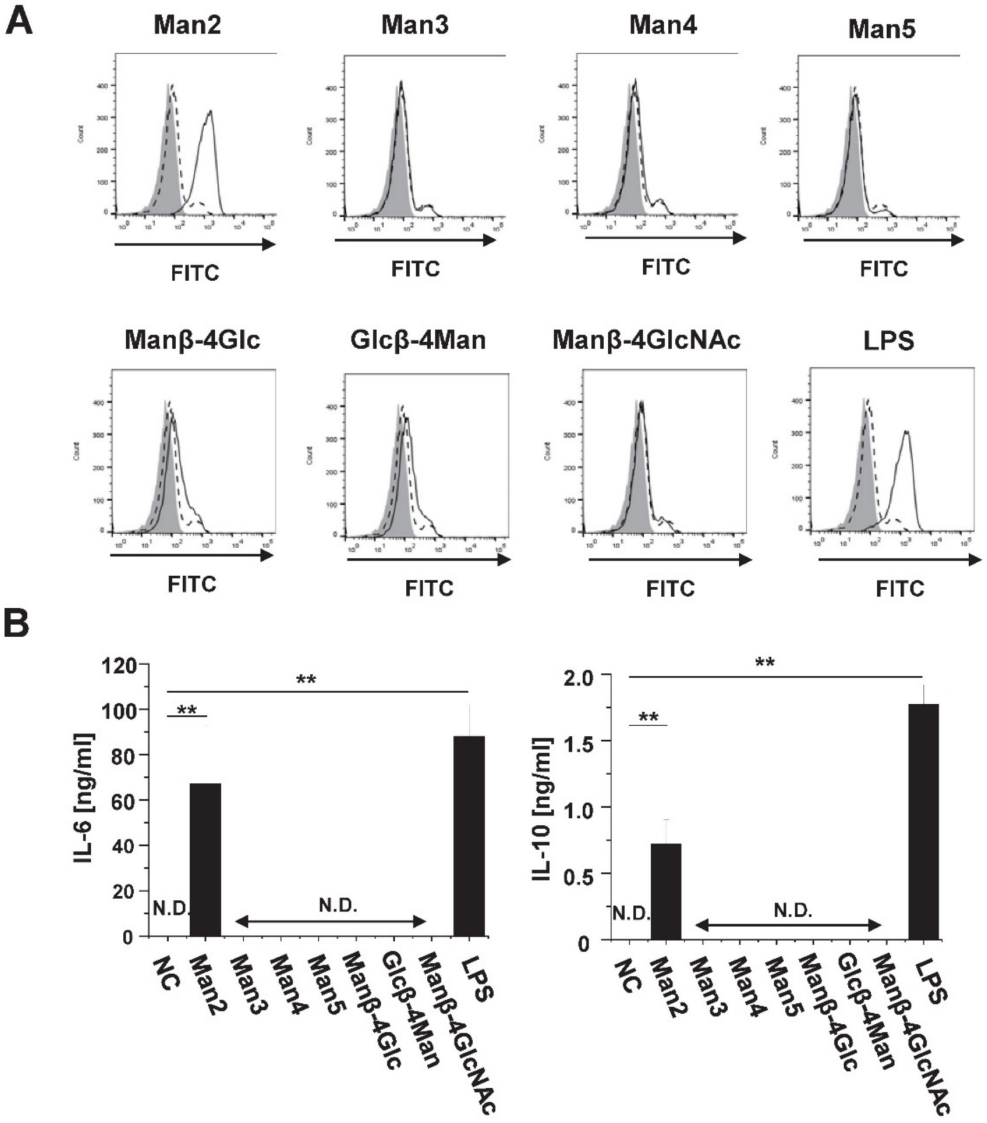

Figure 2. The stimulatory effect of mannooligosaccharides on RAW264.7 cells. (**A**) RAW264.7 cells (1×10^6 cells/mL) were stimulated with either 200 μM of the indicated mannooligosaccharides, 1.0 μg/mL of LPS or cultured only in medium for 24 h. Expression levels of CD40 on the cell surface were analyzed by FACS. Grey area: unstimulated and unstained cells, dashed lines: unstimulated and mAb-stained cells, solid lines: stimulated and mAb-stained cells. (**B**) The concentrations of IL-6 and IL-10 in the cell culture supernatants of RAW264.7 cells (1×10^6 cells/mL) upon stimulation with either 50 μM mannooligosaccharides or 1.0 μg/mL of LPS were determined by ELISA. ** $p < 0.01$ in Dunnett's test. The data are representative for three independent experiments. All bar graphs show mean ± standard deviation (SD). N.D. (not detectable): <31.25 pg/mL (IL-6 and IL-10).

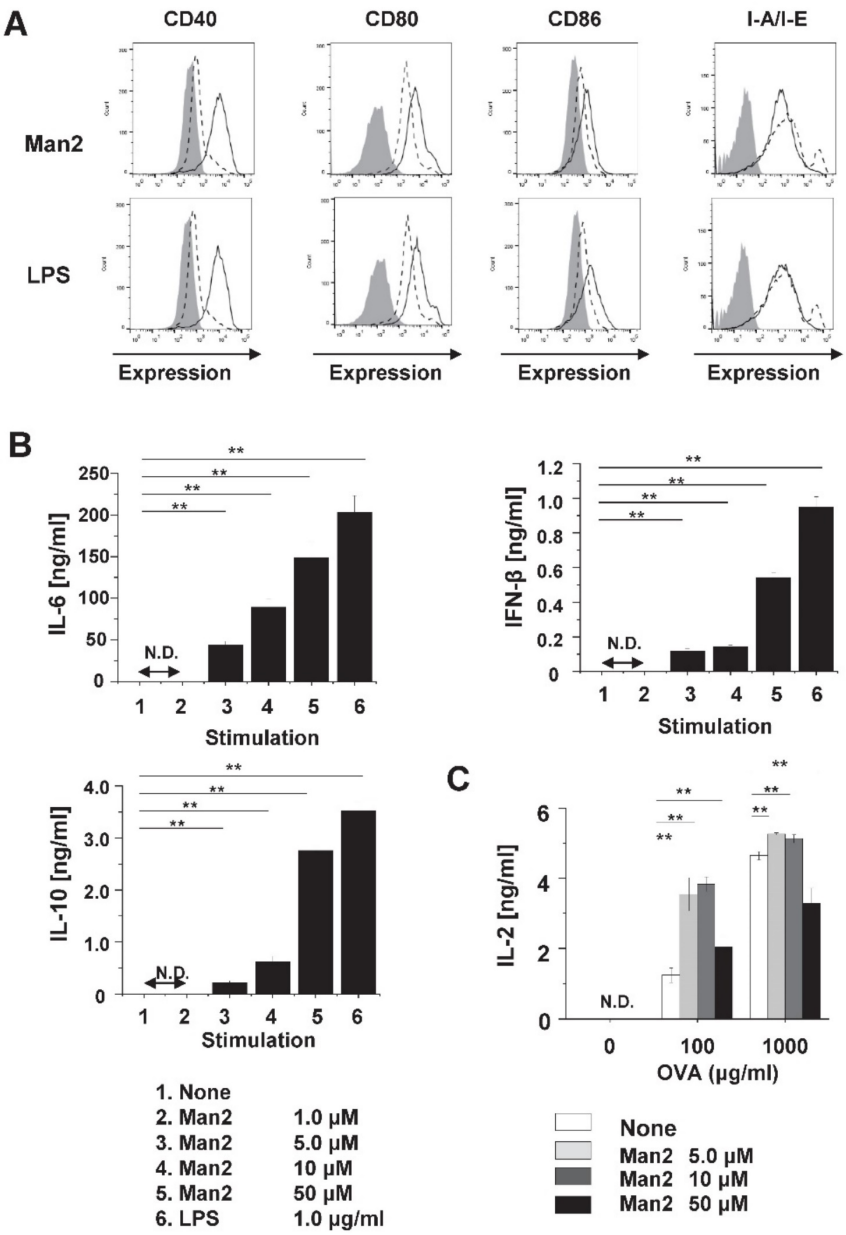

Figure 3. The effect of Man2 on activation and cytokine production of BMDCs. BMDCs (1×10^6 cells/mL) derived from C57BL/6 mice were stimulated with the indicated concentrations of Man2 or 1.0 μg/mL of LPS for 24 h. (**A**) Expression of CD40, CD80, CD86, and MHC class II molecules on the surface of cells upon stimulation with 50 μM Man2 were analyzed by FACS. Grey area: unstimulated and unstained cells, dashed lines: unstimulated and mAb-stained cells, solid lines: stimulated and mAb-stained cells. (**B**) The concentrations of IL-6, IL-10, and IFN-β in the cell culture supernatants were determined by ELISA. (**C**) BMDCs (8.0×10^5 cells/mL) were co-cultured with OT-II cells (1.6×10^5 cells/mL) and stimulated with Man2 and OVA for 24 h. IL-2 concentrations in culture supernatants were measured by ELISA. ** $p < 0.01$ in Dunnett's test. The data are representative for two independent experiments. All bar graphs show mean ± SD. N.D. (not detectable): <31.25 pg/mL (IL-6 and IL-10), <15.6 pg/mL (IFN-β), <25.0 pg/mL (IL-2).

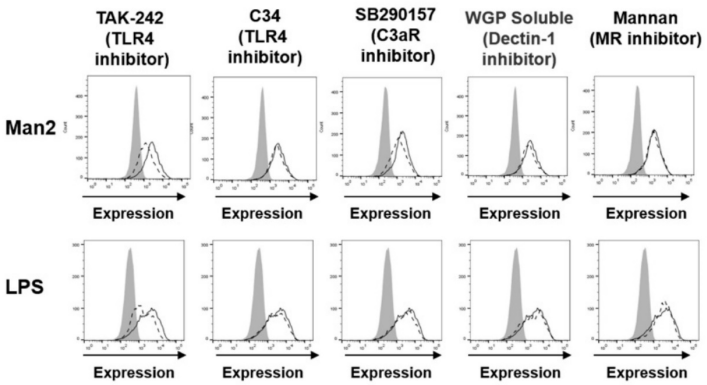

Figure 4. Effect of receptor inhibitors on activation of Man2-treated BMDCs. BMDCs derived from C57BL/6 mice (1×10^6 cells/mL) were treated with either 100 nM TAK-242, 50 μM C34, 100 μM SB290157, 100 μg/mL of WGP® Soluble, or 200 μg/mL of mannan for 30 min, and subsequently stimulated with 50 μM Man2 or 5.0 ng/mL of LPS for 24 h. Expression levels of CD40 on the surface of cells were measured by FACS. Grey lines: unstimulated and unstained cells, dashed lines: inhibitor-and Man2-treated and mAb-stained cells, and black lines: inhibitor-untreated, Man2-treated and mAb-stained cells. The data are representative for two independent experiments.

TAK-242 also significantly reduced Man2-induced IL-6, IL-10, TNF-α, IL-1β, and IFN-β production (Figure 5, Figure S7 and S8). C34 and SB290157 moderately reduced Man2-induced IL-6, TNF-α, IL-1β, and IFN-β production (Figure 5A, Figures S7 and S8), but did not affect IL-10 production in Man2-stimulated BMDCs (Figure 5B). The TLR4- and C3aR-inhibitors also reduced cytokine production in LPS-stimulated BMDCs (Figure 5 and Figure S8). Statistical analysis indicated that both WGP® Soluble and α-mannan suppressed IL-6 production in Man2- or LPS-stimulated BMDCs (Figure 5A). However, the reduction level by WGP is very moderate and probably lacks biological significance. The WGP® Soluble did not suppress IL-10 production in the BMDCs (Figure 5B), whereas α-mannan enhanced it (Figure 5B). The viability assay based on WST-8 (2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt) showed, that the mannooligosaccharides and the inhibitors did not reduce the viability of BMDCs at the tested concentrations (Figure S9). These results suggest, that TLR4 and C3aR are involved in Man2-stimulated BMDC activation.

3.4. Man2 Activates MAPKs and NF-κB in BMDCs

To gain insight into the mechanism of Man2-driven activation of BMDCs, we stimulated the cells with either Man2 or LPS and analyzed the signaling pathways (Figure 6). LPS induced the phosphorylation of p38 kinase, p42/44 kinases, stress-activated protein kinase/c-Jun N-terminal kinase (SAP/JNK), NF-κB/p65, and mTOR-related p70 ribosomal protein S6 kinase (p70S6K), and reduced expression of NF-κB inhibitor α (IkBα) (Figure 6). Man2 also induced dose-dependently higher phosphorylation of all tested MAPKs (p38, p42/44 kinases, and SAP/JNK). The levels of phosphorylation of the tested kinases (except NFkB/p65 and p70S6K) in BMDCs stimulated at 50 μM Man2 were significantly higher than those in non-stimulated cells. Man2 induced dose-dependently, but not significantly higher phosphorylation of NF-kB/p65, and reduced IkBα expression, while phosphorylation of p70S6K was induced only marginally (Figure 6). These results suggest that Man2 mainly activates the kinase- and NF-kB-related signaling cascades, but not the mTOR/p70S6K cascade, in contrast to this LPS activates all these cascades.

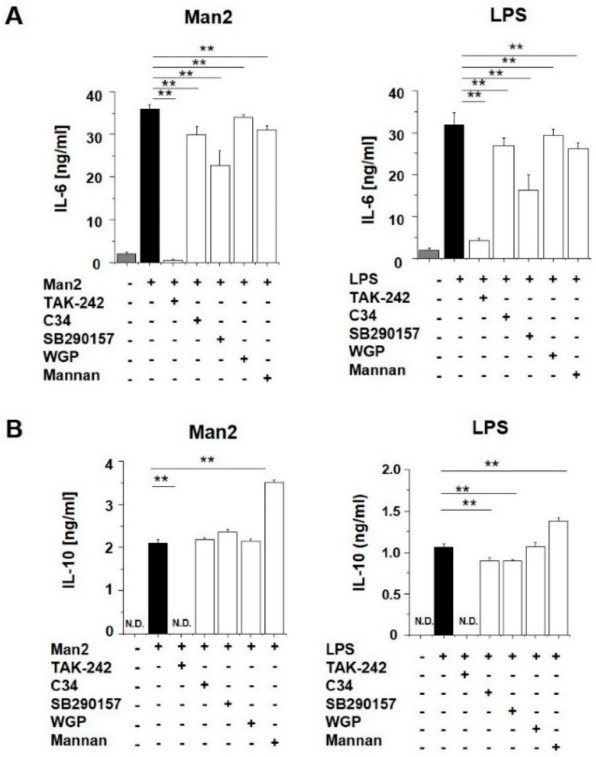

Figure 5. Effect of receptor inhibitors on IL-6 and IL-10 production of Man2-treated BMDCs. BMDCs derived from C57BL/6 mice (1×10^6 cells/mL) were treated with either 10 μM TAK-242, 50 μM C34, 100 μM SB290157, 100 μg/mL of WGP® Soluble, or 200 μg/mL of mannan for 30 min, and subsequently stimulated with 10 μM Man2 or 1.0 μg/mL of LPS for 24 h. The concentrations of (**A**) IL-6 and (**B**) IL-10 in the cell culture supernatants were determined by ELISA. ** $p < 0.01$ in Dunnett's test. The data are representative for two independent experiments. All bar graphs show mean ± SD. N.D. (not detectable): <31.25 pg/mL (IL-6 and IL-10).

3.5. Man2 Activates Glucose Metabolism in BMDCs

LPS influences the glucose metabolism in BMDCs [18,19]. To analyze the impact of Man2 on glucose metabolism, BMDCs were stimulated with either Man2 or LPS for 72 h and induced Warburg effect, cell metabolic state, and cytokine secretion were analyzed. Interestingly, 50 μM Man2 induced a strong Warburg effect in BMDCs, as observed in LPS-stimulated cells (Figure 7A). Man2 also increased glucose consumption and metabolic rate (Figure 7B). The concentrations of the IL-6 and TNF-α pro-inflammatory cytokines in the culture supernatant of Man2-stimulated cells were comparable to those in LPS-stimulated cells, although Man2-induced levels of IL-1ß were found to not be significantly different from unstimulated cells (Figure 7C). In contrast, the concentration of the IL-10 anti-inflammatory cytokine in the cell culture supernatant of Man2-stimulated cells was significantly lower than that in LPS-stimulated cells (Figure 7C). These results suggest that Man2 prolongs the secretion of pro-inflammatory cytokines, associated with increased glucose metabolism.

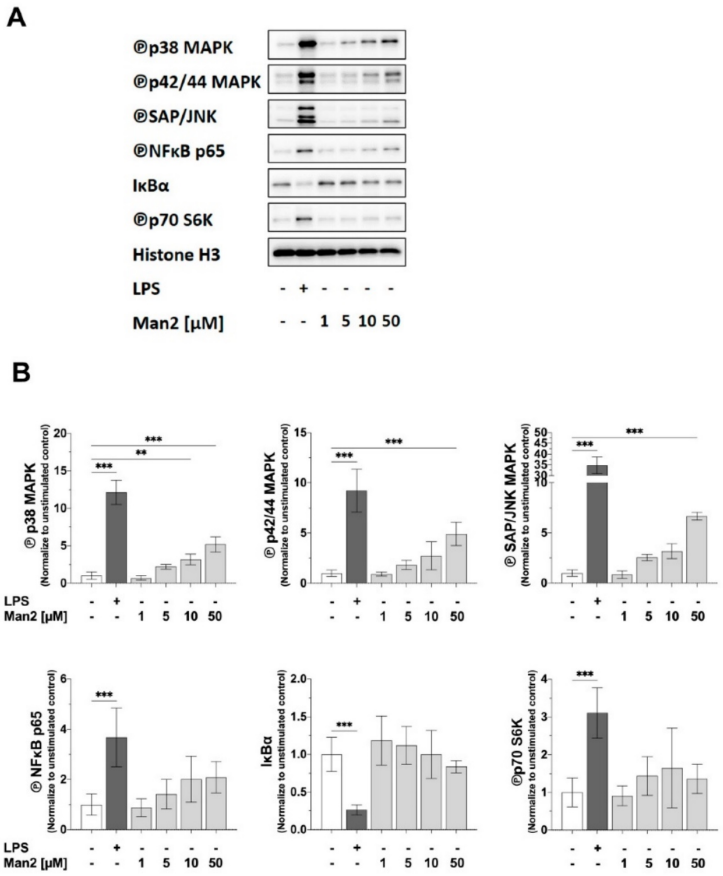

Figure 6. Effect of Man2 on activation of intracellular MAPK-, NFκB-, and mTOR-signaling pathways in BMDCs. BMDCs derived from C57BL/6 mice (1×10^6 cells/mL) were stimulated with either 1.0 to 50 μM Man2 or 10 μg/mL of LPS. (**A**) After 30 min of stimulation, 1×10^6 cells were lysed and target proteins in lysates were detected by Western blotting. (**B**) The intensities of protein bands were quantitated by Image J software. Data are mean values ± SD of four independent experiments. ** $p < 0.01$, *** $p < 0.001$ in 2-way ANOVA tests.

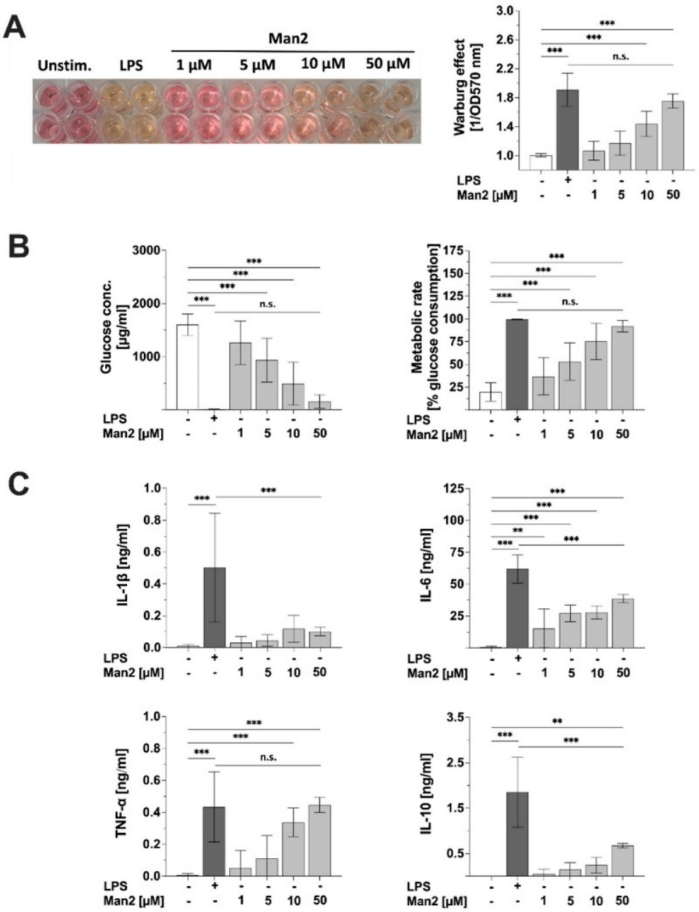

Figure 7. Effect of Man2 on glucose metabolism and cytokine secretion from BMDCs. BMDCs derived from C57BL/6 mice (1×10^6 cells/mL) were stimulated with either 1.0 to 50 μM Man2 or 10 μg/mL of LPS. (**A**) After 72 h of stimulation, the induced Warburg effect was quantified both optically and photometrically. In addition, (**B**) glucose consumption and metabolic rate and from culture medium were determined, whereas (**C**) cytokine concentrations in culture supernatants were determined by ELISA. Data are mean values ± SD of four independent experiments. ** $p < 0.01$, *** $p < 0.001$ in Tukey's HSD multiple comparison test. n.s. = not significant.

4. Discussion

In this study, we demonstrated that Man2, but not other tested mannooligosaccharides with the β-1,4-linkage, activates murine BMDCs via both TLR4- and C3aR-engagement and enhances the T-cell stimulatory capacity of the cells. The enhanced IL-2 production by OT-2 cells in a co-culture of Man2-stimulated BMDCs would be associated with up-regulation of co-stimulatory molecules, CD40, CD80, and CD86 on the cell surface of the stimulated DCs. Compared to that for short time frames (24 h), culture of Man2-stimulated BMDCs for extended timeframes (72 h) appeared to retain the secretion of pro-inflammatory cytokines, but reduced the secretion of anti-inflammatory cytokine remarkably. These results suggest that Man2 could be used as an immunostimulatory molecule to activate DCs and enhance subsequent antigen-specific $CD4^+$ T-cell activation.

Our previous studies showed that MAPK-mediated activation of both NFκB- and mTOR-signaling likely is a key pathway for IL-10 secretion in TLR5-engaged BMDCs with

a flagellin:allergen fusion protein [20,21]. However, in the present study, mechanistically, Man2 activated MAPKs (p38, p42/44, SAP/JNK) and NF-κB at certain levels, but only marginally activated p70S6K in BMDCs. Poncini et al. showed that high levels of IL-10 production are associated with p42/44 and NF-κB signaling in TLR4 engaged DCs with heat-killed trypomastigotes [22]. The results suggest, that Man2 could induce initial IL-10 production mainly via MAPK and NF-κB, but not the mTOR pathway.

In the present study, BMDCs secreted IL-10 during the first 24 h of culture upon stimulation with Man2 with secretion apparently being reduced thereafter. The decreased activation of p70S6K might be linked to the reduced IL-10 secretion in Man2-treated BMDCs. However, in contrast to IL-10 secretion, secretions of IL-6 and TNF-α were detectable after 72 h culture of Man2-treated BMDCs, similar to the observations in LPS-stimulated cells. Several studies have reported TLR-ligands to induce IL-6 and TNF-α production by activating MAPKs and NF-κB in BMDCs [16,21,23]. In addition, recent studies described that a shift from oxidative phosphorylation to anaerobic glycolysis in the metabolism of macrophages and DCs is important for pro-inflammatory cytokine production and effector function of the respective cells [18,19]. Interestingly, a potent glucose metabolism characterized by induction of the Warburg effect and increased glucose consumption from the culture medium was also detected in either LPS- or Man2-stimulated BMDCs. These results suggest that, in addition to the activation of MAPKs and NF-κB, pronounced glucose metabolism is associated with pro-longed IL-6 and TNF-α secretion in Man2-stimulated BMDCs.

To assess the involvement of TLR4 in Man2-mediated cell activation, we pre-treated BMDCs with the two TLR-4 inhibitors TAK-242 [24], and C34 [25]. TAK-242 remarkably reduced Man2-mediated CD40 expression and cytokine production in BMDCs. This is consistent with the previous observation that TAK-242 inhibited Man2-mediated TNF-α production in RAW264.7 cells [10]. TAK-242 is a small-molecule compound that selectively inhibits TLR4 signaling by binding to its intracellular domain [24]. C34 inhibits TLR4 signaling in a different manner by binding to a hydrophobic pocket of myeloid differentiation protein-2 (MD-2), a co-receptor of TLR4 expressed on the cell surface [25]. Although the inhibitory effects of C34 on Man2-mediated BMDC activation were moderate, this MD-2 inhibitor reduced Man2-, or LPS-induced cytokine production in BMDCs. LPS binds the MD-2/TLR4 complex via lipid A molecule, thereby triggering the activation of downstream signaling pathways of TLR4 [26,27]. Several studies have shown that various types of small molecules, such as palmitic acid and neoseptins (chemically synthesized peptidomimetics) primarily bind to the TLR4-MD2 complex and triggers TLR4-mediated signaling in both DCs and macrophages [28,29]. Taken together, the findings indicate, that it is likely that Man2 binds to the TLR4/MD-2 complex, and triggers TLR4-mediated signaling and activation of BMDCs.

It is interesting that only Man 2, but not other tested mannooligosaccharides, is capable of inducing BMDC activation. Compared to galactose and glucose, the hydrophobicity of mannose is higher [30]. In addition, the beta-face of D-mannose is basically hydrophobic, whereas the alpha-face is hydrophilic because OH groups of this hexamer orient in this alpha side due to axial configuration of the C2-hydroxyl group (2-OH) [31]. As described above, some small molecules bind to a hydrophobic pocket of MD-2, and thereby trigger or antagonize activation of TLR4-mediated signals in DCs and macrophages [28,29]. Man2 might trigger BMDC activation by binding the hydrophobic pocket of MD-2 with its hydrophobic beta-face. Other mannooligosaccharides might not be able to bind the pocket of MD-2 due to steric hindrance.

Compared to TAK-242, the inhibitory effect of C34 on Man2-stimulated BMDC activation is low. C34 suppressed IL-6, but not IL-10 production in the cells. In silico analyses showed, that C34 is a hydrophobic compound, and binds mainly to hydrophobic amino acid side chains in the pocket of MD-2, including F121, F119, L61, I117, Y102, I94, V93, F76, V135, and I78, with the exception of E92 [25]. Man2 has hydrophilic alpha-face due to the presence of several OH groups, although its beta-face is hydrophobic. Therefore, the

binding sites of C34 and Man2 could be different. C34 may cover only some of the space of Man2-binding sites in MD-2 that is otherwise occupied by Man2, and not inhibit the effect of Man2 effectively.

In addition to TLR4- and MD2-inhibitors, the C3aR inhibitor SB 290157 [32] also reduced Man2-mediated IL-6 production, but not IL-10 production, suggesting that Man2-mediated C3aR engagement is mainly involved in the induction of IL-6 in BMDCs. C3aR is expressed on leukocytes of the myeloid lineages, such as DCs, macrophages, monocytes, neutrophils, basophils, and mast cells. C3aR is a seven-transmembrane G protein-coupled receptor with a large second extracellular loop [33], which plays an important role in the interaction with its ligands including C3a, LPS, and probably Man2. Several studies have shown that C3aR engagement prevents IL-10 production in monocytes, macrophages, and T cells, and induces pro-inflammatory responses dominantly [34,35]. Engagement of the C3a receptor by Man2 might be at least in part involved in the prolonged secretion of IL-6 and the reduction of IL-10 during prolonged culture time of BMDCs.

α-Mannan significantly enhanced Man2-stimulated IL-10 production, while reducing IL-6 production moderately in BMDCs. We intended to use α-mannan as an inhibitor of the mannose receptor initially. However, Sirvent et al. showed that α-mannan induces IL-10 production predominantly in human DCs via DC-SIGN (Dendritic Cell-specific ICAM-3 Grabbing Non-integrin: CD209) engagement [6]. IL-10 inhibits the function of APCs, including production of pro-inflammatory cytokines [36]. It is likely that α-mannan enhanced IL-10 production and thereby suppressed that IL-6 production in Man2-stimulated BMDCs.

In conclusion, an exclusive stimulatory effect of Man2 on DC activation was demonstrated in an *in vitro* culture system. This is the first study to show, that Man2-drives DC activation occurring via binding of TLR4 and C3aR and subsequent intracellular signaling events. Our results suggest, that Man2 is an immunostimulatory molecule inducing production of pro-inflammatory cytokines and type I IFN in DCs. Man2 also enhances T-cell stimulatory capacity of BMDCs. The results suggest, that Man2 has potential as a vaccine adjuvant and an immunostimulatory component for functional foods. Further studies, e.g., in vivo immunization, or feeding study would be necessary to establish the application of Man2 for health benefits.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/cells10071774/s1, Materials and Methods, Figure S1. Stimulatory effect of mannooligosaccharides on RAW264.7 cells. Figure S2. Stimulatory effect of mannooligosaccharides on expressions of co-stimulatory and MHC class II molecules in BMDCs. Figure S3. Dose dependent effect of Man2 on CD40 expression in BMDCs. Figure S4. Stimulatory effect of mannooligosaccharides on cytokine production in BMDCs. Figure S5. Stimulatory effect of Man2 on TNF-α and IL-1β production in BMDCs. Figure S6. Effect of receptor inhibitors on expressions of co-stimulatory molecules in Man2-treated BMDCs. Figure S7. Effect of receptor inhibitors on expressions of IFN-β production in Man2-treated BMDCs. Figure S8. Effect of receptor inhibitors on expressions of TNF-α and IL-1β production in Man2-treated BMDCs. Figure S9. The influence of mannooligosaccharides and receptor inhibitors on viability of BMDCs.

Author Contributions: Conceptualization, M.T. and S.S. (Stefan Schülke); investigation, T.-Y.C., Y.-J.L., S.S. (Stefan Schülke); Resource, W.S.; data curation, M.T.; data interpretation S.V., S.S. (Stephan Scheurer), S.S. (Stefan Schülke), M.T.; writing—original draft preparation, M.T. and T.-Y.C.; writing—review and editing, All authors; visualization, M.T. and S.S. (Stefan Schülke); supervision, M.T. and S.S. (Stefan Schülke); project administration, M.T.; funding acquisition, M.T., S.S. (Stephan Scheurer), and S.S. (Stefan Schülke). All authors have read and agreed to the published version of the manuscript.

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Informed Consent Statement: Not applicable.

Data Availability Statement: The data that support the findings of this study are available from the corresponding authors, upon reasonable request.

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Conflicts of Interest: The authors declare following competing interests: Dr. Stefan Vieths reports personal fees from Schattauer Allergologie Handbuch, personal fees from Elsevier Nahrungsmittelallergien und Intoleranzen, personal fees from Karger Food Allergy: Molecular Basis and Clinical Practice, non-financial support from German Research Foundation, non-financial support from European Directorate for the Quality of Medicines and Health Care, non-financial support from European Academy of Allergy and Clinical Immunology, non-financial support from German Chemical Society (GDCh), non-financial support from AKM Allergiekongress, non-financial support from International Union of Immunological Societies, non-financial support from Spanish Society for Allergy and Clinical Immunology (SEAIC), outside the submitted work.

Abbreviations

APCs	Antigen presenting cells
BMDCs	Bone marrow derived murine dendritic cells
C3aR	Complement 3a receptor
FITC	Fluorescein isothiocyanate
mAb	Monoclonal antibody
TLR4	Toll-like receptor 4
Man2	β-Man-(1→4)-Man
Man3	β-Man-(1→4)-Man2
Man4	β-Man-(1→4)-Man3
Man5	β-Man-(1→4)-Man4
Manβ-4Glc	β-Man-(1→4)-Glc
Glcβ-4Man	β-Glc-(1→4)-Man
Manβ-4 GlcNAc	β-Man-(1→4)-GlcNAc
MD-2	myeloid differentiation protein-2

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Supporting information

β-(1→4)- Mannobiose acts as an immunostimulatory molecule in murine dendritic cells by binding the TLR4/MD-2 complex

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Materials and Methods

Preparation of Manβ-4GlcNAc

Manβ-4GlcNAc was prepared from α-mannose 1-phosphate (α-Man1P) and N-acetyl-D-glucosamine through the reverse phosphorolysis catalyzed by 1,4-β-mannosyl-N-acetylglucosamine phosphorylase (MNP, EC 2.4.1.320). Recombinant MNP from Bacteroides fragilis NCTC 9343 (BfMNP; GenBank number, CAH07033.1) was prepared as described below. The BfMNP gene was amplified from the genomic DNA of B. fragilis NCTC 9343 by PCR using Primestar HS DNA polymerase (Takara Bio, Kusatsu, Japan) and primers, 5´-AATATTAATTTAATCGAATC-3´ (sense) and 5´-CCCTTTCTGTTTTATTCGAT-3´ (antisense). Amplified DNA fragment was used as the template for the second PCR, in which primers, 5´-TTAACTTTAAGAAGGAGATATACATATGGAAGAAATTAAAATTGC-3´ (sense) and 5´-GATCTCAGTGGTGGTGGTGGTGGTGTCAGATGATACTTGTACGTT-3´ (antisense), were used. The PCR product and linear pET-23a, prepared by PCR using primers, 5´-CACCACCACCACCACCACTGAGATC-3´ (sense) and 5´-ATGTATATCTCCTTCTTAAAGTTAA-3´ (antisense), were connected with In-Fusion HD Cloning Kit (Takara Bio). The expression plasmid was propagated in Escherichia coli DH5α. The DNA sequence of the inserted DNA including its flanking regions was determined with an Applied Biosystems 3130 Genetic Analyzer (Foster City, CA, USA). Recombinant BfMNP was produced in E. coli BL21 (DE3) transformant, harboring the expression plasmid. Bacterial cells were incubated in 2.0 L of LB medium containing 100 μg/mL ampicillin with vigorous shaking at 37°C until OD600 reached 0.6. Production of the recombinant protein was induced by the addition of 0.1 M isopropyl β-thiogalactoside (IPTG) at a final concentration of 0.1 mM, and the induction culture was carried out at

18°C for 24 h. Bacterial cells, harvested by centrifugation at 6,000 ×g at 4°C for 10 min, were disrupted by sonication in 100 mL of 10 mM 2-morpholinoethanesulfonic acid (MES)-NaOH buffer (pH 6.5). Cell-free extract after removal of cell debris by centrifugation at 6,000 ×g at 4°C for 10 min was subjected to anion exchange column chromatography using DEAE Sepharose Fast Flow (2.8 cm I.D. × 20 cm; GE Healthcare, Uppsala, Sweden). After eluting non-adsorbed protein with 10 mM MES-NaOH buffer (pH 6.5), adsorbed protein was eluted by a linear gradient of NaCl from 0 to 0.4 M (total elution volume, 600 mL). The active fraction obtained was further separated by hydrophobic column chromatography using Toyopearl Butyl 650-M (2.8 cm I.D. × 10 cm; Tosoh, Tokyo, Japan). Non-adsorbed protein was eluted with 10 mM MES-NaOH buffer (pH 6.5) containing 25% saturation ammonium sulfate, and adsorbed protein was eluted by a descending linear gradient of ammonium sulfate (25–0% saturation; total elution volume, 600 mL). The fractions containing highly purified BfMNP, judged by SDS-PAGE, were pooled and dialyzed against 10 mM MES-NaOH buffer (pH 6.5).

A reaction mixture (200 mL), containing 7.94 μg/mL BfMNP, 50 mM Man1P, 100 mM N-acetyl-D-glucosamine (Nacalai Tesque, Kyoto, Japan), and 50 mM MES-NaOH buffer (pH 6.5), was incubated at 37°C for 24 h. Man1P bis(cyclohexylammonium) salt, prepared by the publication of Liu et al. [Carbohydrate Research, 401, 1-4 (2015)], was kindly gifted by Dr. Motomitsu Kitaoka (Niigata University, Niigata, Japan). Sample, passed through a membrane filter Amicon Ultra 30,000 nominal molecular weight limit (Merck, Darmstadt, Germany), was concentrated to 20 mL under reduced pressure. The reaction product was purified by gel filtration column chromatography under following conditions: column, Toyopearl HW-40S (5.0 cm I.D. × 95 cm, Tosoh); elution, water; fraction volume, 5 mL. The fractions, containing highly purified Manβ-4GlcNAc, were

pooled and lyophilized (629 mg of Manβ-4GlcNAc was yielded). Chemical structure of Manβ-4GlcNAc was verified by ESI-MS and NMR. NMR spectra were recorded in D2O at 27°C using an AMX500 (500 MHz; Bruker, Billerica, MA, USA). A series of two-dimensional homo- and heteronuclear correlated spectra [correlated spectroscopy, heteronuclear single quantum correlation (HSQC), non-decoupling HSQC, HSQC total correlation spectroscopy, and heteronuclear multiple bond correlation correlated spectroscopy (HMBC)] were acquired.

Cytokine ELISA

The concentrations of cytokines in cell culture supernatants of RAW264.7 cells and BMDCs were measured by ELISA using antibodies and ELISA kits listed below. Following incubation with the respective detection antibodies, ELISA plates were incubated with streptavidin horseradish peroxidase for 30 minutes at room temperature. Detection was performed with 100 μL 3,3′ ,5,5′ -tetramethylbenzidine (Carl Roth Chemikalien, or BioLegend) and incubation at room temperature. The reaction was stopped with 1 M sulfuric acid (Carl Roth Laborbedarf, or FUJIFILM Wako Pure Chemical Corporation). Optical density was measured at 450 nm by SpectraMAX340PC (Molecular Devices), or iMark microplate absorbance reader (Bio-Rad).

<u>List of antibodies and ELISA kits</u>

Purified anti-mouse IL-1β mAb (1:500 dilution: eBioscience, cat.14-7012-85)

Biotin-conjugated anti-mouse IL-1β polyclonal antibody (1:500 dilution: eBioscience, cat.13-7112-81)

Purified anti-mouse IL-6 mAb (1:1000 dilution: eBioscience, cat.14-7061-85)

Biotin-conjugated IL-6 mAb (1:1000 dilution: eBioscience, cat.13-7062-85)

Purified anti-mouse TNF-α (1:500 dilution: eBioscience, cat.14-7325-85)

Biotin-conjugated TNF-α mAb (1:250 dilution: eBioscience, cat. 13-7326-85)

Streptavidin horseradish peroxidase (1:2000 dilution: eBioscience, cat. 554066)

ELISA MAX™ Standard Set Mouse IL-1β (BioLegend, cat. 432601)

ELISA MAX™ Standard Set Mouse IL-6 (BioLegend, cat.431301)

ELISA MAX™ Standard Set Mouse IL-10 (BioLegend, cat. 431411)

Mouse IL-10 ELISA Development Kit (PeproTech, cat.900-T53)

ELISA MAX™ Standard Set Mouse TNF-α (BioLegend, cat. 430901)

Mouse IFN-beta DuoSet ELISA (R&D systems: cat. DY8234-05)

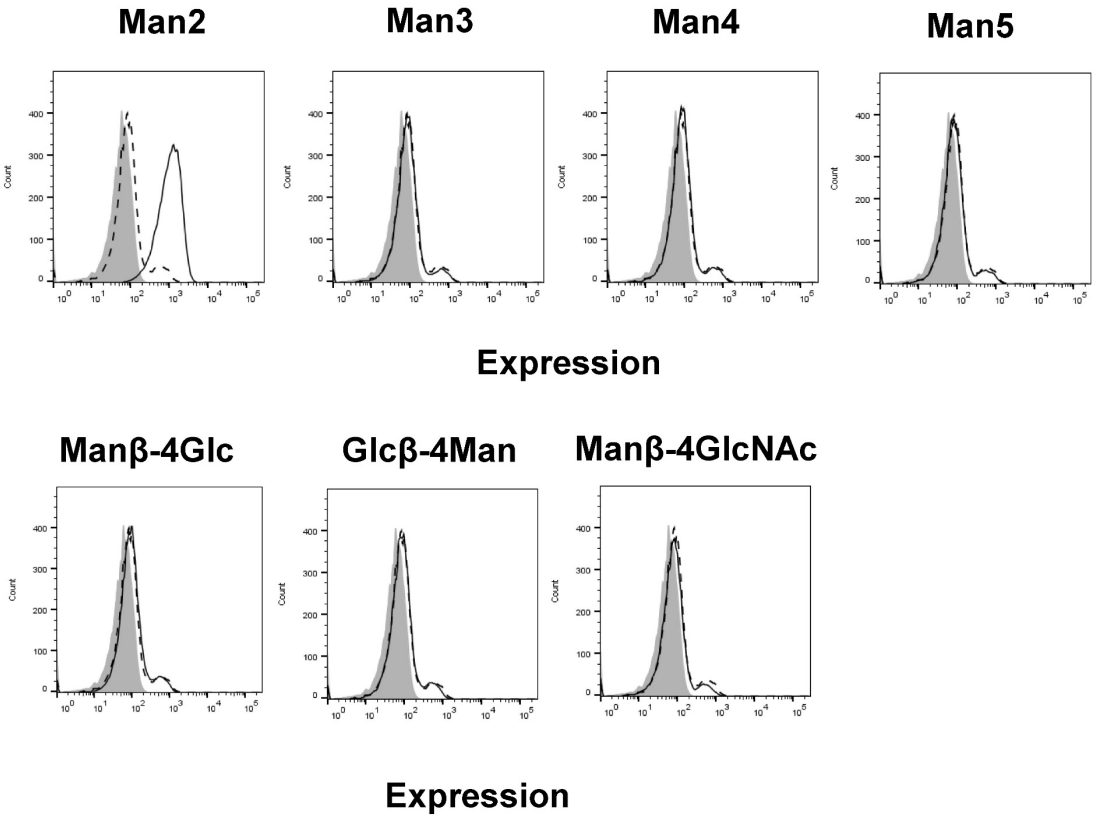

Fig. S1. Stimulatory effect of mannooligosaccharides on CD40 expression in RAW264.7 cells. RAW264.7 cells ($1x10^6$ cells/mL) were stimulated with 50 μM of the indicated mannooligosaccharides or 1.0 μg/mL of LPS. Expression levels of CD40 on the cell surface were analyzed by FACS. Grey area: unstimulated and unstained cells, dashed lines: unstimulated and mAb-stained cells, solid lines: stimulated and mAb-stained cells. The data are representative for two independent experiments.

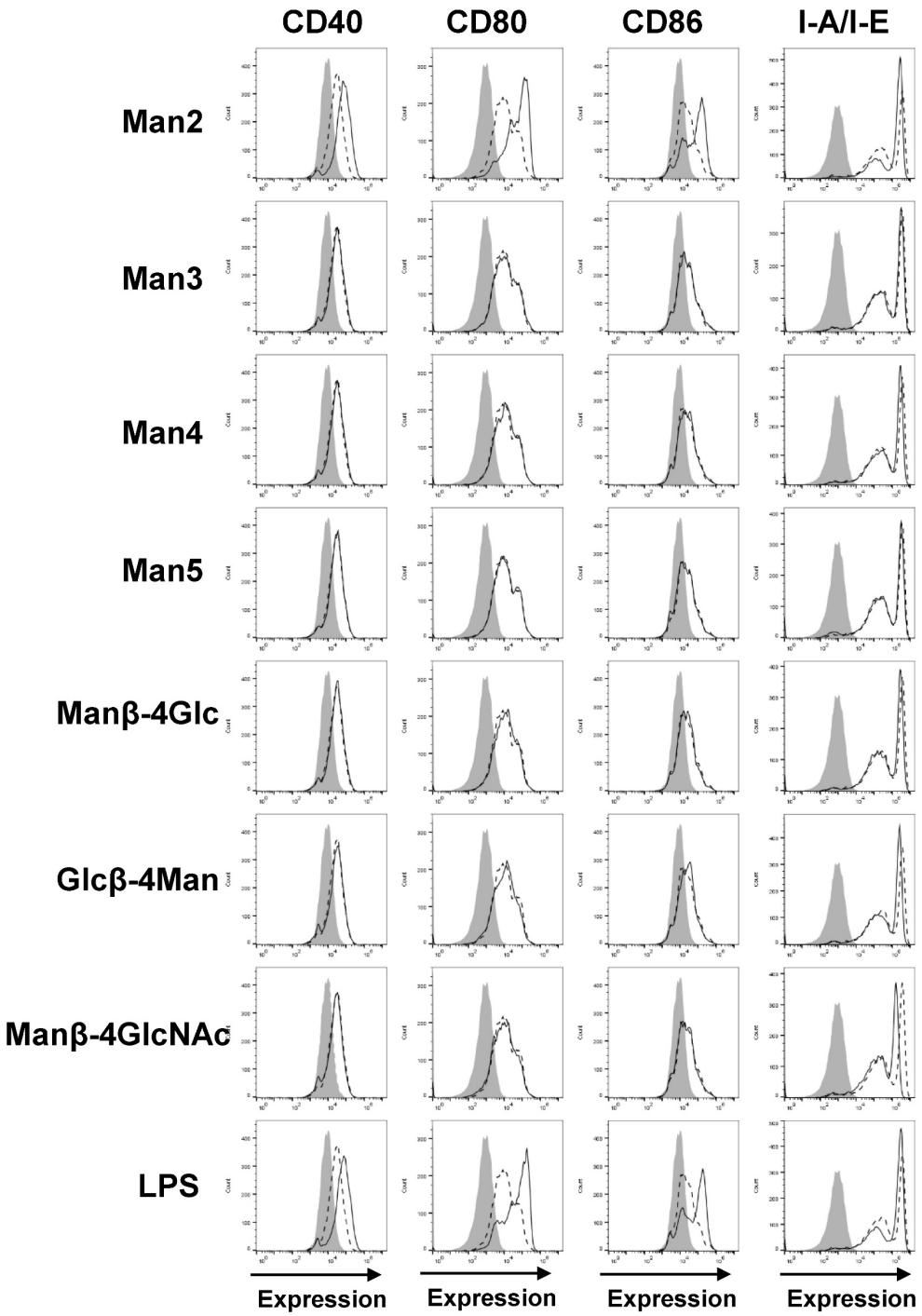

Fig. S2. Stimulatory effect of mannooligosaccharides on expressions of co-stimulatory and MHC class II molecules in BMDCs. BMDCs from BALB/c mice ($1x10^6$ cells/mL) were stimulated with 50 μM of the indicated mannooligosaccharides or 1.0 μg/mL of LPS. Expression levels of CD40, CD80, CD86 and I-A/I-E on the cell surface were analyzed by FACS. Grey area: unstimulated and unstained cells, dashed lines: unstimulated and mAb-stained cells, solid lines: stimulated and mAb-stained cells. The data are representative for two independent experiments.

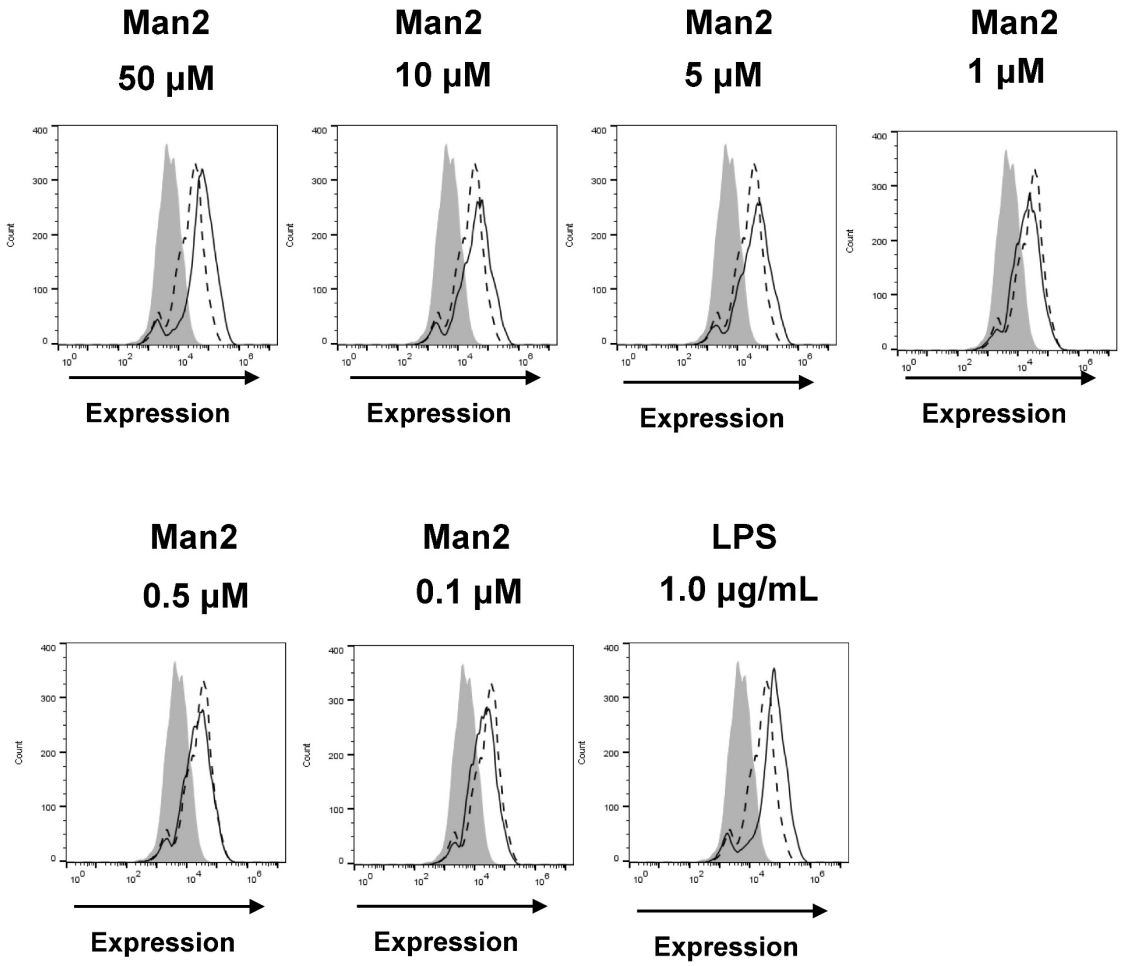

Fig. S3. Dose dependent effect of Man2 on CD40 expression in BMDCs. BMDCs derived from BALB/c mice ($1x10^6$ cells/mL) were stimulated with different concentrations of Man2, or 1.0 µg/mL of LPS. Expression levels of CD40 on the cell surface were analyzed by FACS. Grey area: unstimulated and unstained cells, dashed lines: unstimulated and mAb-stained cells, solid lines: stimulated and mAb-stained cells. The data are representative for two independent experiments.

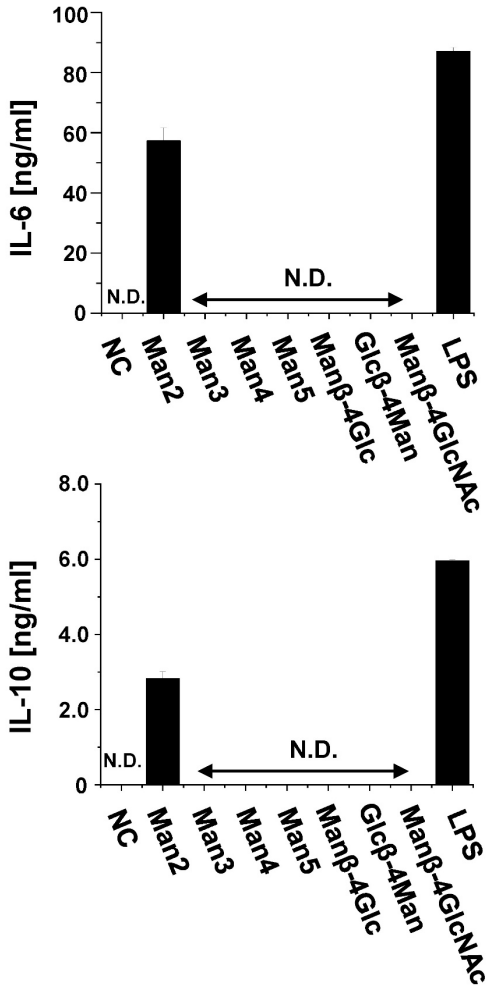

Fig. S4. Stimulatory effect of mannooligosaccharides on cytokine production in BMDCs. BMDCs ($1x10^6$ cells/mL) derived from BALB/c mice were stimulated with 50 μM of the indicated mannooligosaccharides or 1.0 μg/mL of LPS. The concentrations of IL-6 (upper graph) and IL-10 (lower graph) in the culture supernatants were measured by ELISA. The experiment was performed once. The similar trend was observed in BMDCs derived from C57BL/6 mice. N.D. (not detectable): <31.25 pg/mL

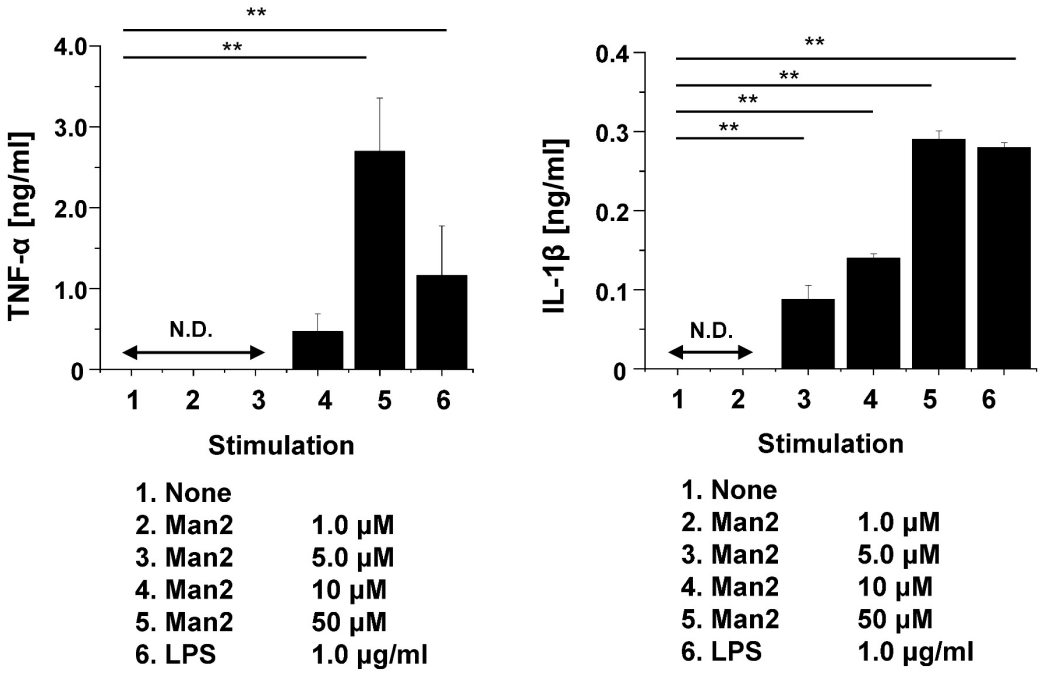

Fig. S5. Stimulatory effect of Man2 on TNF-α and IL-1β production in BMDCs. BMDCs derived from C57BL/6 mice ($1x10^6$ cells/mL) were stimulated with various concentrations of Man2 or 1.0 μg/mL of LPS. The concentrations of TNF-α (left graph) and IL-1β (right graph) in the cell culture supernatants were measured by ELISA. The data are representative for two independent experiments. **P<0.01 in Dunnett's test. All bar graphs show mean +/- SD. N.D. (not detectable): <31.25 pg/mL (TNF-α), <15.6 pg/ml (IL-1β)

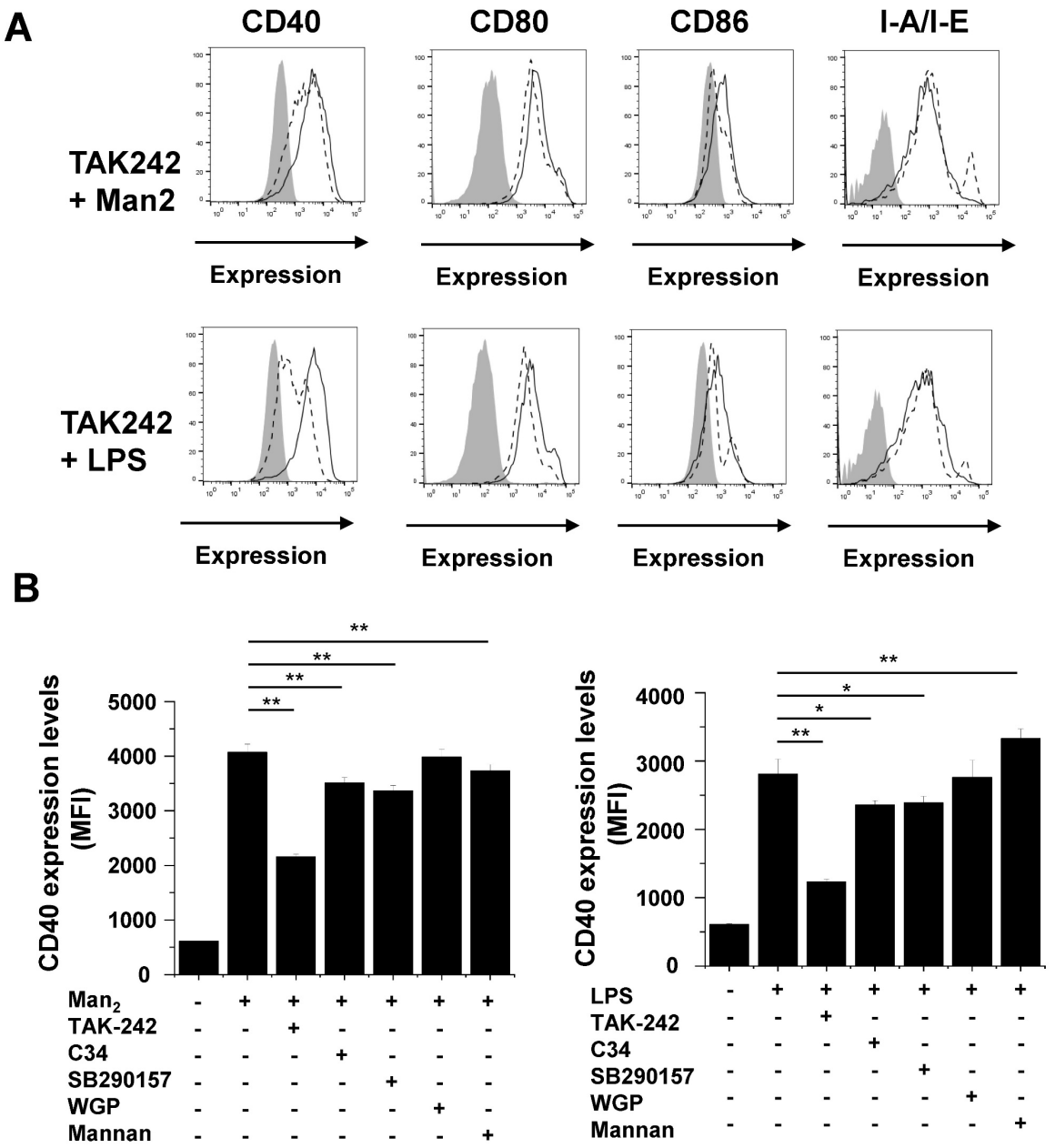

Fig. S6. Effect of receptor inhibitors on expressions of co-stimulatory molecules in Man2-treated BMDCs. BMDCs derived from C57BL/6 mice (1x10^6 cells/mL) were treated with either 100 nM TAK-242, 50 μM C34, 100 μM SB290157, 100 μg/mL of WGP® Soluble, or 200 μg/mL of mannan for 30 min and subsequently stimulated with 50 μM Man2 or 5.0 ng/mL of LPS for 24 hours. (A) Expression levels of CD40, CD80, CD86 or I-A/I-E on the cell surface of TAK-242-treated and Man2- or LPS-stimulated cells were analyzed by FACS. Grey area: unstimulated and unstained cells, dashed lines: inhibitor-and Man2- or LPS-treated and mAb-stained cells, solid lines: inhibitor-untreated, Man2- or LPS-treated and mAb-stained cells. (B) Mean fluorescence intensity (MFI) of CD40 expression in inhibitor-treated and Man2, or LPS-stimulated cells were estimated. The data are representative for two independent experiments. *P<0.05 , **P<0.01 in Dunnett's test. All bar graphs show mean +/- SD.

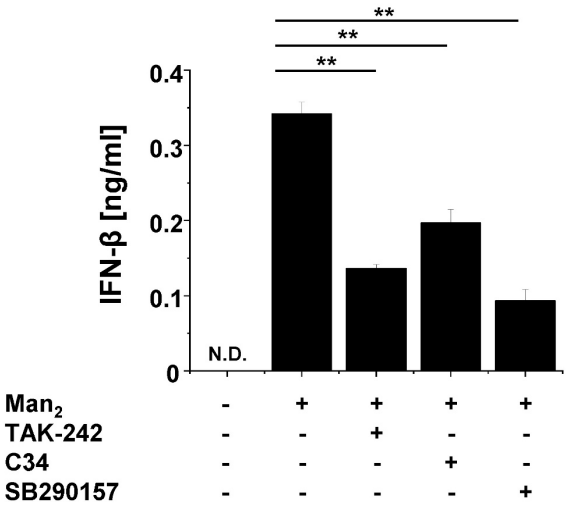

Fig. S7. Effect of receptor inhibitors on expressions of IFN-β production in Man2-treated BMDCs. BMDCs derived from C57BL/6 mice ($1x10^6$ cells/mL) were treated with 100 nM TAK-242, 50 μM C34, or 100 μM SB290157 for 30 min, and subsequently stimulated with 50 μM Man2 for 24 hours. The concentrations of IFN-β in the culture supernatants were measured by ELISA. The data are representative for two independent experiments. **P<0.01 in Dunnett's test. All bar graphs show mean +/- SD. N.D. (not detectable): <25.0 pg/mL

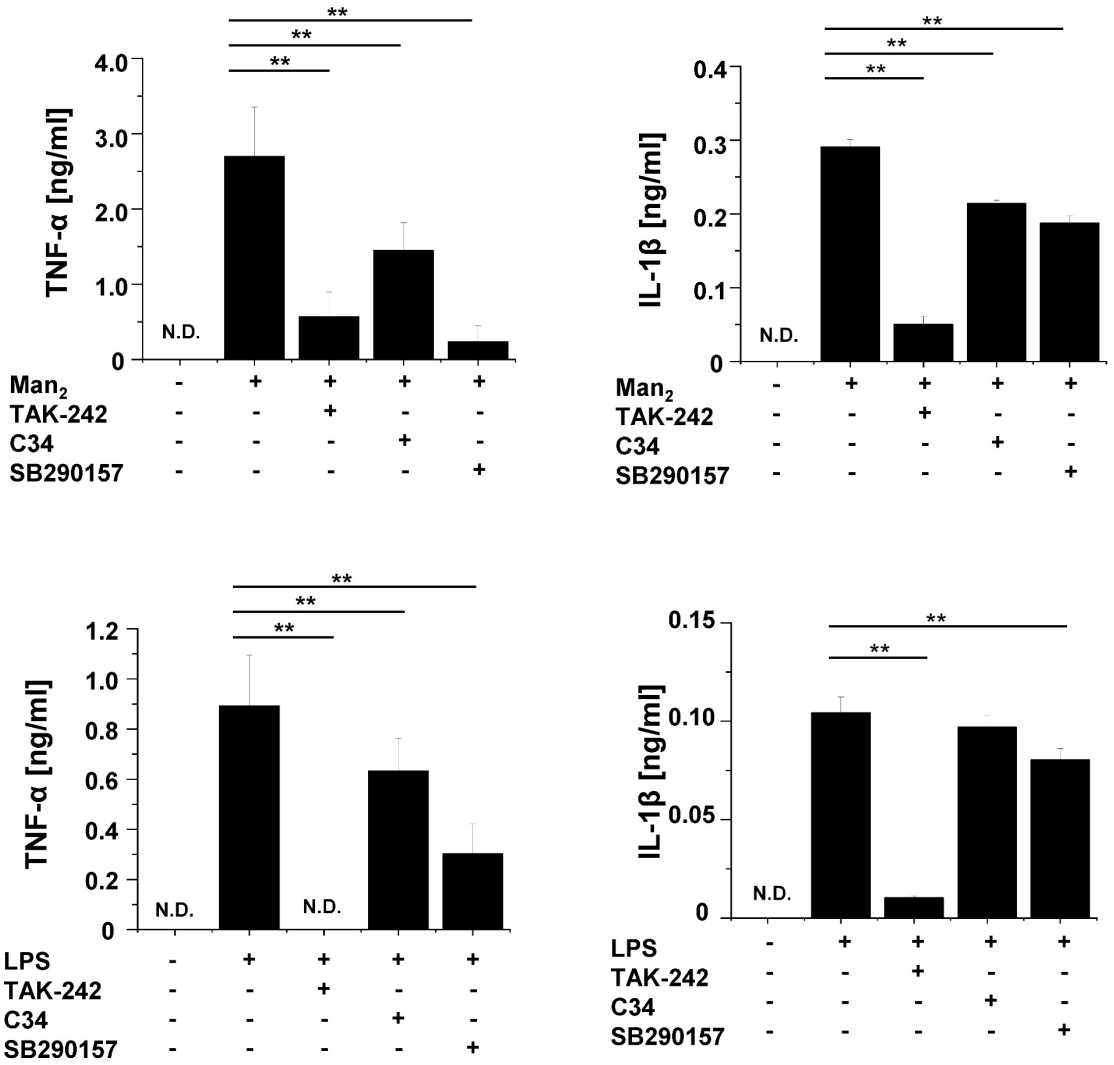

Fig. S8. Effect of receptor inhibitors on expressions of TNF-α and IL-1β production in Man2-treated BMDCs. BMDCs derived from C57BL/6 mice (1x10^6 cells/mL) were treated with 100 nM TAK-242, 50 μM C34, or 100 μM SB290157 for 30 min, and subsequently stimulated with 50 μM Man2 or 5.0 ng/mL of LPS for 24 hours. (A) The concentrations of TNF-α and (B) IL-1β in the culture supernatants were measured by ELISA. The data are representative for two independent experiments. All bar graphs show mean +/- SD. N.D. (not detectable): <31.25 pg/mL (TNF-α), <15.6 pg/mL (IL-1β)

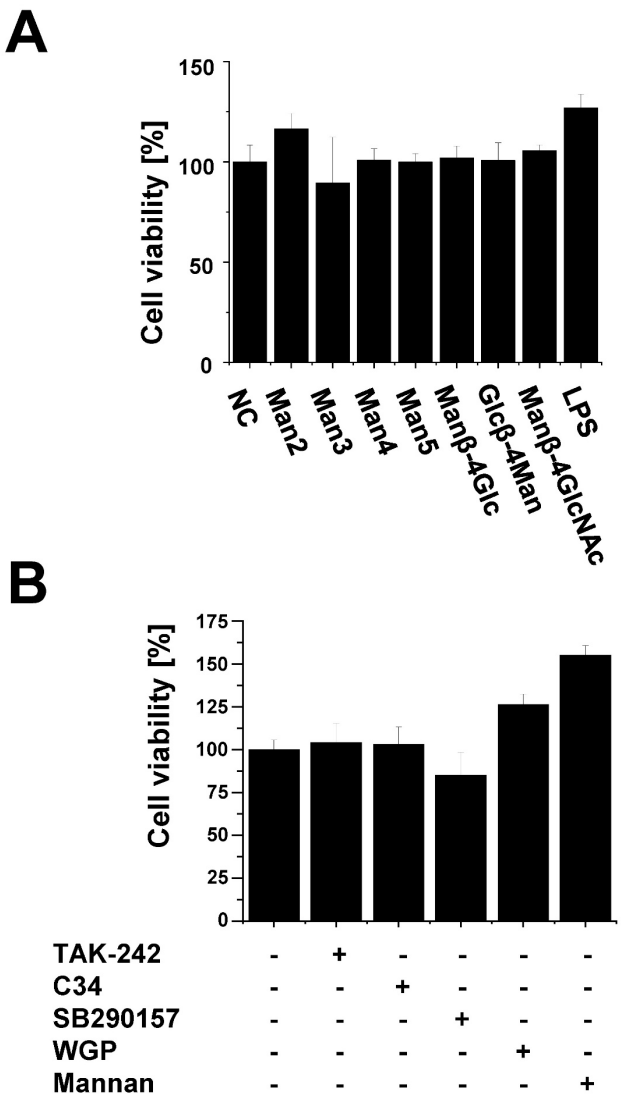

Fig. S9. The influence of receptor inhibitors on viability of BMDCs. (A) BMDCs derived from C57BL/6 mice ($1x10^6$ cells/mL) were stimulated with 50 μM mannooligosaccharide or 1.0 μg/mL of LPS for 24 hours. (B) BMDCs derived from C57BL/6 mice ($1x10^6$ cells/mL) were treated with 100 nM TAK-242, 50 μM C34, 100 μM SB290157, 100 μg/mL of WGP® Soluble, or 200 μg/mL of mannan for 30 min, and subsequently stimulated with 10 μM Man2 or 1.0 μg/mL of LPS for 24 hours. CCK-8 assay was performed to assess the influence of mannooligosaccharides and receptor inhibitors on the viability of BMDCs. The data are representative for two independent experiments. All bar graphs show mean +/- SD.

4 Chapter 2

The Fusion Protein rFlaA:Betv1 Modulates DC Responses by a p38-MAPK and COX2-Dependent Secretion of PGE_2 from Epithelial Cells

Yen-Ju Lin[1], Adam Flaczyk[1,2], Sonja Wolfheimer[1], Alexandra Goretzki[1], Annette Jamin[1], AndreaWangorsch[1], Stefan Vieths[1], Stephan Scheurer[1] and Stefan Schülke[1,*]

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Figures contributed by Yen-Ju Lin:

- All figures except Figure 3B and supplementary Figure S1A, which were contributed by Adam Flaczyk.

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MDPI

Article

The Fusion Protein rFlaA:Betv1 Modulates DC Responses by a p38-MAPK and COX2-Dependent Secretion of PGE_2 from Epithelial Cells

Yen-Ju Lin [1], Adam Flaczyk [1,2], Sonja Wolfheimer [1], Alexandra Goretzki [1], Annette Jamin [1], Andrea Wangorsch [1], Stefan Vieths [1], Stephan Scheurer [1] and Stefan Schülke [1,*]

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Abstract: Developing new adjuvants/vaccines and better understanding their mode-of-action is an important task. To specifically improve birch pollen allergy treatment, we designed a fusion protein consisting of major birch pollen allergen Betv1 conjugated to the TLR5-ligand flagellin (rFlaA:Betv1). This study investigates the immune-modulatory effects of rFlaA:Betv1 on airway epithelial cells. LA-4 mouse lung epithelial cells were stimulated with rFlaA:Betv1 in the presence/absence of various inhibitors with cytokine- and chemokine secretion quantified by ELISA and activation of intracellular signaling cascades demonstrated by Western blot (WB). Either LA-4 cells or LA-4-derived supernatants were co-cultured with BALB/c bone marrow-derived myeloid dendritic cells (mDCs). Compared to equimolar amounts of flagellin and Betv1 provided as a mixture, rFlaA:Betv1 induced higher secretion of IL-6 and the chemokines CCL2 and CCL20 from LA-4 cells and a pronounced MAPK- and NFκB-activation. Mechanistically, rFlaA:Betv1 was taken up more strongly and the induced cytokine production was inhibited by NFκB-inhibitors, while ERK- and p38-MAPK-inhibitors only suppressed IL-6 and CCL2 secretion. In co-cultures of LA-4 cells with mDCs, rFlaA:Betv1-stimulated LA-4 cells p38-MAPK- and COX2-dependently secreted PGE_2, which modulated DC responses by suppressing pro-inflammatory IL-12 and TNF-α secretion. Taken together, these results contribute to our understanding of the mechanisms underlying the strong immune-modulatory effects of flagellin-containing fusion proteins.

Keywords: flagellin; Betv1; epithelial cells; vaccine; fusion protein

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1. Introduction

The current SARS-CoV-2 pandemic has clearly demonstrated the need to develop both safe and efficacious vaccines. Protein-antigen-based vaccines that typically have a low immunogenicity often need to be adjuvanted in order to induce robust immune responses.

In this context, some "Toll"-like receptor (TLR) ligands with intrinsic immune-activating properties are attractive adjuvant candidates. They act as pathogen-associated molecular patterns (PAMPs) which bind conserved pattern recognition receptors (PRRs) on immune cells to induce strong immune responses.

Currently, monophosphporyl lipid A (MPLA), a modified TLR4 ligand derived from the lipopolysaccharide of *Salmonella minnesota* R595 is the only TLR ligand that is already used as an adjuvant in licensed vaccines. Here, several vaccines containing MPLA as a component of more complex adjuvant systems have been licensed or are undergoing phase III clinical trials. These include Fendrix® (for the prevention of hepatitis B), Cervarix®

(human papillomavirus-16 and papillomavirus-18), RTS,S(R) (malaria), and the allergen product Pollinex® Quattro (pollen allergies) [1–3].

The success of MPLA as a vaccine adjuvant clearly demonstrates the immune-modulating potential of TLR-ligands. However, other TLR-ligands were less successful in pre-clinical trials. For example, nucleic acid-based TLR-ligands, such as CpG (activating TLR9), R848 (TLR7/8), or Poly I:C (TLR3), are potent immune activators, but are limited in their clinical efficacy due to problems with both toxicity and stability in vivo [4].

So far, the TLR5 ligand flagellin, a bacterial motility protein that forms the main body of the bacterial flagellum [5], is the only purely protein-based TLR ligand. In addition to TLR5, flagellin activates intracellular NOD-like receptor 4 (NLRC4) resulting in inflammation and IL-1β production [6]. Consequently, flagellin has repeatedly demonstrated mucosal adjuvant properties resulting in the induction of protective immune responses [7–9].

Flagellin being a protein allows for the generation of recombinant flagellin antigen fusion proteins by recombinant DNA technology. Such flagellin-containing fusion proteins have been investigated as vaccine candidates in pre-clinical models for the prevention of influenza [10–12], poxvirus [13], West–Nile virus [14], tetanus [15], and *Pseudomonas* infections [16], as well as for the treatment of allergic diseases [17–23]. Moreover, fusion proteins containing *Salmonella* flagellin C and influenza antigens have been shown to be both safe and well-tolerated in clinical trials [24,25].

Mechanistically, TLR ligand:antigen fusion proteins effectively target the fused antigen to immune cells in vivo that express their respective TLR, resulting in more effective processing and simultaneous presentation of the enclosed antigen in the context of the TLR ligand-mediated immune cell activation [9,26].

We recently generated a fusion protein consisting of flagellin A from *Listeria monocytogenes* and the major birch pollen allergen Betv1 (rFlaA:Betv1) that was able to suppress allergic sensitization in vivo [22]. In vitro analyses in myeloid dendritic cells (mDCs) showed rFlaA:Betv1 to induce a pronounced mDC activation characterized by the secretion of both pro- (IL-6, IL-12, and TNF-α) and anti-inflammatory (IL-10) cytokines as well as pronounced surface expression of co-stimulatory molecules [22]. While the effects of flagellin antigen fusion proteins on DCs are well investigated, their effects on other cell types require further investigation.

Epithelial cells, which similarly express high levels of TLR5 [27] are the first line of defense against invading pathogens [28]. In addition to their function as part of the mechanical barrier, epithelial cells engage in the initiation and maintenance of immune responses [29,30]. Just as other immune cells, epithelial cells express pattern recognition receptors, become activated, and secrete both cytokines and chemokines to alert other immune cells to invading pathogens [28]. Therefore, epithelial cells play an integral part in the establishment of immune responses.

To our knowledge, the effects of fusion proteins on epithelial cells have been poorly studied. Maffía et al. showed that fusion of Cementoin to the secretory leukocyte proteinase inhibitor (SLPI) increased both SPLI's preoteolytic stability and surface binding to TNF-α- or LPS-pretreated A549 alveolar epithelial cells [31]. Moreover, Savar et al. in 2014 generated a FimH/tFliC fusion protein consisting of a truncated form of flagellin C (FliC) from enteroaggregative *E. coli* fused to FimH, the tip adhesion on type 1 fimbria, from uropathogenic *E. coli* to create a vaccine candidate against urinary tract infections [32]. Despite showing that the truncated form of FliC alone could induce IL-8 in the HT-29 epithelial cell line [32], no experiments using this fusion protein on epithelial cells were performed [32].

Therefore, as far as we know, the effects of flagellin:antigen fusion proteins on epithelial cells and intracellular signal transduction are currently unknown.

In the present study, we analyzed the effects of rFlaA:Betv1 on the mouse lung epithelial cell line LA-4. Here, we show that rFlaA:Betv1 is taken up more readily than the mixture of both proteins and induces a MAPK- and NFκB-dependent secretion of both cytokines and chemokines. Moreover, rFlaA:Betv1-stimulated LA-4 cells modulated

cytokine secretion from BALB/c mDCs stimulated with rFlaA:Betv1. Supernatants derived from rFlaA:Betv1-stimulated LA-4 cells selectively suppressed rFlaA:Betv1-induced, mDC-derived IL-12 and TNF-α secretion. These immune-modulatory effects of LA-4 cells were shown to depend on the p38-MAPK- and cyclooxygenase-2 (COX2)-dependent production of prostaglandin E2 (PGE_2).

2. Materials and Methods

2.1. Flow Cytometry

For characterisation of LA-4 cells, the following antibodies were used: PE-conjugated anti-mouse TLR5 (clone: 85B152.5, 1:100, Abcam, Cambridge, UK), PE-conjugated anti-mouse MHCI (clone: 31-1-2S, 1:100, eBiosciences, Frankfurt, Germany), AF488-conjugated anti-mouse Pan-cytokeratin (clone: AE1/AE3, 1:50, eBiosciences), AF488-conjugated anti-human mucin 1 (clone: SM3, 1:25, eBiosciences), APC-conjugated anti-mouse EpCAM (clone: G8.8, 1:50, eBiosciences), and APC-conjugated anti-mouse CEACAM1 (clone: CC1, 1:50, eBiosciences). Bone marrow-derived mDCs were characterized using anti-mouse pacific blue-conjugated CD11b (clone: M1/70.15, 1:50, Invitrogen, ThermoFisher Scientific, Dreieich, Germany), APC-conjugated CD11c (clone: HL3, dilution: 1:500, BD Bioscience), and PE-Cy5-conjugated B220 (clone: RA3-6B2, dilution: 1:100, BD Bioscience), anti-mouse FITC-conjugated CD3 antibody (clone: 145-2C11, 1:50, BD Biosciences), anti-mouse FITC-conjugated CD19 antibody (clone: 6D5, 1:50, Southern Biotech), and anti-mouse FITC-conjugated CD49b pan NK cell antibody (clone: Dx5, 1:100, BioLegend). Cells were analyzed by flow cytometry using a LSR II flow cytometer (BD Bioscience). Data were analyzed using FlowJo either V.7 or V.10 (Treestar Inc., Ashland, OR, USA).

2.2. Generation of Recombinant Proteins

Recombinant flagellin A from *Listeria monocytogenes* (rFlaA, Acc. No: NC_003210) was generated according to [19], and recombinant birch pollen allergen Betv1 (Acc. No: X15877.1) according to [33]. The fusion protein of rFlaA and rBetv1 (rFlaA:Betv1) was generated according to [22] by cDNA fusion using the cDNAs of both rFlaA and rBet v 1 as templates. For the generation of $rFlaA^{*D1}$ and $rFlaA^{*D1}$:Betv1 mutants, amino acids at position 87–94 of the *Listeria monocytogenes* flagellin A D1 domain (QRMRQLAV) were substituted by PCR mutagenesis strategy (Q5® Site-Directed Mutagenesis Kit, NEB, Frankfurt, Germany) with the corresponding sequence stretch from *Helicobacter pylori* flagellin (DTVKVKAT), which was reported to avoid TLR5 binding [34]. Protein expression and purification strategy of the mutants was performed according to [19]. Briefly, BL21 star DE3 cells (Invitrogen, Karlsruhe, Germany) transformed with the cloned mutant sequences in pET15b (Novagen, Darmstadt, Germany) were cultured in 4L of LB-medium supplemented with 50 mg/L of carbenicillin (Roth, Karlsruhe, Germany). Protein expression was induced by 1 mM IPTG at OD_{600} = 0.5, and cells were incubated for 20 h at 25 °C, 180 rpm. Subsequently, cells were harvested, lysed by sonication, and repeated freeze–thawing cycles. Inclusion bodies were solubilized with 50 mM phosphate buffer plus 6 M urea, and proteins were re-folded and purified with His GraviTrap columns (GE Healthcare, Freiburg, Germany). To further improve protein purity and remove endotoxins, a size exclusion chromatography using a HiLoad® 26/600 Superdex® 75 pg column (Cytiva, Freiburg (Breisgau), Germany) was performed. All proteins displayed a purity greater than 98%, the correct folding of secondary structure elements as determined by circular dichroism spectroscopy, and endotoxin contents of 1.14 pg/µg protein (rFlaA), <0.48 pg/µg protein (rBetv1), and 1.7 pg/µg protein (rFlaA:Betv1) <0.963 pg/µg protein ($rFlaA^{*D1}$), and 2.7 pg/µg protein ($rFlaA^{*D1}$:Betv1), respectively (data not shown).

2.3. Culture, Stimulation, Viability of LA-4 Cells

The mouse lung epithelial cell line LA-4 (ATCC CCL-196TM) was cultured in DMEM medium (Gibco, Karlsruhe, Germany), supplemented with 15% FCS (Sigma-Aldrich, Taufkirchen, Germany), 100 U/mL penicillin, 100 µg/mL streptomycin, and 1 mM L-glutamine.

Cells were passaged every 3–4 days using 0.05% trypsin-EDTA. For stimulations, LA-4 cells were seeded overnight at 6.25×10^4 cells/mL in 24-well plates (Thermo Scientific, Dreieich, Germany) and on the following morning stimulated with the indicated equimolar concentrations of either rFlaA + rBetv1, or rFlaA:Betv1 for either 2, 4, or 24 h. LPS (#L5886, Sigma-Aldrich) at 10 µg/mL served as a positive control.

2.4. ELISA

Cytokines in the supernatants were analyzed by ELISA using the following antibody combinations: IL-1β (capture antibody: anti-IL-1β monoclonal mouse antibody (#14-7061-85, eBioscience, Frankfurt, Germany, 1:500); plus detection antibody anti-IL-1β monoclonal mouse biotin-conjugated antibody (#13-7112-81, eBioscience, 1:500)), IL-6 (capture antibody: anti-IL-6 monoclonal mouse antibody (#14-7061-85, eBioscience, Frankfurt, Germany, 1:500); plus detection antibody anti-IL-6 monoclonal mouse biotin-conjugated antibody (#13-7062-85, eBioscience, 1:500)), TNF-α (capture antibody: anti-TNF-α monoclonal mouse antibody (#14-7325-85, eBioscience, 1:500); plus detection antibody anti-TNF-α monoclonal mouse biotin-conjugated antibody (#13-7326-85, eBioscience, 1:500), IL-12 p70 (capture antibody: anti-IL-12 monoclonal mouse antibody (#14-7122-85, eBioscience, 1:500); plus detection antibody: anti-IL-12 monoclonal mouse biotin-conjugated antibody (#MM121B, Invitrogen, 1:500). Following incubation with the respective detection antibodies, plates were incubated with 50 µL of diluted streptavidin horseradish peroxidase (#554066, eBioscience, 1:2000) for 30 min at room temperature. Detection was performed with 100 µL 3,3′,5,5′-tetramethylbenzidine (Carl Roth Chemikalien, Karlsruhe, Germany) and incubation for 3 to 5 min at room temperature. The reaction was stopped with 50 µL per well of 1 M sulfuric acid (Carl Roth Laborbedarf, Karlsruhe, Germany). Optical density was measured at 450 nm by SpectraMAX340PC (Molecular Devices, CA, USA). Levels of IL-10, CCL2, CCL20, and PGE_2 were measured according to the manufacturer's recommendations using either the Murine IL-10 standard ABTS ELISA development kit (Peprotech, Hamburg, Germany), mouse CCL2 ELISA development kit from Invitrogen (#900-T53), CCL20/MIP-3 alpha DuoSet (#DY760, R&D Systems, Wiesbaden-Nordenstadt, Germany), or the PGE_2 ELISA kit (#KA4522, Abnova, Taipei, Taiwan), respectively.

2.5. Inhibitors

LA-4 cells were pre-incubated with the indicated amounts of either the mTOR inhibitor rapamycin (Invivogen, Toulouse, France), MAPK-inhibitors U0126 (MEK1/2 MAPK inhibitor, Cell Signaling Technologies, Leiden, The Netherlands), SP600125 (SAP/JNK MAPK inhibitor, Invivogen), SB-202190 (p38α/β MAPK inhibitor, Invivogen), the NFκB- and MAPK-inhibitor dexamethasone (Invivogen), the IKK-β-inhibitor TPCA-1 (Abcam, Berlin, Germany), the inhibitor of actin polymerization cytochalasin A (Sigma-Aldrich), the inhibitor of endosomal acidification chloroquine (InvivoGen), or the COX2-inhibitor NS-398 (Sigma-Aldrich, Steinheim, Germany) for 90 min and subsequently stimulated with rFlaA:Betv1 for 24 h. For analyzing cell viability, LA-4 cells were treated as indicated and stained for dead cells using the fixable viability dye eFlour450 (#65-0865-14, eBioscience) and measured by FACS. Data were analyzed using FlowJo V.7 (Treestar Inc., Ashland, OR, USA) and GraphPad PRISM (GraphPad Software, San Diego, CA, USA).

2.6. Western Blot

For Western blot experiments, LA-4 cells were seeded overnight at 1.66×10^5 cells/2 mL in 6-well plates (Thermo Scientific) with culture medium. On the next morning, cells were cultured for 3 h at 37 °C, 5% CO_2 in DMEM supplemented with 2% FCS (Sigma-Aldrich) for starvation, and subsequently, stimulated with the indicated proteins in DMEM for 30 min, washed with ice-cold PBS, and lysed with 200 µL lysis buffer (62.5 mM Tris-HCl (pH 6.8), 2% *w/v* SDS, 10% glycerol, 50 mM DTT, 0.01% *w/v* bromophenol blue) for 10 min on ice. Target proteins in lysates were separated by SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes. After blocking with 5% non-fat milk, the

membranes were incubated with the following primary antibodies from Cell Signaling Technologies overnight at 4 °C: phospho-MAPK family antibody sampler kit (#9910), NF-κB pathway sampler kit (#9936), mTOR Substrates Antibody Sampler Kit (#9862) and loading control anti-H3 antibody (#12648, HRP Conjugate). Detection was performed with the provided secondary antibodies using immobilon crescendo Western HRP substrate (#WBLUR0500, Merck, Darmstadt, Germany), and images were captured with either a Fusion-Fx7 spectra reader (Vilber Lourmat, Eberhardzell, Germany) or iBright™ CL1500 system (Thermo Fischer Scientific). Band intensities in Western blots were quantified with ImageJ software (imagej.nih.gov, version: 1.52a, accessed on 1 December 2021) as relative light unit (RLU) normalized to histone H3 loading control.

2.7. Fluorescence Labeling, Uptake, and Microscopy

Recombinant proteins were labeled with the Alexa Flour 488 microscale protein labeling kit (Invitrogen) according to the manufacturer's recommendations. Comparable degrees of fluorescence staining were confirmed by determining the protein concentration based on absorbance at 280 and 494 nm with a NanoDrop ND-1000 (NanoDrop Technologies, Rockland, Del, USA) according to the manufacturer's recommendations and calculating the degree of labeling (DOL) with the following formula:

$$\text{DOL} = (A_{494} \times \text{Dilution factor})/(71{,}000 \times \text{Protein concentration [M]})$$

Protein concentrations after Alexa Flour 488 labeling was confirmed by BCA (micro BCA protein assay kit; Pierce, Rockford, IL, USA). Subsequently, 6.25×10^4 LA-4 cells/mL were stimulated with equimolar amounts of the labeled proteins (rBetv1, rFlaA, rFlaA plus rBetv1, or rFlaA:Betv1) for 15 min at 37 °C either with or without 90 min of pre-stimulation with chloroquine or cytochalasin A and extensively washed with FACS buffer (PBS, 1% BSA, 0.3% sodium azide, and 24 mmol/L EDTA, pH 8.0). Unspecific binding was blocked using incubation of the cells with Fc-Block (eBioscience) for 30 min and then analyzed for protein uptake in LA-4 cells by means of flow cytometry.

For microscopy analysis, LA-4 cells were seeded overnight at 6.25×10^4 cells/mL in 24-well plates. The next day cells were pre-incubated with either cytochalasin A (1 µg/mL) or chloroquine (10 µM) for 90 min and subsequently stimulated with Alexa Fluor 488 labeled proteins for 15 min in DMEM supplemented with 2% FCS. Cells were then washed three times with PBS and fixed for 10 min at room temperature with 4% (*w/v*) paraformaldehyde solution (Thermo Scientific, Dreieich, Germany). After fixation, cells were washed three times with PBS, and stained with 2 µg/mL of the nuclear marker 4′,6-diamidino-2-phenylindole, dilactate (DAPI) (Thermo Scientific) for 5 min and subsequently washed three times with PBS. The uptake of the Alexa Fluor 488 labeled proteins was visualized under a BZ-X800 fluorescence microscope (Keyence, Neu-Isenburg, Germany) at 26× magnification, the addition of the scale bars and merging of figures were achieved using a BZ-X800 analyzer (Keyence, Neu-Isenburg, Germany).

2.8. Mice

BALB/c mice (Jackson Laboratories, Bar Harbor, ME, USA) were bred at the animal facility of the Paul-Ehrlich-Institut under specific pathogen-free conditions.

2.9. Preparation of LA-4:DC Co-Cultures

mDCs were generated from BALB/c bone marrow as described previously [19]. mDC preparations were shown to be free of $CD49b^+$ NK cells, $CD3^+$ T cells, and $CD19^+$ B cells (data not shown). BALB/c mDCs (5×10^5 cells/well) were cultured with either (I) LA-4 cells (6.25×10^4 cells/well) alone, (II) 1, 10, or 100 µL of supernatant derived from, respectively, stimulated LA-4 cells, or (III) LA-4 cells plus supernatants in 24-well plates (see above). For lung DC isolation, BALB/c mice were killed by CO_2 and cardiac perfusion was performed by injecting 20 mL PBS before harvesting the lung tissue. Lungs were digested in a DNase/collagenase (Sigma-Aldrich) solution, and DCs were isolated

using CD11c (N418) magnetic microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany). $CD11c^+$ lung DCs were either cultured alone (1×10^5 cells/well), or co-cultured with LA-4 cells (3.125×10^4 cells/well) in 48-well plates. Cultures were then stimulated with equimolar amounts of either rFlaA + rBetv1 or rFlaA:Betv1 for 24 h. Subsequently, cytokine secretion into culture supernatants was determined by ELISA (see Section 2.4 above).

2.10. Statistical Analysis

Statistical analysis was performed with GraphPad Prism v6 to v8 for Mac or Windows using 2-way ANOVA tests with confidence intervals adjusted for multiple comparisons according to either Bonferroni or Turkey. For statistically significant results the following convention was used: *—p-value < 0.05, **—p-value < 0.01, ***—p-value < 0.001.

3. Results

3.1. rFlaA:Betv1 Induces Chemokine and IL-6 Secretion from LA-4 Epithelial Cells

Initially, LA-4 cells were characterized for their expression of typical epithelial cell surface markers as well as for their expression levels of TLR5 and MHC I (Supplementary Figure S1). LA-4 cells expressed the type II alveolar epithelial markers EpCAM and CEACAM 1, as well as MHC I and TLR5, while only expressing low levels of both mucin 1 and pancytokeratin (Supplementary Figure S1).

In the first set of experiments, LA-4 cells were stimulated with equimolar amounts of either the mixture of rFlaA + rBetv1 or the fusion protein rFlaA:Betv1 for 2, 4, or 24 h, and checked for cytokine and chemokine secretion (Figure 1A).

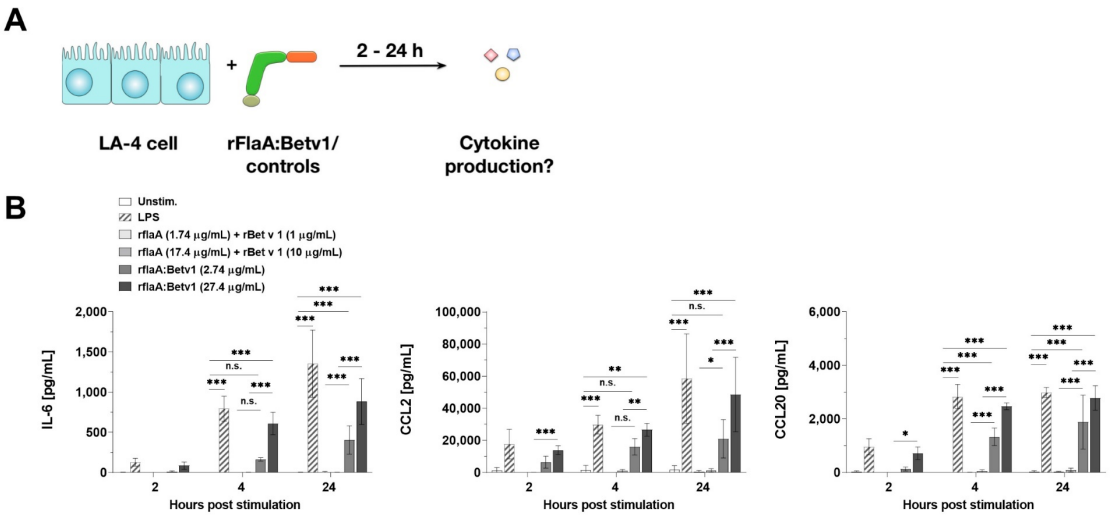

Figure 1. rFlaA:Betv1 induces chemokine and IL-6 secretion from LA-4 epithelial cells. LA-4 cells were stimulated with LPS as a positive control or the indicated equimolar amounts of either rFlaA + rBetv1 or rFlaA:Betv1 for 2, 4, or 24 h (**A**). Supernatants were collected after the indicated time points and checked for the secretion of IL-6, CCL2, and CCL20 by ELISA (**B**). Data are mean results ± SD from three independent experiments with two technical replicates per experiment. Statistical significance indicated as: n.s.: p-value > 0.05, * p-value < 0.05, ** p-value < 0.01, *** p-value < 0.001.

Compared to the mixture of both proteins, rFlaA:Betv1 induced a significantly increased secretion of the pro-inflammatory cytokine IL-6 as well as the chemokines CCL2 and CCL20 (for the stimulation concentration of 27.4 µg/mL rFlaA:Betv1 induced a 562-fold higher secretion of IL-6, a 37-fold higher secretion of CCL2, and a 30-fold higher secretion of CCL2 24 h post-stimulation) (Figure 1B). IL-6 secretion was detected as early as 4 h post-stimulation, while chemokine secretion was already detected 2 h post-stimulation (Figure 1B).

In this experimental setting no secretion of either GM-CSF, G-CSF, IL-33, TSLP, IL-10, TNF-α, IL-12, or IL-1β was detected from stimulated LA-4 cells (data not shown).

To exclude that the minute amounts of residual LPS contained within the used protein preparations may be responsible for the observed LA-4 activation, both LPS titration curves and stimulations of LA-4 cells were performed applying the amounts of endotoxin contained within the used stimulation concentrations (Supplementary Figure S2). Here, initial LPS-mediated LA-4 cell activation was observed with LPS concentrations around 10 ng/mL (Supplementary Figure S2) which is far above the low pg amounts contained within our protein preparations. Moreover, stimulation of LA-4 cells with the endotoxin amounts contained within the used rFlaA:Betv1 concentrations did not result in secretion of any of the investigated cytokines (Supplementary Figure S2).

Moreover, as previously reported, both rFlaA and rFlaA:Betv1, but not rBetv1, were shown to dose-dependently activate TLR5-expressing HEK293 reporter cells ([22]; data not shown). In addition, rFlaA:Betv1 (or potential contaminations contained within in the preparation) did neither activate TLR2- nor TLR4-expressing HEK293 reporter cells ([35]; data not shown).

3.2. rFlaA:Betv1-Mediated LA-4 Cell Activation Is TLR5-Independent

To address whether the observed LA-4 activation by the fusion protein is TLR5-dependent, we generated a mutant fusion protein that harbors a modified sequence stretch of 8 amino acids (QRMRQLAV on position 87 to 94 replaced with DTVKVKAT) in the flagellin D1 domain (rFlaA^{*D1}:Betv1). This sequence alteration was recently described to abolish TLR5 activation [34]. Indeed, both rFlaA^{*D1} and rFlaA^{*D1}:Betv1 were unable to activate TLR5-expressing HEK293 reporter cells (Figure 2A).

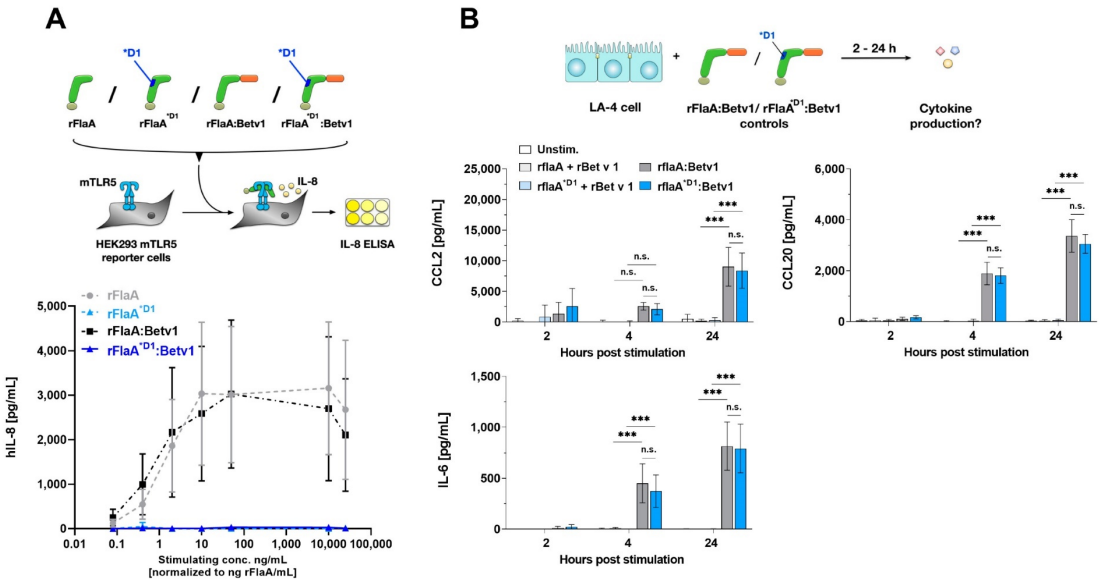

Figure 2. rFlaA:Betv1-mediated LA-4 activation is TLR5-independent. To investigate the TLR5-dependency of rFlaA:Betv1-mediated LA-4 activation, a mutant fusion protein lacking a described TLR5-activation motif was generated (rFlaA^{*D1}:Betv1). Wildtype and mutant rFlaA and rFlaA:Betv1 were tested for their capacity to activate TLR5-expressing HEK293 reporter cells (**A**). LA-4 cells were stimulated with protein amounts of both wildtype and mutant rFlaA + rBetv1 and rFlaA:Betv1 equimolar to 10 μg/mL rBetv1 and checked for chemokine and cytokine secretion after the indicated time points post-stimulation (**B**). Data are mean results of three independent experiments ± SD with two technical replicates per experiment. Statistical significance indicated as: n.s. p-value > 0.05, *** p-value < 0.001.

When LA-4 cells were stimulated with either the wild-type or mutant proteins, we observed no difference in CCL2, CCL20, or IL-6 secretion (Figure 2B), indicating the observed LA-4 activation by rFlaA:Betv1 to be TLR5-independent.

3.3. *rFlaA:Betv1 Is Taken up More Strongly by LA-4 Cells*

Since the activation of LA-4 cells by rFlaA:Betv1 was shown to be TLR5 independent (Figure 2), we checked if an increased uptake of the fusion protein compared to the equimolar mixture of both single proteins might account for the observed stronger cell activation (Figure 3). For this, we labeled the fusion protein and the respective controls with Alexa Fluor 488 (achieving comparable degrees of labeling) and checked for the uptake of the different proteins into LA-4 cells by either flow cytometry or microscopy (Figure 3A). Here, flow cytometry showed the fusion protein to be taken up with a higher frequency than the equimolar mixture of both proteins (Figure 3B). These results were confirmed by microscopy analysis where we observed extensive uptake of the fusion protein but not the respective controls into LA-4 cells (Figure 3C).

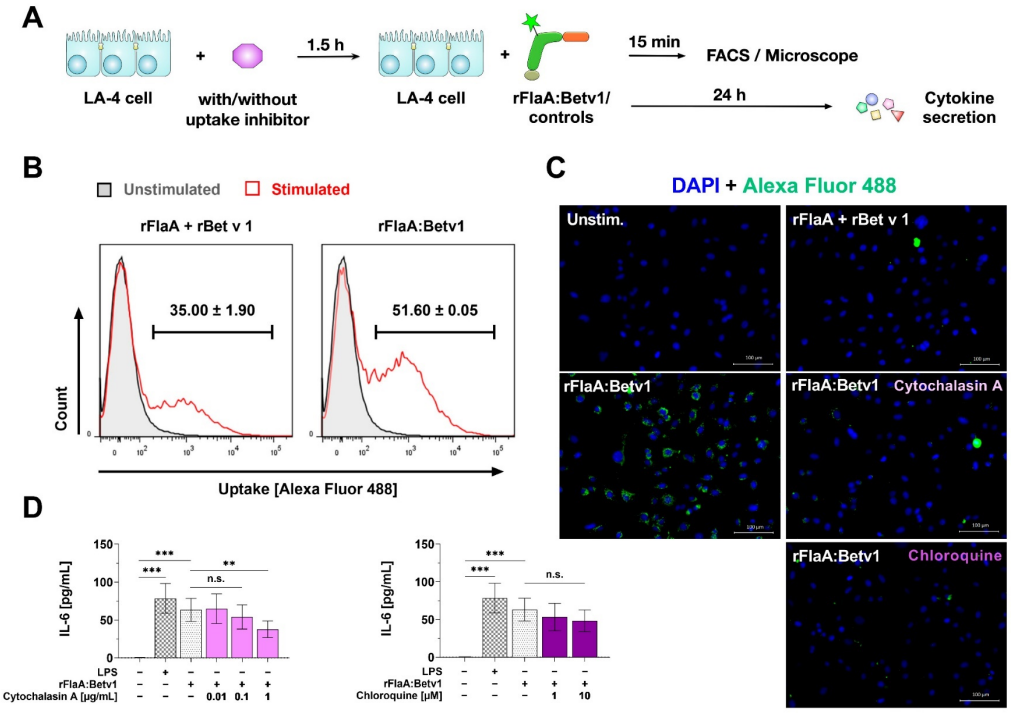

Figure 3. rFlaA:Betv1 is taken up more strongly than the mixture of both single proteins. To investigate the uptake of rFlaA:Betv1, LA-4 cells were stimulated with Alexa Fluor 488 labeled proteins (**A**) and checked for their uptake by either flow cytometry (**B**) or fluorescence microscopy (**C**). In addition, cells were pre-incubated for 90 min with uptake inhibitors and stimulated with the fusion protein for an additional 24 h. The effect of both inhibitors on IL-6 secretion was analyzed 24 h post-stimulation by ELISA (**D**). Data are either representative (**B**,**C**) or mean (**D**) results of three independent experiments ± SD with two technical replicates per experiment. Statistical significance indicated as: n.s.: no statistically significant difference, p-value > 0.05, ** p-value < 0.01, *** p-value < 0.001.

Pre-treatment of LA-4 cells with non-toxic concentrations (Supplementary Figure S3A,B) of the inhibitor of actin polymerization cytochalasin A and the inhibitor of endosomal acidification chloroquine strongly inhibited rFlaA:Betv1 uptake (Figure 3C) and dose-dependently (albeit not significantly for chloroquine) reduced rFlaA:Betv1-induced IL-6 secretion (Figure 3D).

In this experimental setting, neither cytochalasin A nor chloroquine affected CCL2 or CCL20 secretion (Supplementary Figure S3C). Controls for the microscopy analysis are shown in Supplementary Figure S4.

3.4. rFlaA:Betv1 Induces MAPK- and NFκB-Phosphorylation in LA-4 Epithelial Cells

To further analyze the intracellular signaling pathways contributing to the observed activation of LA-4 epithelial cells by rFlaA:Betv1, LA-4 cells were stimulated with either LPS, rFlaA, rBetv1, rFlaA + rBetv1, or rFlaA:Betv1 for 30 min, lysed, and analyzed by Western blot (Figure 4A).

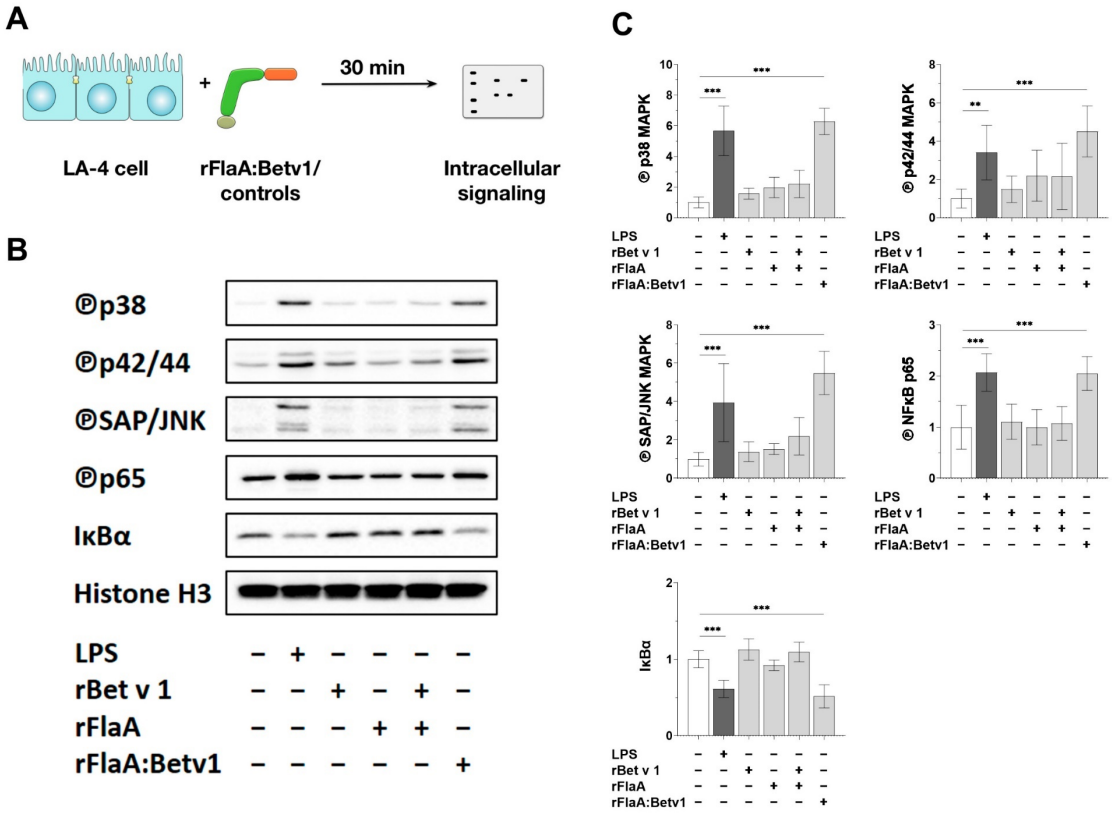

Figure 4. rFlaA:Betv1 induces MAPK- and NFκB-phosphorylation in LA-4 epithelial cells. LA-4 cells were stimulated with either LPS as a positive control, rFlaA, rBetv1, rFlaA + rBetv1, or rFlaA:Betv1 (all equimolar to 10 µg of rBetv1) for 30 min (**A**). Cells were lysed and analyzed by Western blot for the expression levels of the indicated proteins (**B**). The intensity of the Western blot bands from three independent experiments was analyzed and normalized to the expression levels of the loading control histone H3 (**C**). Data are either representative (**B**) or mean (**C**) results from three independent experiments with one lysate generated per experiment. Statistical significance indicated as: ** *p*-value < 0.01, *** *p*-value < 0.001.

Both LPS and rFlaA:Betv1 induced a pronounced phosphorylation of all investigated MAP kinases (p38 MAPK, p42/44 MAPK (also called ERK1/2), and SAP/JNK MAPK) (Figure 4B,C). Moreover, the NFκB subunit p65 was phosphorylated while IκBα levels were strongly reduced (Figure 4B,C), concordant with an activation of NFκB signaling. In contrast, at equimolar concentrations, the controls rFlaA, rBetv1, and rFlaA + rBetv1 showed no changes in the levels of investigated proteins (Figure 4B,C).

Our previous results from myeloid dendritic cells (mDCs) showed rFlaA:Betv1 to induce both mammalian target of rapamycin (mTOR)-dependent metabolic changes and IL-

10 secretion [22]. Therefore, we also checked if mTOR-signaling is activated by rFlaA:Betv1 in epithelial cells. However, there was no pronounced difference in the phosphorylation levels of the mTOR target protein p70 S6 kinase in the different stimulation groups compared to unstimulated controls (Supplementary Figure S5). Moreover, the mTOR inhibitor rapamycin had no significant effect on rFlaA:Betv1-induced cytokine and chemokine secretion (Supplementary Figure S6).

3.5. MAP Kinase-Signaling Contributes to Both rFlaA:Betv1-Induced Pro-Inflammatory IL-6 and CCL20 Secretion from LA-4 Cells

To further dissect the contribution of MAPK signaling to the observed IL-6, CCL2, and CCL20 secretion, LA-4 cells were dose-dependently pre-treated with inhibitors of either SAP/JNK MAPK (SP600125), p42/44 MAPK (U0126), or p38 MAPK (SB202190) for 90 min, stimulated with rFlaA:Betv1 for 24 h, and subsequently checked for cytokine and chemokine secretion into the culture supernatants (Figure 5A). Toxic effects of the investigated MAPK inhibitor concentrations on the LA-4 cells were excluded by live/dead staining (Supplementary Figure S7).

All tested MAPK-inhibitors dose-dependently and (except for SP600125) significantly inhibited rFlaA:Betv1-induced IL-6 secretion (Figure 5B). In contrast, CCL2-secretion was unaffected by MAPK inhibition, and CCL20 secretion was only inhibited by high concentrations of U0126 and SB202190, but not by SP600125 (Figure 5B).

3.6. NFκB-Signaling Contributes to Both rFlaA:Betv1-Induced Pro-Inflammatory IL-6 and CCL2/CCL20 Chemokine Secretion in LA-4 Cells

The contribution of NFκB-signaling to rFlaA:Betv1-mediated cytokine and chemokine secretion was analyzed by pre-treatment of LA-4 cells with either the NFκB-inhibitor dexamethasone or the IκBα-inhibitor TPCA-1 (Figure 6A). Toxic effects of the applied inhibitor concentrations on the LA-4 cells were excluded by live/dead staining (Figure S8).

Dexamethasone dose-dependently and significantly inhibited rFlaA:Betv1-induced IL-6 (by 95% in the highest inhibitor concentration), CCL2- (by 82%) and CCL20-secretion (by 64%, Figure 6B, left panels), respectively. Similarly, the IκBα-inhibitor TPCA-1 dose-dependently inhibited rFlaA:Betv1-induced IL-6 (by 85% in the highest inhibitor concentration), CCL2- (by 71%) and to a lower extend CCL20-secretion (by 38%, Figure 6B, middle panels).

3.7. In Co-Cultures with BALB/c mDCs LA-4 Cells Modulate mDC-Derived, rFlaA:Betv1-Induced Cytokine Secretion

To investigate the functional consequences of rFlaA:Betv1-mediated epithelial cell activation, we checked for the effects of rFlaA:Betv1-stimulated LA-4 cells on mDCs. For this, BALB/c mDCs or LA-4 cells were either cultured alone or as a co-culture, stimulated with rFlaA:Betv1 or their respective controls, and checked for their respective cytokine- and chemokine-secretion (Figure 7A).

Compared to LA-4 cells, mDCs produced significantly higher levels of IL-6 upon stimulation with either rFlaA + rBetv1 or rFlaA:Betv1 in all tested dose ranges (Figure 7B). Here, IL-6 secretion was not significantly different between mDCs cultured alone or together with LA-4 cells (Figure 7B). Moreover, levels of either IL-1β- and IL-10-secretion induced by rFlaA:Betv1 did not differ between mDCs cultured alone or in the presence of LA-4 cells, while LA-4 cells did not produce these cytokines upon stimulation with the tested proteins (Figure 7B).

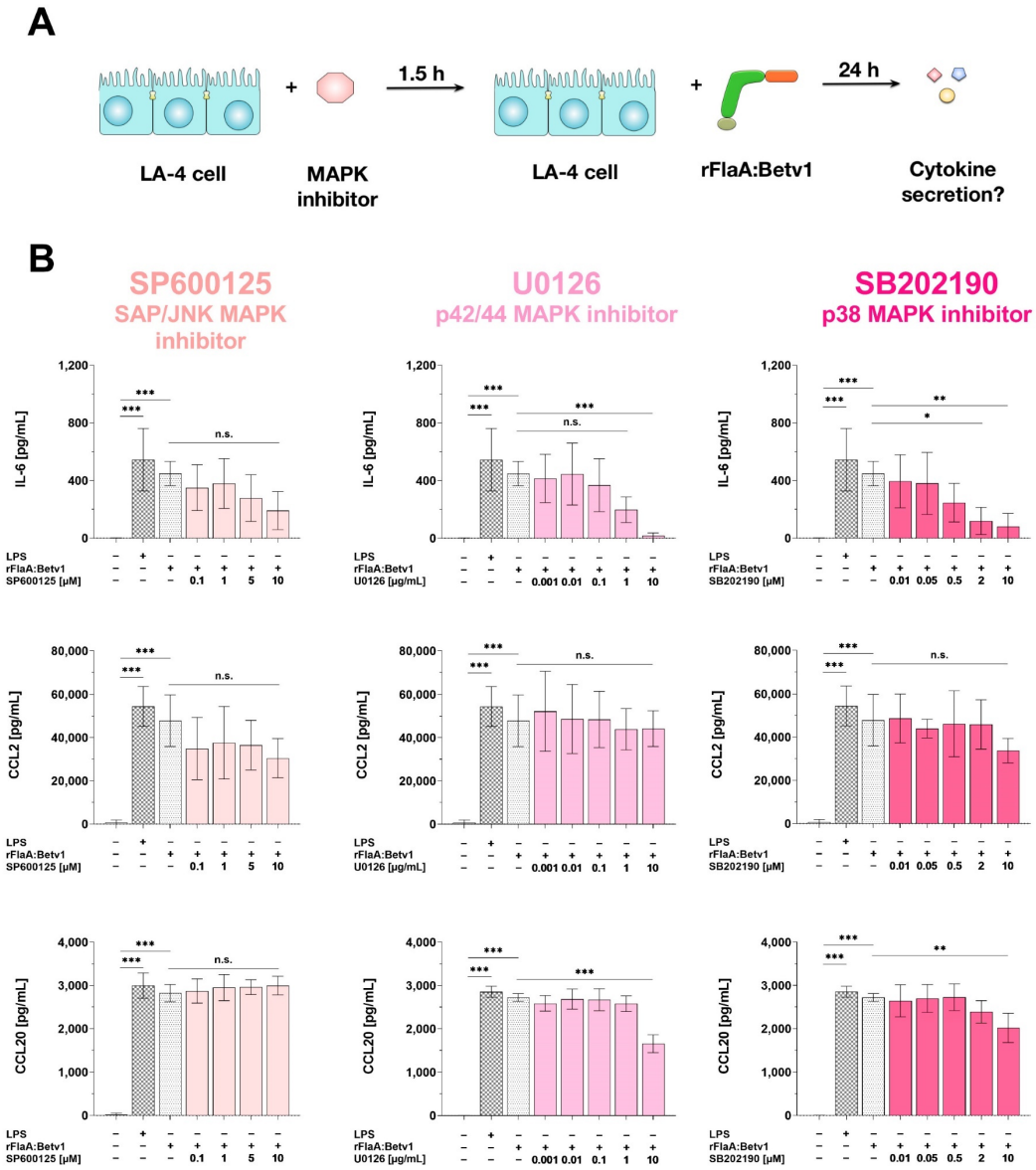

Figure 5. MAPK-signaling contributes to both rFlaA:Betv1-induced pro-inflammatory IL-6 cytokine and chemoattractant CCL20 secretion from LA-4 cells. LA-4 cells were pre-treated with the indicated inhibitor concentrations for 90 min and subsequently stimulated with 27.4 µg/mL rFlaA:Betv1 for 24 h (**A**). Supernatants were collected and checked for the secretion of IL-6, CCL2, and CCL20 by ELISA (**B**). Data are mean results ± SD from three independent experiments with two technical replicates per experiment. Statistical significance indicated as: n.s.: no statistically significant difference, p-value > 0.05, * p-value < 0.05, ** p-value < 0.01, *** p-value < 0.001.

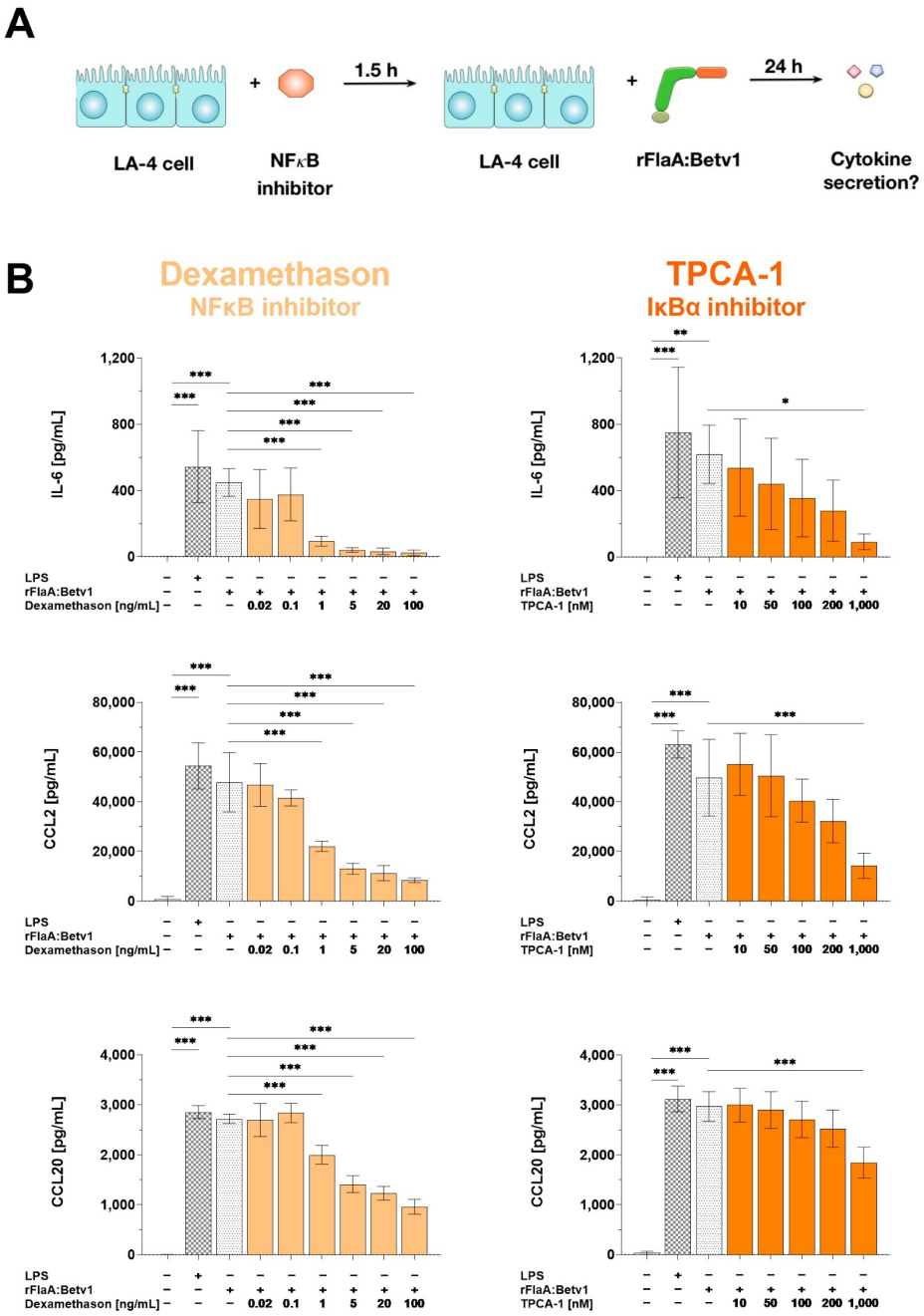

Figure 6. NFκB-signaling contributes to both rFlaA:Betv1-induced pro-inflammatory IL-6 cytokine and CCL2/CCL20 chemokine secretion from LA-4 cells. LA-4 cells were pre-treated with the indicated inhibitor concentrations for 90 min and subsequently stimulated with 27.4 µg/mL rFlaA:Betv1 for 24 h (**A**). Supernatants were collected and checked for the secretion of IL-6, CCL2, and CCL20 by ELISA (**B**). Data are mean results ± SD from three independent experiments with two technical replicates per experiment. Statistical significance indicated as: * p-value < 0.05, ** p-value < 0.01, *** p-value < 0.001.

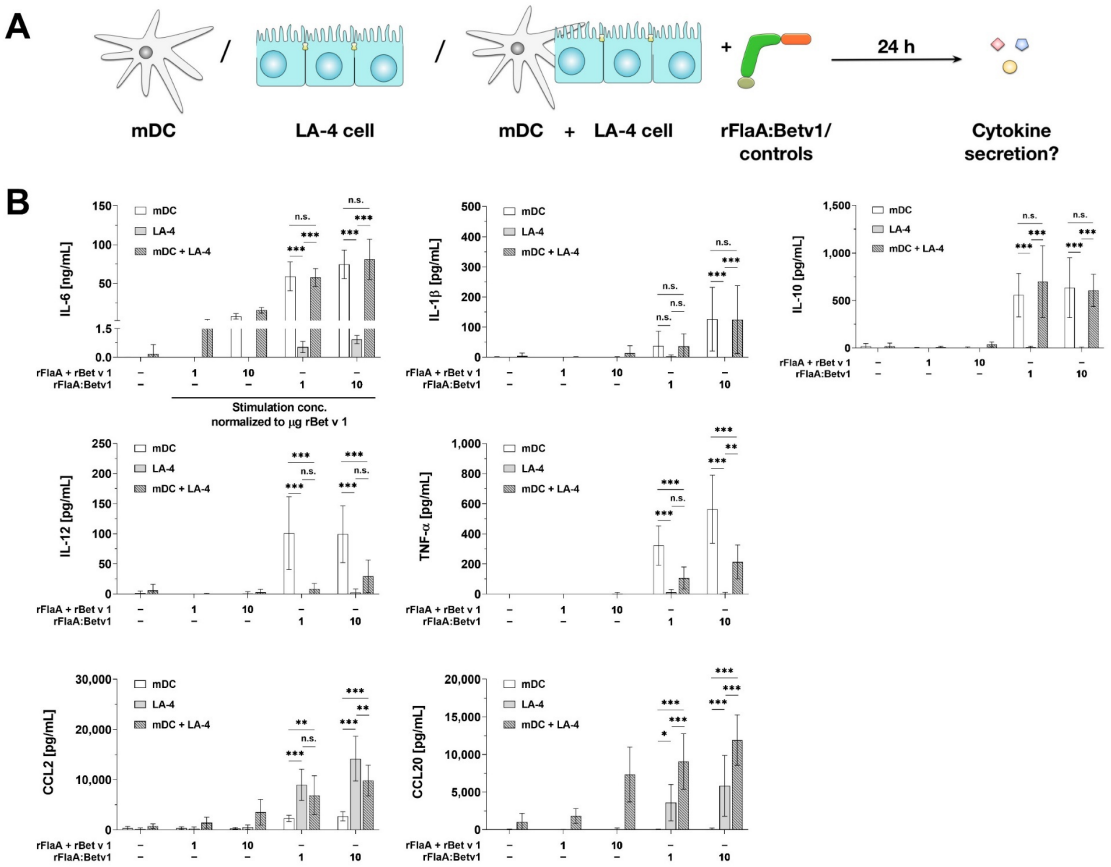

Figure 7. LA-4 cells modulate rFlaA:Betv1-induced cytokine secretion from BALB/c mDCs. BALB/c mDCs and LA-4 cells were cultured either alone or together and stimulated with the indicated equimolar amounts of either rFlaA + rBetv1 or rFlaA:Betv1 (**A**). Cultures were checked for LA-4- and mDC-derived cytokine secretion after 24 h by ELISA (**B**). Data are mean results ± SD from four independent experiments with two technical replicates per experiment. Statistical significance indicated as: n.s.: no statistically significant difference, p-value > 0.05, * p-value < 0.05, ** p-value < 0.01, *** p-value < 0.001.

Secretion of the cytokines IL-12 and TNF-α was exclusively observed from mDCs stimulated with rFlaA:Betv1 (Figure 7B). Interestingly, co-culture of mDCs with LA-4 cells resulted in highly significant reductions in the secretion of both cytokines compared to mDCs stimulated alone (IL-12 reduction by 70% to 92%, TNF-α reduction by 62% to 67%, Figure 7B).

In contrast, the chemokines CCL2 and CCL20 were produced in higher quantities by LA-4 cells (Figure 7B), while mDCs produced a maximum of 2 ng of CCL2 after stimulation with rFlaA:Betv1 and no CCL20 secretion was detected (Figure 7B). While CCL20 levels were consistently higher in co-cultures of mDCs and LA-4 cells compared to LA-4 cells alone, CCL2 levels were slightly decreased upon co-culture of both cell types (Figure 7B).

3.8. Epithelial Cell-Derived Soluble Factors Modulate mDC-Responses to rFlaA:Betv1

We were interested, if the immune-modulating properties of rFlaA:Betv1-stimulated LA-4 cells on mDCs (reduction in IL-12 and TNF-α secretion, see Figure 7B) depend on secreted factors rather than cell-cell contact-dependent mechanisms. Therefore, we incubated mDCs with either 1, 10, or 100 µL of supernatant derived from LA-4 cells that

were either unstimulated or stimulated with rFlaA:Betv1. Afterwards, we re-stimulated the mDCs with rFlaA:Betv1 and checked for the effects of LA-4 supernatants on mDC-derived cytokine secretion (Figure 8A).

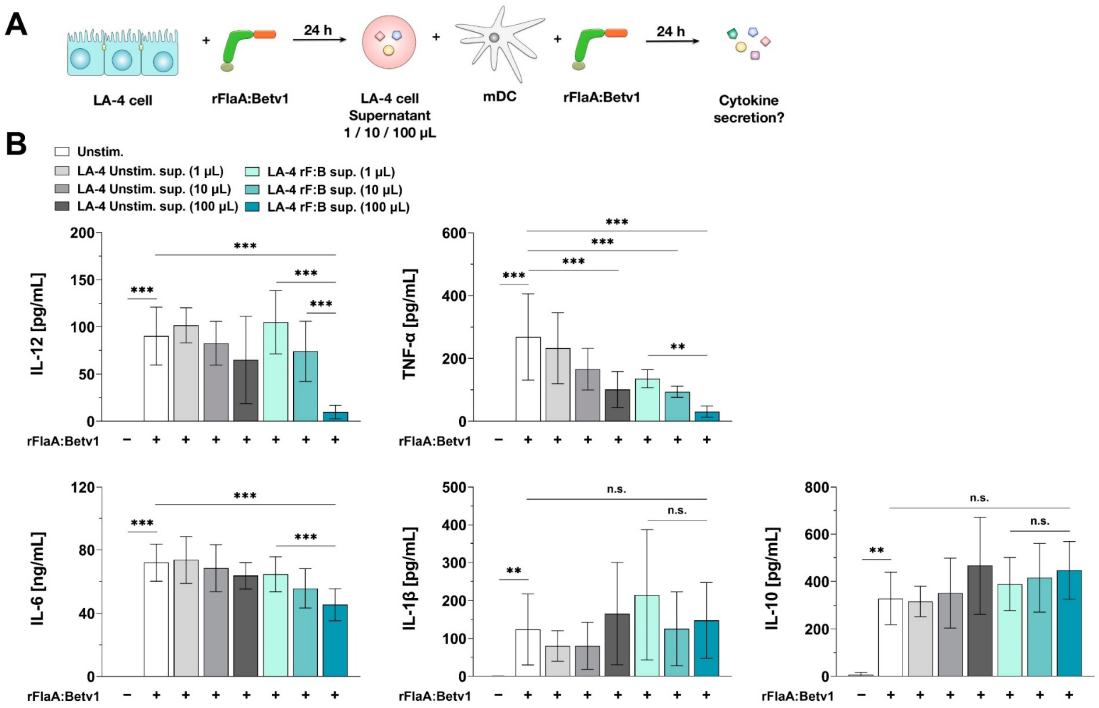

Figure 8. Epithelial cell-derived soluble factors modulate mDC responses to rFlaA:Betv1. BALB/c mDCs stimulated with 27.4 µg/mL rFlaA:Betv1 were incubated for 24 h with 1, 10, or 100 µL of supernatant derived from either unstimulated (LA-4 unstim. sup.) or rFlaA:Betv1-stimulated LA-4 cells (LA-4 rF:B sup.) (**A**). Subsequently, supernatants were collected and checked for the secretion of TNF-α, IL-12, IL-6, IL-1β, and IL-10 by ELISA (**B**). Data are mean results ± SD from three independent experiments with two technical replicates per experiment. Statistical significance indicated as: n.s.: no statistically significant difference, p-value > 0.05, ** p-value < 0.01, *** p-value < 0.001.

Supernatant derived from unstimulated LA-4 cells slightly, but not statistically significantly, suppressed rFlaA:Betv1-induced secretion of both IL-12 and IL-6 from mDC cultures, while having no effect on rFlaA:Betv1-induced IL-1β secretion (Figure 8B). In contrast, supernatants of unstimulated LA-4 cells dose-dependently and significantly suppressed rFlaA:Betv1-induced TNF-α secretion (62% reduction for the addition of 100 µL supernatant compared to mDC cultures stimulated with rFlaA:Betv1 in the absence of supernatant) (Figure 8B).

Interestingly, supernatants obtained from rFlaA:Betv1-stimulated LA-4 cells significantly and dose-dependently suppressed rFlaA:Betv1-induced secretion of either IL-6 (by 42%), IL-12 (by 91%), or TNF-α (by 88%) compared to mDC cultures stimulated with rFlaA:Betv1 in the absence of supernatant (Figure 8B). In accordance with the results obtained from mDC:LA-4 co-culture experiments (Figure 7B), LA-4-derived supernatants had no effect on rFlaA:Betv1-induced IL-1β and IL-10 secretion (Figure 8B). These results demonstrate the modulation of mDC responses by rFlaA:Betv1-stimulated LA-4 cells to depend on secreted factors rather than requiring direct cell–cell contact.

3.9. The Modulation of mDC Responses by rFlaA:Betv1-Stimulated LA-4 Cells Is Dependent on the p38-MAPK-Signaling Pathway

To further investigate the intracellular signaling pathways involved in the production of DC-modulating factors by rFlaA:Betv1-stimulated LA-4 cells, mDCs were treated with supernatants from rFlaA:Betv1-stimulated LA-4 cells that had also been pre-treated with the MAPK- or NFκB-inhibitors previously investigated in Figures 5 and 6 (Figure 9A).

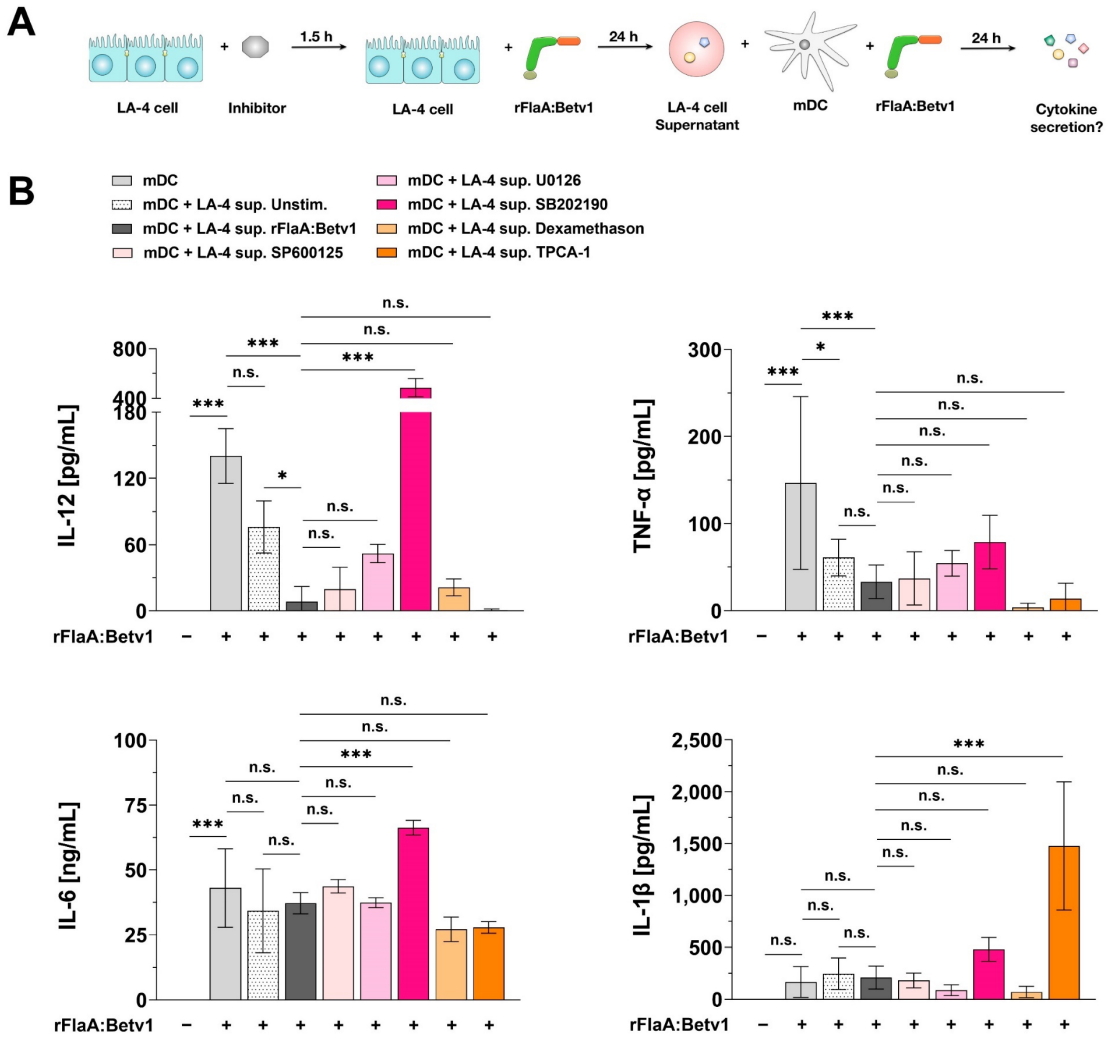

Figure 9. The modulation of mDC responses by rFlaA:Betv1-stimulated LA-4 cells depends on the p38 MAPK-signaling pathway. BALB/c mDCs were either cultured alone or together in the presence or absence of rFlaA:Betv1-re-stimulation. In addition, mDCs were incubated with 100 µL of supernatant (sup.) derived from: (I) unstimulated LA-4 cells (+LA-4 sup. Unstim.), (II) rFlaA:Betv1-stimulated LA-4 cells (+LA-4 sup. rFlaA:Betv1), or (III) LA-4 cells that were pre-treated with the indicated inhibitors for 1.5 h followed by stimulation with 27.4 µg/mL rFlaA:Betv1 for 24 h (+LA-4 sup. (inhibitor name)) (**A**). Supernatants were collected and checked for the secretion of TNF-α, IL-12, IL-6, and IL-1β by ELISA (**B**). Data are mean results ± SD from three independent experiments with two technical replicates per experiment. Statistical significance indicated as: n.s.: no statistically significant difference, *p*-value > 0.05, * *p*-value < 0.05, *** *p*-value < 0.001.

Among the tested supernatants, LA-4 cell-derived supernatants pre-treated with the p38 MAPK-inhibitor SB202190 lost their capacity to suppress mDC-derived cytokine secretion in terms of TNF-α, IL-6, and IL-12 (58.8-fold higher IL-12, 2.4-fold higher TNF-α, and 1.8-fold higher IL-6 levels in mDC + SB202190 sup. vs. rFlaA:Betv1 sup.) (Figure 9B).

In addition to p38 MAPK inhibition, p42/44 MAPK-inhibition by U0126 pre-treatment also slightly reversed the inhibitory effect of rFlaA:Betv1-stimulated LA-4 supernatants on mDC IL-12 production (6.3-fold higher IL-12 levels in mDC + SB202190 sup. vs. rFlaA:Betv1 sup.) (Figure 9B). In this experimental system, neither inhibition of SAP/JNK MAPK by SP600125, NFκB by dexamethasone, nor IκBα-inhibition by TPCA-1 had a significant effect on the suppression of the tested, mDC-derived cytokines (Figure 9B).

Moreover, mDC-derived, rFlaA:Betv1-induced IL-1β secretion was unaffected by supernatants derived from LA-4 cells treated with either rFlaA:Betv1 or pre-treated with MAPK inhibitors or the NFκB inhibitor dexamethasone (Figure 9B). Here, only IκBα-inhibition by TPCA-1 pre-treatment resulted in strongly increased IL-1β production (Figure 9B).

3.10. Modulation of mDC Responses by rFlaA:Betv1-Stimulated LA-4 Cells Is Dependent on the COX2/PGE_2 Pathway

Prostaglandin E2 (PGE_2) is a naturally occurring lipid mediator generated from arachidonic acid. This reaction is catalyzed by either the cyclooxygenase-1 or -2 (COX1/2) enzymes [36]. As an inflammatory mediator, PGE_2 is also known to influence DC activation, migration, and stimulatory capacity [37].

Previously, epithelial cells were shown to secrete PGE_2 COX2-dependently upon LPS stimulation [38,39]. Moreover, both PGE_2 release and COX2 expression were reported to be reduced by the p38-MAPK inhibitor SB202190 upon *Streptococcus pneumoniae* infection in human lung epithelium [40]. Interestingly, PGE_2 was shown to suppress TLR2- and TLR4-induced IL-12 and TNF-α secretion from both DCs and macrophages in vitro [39,41,42].

Therefore, we next analyzed the contribution of the p38-MAPK/COX2/PGE_2 axis to the DC modulating properties of rFlaA:Betv1-stimulated LA-4 cells. We first analyzed if rFlaA:Betv1 could induce p38-MAPK/COX2 dependent PGE_2 production from LA-4 cells (Figure 10A). Results showed that rFlaA:Betv1 induced a higher PGE_2 secretion compared to either the unstimulated control (13.4 fold) or the mixture of rFlaA + rBetv1 (7.4 fold) (Figure 10B). Furthermore, both the p38-MAPK inhibitor SB202190 and the COX2 inhibitor NS-398 dose-dependently and significantly inhibited rFlaA:Betv1-induced PGE_2 release (by 98.5% and 99.2% in the highest inhibitor concentration, respectively) (Figure 10C). We next analyzed the contribution of COX2 to rFlaA:Betv1-induced cytokine and chemokine secretion from LA-4 cells. For this, we pre-treated LA-4 cells with the COX2-inhibitor NS-398 for 90 min, subsequently stimulated with rFlaA:Betv1 for 24 h, and checked for cytokine and chemokine secretion in the supernatant (Supplementary Figure S9A). Toxic effects of the used NS-398 concentrations on LA-4 cells were excluded by live/dead staining (Supplementary Figure S9B). Here, NS-398 dose-dependently inhibited rFlaA:Betv1-induced IL-6 secretion by approx. 30% (Supplementary Figure S9C), but had no effect on chemokine production (Supplementary Figure S9D).

Finally, we incubated mDCs with 100 µL of supernatant derived from LA-4 cells which were pre-treated with increasing concentrations of NS-398, and then checked for mDC-derived cytokine secretion upon re-stimulation with rFlaA:Betv1 (Figure 10D).

Suppression of COX2 in LA-4 was accompanied by an increase of cytokine (IL-12, TNF-α and IL-6) secretion from mDC induced by rFlaA:Betv1 (Figure 10E). Therefore, COX2-inhibition completely reversed the suppressive effect of the supernatant from rFlaA:Betv1-treated LA-4 cells.

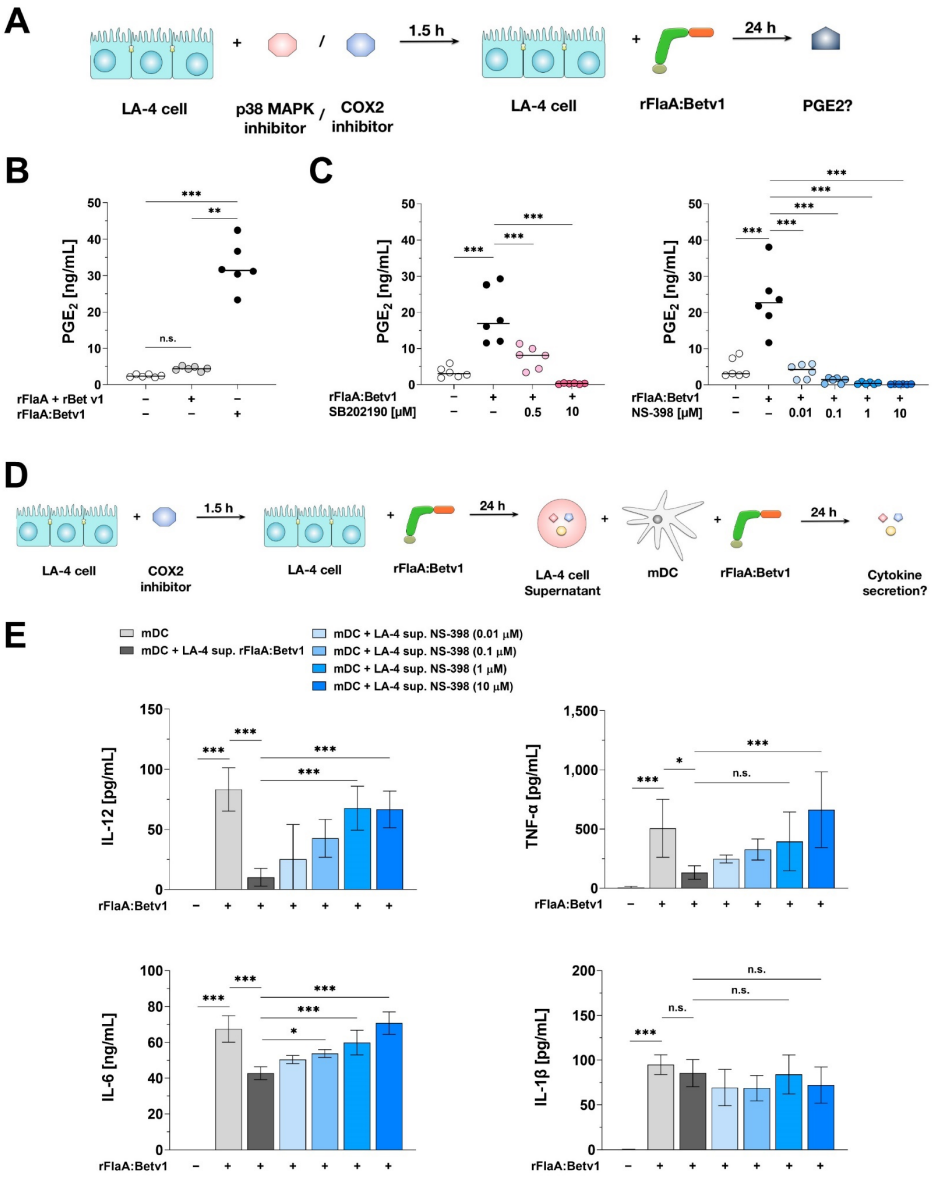

Figure 10. p38-MAPK- and COX2-dependent PGE_2 production contributes to the modulating effects of rFlaA:Betv1-stimulated LA-4 cells on mDCs. LA-4 cells were pre-treated with either the p38-MAPK inhibitor SB202190 or the COX2 inhibitor NS-398 for 90 min and subsequently stimulated with 27.4 µg/mL rFlaA:Betv1 for 24 h (**A**). Supernatants were collected and checked for the secretion of PGE_2 by ELISA (**B**,**C**). BALB/c mDCs were cultured in the presence or absence of rFlaA:Betv1-stimulation. In addition, mDCs were incubated with 100 µL of supernatant (sup.) derived from: (I) rFlaA:Betv1-stimulated LA-4 cells (+LA-4 sup. rFlaA:Betv1), or (II) LA-4 cells that were pre-treated with the indicated amounts of the COX2-inhibitor NS-398 for 1.5 h followed by stimulation with 27.4 µg/mL rFlaA:Betv1 for 24 h (+LA-4 sup. NS-398) (**D**). Supernatants were collected and checked for the secretion of TNF-α, IL-12, IL-6, and IL-1β by ELISA (**E**). Data are mean results ± SD from three independent experiments with two technical replicates per experiment. Statistical significance indicated as: n.s.: no statistically significant difference, p-value > 0.05, * p-value < 0.05, ** p-value < 0.01, *** p-value < 0.001.

4. Discussion

In the present study, we analyzed the immune modulating properties of a flagellin:antigen fusion protein on epithelial cells. While the effects of flagellin on epithelial cells are well-known [43–51] and the interaction of flagellin with epithelial cells has often been described as a key factor for its adjuvant activity [52], our study is the first to characterize the response of epithelial cells to a flagellin:antigen fusion protein. We could show that, upon stimulation with a flagellin:antigen fusion protein, epithelial cells can modulate pro-inflammatory mDC responses by a both p38 MAPK- and COX2-dependent production of PGE_2.

Several studies reported flagellin to induce the secretion of chemokines (IL-8, CXCL1, and CCL20) and cytokines (TGF-β1, G-CSF, GM-CSF, and IL-6) from both airway and intestinal epithelial cells [43–51]. Mechanistically, flagellin-induced CCL20, TGF-β1, IL-8, and IL-6 production from epithelial cells were shown to be both MAPK- [44,45,50,53] and at least in part NFκB-dependent [51,53,54]. Moreover, Ramirez-Moral et al. found that *Pseudomonas aeruginosa* flagellin induced a mTOR-dependent activation of glycolysis, paralleled by the secretion of CXCL1, CXCL8, CCL20, and G-CSF in primary human bronchial epithelial cells [48]. Interestingly, a recent publication investigated the role of flagellin type C (FliC) from *Salmonella enterica* as a vaccine adjuvant. Here, FliC induced a TLR5-dependent secretion of GM-CSF from airway epithelial cells and further promoted the expansion and activation of type 2 conventional dendritic cells (cDC2s), which led to increased Ag-specific $CD4^+$ T cell activation and antibody responses in vivo [47]. These findings prompted us to investigate the interplay between epithelial cell activation and mDC responses to our flagellin:antigen conjugate which is suggested for the intervention of IgE-mediated allergies.

In our study, we observed that compared to the mixture of both proteins, the flagellin:allergen fusion protein rFlaA:Betv1 induced a significantly increased secretion of the myeloid chemo-attractants CCL2 and CCL20 as well as the cytokine IL-6 as early as two hours post-stimulation. This strongly enhanced epithelial cell activation is in accordance with our previous results showing rFlaA:Betv1 to also activate mDCs [22,23] and macrophages [35] more strongly than equimolar amounts of both single proteins. Of note, we observed no secretion of either GM-CSF, G-CSF, IL-33, TSLP, IL-10, TNF-α, IL-12, or IL-1β from LA-4 cells after rFlaA:Betv1-stimulation (data not shown).

Mechanistically, rFlaA:Betv1 was taken up into LA-4 cells more strongly than the mixture of both proteins and rFlaA:Betv1-mediated epithelial cell activation was shown to result from activation of both intracellular MAPK- (p38-, p42/44-, and SAP/JNK-) and NFκB-signaling as inhibition of both pathways prevented cytokine and chemokine secretion. Here, inhibition of NFκB, IκBα, and all types of MAPK dose-dependently inhibited rFlaA:Betv1-induced IL-6 secretion. Interestingly, rFlaA:Betv1-induced CCL2 and CCL20 secretion were shown to be suppressed by either NFκB or IκBα inhibition. In contrast, MAPK-signaling did not contribute to the secretion of the investigated chemokines (the suggested molecular mechanism of rFlaA:Betv1-mediated epithelial cell activation is shown in Figure 11). Interestingly, treatment with both proteins alone or as a mixture did not result in comparable MAPK- or NFκB-activation, cytokine-, or chemokine secretion.

Van Maele et al. showed that non-hematopoietic cells stimulated with flagellin to play a key role in TLR5-dependent, flagellin-mediated adjuvant activity by triggering CCL20 secretion from epithelial cells [55]. In line with this, titration rFlaA on LA-4 cells also resulted in the dose-dependent secretion of both CCL2 and CCL20 when applying higher amounts of FlaA (>17.4 μg/mL, data not shown). These results show, that in higher stimulation concentrations, rFlaA can also activate the investigated epithelial cells. However, it needs to be considered that both studies were performed with different subtypes of flagellin.

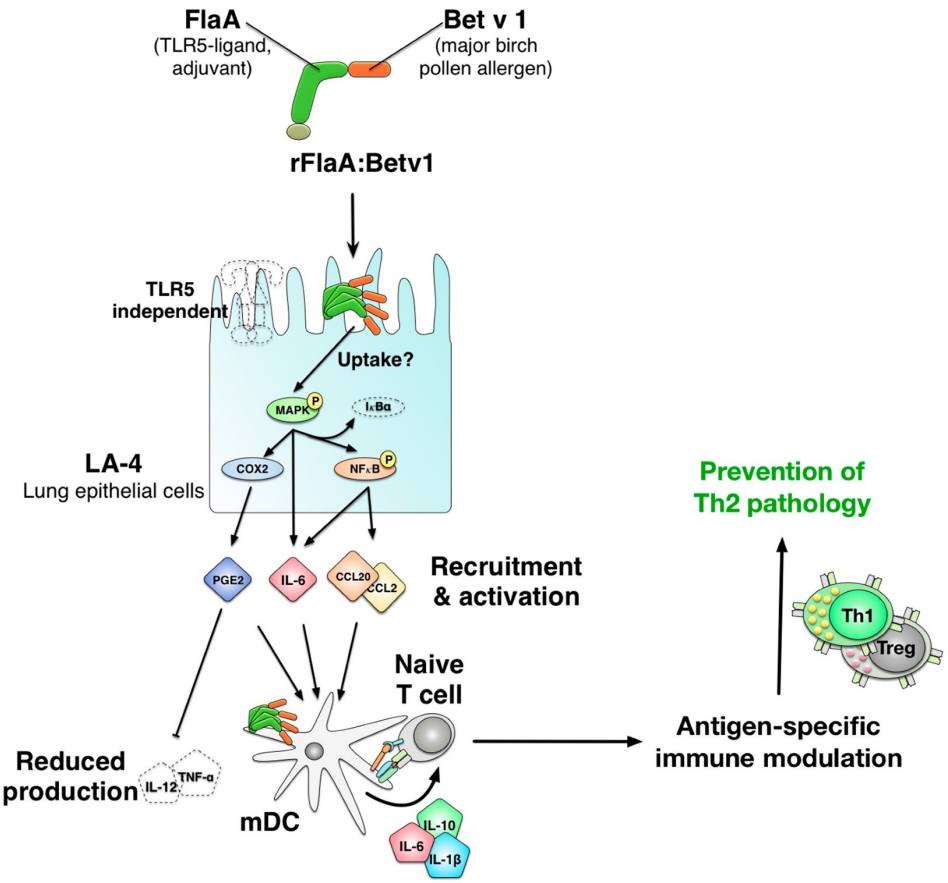

Figure 11. Proposed mechanism of rFlaA:Betv1-mediated LA-4 epithelial cell activation and subsequent modulation of rFlaA:Betv1-induced immune responses by dendritic cells. Stimulation of the mouse lung epithelial cell line LA-4 with the fusion protein rFlaA:Betv1 (combining the TLR-ligand flagellin with the major birch pollen allergen Betv1), resulted in an activation of MAPK- (driving IL-6 secretion), and NFκB-signaling (driving both IL-6 and chemokine secretion). In co-culture with mDCs, LA-4 cell-derived soluble factors/PGE_2 modulated mDC responses induced via rFlaA:Betv1 by reducing their secretion of the pro-inflammatory cytokines IL-12 and TNF-α. This reduction of IL-12 and TNF-α secretion was dependent on both p38-MAPK-signaling and COX2-mediated PGE_2 secretion from rFlaA:Betv1-stimulated LA-4 epithelial cells. Therefore, it is possible that modulation of mDC responses by rFlaA:Betv1-stimulated epithelial cells may contribute to the antigen-specific immune-modulatory effects of flagellin:antigen fusion proteins observed before in vivo (prevention of allergen-specific Th2 responses in our case).

Using a fusion protein mutant unable to activate TLR5 we showed the rFlaA:Betv1-mediated LA-4 cell activation to be TLR5 independent. Although flagellin is a TLR5 ligand, flagellin may achieve its effects TLR5 independently. In previous studies, TLR5 activation by flagellin was found to be MyD88-, MAPK-, and NFκB-dependent [44,45,50,51,53]. Nevertheless, while some TLR5-expressing epithelial cell lines showed a strong activation after flagellin-stimulation (HT29 and A549), others showed only a weak (HeLa, 293T) or no response (T98G) to flagellin [53]. In line with these results, Tallant et al. suggested that the activation of other factors besides TLR5 could be necessary for a complete response to flagellin [53]. Recent studies indicate, for example, that TLR11, a TLR that is highly expressed in various epithelial cells [56,57], can recognize flagellin TLR5 independently [58]. Interestingly, Hatai and colleagues found that TLR11 only acted as a binding receptor for

FliC under acidic conditions (pH 6.0), whereas TLR5 interacted with FliC at pH 6.0 and pH 7.0, suggesting that TLR11 recognizes FliC endolysosomatically [59]. As intracellular processing of the fusion protein was not the focus of this study, further research will be necessary to determine more precisely how flagellin:allergen fusion proteins like rFlaA:Betv1 MAPK-, NFκB-, and COX2-dependently activate epithelial cells after their uptake.

According to previous reports, the stronger LA-4 cell activation by the fusion protein may result from the observed high-molecular aggregation of rFlaA:Betv1 [22], potentially resulting in higher densities of the fusion protein on the cell surface, facilitating protein uptake, and thus stronger activating signals being transmitted to the respective cell's nucleus. In line with this, inhibition of rFlaA:Betv1 uptake into LA-4 cells reduced fusion protein-induced IL-6 secretion but had no effect on chemokine production. Therefore, we speculate that other, yet unknown mechanisms are likely also engaged in rFlaA:Betv1-mediated epithelial cell activation.

In our previous studies, stimulation of mDCs and macrophages with rFlaA:Betv1 also resulted in an mTOR-dependent metabolic shift towards Warburg metabolism (also called aerobic glycolysis) [22,23,35]. However, in rFlaA:Betv1-stimulated LA-4 cells, we did not observe activation of mTOR signaling. Moreover, the mTOR inhibitor rapamycin had no significant effect on rFlaA:Betv1-induced cytokine and chemokine secretion, suggesting only a minor contribution of increased glucose metabolism to rFlaA:Betv1-mediated epithelial cell activation. These results might be explained by our observation, that LA-4 cells predominantly rely on oxidative phosphorylation for energy generation, while the overall metabolism of both mDCs and macrophages is rather glycolytic (data not shown).

Both CCL2 and CCL20 are myeloid chemo-attractants that alert other immune cells to the site of infection [60,61]. The pronounced activation of and chemokine secretion from rFlaA:Betv1-stimulated LA-4 cells prompted us to investigate, if rFlaA:Betv1-activated LA-4 cells could modulate subsequent immune responses induced by the fusion protein. To investigate the functional consequences of fusion protein-mediated epithelial cell activation, we co-cultured BALB/c mDCs with either LA-4 cells or supernatants derived from rFlaA:Betv1-stimulated LA-4 cell cultures.

Upon co-culture of the two cell types, both rFlaA:Betv1-induced IL-12 and TNF-α secretion from mDCs were reduced while the levels of other mDC-derived cytokines (IL-1β, IL-6, and IL-10) remained unchanged. Furthermore, levels of LA-4 cell-derived CCL2 were slightly decreased upon co-culture of both cell types, suggesting that mDCs might metabolize some of the CCL2 produced by the epithelial cells.

Of note, we also observed a suppression tendency of rFlaA:Betv1-induced TNF-α secretion from *ex vivo*-isolated lung DCs (Supplementary Figure S10), suggesting that our findings can at least in part be transferred to the in vivo situation. Here, lung DCs did not produce IL-12 upon stimulation with rFlaA:Betv1 (Supplementary Figure S10).

To further investigate if the immune modulation by LA-4 cells depends on either direct cell–cell contact or soluble factors, mDCs were incubated with cell-free supernatants of rFlaA:Betv1-stimulated LA-4 cells. Our results showed the suppression of IL-12 and TNF-α from BALB/c mDCs to be dependent on LA-4-derived soluble factors. Interestingly, the lower secretion of rFlaA:Betv1-induced cytokines IL-12 and TNF-α from BALB/c mDCs was paralleled by unchanged levels of IL-1β and IL-10. Taken together, these results suggest, that factors secreted from rFlaA:Betv1-stimulated LA-4 cells can specifically modulate mDC responses.

Since rFlaA:Betv1-induced CCL2, CCL20, and IL-6 secretion from LA-4 cells were not responsible for the modulation of mDC responses, other yet unidentified factors might be engaged. PGE_2 is a naturally occurring lipid mediator generated by COX2-mediated conversion of arachidonic acid [36]. PGE_2 is known as an inflammatory mediator that influences DC activation, migration, and stimulatory capacity [37]. In allergy, it was shown to suppress allergic airway responses by inhibiting group 2 innate lymphoid cell (ILC2) activation [62], and decreasing the production of the Th2 cytokine IL-13 [63].

PGE_2 was reported to be secreted by epithelial cells upon LPS stimulation in a COX2-dependent manner [38,39]. Here, COX2 activation was shown to be located downstream of the p38-MAPK pathway [40,64]. In accordance with the results presented in our study, PGE_2 was shown to suppress TLR2- and TLR4-induced IL-12 and TNF-α secretion from both DCs and macrophages in vitro [39,41,42].

In our study, rFlaA:Betv1 induced a higher secretion of PGE_2 from LA-4 cells compared to either unstimulated controls or the mixture of both proteins. Finally, pre-treatment of LA-4 cells with either the COX2-inhibitor NS-398 or the p38 MAPK-inhibitor SB202190 dose-dependently suppressed rFlaA:Betv1-induced PGE_2 production and significantly reduced the inhibitory capacity of supernatants from FlaA:Betv1-treated LA-4 cells on mDCs. Of note, inhibition of either p42/44 MAPK-, SAP/JNK MAPK-, NFκB-, or IκBα-activation in LA-4 cells did not have comparable effects, suggesting that these pathways do not contribute to the production of immune-modulating, soluble factors from LA-4 cells.

It is tempting to speculate that COX2 in epithelial cells is either directly or indirectly engaged in the immune modulating capacity of rFlaA:Betv1. The importance of COX2 activation is highlighted by the current discussion if administration of COX2 inhibitors such as ibuprofen around the time of vaccination decreases long-term responses to the COVID-19 vaccines.

In this context, it would be possible that the pronounced anti-allergic effects of different flagellin:allergen fusion proteins observed before [17,18,20–22] may at least in part result from a reduced production of pro-inflammatory cytokines (IL-12 and TNF-α) from DCs if these cells are confronted with epithelial cell-secreted factors in flagellin:allergen fusion protein-treated animals. This effect could be further promoted by the unchanged secretion of the anti-inflammatory cytokine IL-10 (Figure 11).

One shortcoming of our study is the exclusive usage of a cell line which may or may not reflect the in vivo situation. Future studies will need to investigate the effects of rFlaA:Betv1 on primary epithelial cells. Moreover, investigating the modulation of rFlaA:Betv1-induced epithelial cell responses in an air-liquid interface setting would be of interest to better understand the effects of the fusion protein, but could not be achieved within the limits of this study.

In summary, we investigated the activation of the mouse lung epithelial cell line LA-4 by a vaccine candidate consisting of the TLR5-ligand flagellin and the major birch pollen allergen Betv1. We observed, that rFlaA:Betv1, but not the mixture of both single proteins, triggered an increased uptake and a MAPK-, NFκB-, and COX2-dependent activation of epithelial cells, characterized by a pronounced secretion of the cytokine IL-6, the myeloid chemo-attractants CCL2 and CCL20, as well as PGE_2. Furthermore, rFlaA:Betv1-stimulated LA-4 cells modulated the activation of mDCs, resulting in lower secretion of the pro-inflammatory cytokines IL-12 and TNF-α, while the levels of the IL-1β and IL-10 remained unchanged. Our current mechanistic understanding of the events contributing to rFlaA:Betv1-mediated LA-4 activation and its potential consequences for subsequent immune responses induced by rFlaA:Betv1 is depicted in Figure 11.

Finally, the results further establish epithelial cells as important target cells for vaccination approaches and increase our understanding of the mechanisms underlying the strong immune-modulatory effects of flagellin-containing fusion proteins observed in vivo. Our work will likely contribute to a potentially safe and efficient application of such vaccines in the future.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/cells10123415/s1, Figure S1: Flow cytometric characterization of the used LA-4 cell line, Figure S2: The amounts of LPS contained within the used rFlaA:Betv1 preparations do not induce LA-4 cells activation, Figure S3. Cytotoxicity and effect on chemokine secretion of the used uptake-inhibitors, Figure S4. rFlaA:Betv1 is taken up more strongly than the mixture of both single proteins, Figure S5: mTOR signaling is not activated by rFlaA:Betv1 in LA-4 cells, Figure S6: mTOR signaling does not contribute to rFlaA:Betv1-induced cytokine and chemokine production from LA-4 cells, Figure S7: Cytotoxicity of the used MAPK-inhibitors on LA-4 cells, Figure S8: Cytotoxicity of the

used NFκB-inhibitors on LA-4 cells, Figure S9: Cytotoxicity and effect of the COX2 inhibitor NS-398 on chemokine and IL-6 secretion from LA-4 cells, Figure S10. LA-4 cells also suppress rFlaA:Betv1-induced TNF-α secretion from lung dendritic cells.

Author Contributions: Conceptualization, Y.-J.L., A.F., S.S. (Stephan Scheurer) and S.S. (Stefan Schülke); Data curation, Y.-J.L. and S.S. (Stefan Schülke); Formal analysis, Y.-J.L., A.F., A.G., S.W. and S.S. (Stefan Schülke); Funding acquisition, S.S. (Stephan Scheurer) and S.S. (Stefan Schülke); Investigation, Y.-J.L., A.F., S.W., A.G., A.J., A.W. and S.S. (Stefan Schülke); Methodology, Y.-J.L., A.F., A.J., A.W. and S.S. (Stefan Schülke); Project administration, S.S. (Stephan Scheurer) and S.S. (Stefan Schülke); Resources, S.V.; Supervision, A.W., S.S. (Stephan Scheurer) and S.S. (Stefan Schülke); Visualization, Y.-J.L. and S.S. (Stefan Schülke); Writing—original draft, Y.-J.L. and S.S. (Stefan Schülke); Writing—review and editing, Y.-J.L., A.F., S.W., A.G., A.J., A.W., S.V., S.S. (Stephan Scheurer) and S.S. (Stefan Schülke). All authors have read and agreed to the published version of the manuscript.

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Abbreviations

cDC2s	Type 2 conventional dendritic cells
COX2	Cyclooxygenase-2
FliC	Flagellin type C
ILC2s	Group 2 innate lymphoid cells
MAPK	Mitogen-activated protein kinase
mDCs	Myeloid dendritic cells
MPLA	Monophosphoryl lipid A
mTOR	Mammalian target of rapamycin
NLRC4	NOD-like receptor 4
PAMPs	Pathogen-associated molecular patterns
PGE_2	Prostaglandin E2
PRRs	Pattern recognition receptors
rBetv1	Recombinant birch pollen allergen Betv1
rFlaA	Recombinant flagellin A from *Listeria monocytogenes*
rFlaA:Betv1	Fusion protein consisting of flagellin A from *Listeria monocytogenes* and the major birch pollen allergen Betv1
RLU	Relative light unit
TLR	Toll-like receptor

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Article

The Fusion Protein rFlaA:Betv1 Modulates DC Responses by a p38-MAPK and COX2-Dependent Secretion of PGE_2 from Epithelial Cells

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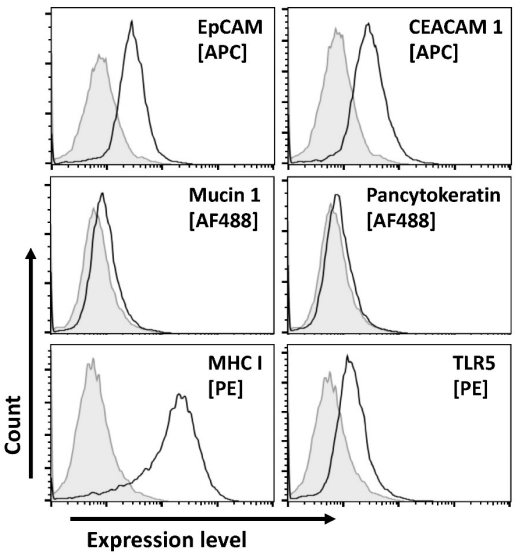
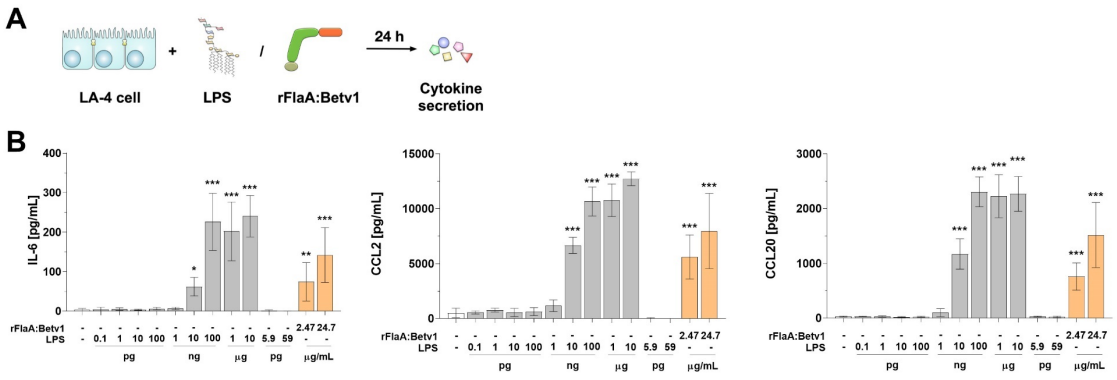
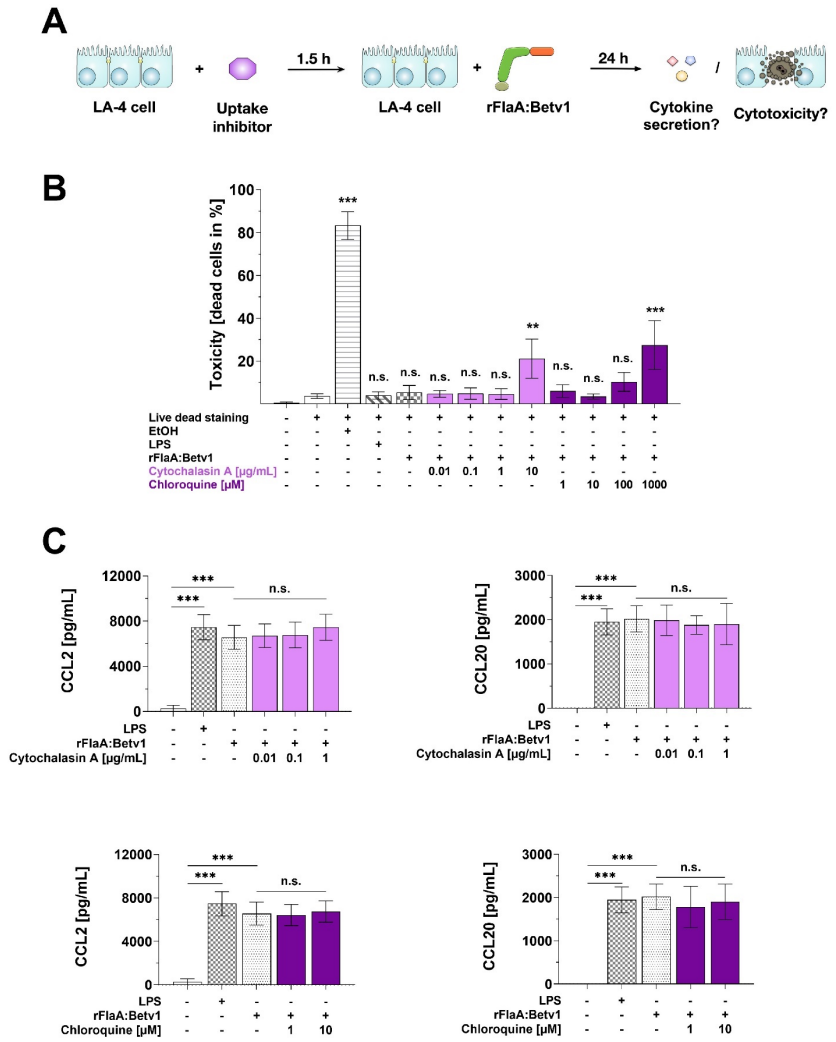
Supplementary information



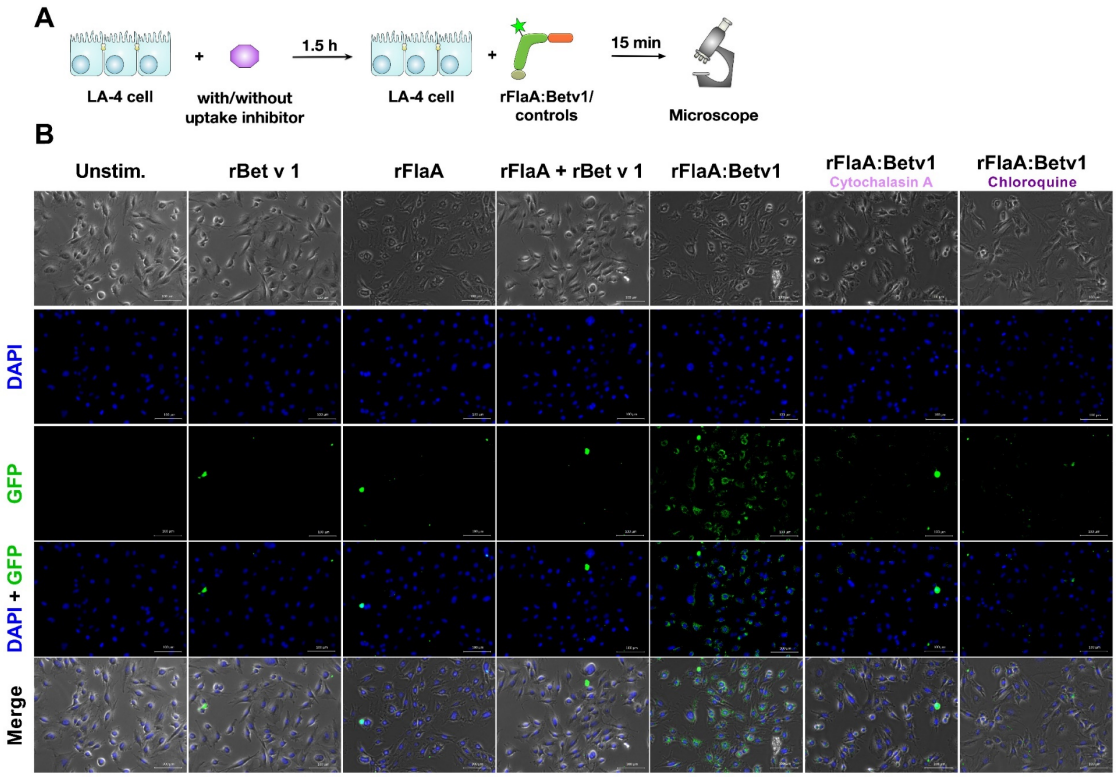
Supplementary Figure S1: Flow cytometric characterization of the used LA-4 cell line. LA-4 cells were stained with the indicated antibodies (black lines) and their expression levels were compared to unstained cells (gray-tinted line). 10,000 cells were measured by flow cytometry using a BD LSR II cytometer. Data are representative results from three independent experiments with 10.000 event recorded per measurement.



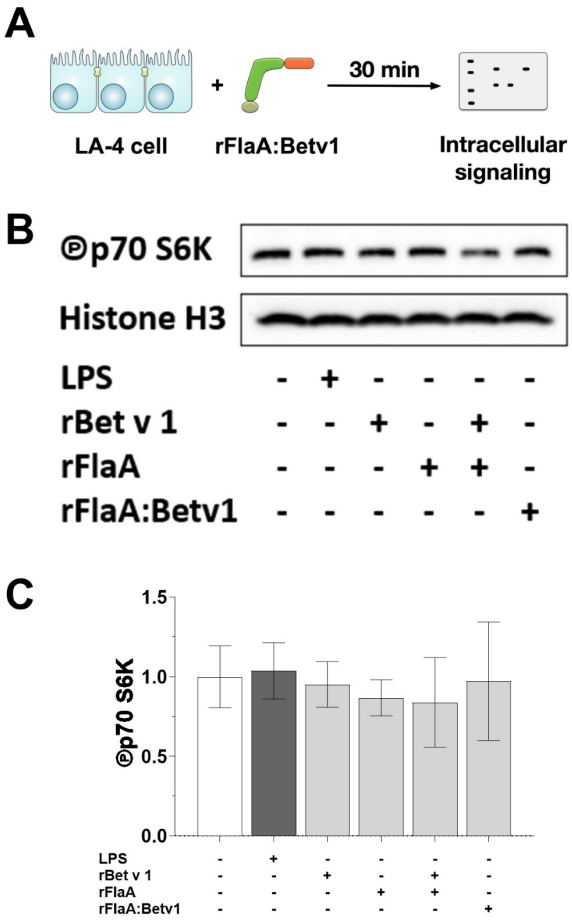
Supplementary Figure S2. The amounts of LPS contained within the used rFlaA:Betv1 preparations do not induce LA-4 cells activation. LA-4 cells were stimulated with either the indicated amounts of LPS to establish dose-response curves (light grey), the residual amounts of LPS contained within the applied concentrations of rFlaA:Betv1 (dark grey), or rFlaA:Betv1 (orange) for 24 h (**A**). Supernatants were analyzed for the induced cytokine and chemokine secretion by ELISA (**B**). Data are mean results of three independent experiments±SD with two technical replicates per experiment.



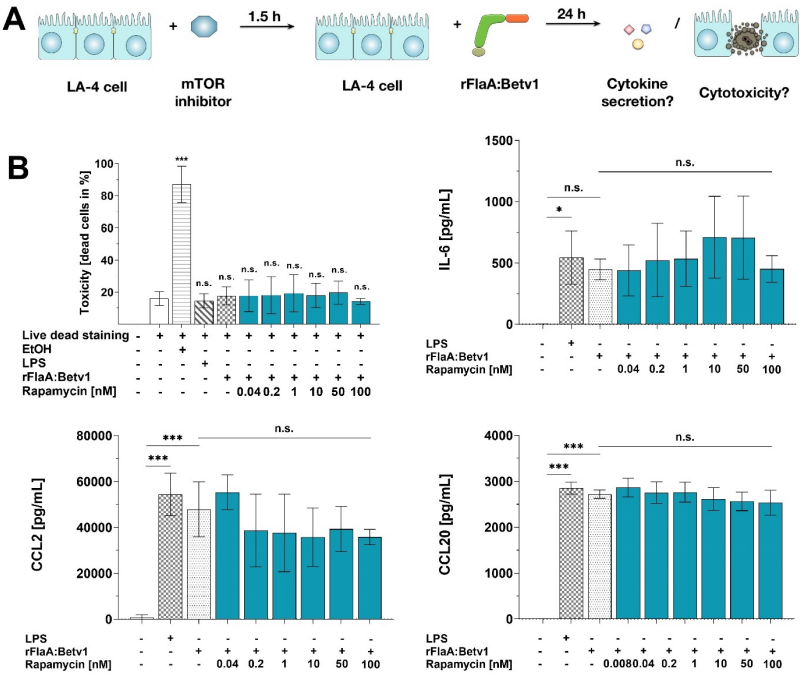
Supplementary Figure S3. Cytotoxicity and effect on chemokine secretion of the used uptake-inhibitors. LA-4 cells were pre-treated with the indicated inhibitor concentrations for 90 min and subsequently stimulated with 27.4 µg/ml rFlaA:Betv1 for 24 h (**A**). Cells killed by incubation for 5 min with 70% ethanol served as positive control. Cells were harvested, stained for dead cells using fixable viability dye, and analyzed for the percentage of dead cells by flow cytometry (**B**). Inhibitor concentrations that showed toxic effects were excluded from the subsequent stimulation experiments. The effect of both inhibitors on chemokine secretion was analyzed 24 h post-stimulation by ELISA (**C**). Data are mean results±SD from three independent experiments with two technical replicates per experiment. Statistical comparisons were performed between indicated samples and unstimulated control samples (**B**) or as indicated (**C**).



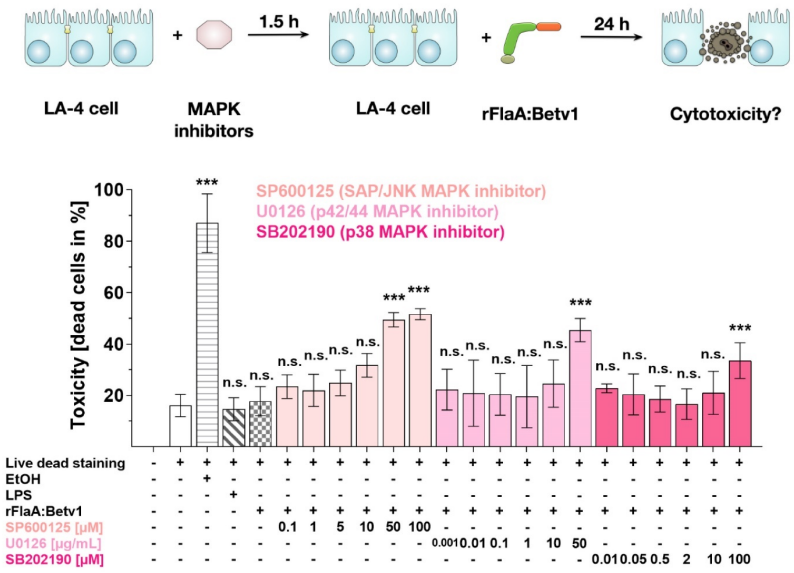
Supplementary Figure S4. rFlaA:Betv1 is taken up more strongly than the mixture of both single proteins. To investigate the uptake of rFlaA:Betv1, LA-4 cells were stimulated with Alexa Flour 488 labeled proteins (**A**) and checked for their uptake by fluorescence microscopy (**B**). In addition, cells were pre-incubated for 90 minutes with the uptake inhibitors cytochalasin A (1 µg/mL) or chloroquine (10 µM) and stimulated with the fusion protein for an additional 15 minutes. Data are representative results of three independent experiments±SD with one technical replicate per experiment.



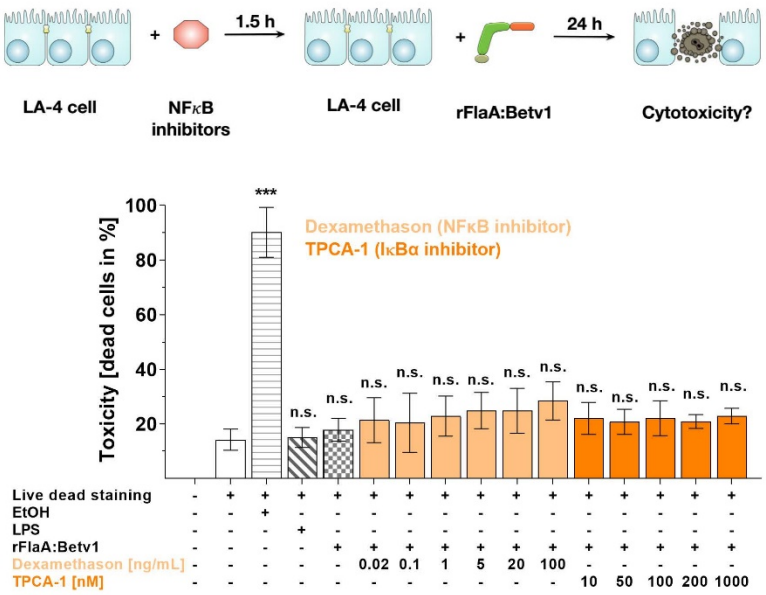
Supplementary Figure S5. mTOR signaling is not activated by rFlaA:Betv1 in LA-4 cells. LA-4 cells were stimulated with either 10 μg LPS as a positive control, rFlaA, rBet v 1, rFlaA + rBet v 1, or rFlaA:Betv1 (all equimolar to 10 μg of rBet v 1) for 30 min (**A**). Cells were lysed and analyzed by Western blot for phosphorylation of the mTOR target protein p70 S6 kinase and expression levels of the internal loading control histone H3 (**B**). The intensities of the Western blot bands were quantified and normalized to the expression levels of the loading control histone H3 (**C**). Data are either representative (**B**) or mean results±SD (**C**) from three independent experiments with one lysate generated per experiment.



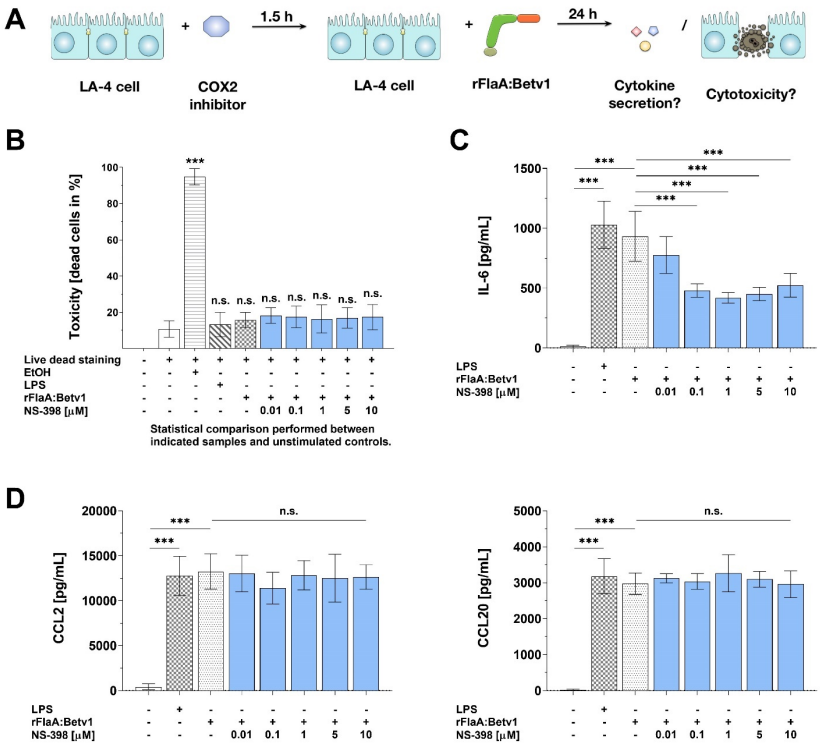
Supplementary Figure S6. mTOR signaling does not contribute to rFlaA:Betv1-induced cytokine and chemokine production from LA-4 cells. LA-4 cells were pre-treated with the mTOR inhibitor rapamycin in the indicated concentrations for 90 min and subsequently stimulated with 27.4 µg/mL rFlaA:Betv1 for 24 h (**A**). Cells were harvested, stained for dead cells using fixable viability dye, and analyzed for the percentage of dead cells by flow cytometry (**B**). As a positive control, cells were killed by incubation with 70% ethanol for 5 min. Furthermore, supernatants were collected and checked for the secretion of IL-6, CCL2, and CCL20 by ELISA (**B**). Data are mean results±SD from three independent experiments with two technical replicates per experiment.



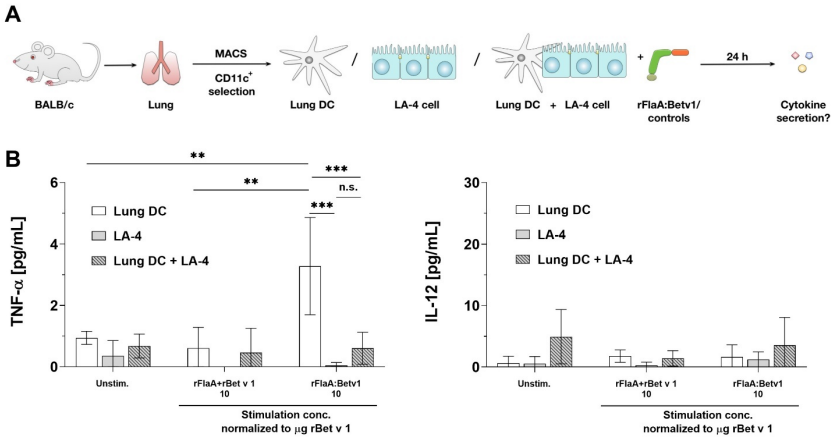
Supplementary Figure S7. Cytotoxicity of the used MAPK-inhibitors on LA-4 cells. LA-4 cells were pre-treated with the indicated inhibitor concentrations for 90 min and subsequently stimulated with 27.4 µg/ml rFlaA:Betv1 for 24 h. Cells killed by incubation for 5 min with 70% ethanol served as positive control. Cells were harvested, stained for dead cells using fixable viability dye, and analyzed for the percentage of dead cells by flow cytometry. Inhibitor concentrations that showed toxic effects were excluded from the subsequent stimulation experiments. Data are mean results±SD from three independent experiments with two technical replicates per experiment. Statistical comparisons were performed between indicated samples and unstimulated control samples.



Supplementary Figure S8: Cytotoxicity of the used NFκB-inhibitors on LA-4 cells. LA-4 cells were pre-treated with the indicated inhibitor concentrations for 90 min and subsequently stimulated with 27.4 µg/mL rFlaA:Betv1 for 24 h. Cells killed by 5 min incubation with 70% ethanol served as a positive control. Cells were harvested, stained for dead cells using fixable viability dye, and analyzed for the percentage of dead cells by flow cytometry. Inhibitor concentrations that showed toxic effects were excluded from the subsequent stimulation experiments. Data are mean results±SD from three independent experiments with two technical replicates per experiment. Statistical comparisons were performed between indicated samples and unstimulated control samples.



Supplementary Figure S9. Cytotoxicity and effect of the COX2 inhibitor NS-398 on chemokine and IL-6 secretion from LA-4 cells. LA-4 cells were pre-treated with the COX2 inhibitor NS-398 in the indicated concentrations for 90 min and subsequently stimulated with 27.4 µg/mL rFlaA:Betv1 for 24 h (**A**). Cells were harvested, stained for dead cells using fixable viability dye, and analyzed for the percentage of dead cells by flow cytometry (**B**). Supernatants were collected and checked for the secretion of IL-6, CCL2, and CCL20 by ELISA (**C**,**D**). As a positive control, cells were killed by 5 min incubation with 70% ethanol. Data are mean results±SD from three independent experiments with two technical replicates per experiment.



Supplementary Figure S10. LA-4 cells also suppress rFlaA:Betv1-induced TNF-α secretion from lung dendritic cells. Dendritic cells were isolated by magnetic cell sorting from the lungs of naïve BALB/c mice. Lung DCs and LA-4 cells were cultured either alone or together and stimulated with the indicated equimolar amounts of either rFlaA + rBet v 1 or rFlaA:Betv1 (**A**). Cultures were checked for IL-12 and TNF-α secretion after 24 h by ELISA (**B**). Data are results from three independent experiments with one technical replicate per experiment.

5 Chapter 3

The Flagellin:Allergen Fusion Protein rFlaA:Betv1 Induces a MyD88- and MAPK-Dependent Activation of Glucose Metabolism in Macrophages

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- Software: C.M.
- Formal analysis: **Y.-J.L.**, G.P., C.M., A.G., J.Z., A.F., S.W., P.C., Z.I., G.v.Z., S.S.
- Investigation: **Y.-J.L.**, G.P., C.M., A.G., J.Z., A.F., S.W., P.C., S.S. (Stefan Schülke)
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- Data curation, **Y.-J.L.**, S.S. (Stefan Schülke)
- Writing—original draft preparation, S.S. (Stefan Schülke), **Y.-J.L.**
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- Supplementary S6 (cooperation with Garibald Papp)
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The Flagellin:Allergen Fusion Protein rFlaA:Betv1 Induces a MyD88− and MAPK-Dependent Activation of Glucose Metabolism in Macrophages

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Abstract: TLR5 ligand flagellin-containing fusion proteins are potential vaccine candidates for many diseases. A recombinant fusion protein of flagellin A and the major birch pollen allergen Bet v 1 (rFlaA:Betv1) modulates immune responses in vitro and in vivo. We studied the effects of rFlaA:Betv1 on bone marrow-derived macrophages (BMDMs). BMDMs differentiated from BALB/c, C57BL/6, TLR5$^{-/-}$, or MyD88$^{-/-}$ mice were pre-treated with inhibitors, stimulated with rFlaA:Betv1 or respective controls, and analyzed for activation, cytokine secretion, metabolic state, RNA transcriptome, and modulation of allergen-specific Th2 responses. Stimulation of BMDMs with rFlaA:Betv1 resulted in MyD88-dependent production of IL-1β, IL-6, TNF-α, IL-10, CD69 upregulation, and a pronounced shift towards glycolysis paralleled by activation of MAPK, NFκB, and mTOR signaling. Inhibition of either mTOR (rapamycin) or SAP/JNK-MAPK signaling (SP600125) resulted in dose-dependent metabolic suppression. In BMDM and T cell co-cultures, rFlaA:Betv1 stimulation suppressed rBet v 1-induced IL-5 and IL-13 secretion while inducing IFN-γ production. mRNA-Seq analyses showed HIF-1a, JAK, STAT, phagosome, NLR, NFκB, TNF, TLR, and chemokine signaling to participate in the interplay of cell activation, glycolysis, and immune response. rFlaA:Betv1 strongly activated BMDMs, resulting in MyD88−, MAPK−, and mTOR-dependent enhancement of glucose metabolism. Our results suggest macrophages are important target cells to consider during restauration of allergen tolerance during AIT.

Keywords: flagellin; TLR5; allergen; Bet v 1; fusion protein; metabolism; Warburg; HIF-1a; immune metabolism

1. Introduction

In the future, novel adjuvants and vaccines may be valuable tools to improve the efficacy, safety, and convenience of allergen immunotherapy. However, for their safe and efficient application, basic knowledge describing their immune modulating capacity, activated cell types, as well as a characterization of the underlying immunological mechanisms is needed.

The Toll-like receptor 5 (TLR5) ligand flagellin is a bacterial motility protein that forms the main body of the bacterial flagellum [1]. Because of its intrinsic immune-activating potential, flagellin was demonstrated to be an effective mucosal adjuvant mediating protective immune responses [2–4].

Since flagellin is the only proteinaceous TLR ligand, its application for the generation of recombinant flagellin:antigen fusion proteins is of special interest in vaccine development. Flagellin-containing fusion proteins have already been shown to be both safe and well-tolerated in clinical trials [5,6]. Consequently, fusion proteins combining flagellin with different antigens have been investigated for their potential to generate immune protection against different diseases including, among others: influenza [7–9], poxvirus [10], West Nile virus [11], and C. *tetani* [12], and *Pseudomonas* infections [13].

Moreover, flagellin:antigen fusion proteins are suggested as treatment options for IgE-mediated type I allergies [14–17]. Kitzmüller and colleagues recently described the enhanced immunogenicity, reduced allergenicity, and intrinsic adjuvant activity of flagellin C:Betv1 fusion proteins containing the major birch pollen allergen Bet v 1 in human monocyte-derived dendritic cells (DCs) and T cells from allergic patients [14].

In theory, adjuvant:antigen fusion proteins have the potential to both boost and modulate antigen-specific immune responses by targeting the fused antigen to immune cells in vivo that express receptors for the respective adjuvant. In addition, adjuvant-mediated immune cell activation influences the processing and increases the presentation of antigen-derived peptides in the context of the adjuvant-mediated immune cell activation [4]. Concordantly, our work has demonstrated that the fusion of allergens to the TLR5-ligand flagellin was able to suppress allergen-specific Th2 responses both in vitro and in vivo via the induction of both IL-10-mediated anti-inflammatory responses and Th1 responses [15–18]. However, the induction of overreaching Th1 responses may also have detrimental effects [19]. Therefore, the balanced induction of both pro- and anti-inflammatory responses by rFlaA:Betv1 may have advantages compared to exclusively triggering Th1-biased responses.

In our previous work, the fusion protein rFlaA:Betv1, consisting of flagellin A from *Listeria monocytogenes* and the major birch pollen allergen Bet v 1, was shown to suppress allergic sensitization in vivo [15]. These immune-modulatory effects were paralleled by a pronounced rFlaA:Betv1-mediated activation of myeloid dendritic cells (mDCs) in vitro, secretion of both pro- (IL-6, IL-12, and TNF-α) and anti-inflammatory (IL-10) cytokines, and a mammalian target of rapamycin (mTOR)-dependent metabolic switch towards glycolysis [15]. In this context, the contribution of both immune cell metabolism to the overall immune responses induced by such fusion proteins and other immune cell types needs further investigation.

Macrophages are both the main effector cells of the innate immune system and professional antigen-presenting cells (together with DCs and B cells). Therefore, macrophages control the induction and regulation of T cell immune responses via the uptake, processing, and presentation of antigens to antigen-specific T cells [20,21]. Epigenetics, cell survival, and the tissue microenvironment are the three main pathways controlling the phenotypical polarization of macrophages [22].

Based on both surface marker expression and their function, macrophages can be divided into classically activated (M1) or alternatively activated (M2) phenotypes [22]. Moreover, M2 macrophages can be further subdivided into the four sub-populations M2a, M2b, M2c, and M2d [23–25]. M1 macrophages are induced by either microbial products or Th1 cytokines, such as LPS, IFN-γ, or granulocyte-macrophage colony stimulating factor (GM-CSF) [22,26]. Functionally, they are capable of secreting pro-inflammatory cytokines, eliminating pathogens, and promoting a local Th1 environment [22,27].

M2a macrophage differentiation is induced by the Th2 cytokines IL-4 and IL-13; IL-1 receptor ligands and immune complexes were reported to induce M2b macrophages. IL-10, TGFβ, and glucocorticoids were reported to induce M2c macrophages, and finally, TLR antagonists were reported to induce M2d macrophage differentiation [28]. Here, M2a

macrophages secrete high levels of the Th2 cytokine IL-13 as well as other chemokines, which promote Th2 responses and induce eosinophil infiltration [28]. Both M2b and M2c macrophages are capable of secreting anti-inflammatory IL-10 in order to regulate immune responses. The M2d phenotype is known as a tumor-associated macrophage that regulates tumor metastasis [26]. While M1 and M2 macrophages are closely related to Th1 and Th2 responses, several studies have indicated the role of macrophages in allergic responses, especially in asthma [28–32]. In general, the M1 phenotype is the major effector macrophage in non-allergic asthma [31], and patients with higher frequencies of M1 cells have less severe clinical allergic reactions [29]. In contrast, M2 cells, especially of the M2a phenotype, are suggested to be the major effector macrophages in allergic asthma because they can promote Th2 responses, promote the secretion of histamine by interaction with antigen-specific Th2 cells, and induce eosinophil migration [28–32]. Therefore, understanding the effects of rFlaA:Betv1 on macrophages could further improve our knowledge of the immune-modulating properties of this promising vaccine candidate for type I allergies.

2. Materials and Methods

2.1. Generation of Recombinant Proteins

Recombinant flagellin A from *Listeria monocytogenes* (rFlaA, Acc. No: NC_003210) was generated according to [33]; recombinant birch pollen allergen Bet v 1 (Acc. No: X15877.1) was generated according to [34]. The fusion protein rFlaA and rBet v 1 (rFlaA:Betv1) was generated according to [15] by cDNA fusion using the cDNAs of both FlaA and Bet v 1 as templates. All proteins displayed an estimated purity greater than 95%, correct folding of secondary structure elements as determined by circular dichroism-spectroscopy, and an endotoxin content of 1.14 pg/µg protein (rFlaA), <0.48 pg/µg protein (rBet v 1), and 1.7 pg/µg protein (rFlaA:Betv1), respectively (data not shown).

2.2. Determination of Beta-Glucans

To exclude the immunological effects of TLR2 ligands in the used preparation of rFlaA:Betv1, the concentration of (1→3) beta-glucans contained within the used rFlaA:Betv1 preparation was determined using the Endosafe-PTS Portable Test System (Charles River Laboratories, Cologne, Germany) according to the manufacturer's recommendations.

2.3. Mice

BALB/c, C57BL/6 mice, TLR5$^{-/-}$, and MyD88$^{-/-}$ mice (C57BL/6 background, all from Jackson Laboratories, Bar Harbor, Maine, ME, USA) were bred at the animal facility of the Paul Ehrlich Institute under specific pathogen-free conditions. All animal experiments were performed in compliance with the German animal protection law (granting authority: RP Darmstadt, Germany, Approval number: F107/131).

2.4. In Vitro Generation of Mouse Bone Marrow-Derived Macrophages, Stimulation, and Flow Cytometry

Mouse BMDMs were generated from bone marrow by culture in RPMI 1640 medium (Gibco, Karlsruhe, Germany) supplemented with 10% FCS, 1 mM sodium pyruvate, 10 mM HEPES, 100 U/mL penicillin, 100 µg/mL streptomycin, 1 mM L-glutamine, 0.1 mM 2-mercaptoethanol, and 20 ng/mL of mouse recombinant macrophage colony-stimulating factor (rmM-CSF, PeproTech, Hamburg, Germany). For differentiation into BMDMs, 2×10^6 cells were seeded per 100 mm dish in 10 mL complete medium. On day 3, 10 mL of complete medium were added per culture dish. On days 6 and 8, 10 mL medium were exchanged and on day 10 M-CSF concentration was reduced to 12.5 ng/mL.

On day 11, BMDMs were harvested, seeded at 3.2×10^5 cells/mL in 24-well plates (Thermo Scientific, Dreieich, Germany), and stimulated with the indicated equimolar concentrations of rFlaA, rBet v 1, rFlaA + rBet v 1, rFlaA:Betv1, or LPS for either 24 h or 96 h, based on the experimental design. As an additional control, BMDMs were stimulated with LPS amounts corresponding to the residual amounts contained within the rFlaA:Betv1

preparation used for BMDM stimulation. Supernatants were analyzed for cytokine secretion by ELISA using the following antibody combinations: IL-1β (capture antibody: IL-1β monoclonal mouse antibody 1:500 (#14-7012-85, eBioscience, Frankfurt, Germany) plus secondary detection antibody: IL-1β polyclonal mouse biotin-conjugated antibody 1:500 (#13-7112-81, eBioscience, Frankfurt, Germany)), IL-6 (capture antibody: IL-6 monoclonal mouse antibody 1:1000 (#14-7061-85, eBioscience) plus detection antibody: IL-6 monoclonal mouse biotin-conjugated antibody 1:1000 (#13-7062-85, eBioscience)), TNF-α (capture antibody: TNF-α monoclonal mouse antibody 1:500 (#14-7325-85, eBioscience) plus detection antibody: TNF-α monoclonal mouse biotin-conjugated antibody 1:250 (#13-7326-85, eBioscience)). Levels of IL-10 were measured using the mouse ELISA Development Kit from PeproTech (#900-T53, PeproTech) according to the manufacturer's recommendations.

For flow cytometric analyses, BMDMs were seeded at 3.2×10^5 cells/mL in 24-well plates (Thermo Scientific) and stimulated with the indicated equimolar concentrations of rFlaA, rBet v 1, rFlaA + rBet v 1, or rFlaA:Betv1 for 24 h. 10 µg/mL lipopolysaccharide (LPS, L5886, Sigma Aldrich, Taufkirchen, Germany) served as a positive control. Cells were stained with anti-mouse, Pacific Blue-conjugated CD11b (clone: M1/70.15, dilution: 1:50; Invitrogen, Thermo Fisher Scientific) and PE-Cy5-conjugated F4/80 (clone: BM8, dilution: 1 to 100, eBioscience). The activation of $CD11b^+F4/80^+$ BMDMs was assessed using anti-mouse PE-Cy7-conjugated CD69 (clone: H1.2F3, dilution: 1 to 100, eBioscience) and the PE-Cy7 intensity was quantified using a LSR II flow cytometer (BD Bioscience). For analysis of cell viability, BMDMs were treated as indicated and stained for dead cells using the fixable viability dye eFlour 780 (eBioscience). Data were analyzed using FlowJo V.7 (Treestar Inc., Ashland, OR, USA).

2.5. Preparation of BMDM:$CD4^+$ T Cell Co-Cultures

BALB/c BMDMs were either cultured alone (5×10^5 cells/mL) or with splenic $CD4^+$ T cells (6.3×10^5 cells/mL) isolated from BALB/c mice immunized with Bet v 1 plus alum (2 times 10 µg rBet v 1 plus 0.5 mg alum i.p., 2 weeks apart) using the $CD4^+$ T Cell Isolation Kit (Miltenyi Biotech, Bergisch Gladbach, Germany). Cultures were stimulated with Bet v 1, rFlaA:Betv1, or rFlaA + rBet v 1 for 72 h. Subsequently, cytokine secretion into culture supernatants was determined using either BD OptEIA ELISA Sets (IFN-γ, IL-5, BD Bioscience), Ready-Set-Go-ELISA kits (IL-13, eBioscience) or the following antibody combination for IL-2: capture antibody: IL-2 monoclonal mouse antibody 1:500 (#503702, BioLegend, Koblenz, Germany) plus detection antibody: IL-2 monoclonal mouse biotin-conjugated antibody 1:500 (#503804, BioLegend).

2.6. Inhibitors

To analyze signaling pathways involved in rFlaA:Betv1-mediated BMDM activation, BMDMs were pre-incubated with the indicated amounts of rapamycin (mTOR inhibitor) or the respective MAPK inhibitors, including either SP600125 (SAP/JNK MAPK inhibitor), SB-202190 (p38α/β MAPK inhibitor, all Invivogen, Toulouse, France), or U0126 (MEK1/2 MAPK inhibitor, Cell Signaling Technologies, Leiden, The Netherlands) for 90 min and subsequently stimulated with rFlaA:Betv1 for either 24 h (ELISA) or 96 h (ELISA and analysis of cell metabolic state).

2.7. Western Blotting

For Western blot experiments, BMDMs were starved in RPMI1640 supplemented with 1% FCS (Sigma-Aldrich) and cultured for 3 h at 37 °C and 5% CO_2 in either T25/75 flasks, cell culture plates, or FACS tubes. Subsequently, 1×10^6 BMDMs were stimulated with the indicated proteins in RPMI1640. 30 min to 48 h post-stimulation, cells were washed with ice-cold PBS and subsequently lysed with 200 µL lysis buffer (62.5 mM Tris-HCl (pH 6.8), 2% w/v SDS, 10% glycerol, 50 mM DTT, 0.01% w/v bromophenol blue) for 10 min on ice. Target proteins in lysates were separated by SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes. After blocking with 5% non-fat milk, the

membranes were incubated with the following primary antibodies from Cell Signaling Technologies overnight at 4 °C: phospho-MAPK Family Antibody Sampler Kit (#9910), NF-κB Pathway Sampler Kit (#9936), mTOR Substrates Antibody Sampler Kit (#9862), Glycolysis Antibody Sampler Kit (#8337), anti-Phospho-Stat3 (Ser727) antibody (#9134), anti-HIF-1a antibody (#14179), anti-ACO2 antibody (#6571), and loading control anti-histone H3 antibody (#12648, HRP Conjugate) or anti-GAPDH antibody (#8884, HRP Conjugate). Detection was performed with the provided secondary antibodies using ACE Glow substrate (VWR, Darmstadt, Germany) and images were captured with either a Fusion-Fx7 Spectra reader (Vilber Lourmat, Eberhardzell, Germany) or an iBright CL1500 system (Thermo Fischer Scientific). Band intensities in Western blots were quantified with ImageJ software (imagej.nih.gov, version: 1.52a) as relative light units (RLU) normalized to the histone H3 loading control.

2.8. Analysis of Cell Metabolic State

The Warburg effect, glucose consumption, and metabolic rate in stimulated BMDM cultures were determined according to [15].

2.9. Metabolic Flux Analysis

For metabolic flux analysis, 1×10^5 BMDMs per well were seeded in Seahorse XF96 cell culture microplates (V3-PS, TC-treated, Agilent, Santa Clara, CA, USA). The next day, the medium was exchanged, and cells were stimulated as indicated using the different proteins. Seahorse XF Real-Time ATP rate assays, Seahorse XF Glycolysis, and Seahorse XF Cell Mito Stress Tests were performed according to the manufacturer's recommendations (Agilent). Cycle numbers were as follows: 4 cycles of baseline measurement, 14 cycles of stimulation with the different proteins, and 8 cycles with either oligomycin, rotenone/ antimycin A (Rot/AA), and 2-deoxy-glucose (2-DG), respectively (1 cycle = 3 min mixing plus 3 min measuring). Post-measurement, samples were normalized to total protein content via BCA (Thermo Fischer Scientific) and analyzed using Wave Desktop software (Agilent) and Graphpad Prism v8. Glycolytic Stress and Mito Stress results were analyzed using the respective report generator sheets according to the manufacturer's recommendations (Agilent).

2.10. RNA-Seq and Bioinformatics

For RNA-Seq analyses, 3.2×10^5 BMDMs/mL were stimulated in 24-well plates with either LPS as a positive control or the indicated equimolar concentrations of rFlaA + rBet v 1 or rFlaA:Betv1 for 48 h. Subsequently, BMDMs were harvested (4 biological replicates per condition) and frozen in Trizol (Invitrogen, Thermo Fisher Scientific) before total RNA isolation with the Direct-zol RNA Miniprep Kit (Zymo Research, Freiburg, Germany). We harnessed a modified version of the NNSR priming method [35] to prepare stranded, Illumina-compatible libraries as follows: we used the NEBNext Poly(A) mRNA Magnetic Isolation Module (NEB) to isolate mRNA from 1 µg total RNA per sample. cDNA was synthesized with Superscript IV in the presence of 4 µg Actinomycin D and RiboLock RNase inhibitor (Thermo Fisher Scientific) with the NNSR_RT primer (see Table 1 below) for 30 min at 45 °C, following the recommendations of the manufacturer. After subsequent magnetic bead purifications and RNase H (NEB) treatment, samples were subjected to second-strand cDNA synthesis with 3′-5′ exo(−) Klenow (NEB) and with the primer NNSR_2 for 30 min at 37 °C. The bead-purified DNA samples were amplified with the NNSRnest_ind_N and NNSR_Illumina primers using the NEBNext Ultra II Master Mix (NEB) to obtain the barcoded libraries with the following cycling conditions: 98 °C 10 s; 5 cycles of 98 °C 10 s, 55 °C 30 s, 68 °C 30 s; 15 cycles of 98 °C 10 s, 65 °C 30 s, 68 °C 30 s. The smears of PCR products were agarose gel-purified, and the libraries were sequenced on a NextSeq instrument with single-end 86 bp settings.

Table 1. Primers used for cDNA synthesis.

Oligo Name	Sequence
NNSR_RT	gctcttccgatctctNNNNNN
NNSR_2	gctcttccgatctgaNNNNNN
NNSRnest_ind_N	CAAGCAGAAGACGGCATACGAGATNNNNNNGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTGA (N stands for a 6mer TruSeq index (Illumina))
NNSR_Illumina	AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATCTCT

The raw sequencing reads were quality-, length-, and adapter-trimmed with *fastp* [36]. We used STAR [37] to align the trimmed reads to the human genome (hg38 assembly) and to count the mapped reads on gene level. The quantitative evaluation of the counts, differential gene expression, and the accompanying statistical analyses were performed in the R environment (https://www.r-project.org, version 3.5.0. (accessed on 23 April 2018) using the DESeq2 package (version 1.28.3)) [38]. KEGG pathway analysis [39] and gene set enrichment analysis were performed with the clusterProfiler package [40]. Data were deposited in the SRA database (accession number: PRJNA755840).

2.11. Statistical Analysis

Statistical analyses were performed with GraphPad Prism v6 to v8 for either Mac or Windows using 2-way ANOVA tests with confidence intervals adjusted for multiple comparisons according to either Bonferroni or Tukey. For statistically significant results, the following convention was used: * p-value < 0.05, ** p-value < 0.01, *** p-value < 0.001.

3. Results

3.1. Activation of BMDM Metabolism by rFlaA:Betv1 Is Accompanied by Both Pro- and Anti-inflammatory Cytokine Secretion

BMDMs differentiated from C57BL/6 mice were stimulated with equimolar amounts of rBet v 1, rFlaA, the mixture of rFlaA and rBet v 1 (rFlaA + rBet v 1), or the fusion protein rFlaA:Betv1 for 96 h and analyzed for their cellular metabolic state and cytokine secretion profile (Figure 1A). Both rFlaA and rFlaA:Betv1 activated BMDM metabolism (Figure 1B). While differences between the rFlaA:Betv1- and either the rFlaA− or rFlaA + rBet v 1-induced Warburg effect were significant, there was no difference in glucose consumption between rFlaA:Betv1− and rFlaA + Bet v 1-stimulated BMDMs (Figure 1B). Activation of BMDM glucose metabolism was paralleled by a highly significantly increased production of both pro- (IL-1β, IL-6, TNF-α) and anti-inflammatory (IL-10) cytokines from rFlaA:Betv1-stimulated BMDMs compared to cells stimulated with either both proteins alone or provided as a non-fused mixture (for a stimulation concentration equimolar to 10 μg/mL rBet v 1, rFlaA:Betv1 induced 1.6-fold higher IL-1β secretion, 3.4-fold higher IL-6 secretion, 5.5-fold higher TNF-α secretion, and 6.1-fold higher IL-10 secretion than the respective mixture of both proteins, Figure 1C). Similar activation of BMDM metabolism (Repository Figure S1) and cytokine secretion profiles were observed for BALB/c BMDMs (Repository Figure S2).

To exclude potential immune-activating effects of the residual amounts of LPS contained within the used rFlaA:Betv1 preparation, we established thresholds for LPS-induced BMDM activation (Repository Figure S3A). Here, the threshold for an LPS-induced Warburg effect was between 1 and 10 ng. Increased glucose consumption and significant secretion of IL-10 were observed starting at 10 ng; secretion of IL-6 started at 1 ng, while IL-1β- and TNF-α-secretion were detected after stimulation with 1 μg of LPS (Repository Figure S3B,C). In addition, stimulation of BMDMs with the amounts of LPS contained within the used concentrations of rFlaA:Betv1 (0.59 pg LPS contained within 0.247 μg/mL rFlaA:Betv1, 5.9 pg LPS in 2.47 μg/mL rFlaA:Betv1, and 59 pg LPS in 24.7 μg/mL rFlaA:Betv1) neither resulted in activation of BMDM metabolism nor cytokine secretion (Repository Figure S3B).

To further exclude effects of LPS or potentially contaminating TLR2 ligands in the used rFlaA:Betv1 preparation, we used HEK293 cell lines stably transfected with either mouse TLR2, TLR4, or TLR5 as well as non-TLR-transfected HEK293 control cells (Repository Figure S4A). Here, rFlaA:Betv1 exclusively induced TLR5 activation, as indicated by a dose-dependent release of IL-8 from these cells (Repository Figure S4B). To further exclude the presence of TLR2 ligands in the rFlaA:Betv1 preparation, levels of (1→3) beta-glucans were determined. Detection of (1→3) beta-glucans in the used rFlaA:Betv1 preparation showed the levels of (1→3) beta-glucans to be below 0.8 pg/µg of protein, which is safely below the low ng amounts of different TLR2-ligands (LPS, mycoplasma diacylated lipoprotein, lipomannan, lipoarabinomannan, and Pam_3CysK_4) that were previously reported to activate TLR2 on BMDMs [41]. Therefore, we could show that neither the residual LPS amounts nor TLR2 ligands contribute to the rFlaA:Betv1-mediated activation of BMDMs.

In summary, stimulation of BMDMs with rFlaA:Betv1, and, to a lower degree, with rFlaA and rFlaA + rBet v 1, resulted in a pronounced activation of both BMDM metabolism and pro- and anti-inflammatory cytokine secretion.

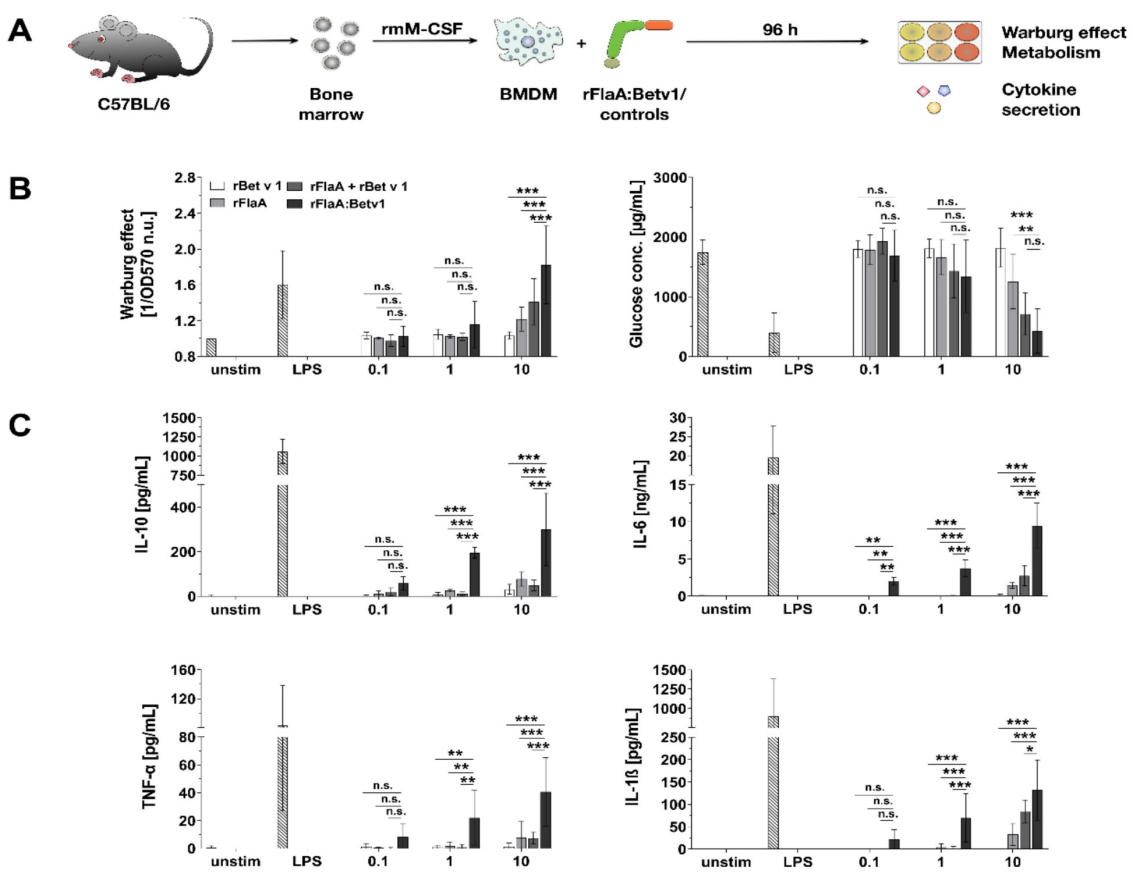

Figure 1. Activation of BMDM metabolism by rFlaA:Betv1 is accompanied by both pro- and anti-inflammatory cytokine secretion. C57BL/6 BMDMs were differentiated from mouse bone marrow and stimulated with the indicated equimolar protein amounts or LPS as a positive control for 96 h (**A**). Supernatants were analyzed for the induced Warburg effect and glucose consumption (**B**) as well as cytokine secretion by ELISA (**C**). Data are mean results of three independent experiments ± SD with two technical replicates per experiment. Statistical significance indicated as: n.s. p-value > 0.05, * p-value < 0.05, ** p-value < 0.01, *** p-value < 0.001.

3.2. rFlaA:Betv1 Triggers a Pronounced Shift Towards Glycolysis in BMDMs

In-depth analysis of the BMDM metabolic state using Seahorse technology (Figure 2A) revealed rFlaA, rFlaA + rBet v 1, and rFlaA:Betv1 to dose-dependently increase glycolysis (detected by enhanced extracellular acidification rates (ECAR)), while at the same time reducing mitochondrial respiration (reduced oxygen consumption rates (OCR), Figure 2B). In contrast, rBet v 1 had only minimal effects on BMDM metabolism (Figure 2B). Further inhibition of the mitochondrial ATP synthase by oligomycin and of electron transport chain complexes by rotenone/antimycin A (Rot/AA) resulted in compensatory increases in ECAR, which were less pronounced in rFlaA:Betv1-stimulated BMDMs compared to the controls (Figure 2B). Treatment with the competitive inhibitor of glucose-6-phosphate, generation of 2-deoxy-glucose (2-DG) completely suppressed extracellular acidification (Figure 2B). In line with these results, analysis of the glycolytic state showed that rFlaA:Betv1-stimulated BMDMs have similar glycolytic capacities (capacity to produce energy by glycolysis) but higher glycolytic rates (amount of energy generated by glycolysis per minute and cell) compared to BMDMs stimulated with rFlaA + rBet v 1. In comparison, BMDMs stimulated with equimolar amounts of rFlaA + rBet v 1 have higher glycolytic reserves (capacity to further increase energy production by glycolysis) while no significant differences in non-glycolytic acidification (acidification of the extracellular medium by processes other than glycolysis) were observed between treatment groups (Figure 2C). In contrast to this, detailed analysis of mitochondrial function via Mito Stress tests revealed no differences in basal respiration, spare respiratory capacity, mitochondrial ATP production, proton leak, and non-mitochondrial respiration between rFlaA + rBet v 1- and rFlaA:Betv1-stimulated cells (Repository Figure S5).

Taken together, the metabolic phenotyping of rFlaA:Betv1-stimulated BMDMs suggested the fusion protein induced a pronounced shift towards increased levels of glycolysis while mitochondrial respiration was suppressed.

3.3. rFlaA:Betv1-Induced BMDM Metabolism and Inflammatory Cytokine Secretion Depend on MyD88 While Only Being Partially TLR5-Dependent

In order to study the signaling mechanisms contributing to rFlaA:Betv1-mediated BMDM activation, BMDMs were differentiated from either C57BL/6, TLR5-, or MyD88-deficient mice, stimulated with the fusion protein and the respective controls, and analyzed to assess their metabolic state and cytokine secretion profile (Figure 3A).

Interestingly, activation of BMDM metabolism (Warburg effect and glucose consumption) by both rFlaA and rFlaA:Betv1 was shown to be partially TLR5-dependent (Figure 3B). MyD88-deficient BMDM did not show increased metabolic activity after stimulation with either of the tested proteins (Figure 3B). In line with these results, cytokine secretion induced by either rFlaA, rFlaA + rBet v 1, or rFlaA:Betv1 was shown to be abrogated in MyD88-deficient BMDMs (Figure 3C). Among the tested cytokines, only rFlaA:Betv1-induced IL-10 and TNF-α showed a substantial TLR5 dependency (41% and 87% reduction in TLR5-deficient mice compared to C57BL/6 BMDMs, respectively) while secretion levels of all other cytokines were unaffected by TLR5 deficiency (Figure 3C).

In summary, using BMDMs differentiated from either TLR5− or MyD88-deficient bone marrow, the activation of glucose metabolism and cytokine secretion was shown to be abrogated in the absence of MyD88 while being partially TLR5 dependent for the secretion of IL-10 and TNF-α.

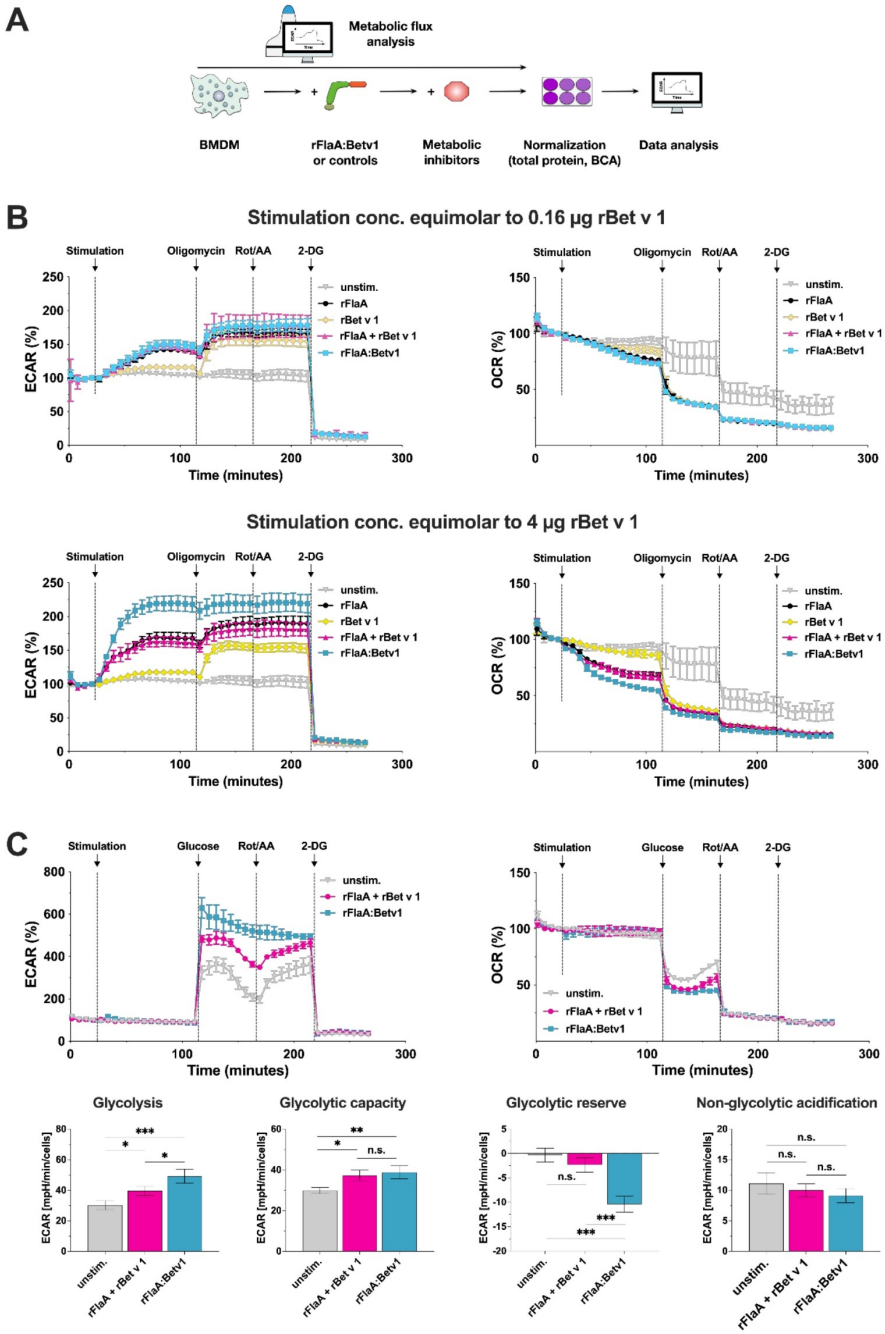

Figure 2. rFlaA:Betv1 triggers a pronounced shift towards glycolysis in BMDMs. Assay scheme for metabolic flux analysis using Seahorse technology (**A**). BMDMs were stimulated with either rFlaA + rBet v 1 or rFlaA:Betv1 in the indicated equimolar concentrations (0.16 µg rBet v 1 = 8 nM, and 4 µg rBet v 1 = 200 nM of protein) and analyzed for extracellular acidification rates (ECAR) and oxygen consumption rates (OCR) using Seahorse technology (**B**). 14 cycles (84 min) post-stimulation, ATP synthase, the electron transfer chain, and glycolysis were inhibited by sequential injection of oligomycin,

rotenone/antimycin A (Rot/AA), and 2-deoxy-glucose (2-DG), for 8 cycles (48 min) each, respectively (**B**). Glycolytic stress test was performed in BMDMs stimulated with either rFlaA + rBet v 1 or rFlaA:Betv1 (both equimolar to 4 µg of rBet v 1) (**C**). Levels of glycolysis, glycolytic capacity, glycolytic reserve, and non-glycolytic respiration were analyzed by Seahorse technology using the "XF Glycolysis Stress Test Report Generator" according to the manufacturer's recommendations (**C**). Data are representative results of three independent experiments (with three to four technical replicates per experiment) that showed similar results. Statistical significance indicated as: n.s. p-value > 0.05, * p-value < 0.05, ** p-value < 0.01, *** p-value < 0.001.

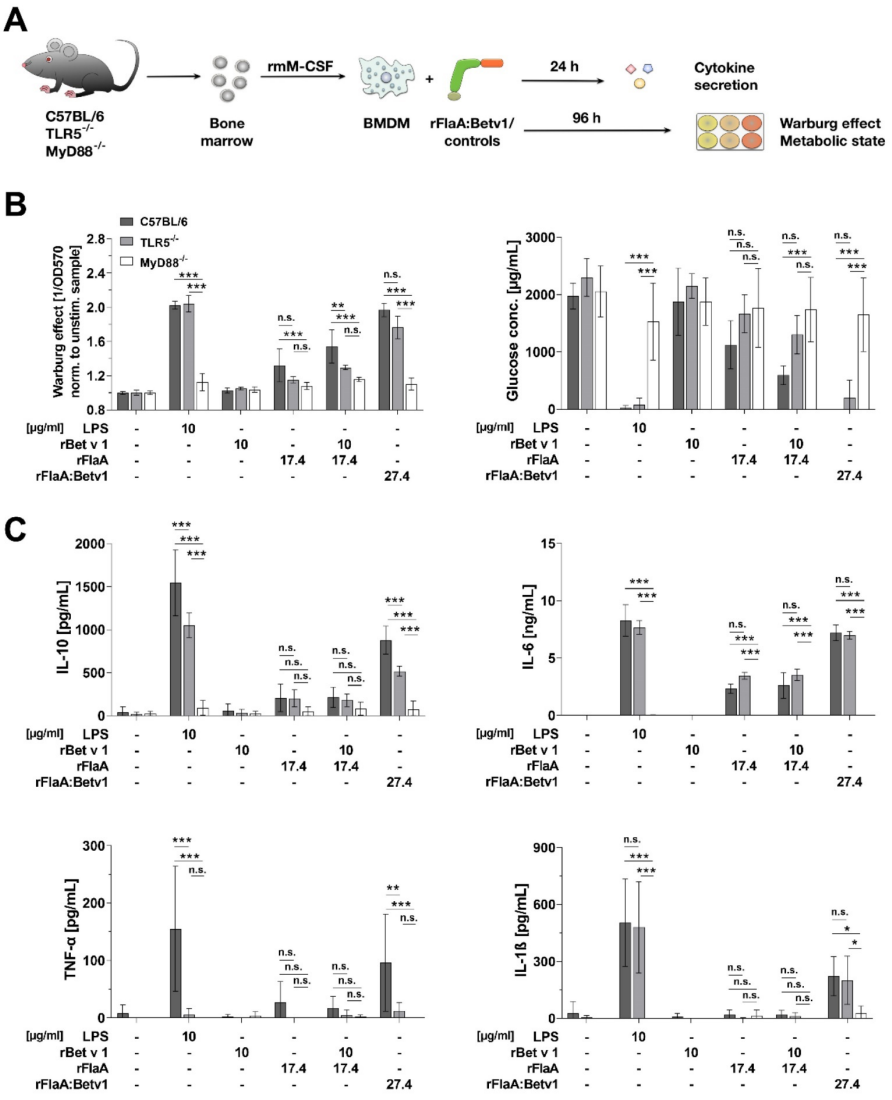

Figure 3. rFlaA:Betv1-induced BMDM metabolism and inflammatory cytokine secretion depend on MyD88 while being only partly TLR5-dependent. C57BL/6, TLR5$^{-/-}$, or MyD88$^{-/-}$ BMDMs were differentiated from mouse bone marrow and stimulated with the indicated equimolar protein amounts or LPS as a positive control for either 24 h (cytokine secretion, **C**) or 96 h (metabolic state, **B**) (**A**). Supernatants were analyzed for the induced Warburg effect and glucose consumption (**B**) as well as cytokine secretion by ELISA (**C**). Data are mean results of three independent experiments ± SD with two technical replicates per experiment. Statistical significance indicated as: n.s. p-value > 0.05, * p-value < 0.05, ** p-value < 0.01, *** p-value < 0.001.

3.4. rFlaA:Betv1 Induces MAPK, NFκB, and mTOR Signaling in BMDMs

Subsequently, the activation status and intracellular signaling cascades of C57BL/6, TLR5$^{-/-}$, or MyD88$^{-/-}$ BMDMs stimulated by rFlaA:Betv1 were analyzed (Figure 4A).

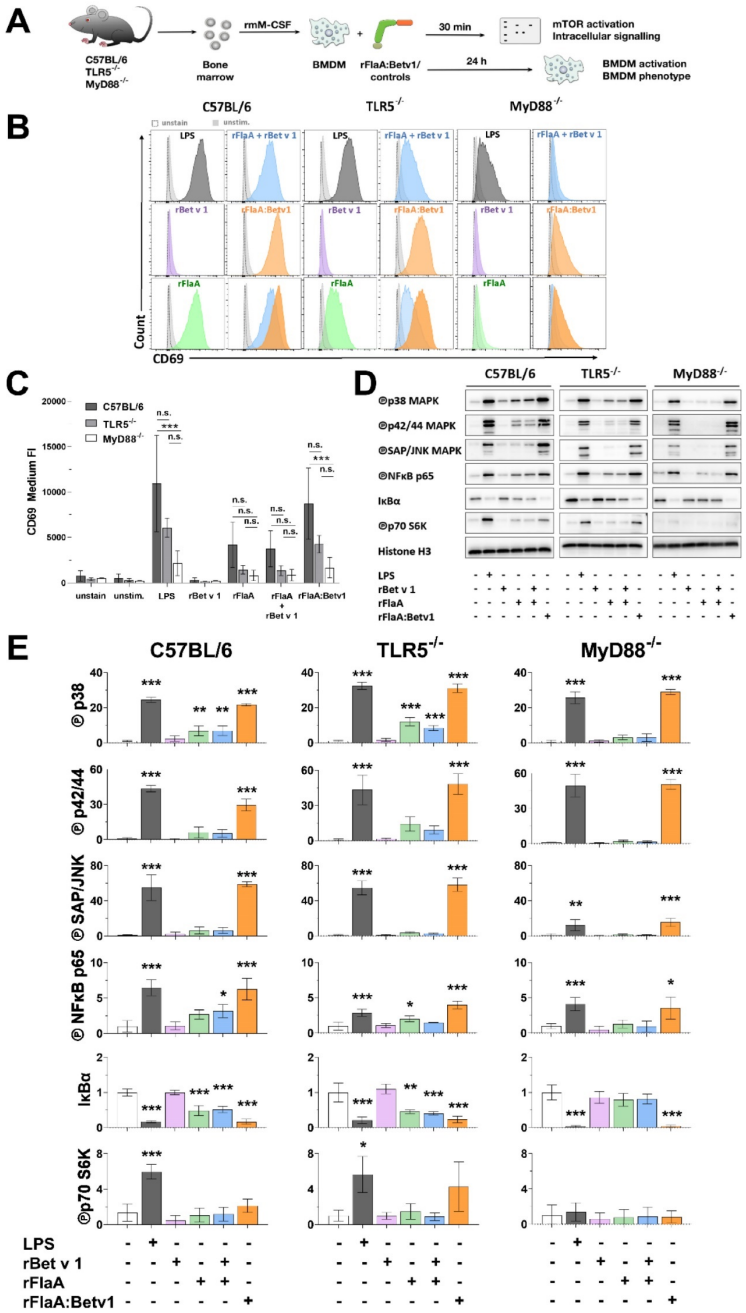

Figure 4. rFlaA:Betv1-stimulation induces stronger BMDM activation characterized by signaling of MAPK, NFκB, and mTOR . C57BL/6, TLR5$^{-/-}$, or MyD88$^{-/-}$ BMDMs were differentiated from

mouse bone marrow and stimulated with the indicated equimolar protein amounts or LPS as a positive control for either 30 min (Western blot, **D**) or 24 h (flow cytometry, **B**,**C**) (**A**). Expression levels of CD69 were analyzed by flow cytometry (exemplary result in **B**, mean fluorescence intensity of three independent experiments depicted in **C**) and activation of MAPK, NFκB, and mTOR1 signaling was analyzed by Western blot (**D**). Western blots from three independent experiments were quantified and normalized to expression levels of histone H3 (**E**). Indicated are statistically significant differences compared to unstimulated controls. Data are either representative results from three independent experiments (**B**,**D**) or mean results of three independent experiments ± SD (**C**,**E**) with either 10.000 BMDMs (**B**,**C**) or one lysate (**D**,**E**) measured per experiment. Statistical significance indicated as: n.s. p-value > 0.05, * p-value < 0.05, ** p-value < 0.01, *** p-value < 0.001.

Stimulation of C57BL/6 BMDMs with rFlaA:Betv1 resulted in higher up-regulation of CD69 compared to either rFlaA− or rFlaA + rBet v 1-stimulation (Figure 4B and quantified in Figure 4C). Of note, both rFlaA− and rFlaA:Betv1-induced CD69 upregulation was partially, but not significantly, reduced in TLR5-deficient BMDMs, while being completely abrogated in MyD88-deficient BMDMs (Figure 4B,C). Stimulation with rBet v 1 alone in either tested mouse strain did not result in increased CD69 expression (Figure 4B,C).

Stimulation of C57BL/6 BMDMs with rFlaA:Betv1 resulted in strongly increased levels of phosphorylated MAP kinases p38, p42/44, and SAP/JNK, phosphorylation of NFκB p65, as well as phosphorylation of the mTOR target protein p70 S6 kinase compared to either rBetv 1−, rFlaA−, or rFlaA + rBet v 1-stimulated BMDMs (Figure 4D,E, left panel). Here, both rFlaA− and rFlaA:Betv1-induced phosphorylation of MAP kinases, NFκB, and p70 S6 kinase was reduced but still detectable in TLR5-deficient BMDMs (Figure 4D,E, middle panel). Interestingly, while rFlaA-induced phosphorylation of p70 S6 kinase and MAP kinases was abrogated in MyD88-deficient BMDMs, rFlaA:Betv1-induced MAP kinase, SAP/JNK, and NFκB activation could still be detected in MyD88-deficient BMDMs (Figure 4D,E, right panel).

Taken together, we observed rFlaA:Betv1 to induce a stronger activation of MAPK, NFκB, and mTOR signaling in BMDMs compared to either rFlaA alone or the mixture of both proteins. rFlaA:Betv1-induced activation of MAPK and NFκB signaling was largely TLR5− and MyD88-independent, while p70 S6 kinase activation was only abrogated in MyD88-deficient BMDMs.

3.5. Activation of BMDM Metabolism and IL-10 Secretion are Partly mTOR-Dependent while Pro-Inflammatory Cytokine Secretion Is mTOR-Independent

To study the contribution of either mTOR and/or different MAP kinases to rFlaA:Betv1-mediated activation of BMDM metabolism and cytokine secretion, BMDMs were pre-incubated with either rapamycin to inhibit mTOR activation (Figure 5A) or different MAPK inhibitors (Figure 6A). Toxicity of all tested inhibitors on BMDMs was excluded by live/dead staining (Repository Figure S6).

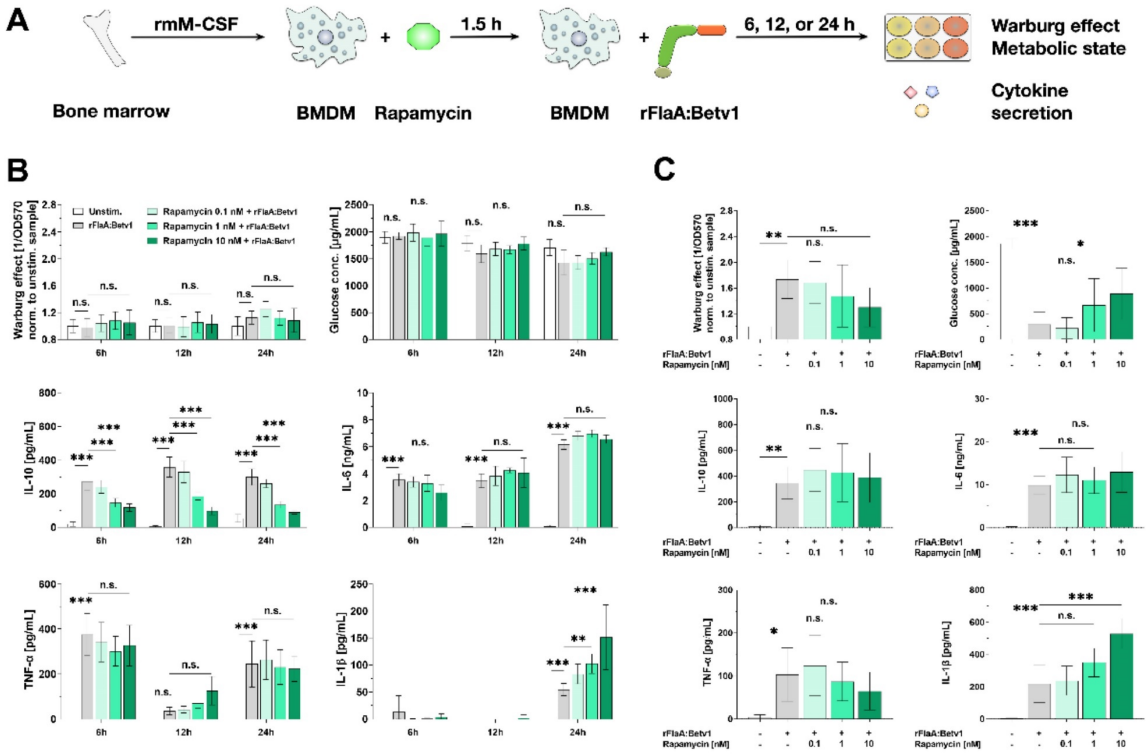

Figure 5. rFlaA:Betv1-induced activation of C57BL/6 BMDM metabolism and IL-10 secretion are partly mTOR-dependent, while pro-inflammatory cytokine secretion is mainly mTOR-independent. C57BL/6 BMDMs were differentiated from mouse bone marrow, pre-treated with the indicated rapamycin (mTOR inhibitor) concentrations for 90 min, and subsequently stimulated with rFlaA:Betv1 for an additional 6, 12, 24, or 96 h (**A**). Cells were analyzed for their metabolic state and cytokine secretion by ELISA after either 6 to 24 h (**B**) and 96 h (**C**). Data are mean results of three independent experiments ± SD, with two technical replicates per experiment. Statistical significance indicated as: n.s. p-value > 0.05, * p-value < 0.05, ** p-value < 0.01, *** p-value < 0.001.

To inhibit mTOR complex 1 (mTORC1) activation, BMDMs were pre-treated with rapamycin and checked for the Warburg effect, glucose consumption, and cytokine secretion after either 6, 12, 24, or 96 h (Figure 5B,C). Interestingly, dependent on the time, the engagement of mTOR had different effects on IL-10 secretion.

While up to 24 h post-stimulation a rFlaA:Betv1-induced Warburg effect and increased glucose consumption were not yet detectable, mTOR inhibition dose-dependently suppressed rFlaA:Betv1-induced IL-10 secretion at all investigated time points. Here, pro-inflammatory cytokine secretion was either unaffected (IL-6 & TNF-α) or even increased (IL-1β, Figure 5B). In contrast to this, at 96 h post-stimulation, inhibition of mTORC1 activation slightly, but not significantly, inhibited the rFlaA:Betv1-induced Warburg effect and reduced glucose consumption from the culture medium (Figure 5C). With the exception of a dose-dependent 2.4-fold increase in rFlaA:Betv1-induced IL-1β secretion, rFlaA:Betv1-induced IL-10, IL-6, and TNF-α secretion were all unaffected by mTOR-inhibition (Figure 5C).

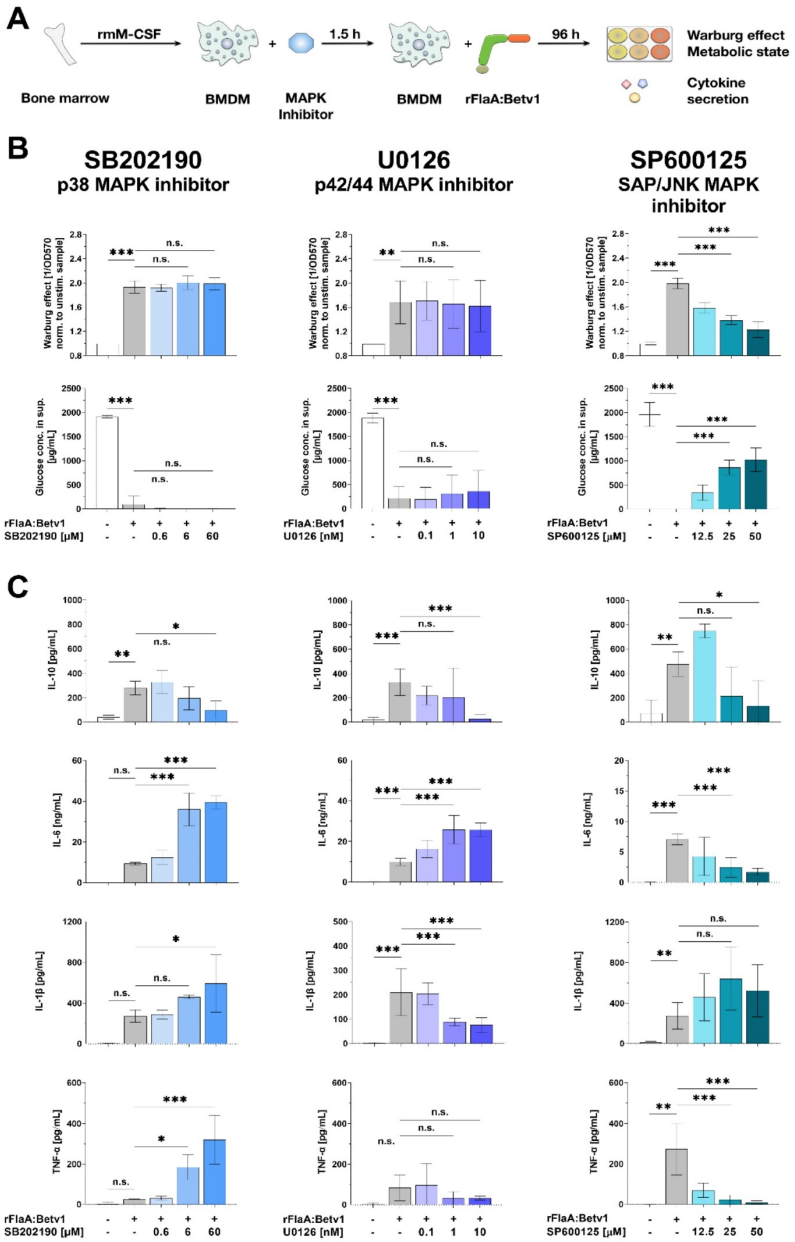

Figure 6. SAP/JNK MAP kinase signaling contributes to rFlaA:Betv1-induced activation of BMDM metabolism as well as both pro- and anti-inflammatory cytokine secretion. C57BL/6 BMDMs were differentiated from bone marrow, pre-treated with the indicated MAPK inhibitors (either SB202190 (p38 MAPK inhibitor), U0126 (p42/44 MAPK inhibitor), or SP600125 (SAP/JNK MAPK inhibitor)) for 90 min, and subsequently stimulated with rFlaA:Betv1 for additional 96 h (**A**). Cells were analyzed for their metabolic state (**B**) and cytokine secretion by ELISA (**C**). Data are mean results of three independent experiments ± SD, with two technical replicates per experiment. Statistical significance indicated as: n.s. p-value > 0.05, * p-value < 0.05, ** p-value < 0.01, *** p-value < 0.001.

To compensate for a potential degradation of rapamycin in the BMDM cultures, we added 2.5 nM of rapamycin daily for 4 days and compared the results to a one-time application of 10 nM rapamycin (as was done before in Figure 5, Repository Figure S7A). Here, in accordance with our previous results, neither a single application of 10 nM rapamycin at the beginning of the culture nor sequential adding of rapamycin over the whole culture time had any significant effects on either the rFlaA:Betv1-induced Warburg effect, glucose consumption, or cytokine secretion (Repository Figure S7B).

Therefore, these data suggest that, while rFlaA:Betv1-induced pro-inflammatory cytokine secretion is largely mTOR-independent, early rFlaA:Betv1-induced IL-10 secretion is mediated via mTOR. At later time points, mTOR-independent factors seem to drive IL-10 secretion.

3.6. MAP Kinase Signaling Contributes to rFlaA:Betv1-Induced Activation of BMDM Metabolism as well as Both Pro- and Anti-Inflammatory Cytokine Secretion

To investigate the contribution of different types of MAPK to rFlaA:Betv1-induced activation of BMDM metabolism and cytokine secretion, BMDMs were pre-treated with either the p38 MAPK inhibitor SB202190, the p42/44 MAPK inhibitor U0126, or the SAP/JNK MAPK inhibitor SP600125 (Figure 6). Interestingly, inhibition of different types of MAP kinases had differential effects on rFlaA:Betv1-induced activation of BMDM metabolism and cytokine secretion (Figure 6A).

Inhibition of p38 MAPK by pre-treatment with SB202190 and p42/44 MAPK by pre-treatment with U0126 had no effect on either the rFlaA:Betv1-induced Warburg effect or glucose consumption (Figure 6B, left and middle lane). In contrast to this, inhibition of SAP/JNK MAPK by SP600125 dose-dependently and highly significantly suppressed the rFlaA:Betv1-induced Warburg effect and glucose consumption (Figure 6B, right lane).

Inhibition of all types of MAPK dose-dependently inhibited rFlaA:Betv1-induced IL-10 secretion (Figure 6C). IL-6 secretion was significantly increased upon inhibition of either p38 MAPK (4.2-fold increase compared to rFlaA:Betv1-stimulated cells) or p42/44 MAPK (2.6-fold increase), but inhibited upon SAP/JNK MAPK-inhibition (4.1-fold reduction) (Figure 6C). rFlaA:Betv1-induced IL-1β secretion was significantly increased upon inhibition of either p38 MAPK inhibition (2.2-fold reduction compared to rFlaA:Betv1-stimulated cells) or SAP/JNK MAPK inhibition (1.9-fold increase), but significantly suppressed upon p42/44 MAPK inhibition (2.7-fold reduction) (Figure 6C). Finally, rFlaA:Betv1-induced TNF-α secretion was 12.6-fold increased by pre-treatment with the p38 MAPK inhibitor SB202190, while SAP/JNK MAPK inhibition (28.9-fold) suppressed TNF-α production (Figure 6C).

In summary, MAPK activation was shown to significantly contribute to rFlaA:Betv1-mediated BMDM activation. While the tested MAPK inhibitors differentially influenced rFlaA:Betv1-mediated cytokine secretion, only SAP/JNK MAPK activation was shown to be involved in the activation of glucose metabolism. Statistical significance indicated as: n.s. p-value > 0.05, * p-value < 0.05, ** p-value < 0.01, *** p-value < 0.001;

3.7. rFlaA:Betv1 Suppresses Th2 Responses from Bet v 1-specific CD4$^+$ T Cells In Vitro

To test the immune-modulatory capacity of rFlaA:Betv1-stimulated BMDMs, cells were cultured with CD4$^+$ T cells isolated ex vivo from rBet v 1 plus alum-sensitized BALB/c mice (Figure 7A). Co-cultures were re-stimulated with either (I) rBet v 1 to induce rBet v 1-specific recall responses, (II) equimolar amounts of rFlaA:Betv1 to check for rFlaA:Betv1-induced metabolic changes and cytokine secretion in BMDM:CD4$^+$ T cell co-cultures, (III) rBet v 1 plus the equimolar mixture of rFlaA + rBet v 1 to analyze the effect of the protein mixture on rBet v 1-induced cytokine responses, or (IV) rBet v 1 plus rFlaA:Betv1 to investigate the effect of the fusion protein on rBet v 1-induced cytokine responses.

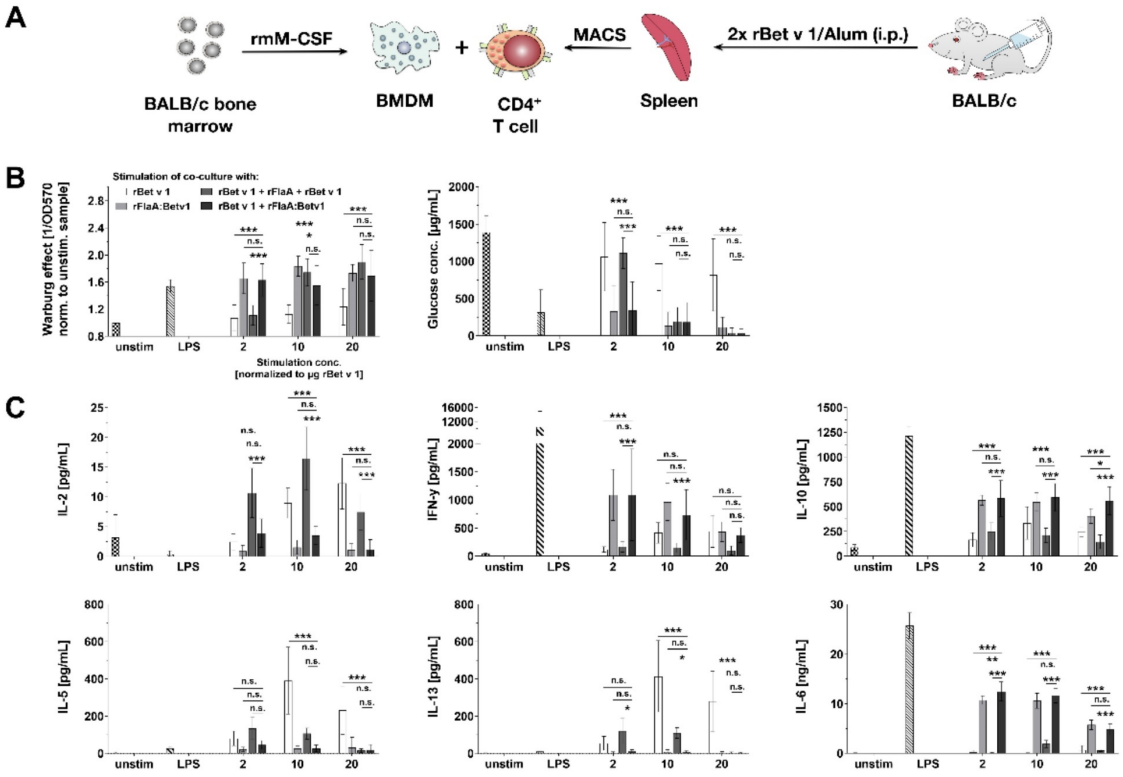

Figure 7. rFlaA:Betv1 suppresses rBet v 1-specific Th2 responses in vitro. BALB/c BMDMs were differentiated from mouse bone marrow, co-cultured with $CD4^+$ T cells isolated by magnetic activated cell sorting (MACS) from rBet v 1 plus Alum-sensitized mice and stimulated with the indicated equimolar protein amounts or LPS as a positive control for 96 h (**A**). Supernatants were analyzed for their metabolic state (**B**) and cytokine secretion by ELISA (**C**). Data are mean results of three independent experiments ± SD with two technical replicates per experiment. Statistical significance indicated as: n.s. p-value > 0.05, * p-value < 0.05, ** p-value < 0.01, *** p-value < 0.001.

Interestingly, stimulation with rBet v 1 only induced a very slight Warburg effect in BMDM:$CD4^+$ T cell co-cultures, which was stronger after stimulation with the mixture of rFlaA + rBet v 1 (equimolar to 10 µg rBet v 1), while rFlaA:Betv1 induced a strong Warburg effect. even in the lowest stimulation concentration (Figure 7B). Results of glucose consumption paralleled the results observed for the Warburg effect (with high Warburg effects resulting in high glucose consumption, Figure 7B).

rBet v 1-induced IL-2 secretion was further boosted upon co-stimulation with the mixture of both proteins, while being suppressed after co-stimulation with rFlaA:Betv1 (Figure 7C). Stimulation of co-cultures with either rFlaA:Betv1 or rBet v 1 + rFlaA:Betv1 resulted in significantly increased levels of IFN-γ or IL-6 secretion (Figure 7C). Here, both levels of rFlaA:Betv1-induced IFN-γ and IL-6 secretion were reduced for the highest stimulation concentration (equimolar to 20 µg rBet v 1/mL) compared to the other stimulation concentrations (Figure 7C). In line with previous results, stimulation of BMDM:$CD4^+$ T cell co-cultures with rFlaA:Betv1 resulted in pronounced anti-inflammatory IL-10 secretion (Figure 7C). Here, levels of secreted IL-10 were approximately 500 pg/mL for all tested stimulation concentrations (Figure 7C). rBet v 1-induced secretion of the Th2 cytokines IL-5 and IL-13 was significantly suppressed by either co-stimulation with either rFlaA + rBet v

1 or rFlaA:Betv1 (Figure 7C). Of note, in contrast to rBet v 1, rFlaA:Betv1 did not induce secretion of Th2 cytokines IL-5- and IL-13 from BMDM:CD4$^+$ T cell co-cultures (Figure 7C). Neither rFlaA + rBet v 1 nor rFlaA:Betv1 directly activated the metabolism or cytokine secretion from naïve CD4$^+$ T cells (data not shown).

Taken together, rFlaA:Betv1-mediated activation of BMDM metabolism was also observed in BMDM:CD4 T cell co-cultures. Here, both addition of rFlaA:Betv1 and rFlaA + rBet v 1 suppressed the rBet v 1-induced secretion of Th2 cytokines, while rFlaA:Betv1 also triggered significant production of IFN-γ, IL-6, and IL-10.

3.8. rFlaA:Betv1 Induces a Transcriptional Shift Towards HIF-1a-Mediated Glycolytic Metabolism in BMDMs

To (I) confirm both the activation of glycolytic metabolism and BMDM activation and (II) investigate the regulation of other signaling pathways in rFlaA:Betv1-stimulated BMDMs in an unbiased way, BMDMs were stimulated with either LPS, rFlaA + rBet v 1, or rFlaA:Betv1 for 48 h, and whole-cell RNA expression profiles were generated using MiSeq technology (Figure 8A). In addition, samples were collected for Western blot analysis 2, 6, 24, and 48 h post-stimulation (Figure 8A).

Principal component analyses showed the biological replicates for each stimulation condition to closely group with each other, while the different stimulation conditions formed clearly separated groups (Figure 8B). Here, rFlaA:Betv1-stimulated BMDMs showed similar cell activation capacity as LPS-stimulated cells (Figure 8B).

The most prominently regulated KEGG pathways for the different stimulation conditions are depicted in Figure 8C. In general, an upregulation of endocytosis, glycolysis, antigen processing and presentation, proteasome, oxidative phosphorylation, and signaling of HIF, JAK-STAT, phagosomes, NLR, NFκB− TNF, TLR, and chemokines was observed 48 h post-stimulation. Instead, signaling pathways mediating the early events of BMDM activation, such as phosphatidylinositol 3 kinase (PI3K)-AKT, MAPK, and mTOR signaling, were down-regulated compared to unstimulated samples (Figure 8C). Here, a general similarity between LPS- and rFlaA:Betv1-stimulated samples was again observed (Figure 8C). Compared to rFlaA + rBet v 1-stimulated BMDMs, rFlaA:Betv1-stimulation resulted in an upregulation of pathways associated with HIF-1a, TNF, TLR, and chemokine signaling, while lysosome-associated signaling was downregulated (Figure 8C).

Finally, activation of JAK-STAT-HIF-1a signaling, glycolysis, and the downregulation of mitochondrial respiration observed in transcriptomic samples were confirmed by Western blot (Figure 8D). Here, we observed a phosphorylation of STAT3 2, 6, and 24 h post-stimulation, which was more pronounced in LPS− and rFlaA:Betv1-stimulated BMDMs, and followed by a strong upregulation of HIF-1a starting 24 h post-stimulation after the peak of STAT3 phosphorylation at 6 h (Figure 8D). We also observed a stronger expression of the glucose transporter Glut-1 in all stimulation conditions 48 h post-stimulation and a stronger expression of the glycolysis-promoting enzyme 6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 3 (PFKFB3) in LPS− and rFlaA:Betv1-stimulated BMDMs 24 h post-stimulation. In accordance with the observed switch to glycolytic metabolism, expression levels of the mitochondrial enzyme aconitase 2 (ACO2) were lower in LPS− and rFlaA:Betv1-stimulated BMDMs both 24 and 48 h post-stimulation (Figure 8D).

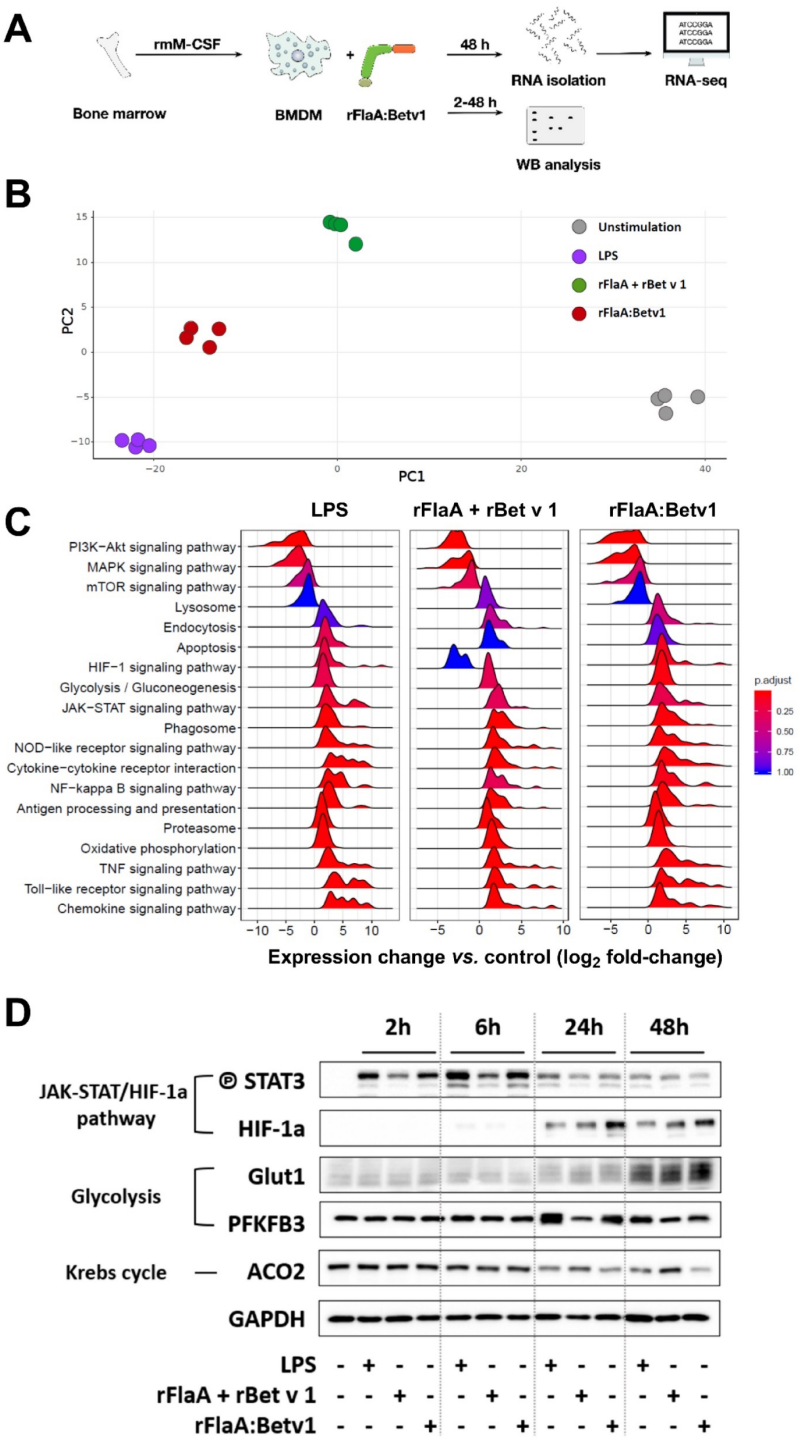

Figure 8. rFlaA:Betv1 induces a transcriptional shift towards HIF-1a-mediated glycolytic metabolism in BMDMs. C57BL/6 BMDMs were differentiated from mouse bone marrow and stimulated with either

LPS as a positive control or equimolar amounts of either rFlaA + rBet v 1 or rFlaA:Betv1 for 2 to 48 h (**A**). Cells were harvested and used for either RNA-seq (**B**,**C**) or Western blot analyses (**D**). To characterize the transcriptional status of the different samples, principal component analysis (**B**) and gene set enrichment analysis of the most significantly regulated KEGG pathways (**C**) was performed. BMDMs stimulated for 2, 6, 24, or 48 h with either LPS, rFlaA + rBet v 1, or rFlaA:Betv1 were analyzed by Western blot for expression levels of the indicated molecules (**D**). Data are either mean (**C**) or representative (**D**) results of three to four independent experiments using either one RNA preparation (**B**,**C**) or one lysate (**D**) per experiment.

Taken together, the results obtained by RNA-seq analyses confirm a pronounced shift towards anaerobic metabolism in rFlaA:Betv1−, LPS−, and, to a lesser extent, rFlaA + rBet v 1-stimulated BMDMs.

Our current mechanistic understanding of the molecular events contributing to rFlaA:Betv1-mediated BMDM activation is depicted in Figure 9.

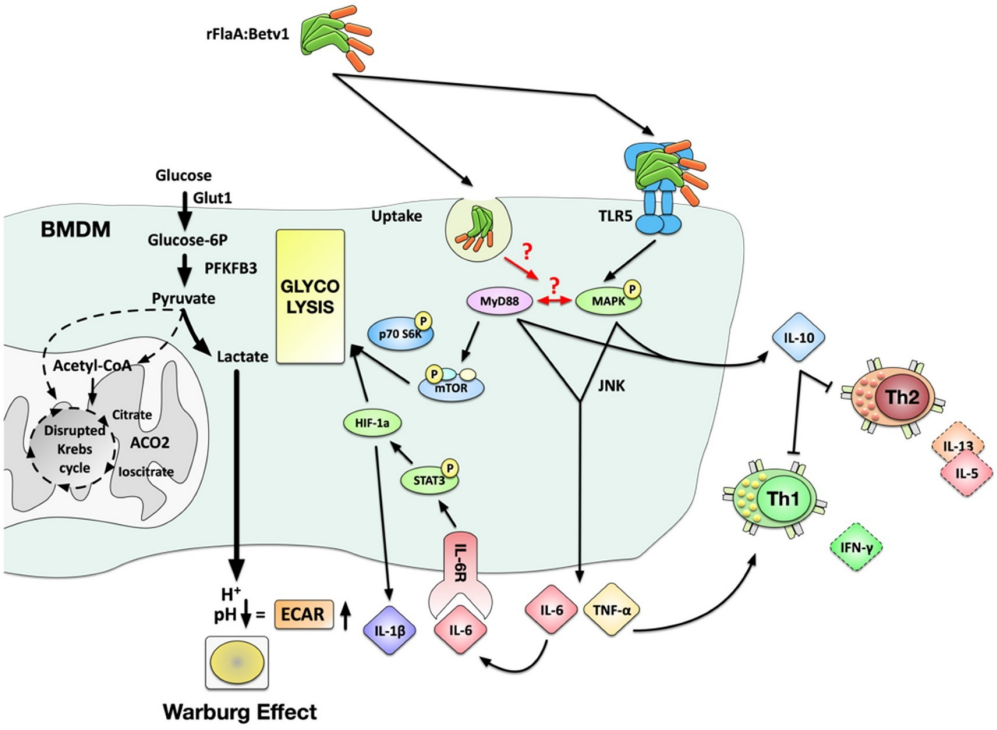

Figure 9. Suggested molecular signaling events contributing to rFlaA:Betv1-mediated activation of BMDMs. Stimulation of BMDM with aggregated rFlaA:Betv1 results in both TLR5-dependent and -independent uptake, leading to the activation of both MyD88− and MAPK-mediated signaling cascades. rFlaA:Betv1-induced pro-inflammatory IL-6 and TNF-α secretion were shown to be MyD88− and MAPK-dependent, likely promoting the induction of IFN-γ-producing Th1 cells in BMDM:TC co-cultures. Western blot analysis confirmed rFlaA:Betv1 to trigger STAT3 activation, increasing the expression of HIF-1a, which is likely to be responsible for the observed IL-1β secretion. Metabolically, we found a MyD88-dependent phosphorylation of the mTOR target protein p70 S6 kinase, which together with HIF-1a likely mediated the observed shift towards enhanced glycolysis with increased levels of the glucose transporter Glut-1 and PFKFB3, and the induction of the Warburg effect while being paralleled by a decreased expression of mitochondrial proteins such as ACO2 and reduced oxygen consumption rates, suggesting a disrupted Krebs cycle in these cells. Finally, TLR5, MyD88, and MAPK activation were shown to be involved in the rFlaA:Betv1-induced production of the anti-inflammatory cytokine IL-10 that likely caused the observed suppression of rBet v 1-induced Th1 and Th2 responses in BMDM:TC co-cultures.

4. Discussion

In the present study, we analyzed the activation, metabolic state, and transcriptional state of BMDMs, as well as their immune-modulatory properties upon stimulation with a flagellin:allergen conjugate (rFlaA:Betv1), which was recently shown in preclinical studies to prevent allergic Th2 responses [15]. Here, the allergen Bet v 1 serves as a model antigen that is conjugated to the TLR5 ligand flagellin FlaA, which acts as an adjuvant activating the immune system. Thereby, FlaA may modulate the immunological properties of the conjugated antigen while also targeting the antigen to innate immune cells.

Currently, the effects of flagellin-containing fusion proteins on macrophages, which serve as important innate immune cells, are less described. So far, only one study by Verma et al. has described the insertion of a cytochrome C molecule into the flagella of *Salmonella dublin* to efficiently trigger processing and presentation of cytochrome-derived peptides by mouse peritoneal macrophages [42]. This prompted us to investigate the effect of FlaA and FlaA-containing fusion proteins on mouse macrophages.

4.1. rFlaA:Betv1 Induces a Partly TLR5-Dependent Activation of BMDMs Characterized by Both Pro- and Anti-Inflammatory Cytokine Secretion

We observed a stronger activation of mouse BMDMs by rFlaA:Betv1 compared to the respective controls. However, both stimulation with rFlaA and rFlaA + rBet v 1 resulted in a weaker, but still clearly detectable, activation of cytokine secretion and glycolytic metabolism in BMDMs. In line with the available literature [2–4], these results confirmed the adjuvant effect of non-conjugated flagellin.

BMDM activation by rFlaA:Betv1 was characterized by strongly increased pro- (IL-1β, IL-6, TNF-α) and anti-inflammatory (IL-10) cytokine secretion. In terms of the net outcome of this mixed pro- and anti-inflammatory response, the observed suppression of rBet v 1-induced Th2 cytokine secretion from BMDM:$CD4^+$ T cell co-cultures suggested rFlaA:Betv1-mediated anti-inflammatory signaling (at least in higher stimulation concentrations) to be more pronounced.

While rFlaA:Betv1-mediated TNF-α secretion was largely TLR5-dependent, IL-10 secretion was partially reduced in $TLR5^{-/-}$ BMDMs, and IL-1β and IL-6 secretion were found to be TLR5-independent. In line with our results, Bao and colleagues reported *S. typhimurium* FliC to induce a TLR5-dependent TNF-α production via the activation of PI3K/AKT /mTOR-dependent NFκB and STAT3 signaling in mouse macrophages [43]. Inhibition of both PI3K by either LY294002 or wortmannin and the mTOR complex 1 (mTORC1) by rapamycin decreased flagellin-induced TNF-α and IL-6 expression as well as macrophage proliferation [43]. In an additional study, flagellin-induced TNF-α production from alveolar macrophages [44] was shown to be TLR-dependent, while FliC-induced IL-1ß secretion from mouse macrophages was shown to be IPAF-dependent, but TLR5-independent [45]. Interestingly, upregulation of CD69 was substantially reduced in $TLR5^{-/-}$ BMDMs, while neither rFlaA:Betv1-induced cytokine secretion nor cell activation were observed in MyD88-deficient BMDMs.

Interestingly, we observed a significantly increased secretion of IL-1β upon inhibition of mTOR in rFlaA:Betv1-stimulated BMDMs both 24 and 96 h post-stimulation. Tang et al. reported similar results from tuberous sclerosis complex 1 (TSC-1)-deficient macrophages, which display constitutively active mTOR signaling [46]. Here, impaired (LPS-induced) IL-1β secretion from tuberous sclerosis complex 1 (TSC-1)-deficient macrophages could be increased by either inhibition of mTOR activation or mTOR deletion [46]. Mechanistically, mTOR-mediated downregulation of the CCAAT enhancer-binding protein (C/EBPβ) was shown to critically contribute to the reduction in IL-1β production [46]. These results suggest that, both in the model of Tang et al. and in rFlaA:Betv1-stimulated BMDMs, TSC-1 may promote IL-1β production via the C/EBPβ pathway, while activation of mTOR suppresses both TSC-1 itself and TSC-1-dependent downstream processes (including IL-1β production) [46].

In our previous publication describing the molecular characterization of rFlaA:Betv1, we reported rFlaA:Betv1 to be aggregated [15]. Here, rFlaA:Betv1 formed high molecular aggregates, which were only visible under native PAGE conditions [15]. Increased uptake of these aggregates in myeloid dendritic cells was shown to be TLR5-independent while being essential for the induction of rFlaA:Betv1-mediated IL-10 and IL-6 secretion [15]. Therefore, aggregation was previously shown to be an important factor determining the immunogenicity of the fusion proteins.

Mechanistically, we assume the stronger BMDM-activating potential of the fusion protein compared to both single proteins to result from: (i) the previously observed aggregation of rFlaA:Betv1 [15], which results in higher densities of the fusion protein on the surface of BMDMs, higher protein uptake, and therefore stronger activating signals transmitted to the respective BMDMs nucleus, (ii) changes in BMDM metabolic state due to the strongly activating signal provided by rFlaA:Betv1, and (iii) (for the induction of adaptive immune responses by rFlaA:Betv1-stimulated BMDMs) differences in antigen processing upon fusion to flagellin already reported for rOva contained within a rFlaA:Ova fusion protein [18].

Taken together, these results suggest that both TLR5-dependent and -independent processes contribute to rFlaA:Betv1-mediated BMDM activation, while MyD88 is a key molecule in rFlaA:Betv1-mediated macrophage activation.

4.2. rFlaA:Betv1-Stimulated BMDMs Efficiently Modulate Allergen-Specific T Cell Responses

Considering that the activation of BMDMs by rFlaA:Betv1 is antigen unspecific, we checked for the effect of rFlaA:Betv1-stimulated BMDMs on Th2-biased Bet v 1-specific T cells in an ex vivo co-culture model. Here, both rFlaA + rBet v 1 and rFlaA:Betv1 efficiently suppressed rBet v 1-induced Th2-cytokine secretion, while only rFlaA:Betv1 boosted IL-10 and IFN-γ secretion, once again confirming the pronounced immune modulatory capacity and Th1-inducing capacity of the fusion protein. One weakness of the employed co-culture setup is that the cellular source of the secreted cytokines (BMDMs or T cells) cannot be exactly determined. However, despite this limitation, our data provide evidence that simultaneous targeting of adjuvant and antigen to BMDMs efficiently promotes modulation of antigen-specific effector T cell responses. While in this study we investigated rBet v 1-specific T cell responses, our previous work on the model antigen ovalbumin [16,18] and the major mugwort allergen Art v 1 [17] suggests that the findings can also be transferred to other antigens.

4.3. rFlaA:Betv1 Activates Both Glycolytic Metabolism and HIF-1a Signaling in BMDMs

A further objective of this study was to explore the mechanisms underlying the observed immune modulation in more detail. Previous reports suggested mTOR to be engaged in rFlaA:Betv1-mediated cytokine responses [15]. rFlaA:Betv1 also triggered changes in the metabolic phenotype of the stimulated BMDMs. In activated immune cells, distinct metabolic changes not only provide the energy needed by these cells to proliferate and fulfill their effector functions, but are also used to generate important immunological active effector molecules (e.g., prostaglandins, ROS, NOS, or itaconate generated from a disrupted Krebs cycle in highly glycolytic cells) [47]. Therefore, cellular metabolism and signaling pathways classically associated with the activation and effector function of immune cells have recently been recognized to be intimately intertwined, regulating each other, and thereby shaping the overall immune responses induced.

We observed a highly significant induction of the Warburg effect in rFlaA:Betv1-stimulated BMDMs. The observed induction of the Warburg effect suggests a cellular shift towards glycolysis with lactate generation and extracellular acidification [48]. The suggested metabolic changes were confirmed using both Seahorse Metabolic Flux and transcriptomics analyses. RNA Seq confirmed the activation of both BMDM glycolytic metabolism (upregulation of key glycolysis enzymes Glut-1/2, phospho-fructo-kinase (PFK), PFKFB3, phosphoglycerate mutase (PGM), pyruvate kinase isozyme (PKM), and

pyruvate dehydrogenase kinase 1 (PDK1)), intracellular signaling (TLR, MAPK, and NFκB signaling), and both cytokine and chemokine secretion (Repository Figure S8). Taken together, the activation status and metabolic phenotype of rFlaA:Betv1-stimulated BMDMs closely relates to M1-polarized macrophages, which switch their metabolism towards glycolysis after being activated by pathogen-associated molecular patterns [49–53]. Increases in glycolytic metabolism enable M1-like macrophages to efficiently fulfill their effector function, including the production of pro-inflammatory cytokines [54] and phagocytosis [55].

In our experimental setting, HIF-1a, which was shown to be essential for glycolytic metabolism in macrophages, seems to be of particular importance. HIF-1a-deficient macrophages lack both lactate and ATP production after LPS stimulation, and impaired glycolysis was shown to profoundly reduce the clearance of bacteria and fungi [56,57]. In contrast to M1 macrophages, activated M2 macrophages were reported to display STAT6— and PGC-1β-dependent decreases in glycolytic activity, while mitochondrial oxygen consumption rates and fatty acid oxidation were increased [58,59]. In line with the suggested M1 phenotype, stimulation of BMDMs with rFlaA:Betv1 resulted in a pronounced activation of HIF-1a signaling, which was not observed in BMDMs stimulated with equimolar amounts of rFlaA + rBet v 1. These results suggest the induction of HIF-1a signaling being critical for rFlaA:Betv1-mediated BMDM activation. Remarkably, succinate accumulating from a disrupted Krebs cycle in LPS-stimulated macrophages was shown to promote both glycolysis and IL-1ß production via the stabilization of HIF-1a (reviewed in [47]), thereby linking the activation of glycolytic metabolism to the activation of HIF-1a signaling. Interestingly, this newly discovered activation of HIF-1a signaling by rFlaA:Betv1 might further explain the previously observed induction of Th1-biased immune responses by flagellin:allergen fusion proteins in vitro and in vivo [15–18], as the activation of HIF-1a in macrophages was recently described to promote induction of Th1 responses [60]. Besides, M1 macrophages are known to produce pro-inflammatory cytokines, such as IL-12, TNF-α, IFN-γ, and IL-6, and can further induce Th1 responses [61]. The production of these cytokines was also reported to be downregulated in macrophage-specific HIF-1a knockout mice upon β-aminopropionitrile stimulation [62]. Indeed, we observed that rFlaA:Betv1 boosted production of the Th1 cytokine IFN-γ secretion even from BMDM:$CD4^+$ T cell co-cultures with Th2-biased T cells (Figure 7).

4.4. MAPK, NFκB, and mTOR Signaling Contribute to rFlaA:Betv1-Mediated BMDM Activation

Finally, we observed a pronounced activation of MAPK, NFκB, and mTOR signaling in rFlaA:Betv1-stimulated BMDMs. Interestingly, the activation of these signaling pathways was TLR5-independent, while MAPK and NFκB (but not mTOR) signaling were still observed in MyD88-deficient BMDMs.

We speculate MAPK signaling and mTOR/HIF-1a-controlled activation of BMDM metabolism to extensively cross-talk in rFlaA:Betv1-stimulated BMDMs. Here, activated p42/44 MAPK signaling can cross-activate mTOR signaling by either (i) activating PI3K [63–67], (ii) phosphorylation of TSC-2, which releases TSC-1/2-mediated mTOR inhibition [67,68], (iii) phosphorylation of regulatory associated protein of mTOR (RAPTOR), which promotes mTORC1 activation, and (iv) direct activation of the mTOR downstream target protein p70 S6 kinase [69–71].

In summary, the presented study reports the immune metabolic effects of a novel type of vaccine candidate generated by combining the TLR5 ligand flagellin with the major birch pollen allergen Bet v 1 (which serves a model antigen). Our results suggest that stimulation of BMDMs with rFlaA:Betv1 triggered a MyD88-dependent, but only partly TLR5-dependent, cytokine production, stronger activation of HIF-1a, MAPK, NFκB, and mTOR signaling (with the activation of MAPK being located upstream of mTOR signaling), and increased glucose metabolism. In BMDM and $CD4^+$ T cell co-cultures, rFlaA:Betv1 stimulation significantly suppressed Bet v 1-induced IL-5 and IL-13 secretion while inducing IFN-γ production (summarized in Figure 9). Therefore, macrophages contribute to the strong immune-modulating effects of rFlaA:Betv1 previously observed in vivo [15] and

should be considered important target cells contributing to the re-establishment of allergen tolerance in AIT.

Finally, our results suggest that both the fusion of antigens to immune-activating molecules and the resulting structural alterations in the fused antigen can increase its immunogenicity, resulting in the induction of distinct immune responses. Therefore, such adjuvant:antigen fusion proteins likely are valuable tools to induce distinct (in our case balanced Th1) immune responses against antigens with otherwise either low immunogenicity or a high risk of triggering potentially harmful Th2 responses.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/cells10102614/s1: Figure S1: Both rFlaA and rFlaA:Betv1 activate BMDM metabolism, Figure S2: Activation of BMDM metabolism is accompanied by both pro- and anti-inflammatory cytokine secretion, Figure S3: The amount of LPS contained within the used rFlaA:Betv1 preparations do not induce BMDM activation, Figure S4: The used rFlaA:Betv1 preparation does not have mTLR2- or mTLR4-activating potential, Figure S5: Both rFlaA + rBet v 1 and rFlaA:Betv1 comparably reduce mitochondrial respiration, Figure S6: Toxicity of the tested inhibitors on C57BL/6 BMDMs, Figure S7: 96 h post-stimulation, rapamycin has only minor effects on rFlaA:Betv1-induced activation of BMDM metabolism and cytokine secretion, Figure S8: rFlaA:Betv1 induces a transcriptional shift towards both increased glycolytic metabolism and higher activation status in BMDMs.

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Abbreviations

2-DG	2-deoxy-glucose
ACO2	Aconitase 2
BMDM	Bone marrow-derived macrophage
CD	Cluster of differentiation
C/EBPβ	CCAAT enhancer-binding protein
ECAR	Extracellular acidification rate
GM-CSF	Granulocyte-macrophage colony-stimulating factor
IFN-γ	Interferon gamma
IL	Interleukin
Ipaf	ICE-protease activating factor

MAP(K)	Mitogen-activated protein (kinase)
(m)DC	(Myeloid) Dendritic cell
mTOR	Mammalian target of rapamycin
mTORC1	mTOR complex 1
MyD88	Myeloid differentiation primary response 88
NFκB	Nuclear factor 'kappa-light-chain-enhancer' of activated B-cells
OCR	Oxygen consumption rate
PI3K	Phosphatidylinositol 3 kinase
PDK:1	Pyruvate dehydrogenase kinase 1
PFK	Phospho-fructo-kinase
PFKFB3	6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 3
PGM	Phosphoglycerate mutase
PKM	Pyruvate kinase isozyme
rBet v 1	Recombinant major birch pollen allergen number one from *Betula verrucosa*
rFlaA	Recombinant *Listeria monocytogenes* flagellin A
rFlaA:Betv1	Recombinant fusion protein of FlaA and Bet v 1
rmM-CSF	Recombinant mouse macrophage colony-stimulating factor
RAPTOR	Regulatory associated protein of mTOR
TSC	Tuberous sclerosis complex
Rot/AA	Rotenone/ antimycin A
SAP/JNK MAPK	Stress-activated protein kinase/c-Jun NH_2-terminal kinase
Th1/2	T helper 1/2 cell
TLR	Toll-like receptor
TNF-α	Tumor necrosis factor alpha

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The Flagellin:Allergen Fusion Protein rFlaA:Betv1 Induces a MyD88– and MAPK-Dependent Activation of Glucose Metabolism in Macrophages

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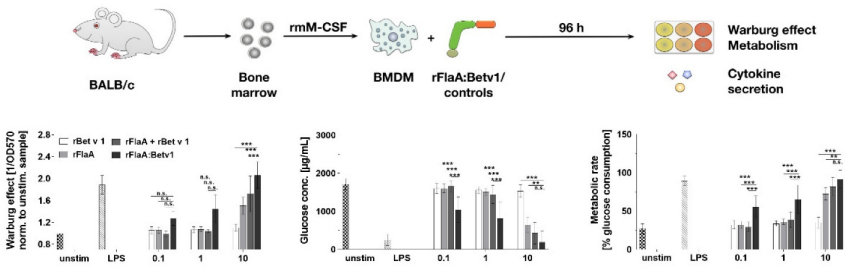
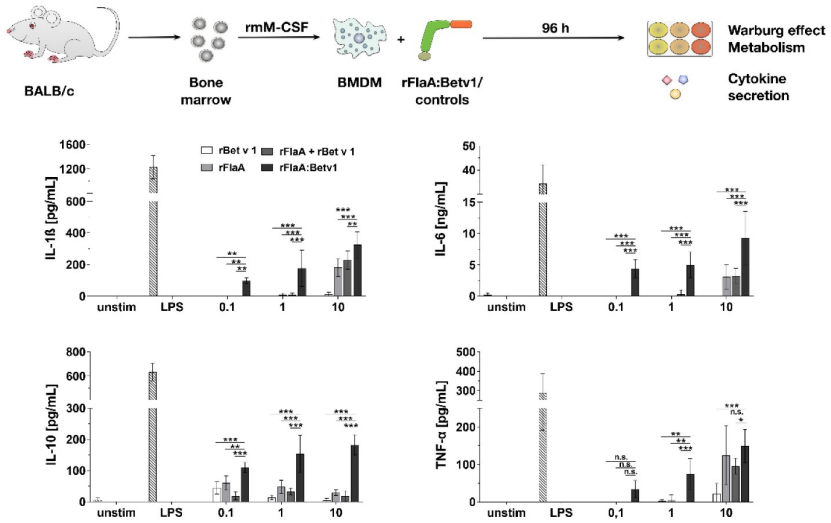
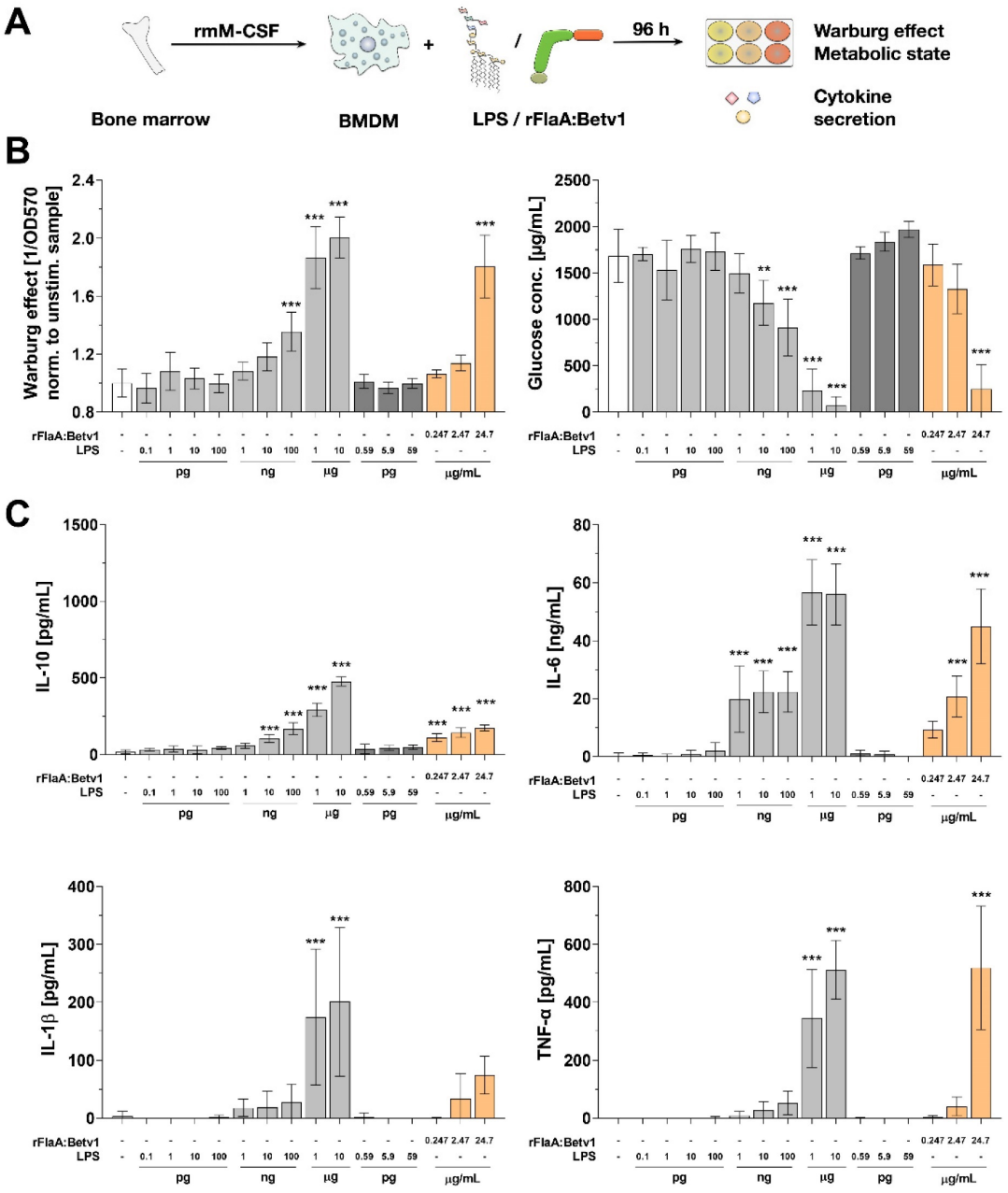
Supplementary information



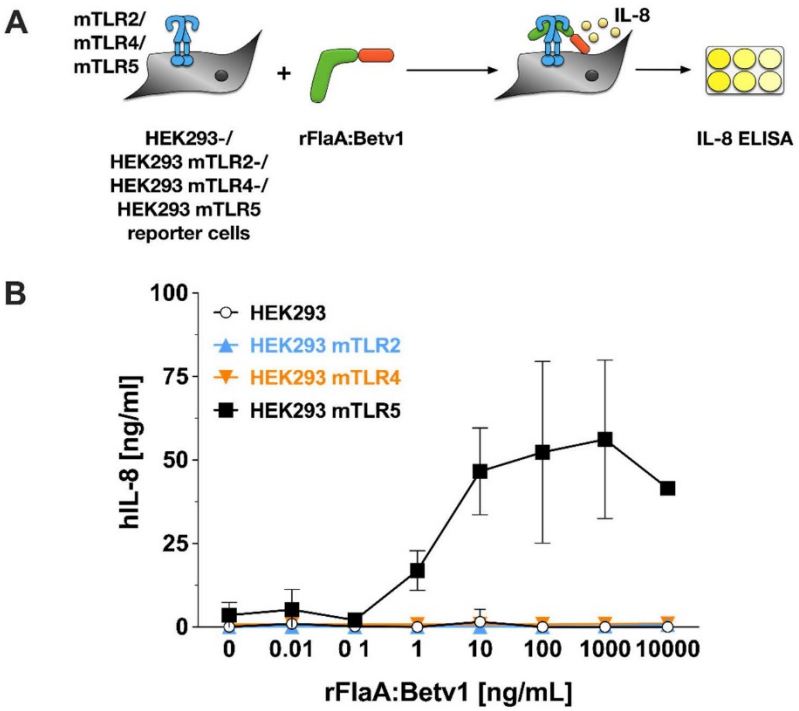
Repository Figure S1. Both rFlaA and rFlaA:Betv1 activate BMDM metabolism. BMDMs were differentiated from BALB/c bone marrow and stimulated with the indicated equimolar protein amounts or LPS as a positive control for 96 h. Supernatants were analyzed for the induced Warburg effect, glucose consumption from the culture medium as well as changes in metabolic rate. Data are mean results of three independent experiments ±SD with two technical replicates per experiment.



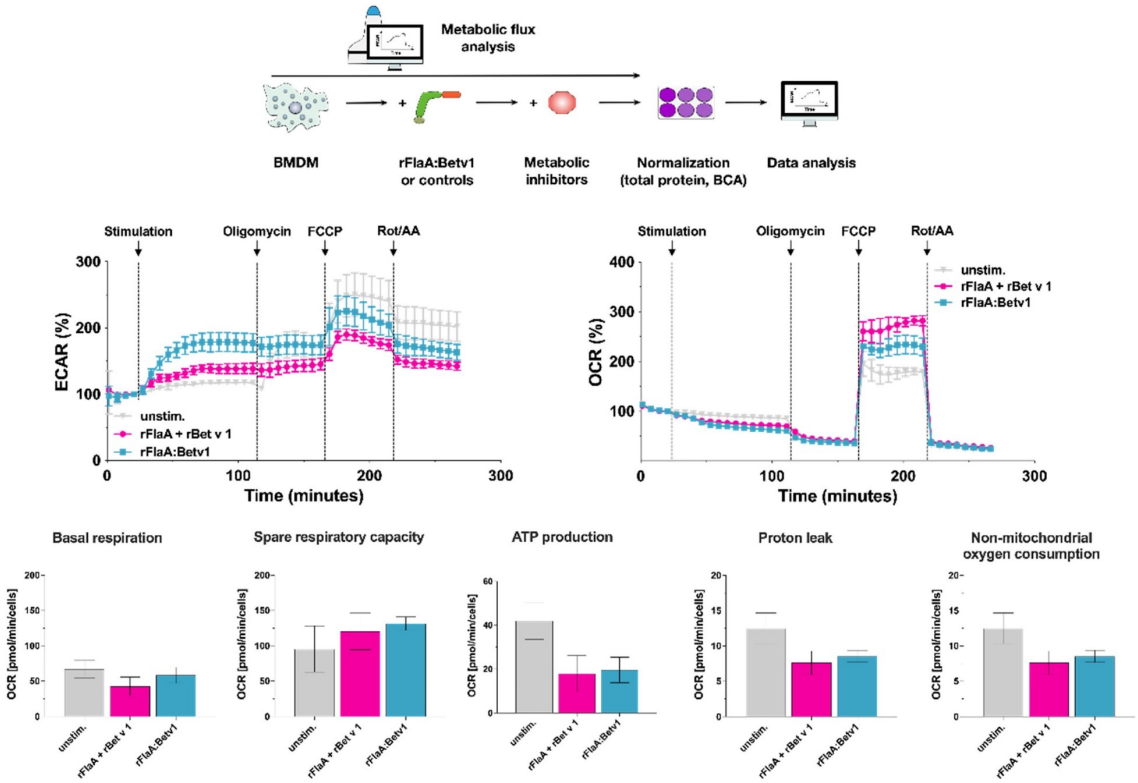
Repository Figure S2. Activation of BMDM metabolism is accompanied by both pro- and anti-inflammatory cytokine secretion. BMDMs were differentiated from BALB/c bone marrow and stimulated with the indicated equimolar protein amounts or LPS as a positive control for 96 h. Supernatants were analyzed for cytokine secretion by ELISA. Data are mean results of three independent experiments ±SD with two technical replicates per experiment.



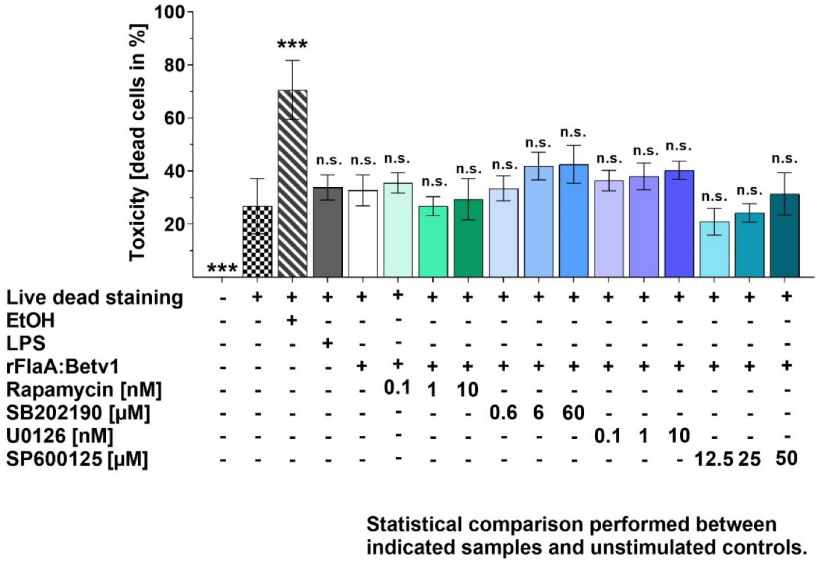
Repository Figure S3. The amounts of LPS contained within the used rFlaA:Betv1 preparations do not induce BMDM activation. C57BL/6 BMDMs were differentiated from mouse bone marrow and stimulated with the indicated amounts of either LPS to establish dose-response curves (light grey) the residual amounts of LPS contained within the applied concentrations of rFlaA:Betv1 (dark grey) to exclude BMDM activating effects, or rFlaA:Betv1 (orange) for 96 h (**A**). Supernatants were analyzed for the induced Warburg effect and glucose consumption (**B**) as well as cytokine secretion by ELISA (**C**). Data are mean results of three independent experiments±SD with two technical replicates per experiment.



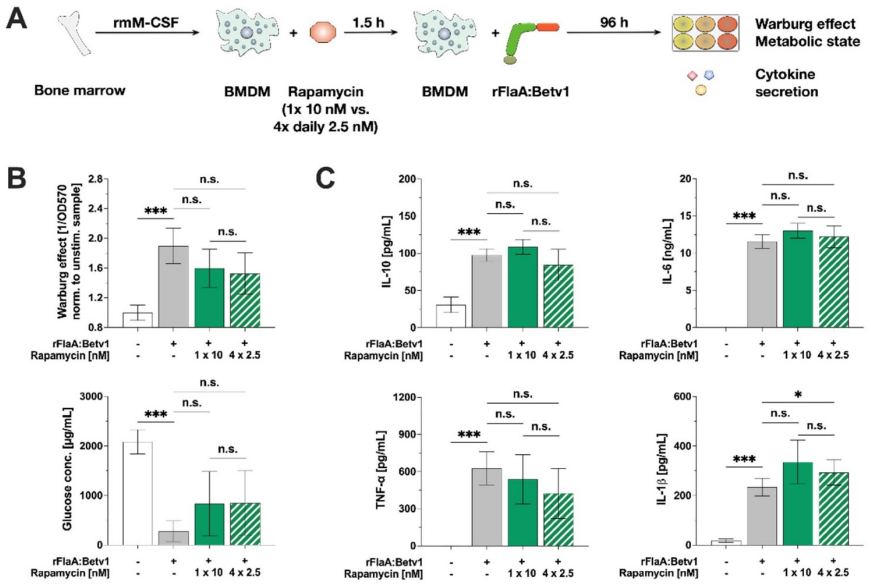
Repository Figure S4: The used rFlaA:Betv1 preparation does not have mTLR2- or mTLR4-activating potential. HEK293 cells stably transfected with either mouse TLR2, TLR4, or TLR5 were stimulated for 22 h with the indicated amounts of rFlaA:Betv1 (**A**). Non-transfected HEK293 cells were used as controls. IL-8 secretion into the culture supernatants was determined by ELISA (**B**). Data are mean of three independent experiments±SD with two technical replicates per experiment.



Repository Figure S5. Both rFlaA + rBet v 1 and rFlaA:Betv1 comparably reduce mitochondrial respiration. Mitochondrial function in either rFlaA + rBet v 1- or rFlaA:Betv1-stimulated BMDMs (both equimolar to 4 µg of rBet v 1) was analyzed by Seahorse Technology. Basal respiration, spare respiratory capacity, mitochondrial ATP production, proton leak, and non-mitochondrial respiration were analyzed using the "XF cell Mito Stress Test Report Generator" according to the manufacturer's recommendations. Data are representative results of three independent experiments (with three to four technical replicates per experiment) that showed similar results.

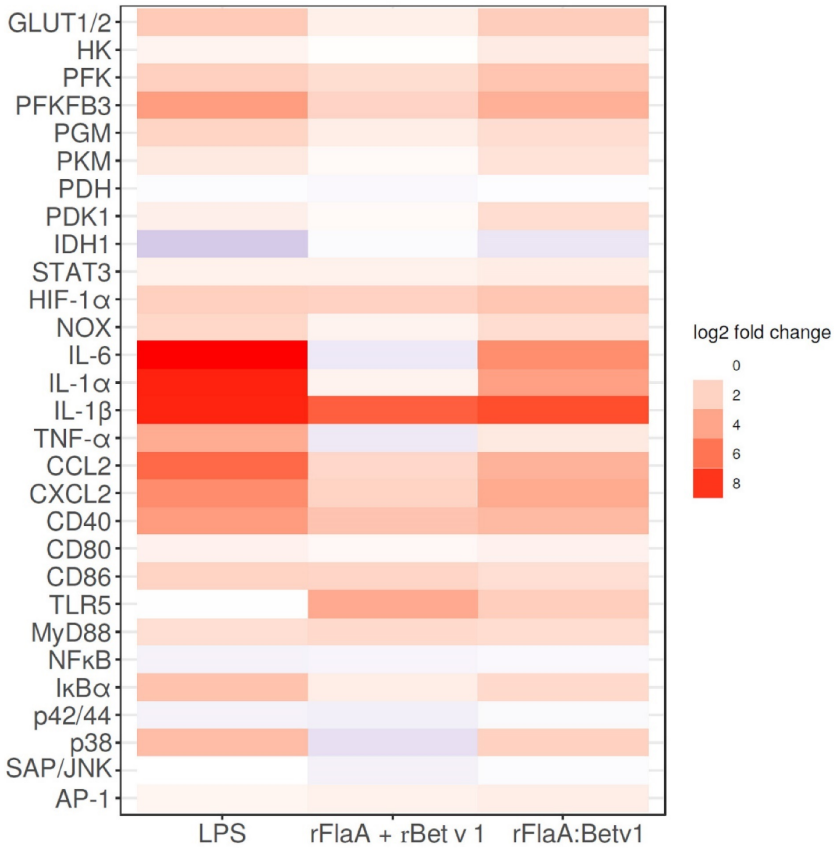


Repository Figure S6. Toxicity of the tested inhibitors on C57BL/6 BMDMs. C57BL/6 BMDMs were differentiated from bone marrow and stimulated with the indicated inhibitor concentrations, LPS, or rFlaA:Betv1, for 24 h. Cells killed by incubation with 70% EtOH served as positive controls. The frequency of dead cells was determined by live dead staining using eBioscience Fixable Viability Dye eFlour780 as recommended by the manufacturer. Data are mean results of three independent experiments ±SD with two technical replicates per experiment.



Repository Figure S7. 96 h post stimulation rapamycin has only minor effects on rFlaA:Betv1-induced activation of BMDM metabolism and cytokine secretion. C57BL/6 BMDMs were differentiated from bone marrow, pre-treated with either 10 nM rapamycin (mTOR inhibitor) once for 90 min or 2.5 nM rapamycin every 24 h, and subsequently stimulated with rFlaA:Betv1 for additional 96 h

(**A**). Cells were analyzed for their metabolic state (**B**) and cytokine secretion by ELISA (**C**). Data are mean results of three independent experiments±SD with two technical replicates per experiment.



Repository Figure S8 rFlaA:Betv1 induces a transcriptional shift towards both increased glycolytic metabolism and a higher activation status in BMDMs. C57BL/6 BMDMs were differentiated from bone marrow and stimulated with LPS as positive control or equimolar amounts of either rFlaA + rBet v 1 or rFlaA:Betv1 for 48 h. Cells were harvested, used for RNA Seq-analyses, and mRNA-expression levels of the indicated molecules compared to the mean of unstimulated BMDMs were analyzed from the different stimulation conditions. Fold changes (log_2 scale) in mRNA expression level compared to unstimulated controls are indicated by color. Shown are mean expressional changes, obtained with four biological replicates per condition.

6 General Discussion & Summary

In the present studies, we investigated the potential of β-(1→4)-mannobiose (Man2) as a novel adjuvant and the immune mechanisms in cell types activated by the therapeutic candidate rFlaA:Betv1 suggested for the intervention of type I allergies. Our overall findings are summarized in Figure 7 and will be discussed here.

6.1 Man2 has potential as a novel adjuvant as it can activate dendritic cells

In chapter 1, we showed that Man2 could activate both dendritic cells and macrophages, two important APCs which play a role during vaccination. Mechanistically, Man2 induced a TLR4/ complement C3a receptor (C3aR)-dependent inflammatory cytokine production and the upregulation of co-stimulatory molecules. More specifically, Man2-induced IL-6, TNF-α, IL-1β, and IFN-β secretion was shown to be both TLR4- and C3aR-dependent, while IL-10 secretion was only mediated by the TLR4 pathway. Distinct activation of pro-and anti-inflammatory cytokines allows the development of improved intervention strategies with predominant anti-inflammatory response. Besides, Man2 treatment-induced activation of the MAPK-/ NFκB-pathways and triggered a switch towards a glycolytic metabolic phenotype in mDCs. Finally, and most importantly, Man2-stimulated mDCs enhanced antigen-specific T cell-derived IL-2 production, demonstrating that Man2 has potential as a vaccine adjuvant (Figure 7A).

6.2 rFlaA:Betv1 can activate both epithelial cells and macrophages which are important target cells for allergy treatment

In our group's previous studies, the flagellin:allergen fusion protein rFlaA:Betv1 was shown to suppress allergic sensitization *in vivo*[77,78,80] and the underlying mechanisms were further studied in mDCs[78,95]. However, the effects of flagellin:allergen fusion proteins on other cell types require further investigation to better understand the immune modulating potential of these constructs. Therefore, in chapters 2 and 3, we analyzed the effect of rFlaA:Betv1 on mouse epithelial cells and macrophages.

During allergic sensitization to birch pollen, airway epithelial cells are the first line of contact with the allergens. They serve as a physical barrier but also release cytokines or chemokines in order to recruit and modulate immune cells like DCs and further promote Th2 response[112]. Therefore, in the development of therapeutic approaches, modifying the response of airway epithelial cells to allergens could be an attractive treatment option. For this, in chapter 2, we checked for the effect of our rFlaA:Betv1 fusion protein on LA-4 cells, a mouse lung epithelial cell-line, and further analyzed the modulatory capacity of rFlaA:Betv1-stimulated LA-4 cells on mDC responses. Our results showed, that rFlaA:Betv1 could induce a MAPK- and NFκB-dependent, but TLR5-independent secretion of the myeloid chemoattractants CCL2 and CCL20, as well as the pro-inflammatory cytokine IL-6. Additionally,

rFlaA:Betv1-activated LA-4 cells could modulate mDC response by decreasing their IL-12 and TNF-α production while maintaining rFlaA:Betv1-stimulated IL-6, TNF-α, and IL-1β secretion. This effect was shown to depend on factors secreted from rFlaA:Betv1-stimulated epithelial cells. Here, we found that rFlaA:Betv1 induced a higher p38-MAPK and cyclooxygenase 2 (COX2)-dependent prostaglandin E2 (PGE_2) production in LA-4 cells. Furthermore, supernatant derived from COX2-inhibitor pre-treated LA-4 cells, which did no longer contain PGE_2, dose-dependently reversed the suppressive effect of LA-4 cells on mDC-derived IL-12 and TNF-α secretion (Figure 7B). Therefore, epithelial cells also contribute to the immune-modulating capacity of our flagellin:allergen fusion protein, as they were shown to both communicate with APCs and modulate APC responses.

APCs play an essential role in connecting innate and adaptive immunity. Not surprisingly, they also have an important function in allergic responses by presenting allergens to T cells and shaping the overall immune reaction toward Th2 responses[113]. Previously, our group found that rFlaA:Betv1 could activate mDCs by inducing the secretion of both pro-(IL-6, IL-12, and TNF-α) and anti-inflammatory (IL-10) cytokines. Interestingly, the fusion protein also induced a JNK-MAPK- and mTOR-dependent metabolic switch towards glycolysis (also called the Warburg Effect) that contributed to the strongly immune modulating IL-10 secretion induced by rFlaA:Betv1[78,95]. However, the effect of flagellin fusion proteins on macrophages, another important type of APC, was so far unknown. Therefore, in chapter 3, we used different approaches to thoroughly investigate the effect of our rFlaA:Betv1 fusion protein on BMDMs. The results showed that (1) rFlaA:Betv1 induced higher secretion of both pro-inflammatory IL-6, TNF-α, IL-1β, and anti-inflammatory IL-10 from BMDMs compared to the mixture of both proteins. (2) By measuring the Warburg Effect and using Seahorse Extracellular Flux technology, we could show that rFlaA:Betv1 triggered a pronounced shift towards glycolysis in BMDMs. (3) The induced cytokine secretion and Warburg Effect were shown to be mainly dependent on MyD88- and JNK-MAPK activation, while only being partially TLR5-dependent. (4) In contrast to the previously analyzed mDCs, mTOR activation could only modulate rFlaA:Betv1-induced IL-10 production in the short-term (up to 24 hours) and only partially mediated the induced Warburg Effect. (5) Using RNA-Seq analyses, we again confirmed the up-regulation of glycolysis-related signaling pathways in rFlaA:Betv1-stimulated BMDMs. Moreover, we identified the significant up-regulation of Janus kinase (JAK)-signal transducer and activator of transcription (STAT)-transcription factor hypoxia-inducible factor 1 α (HIF-1α) signaling in transcriptomic samples, which was confirmed in protein expression analyses by Western Blot. (6) Finally, rFlaA:Betv1-stimulated BMDMs showed a pronounced T cell-modulatory capacity by suppressing the secretion of Th2 cytokines (IL-5 and IL-13) from Bet v 1-specific Th2-biassed $CD4^+$ T cells while at the same time increasing both Th1- (IFN-γ) and anti-inflammatory (IL-10) cytokine secretion (Figure 7C).

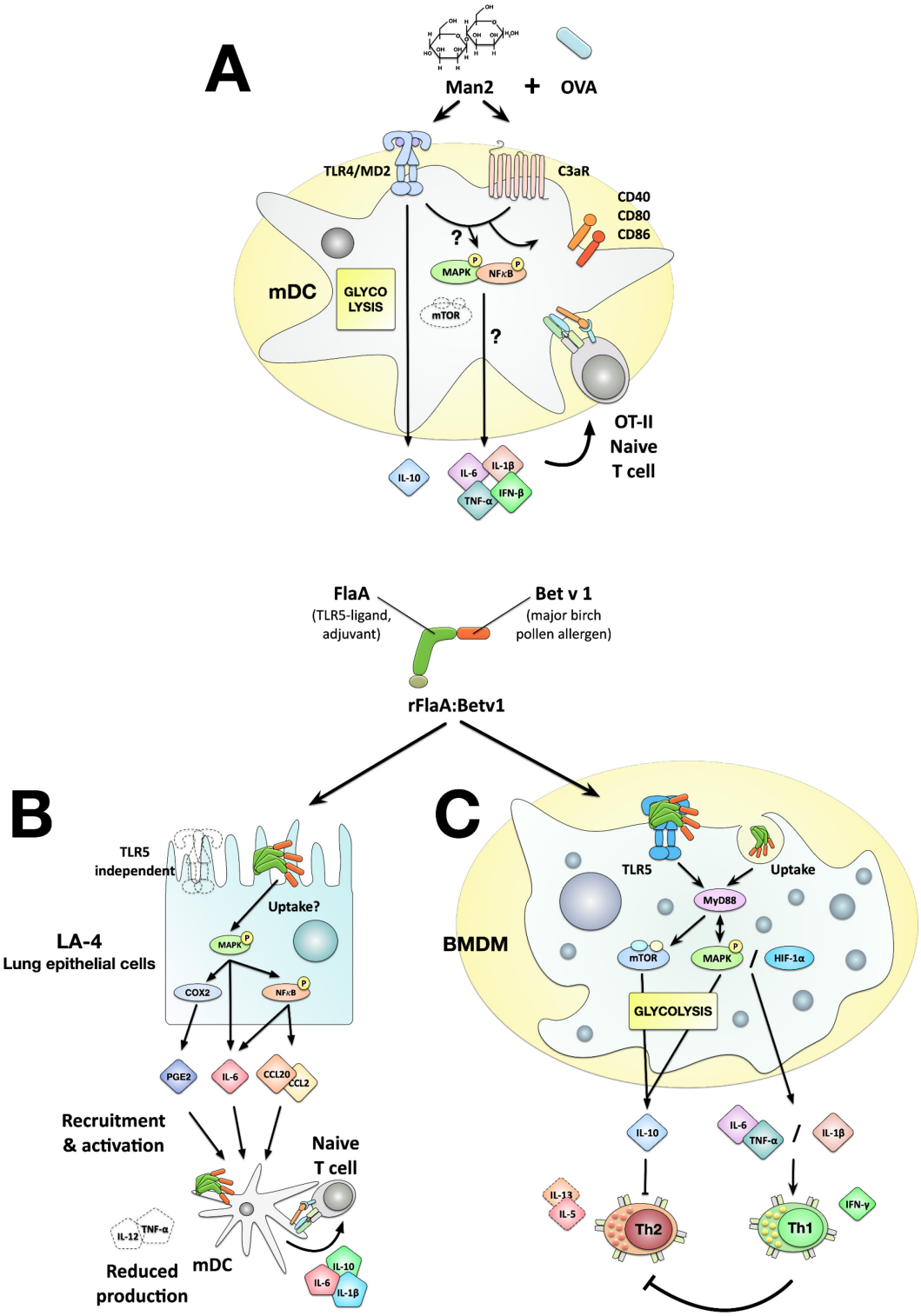


Figure 7: Summary of the findings obtained in this thesis

The proposed molecular signaling events underlying Man2-induced mDC activation (A). Suggested mechanisms contributing to rFlaA:Betv1-mediated activation of LA-4 epithelial cells (B) and BMDMs (C). For more information, see the text above. Abbreviations: mDCs: myeloid dendritic cells, BMDMs: bone marrow-derived macrophages, Man2: β-(1→4)-mannobiose, OVA: egg allergen ovalbumin, TLR: Toll-like receptor, MD2: myeloid differentiation factor 2, C3aR: complement C3a receptor, MAPK: mitogen-activated protein kinase, NFκB: nuclear factor kappa-light-chain-enhancer of activated B cells, mTOR: mammalian target of rapamycin, COX2: cyclooxygenase 2, PGE_2: prostaglandin E2, HIF-1α: transcription factor hypoxia-inducible factor 1 α.

6.3 Man2 and rFlaA:Betv1 are promising tools to modulate allergen-specific immune responses

Taken together, in this thesis we found that: (1) Man2 could drive mDC activation with enhanced T-cell stimulatory capacity, showing its potential as a vaccine adjuvant. And that (2) rFlaA:Betv1 could activate both epithelial cells and macrophages, demonstrating these two cell types to also contribute to the immune-modulatory effects of flagellin-containing fusion proteins previously observed *in vivo* (Figure 7). Therefore, epithelial cells and macrophages likely are important target cells which should be considered during therapy development for type I allergies.

In the following sections, I would like to further discuss our findings in more detail, including what is the difference between using a carbohydrate-based compound (Man2) as a potential adjuvant compared to a fusion protein containing the TLR5-ligand flagellin; why it is important to investigate different cell types when developing immunotherapy for allergies; what are the differences between rFlaA:Betv1-activated mDCs and BMDMs; why is it important to study changes in the respective cells' metabolic phenotype during treatment development; what are the strengths of either Man2 as an adjuvant or flagellin:allergen fusion proteins as potential therapeutics for type I allergy; and finally, what is the outlook of this thesis.

6.4 The differences between using either Man2 or flagellin-conjugate as novel adjuvants

This study tested two different adjuvants, the carbohydrate-based Man2 (chapter 1) and the bacterial protein flagellin (chapter 2&3). In the second case, we genetically fused flagellin with antigens to combine them into a single molecule in order to improve both targeting of the antigen to the desired cells *in vivo* and increasing the fused antigen's immunogenicity. There were several different features when using these two adjuvants (summarized in Figure 8) which are discussed below:

6.4.1 Man2-mediated cell activation through the TLR4/MD2 pathway, while flagellin:allergen fusion protein showed TLR5-independent, MAPK-mediated cell activation

In chapter 1, we found that Man2 activated mDCs mainly via the TLR4/ myeloid differentiation factor 2 (MD2) pathway. Stimulation with Man2 led to a pronounced MAPK- and NFκB-activation, and by using the TLR4 inhibitor TAK-242, we demonstrated the secretion of pro-inflammatory cytokines and expression of CD markers to be TLR4-dependent. These responses were probably MyD88-dependent, as MyD88 is essential for TLR4-signaling, and TAK-242 was shown to inhibit the recruitment of both the TLR adaptor proteins MyD88 and TRIF by binding to Cys747 in the intracellular domain of TLR4[114]. Besides, the overall levels of Man2-induced DC activation were also comparable to another well-characterized TLR4-ligand Lipopolysaccharide (LPS).

In contrast, the investigated flagellin:allergen fusion protein rFlaA:Betv1 activated mDCs in a TLR5-independent manner[78], which was also demonstrated in epithelial cells (chapter 2). Mechanistically, we excluded the possibility, that fusing Bet v 1 to flagellin might affect the TLR5 binding affinity of the resulting fusion protein (data shown in chapter 3). Furthermore, a recent structural study described that fusion of *Bacillus cereus* flagellin (that similarly to our *Listeria monocytogenes* FlaA, contains only the D0 and D1 domains and lacks the hypervariable D2 and D3 domains) to T4 lysozyme at the C-terminal also didn't affect the binding capacity to human TLR5[115]. One of the potential mechanisms how flagellin:allergen fusion proteins activate their target cells was to induce a strong uptake based on their high molecular aggregation[78] (chapter 2 & 3). This theory was confirmed by treating cells with Alexa Flour 488-labeled rFlaA:Betv1. Both FACS- or fluorescence microscopy analyses previously demonstrated a higher uptake of rFlaA:Betv1 into mDCs[78]. This was also confirmed in this thesis for LA-4 epithelial cells (chapter 2). Furthermore, this enhanced uptake was again shown to be TLR5-independent in mDCs[78]. Aggregates were recently also shown to be beneficial in an allergy treatment approach. A recent study from Najafi *et al.* showed, that a recombinant fusion protein consisting of Bet v 1 and Phl p 5 also forms aggregates, which could induce production of allergen-specific IgG antibodies *in vivo*, while efficiently reducing mast cell activation (reduced β-hexosaminidase release from RBL cells) *in vitro* compared to the mixture of both allergens[116].

The second possible mechanism by which rFlaA:Betv1 more strongly activates immune cells compared to the mixture of both single proteins was the activation of other receptors than TLR5 by the flagellin:allergen fusion protein. It is well known that flagellin can not only activate TLR5 but also bind to the intracellular NLR family CARD domain containing 4 (NLRC4) (also named ICE-protease activating factor (IPAF))[117] and further induce inflammasome activation. In more detail, NLRC4 together with NLR family apoptosis inhibitory protein (NAIP) can bind to intracellular flagellin, leading to oligomerization of NLRC4 and its subsequent interaction with apoptosis-associated speck-like protein (ASC) forming the inflammasome complex[118]. Once this complex is formed, ASC and the CARD domain of NLRC4 can catalyze the cleavage of pro-Caspase-1 protein to its active form, resulting in the cleavage of pro-IL-1β and pro-IL-18 to their respective active forms which are secreted by activated immune cells to modulate immune responses[118]. IL-1β produced by inflammasome activation is known to modulate secretion of other cytokines from immune cells, such as IL-6, TNF-α, and IL-1α[119]. In another, currently still unpublished side-project of this thesis, rFlaA:Betv1 could indeed induce inflammasome activation, but surprisingly not only NLRC4-, but also NLRP3-dependently (Lin *et al.*, in preparation). Currently, it is well studied that different stimuli can activate the NLRP3-inflammasome[120]. Two proposed mechanisms by which rFlaA:Betv1 might activate the NLRP3-inflammasome are: (1) the aggregated structure of rFlaA:Betv1 may lead to lysosomal disruption as already described for crystal particles like uric acid or alum, two well-known NLRP3 activators[121]. (2) Research showed that immune cells shift their metabolic phenotype toward glycolysis, which could lead to a disrupted Krebs cycle and release mitochondrial reactive oxygen species (ROS), mitochondrial DNA, or Krebs cycle intermediates (e.g. succinate) to the cytosol which further activate the NLRP3-inflammasome[122–124]. Our studies on

mDCs[78,95] and BMDMs (chapter 3) also demonstrated higher rates of glycolysis in both cell types after rFlaA:Betv1 stimulation, indicating the possibility of rFlaA:Betv1 to promote NLRP3 activation. These results suggest, that in addition to signaling pathways traditionally known to be triggered by flagellin alone, aggregated flagellin:allergen fusion proteins might induce additional signaling pathways that contribute to their superior immune activating capacity.

Besides NLRs, another intracellular TLR, TLR11, which localizes in the endolysosomal compartment, was recently reported to bind flagellin[125]. The activation of TLR11 was reported to be independent of TLR5[126,127], and TLR5$^{-/-}$ mice express higher levels of TLR11, which could explain that TLR5$^{-/-}$ mice showed improved resistance to *S. typhimurium* infection[126,128]. More interestingly, TLR11 could also recognize another major birch pollen allergen Bet v 2 (a profilin)[129]. Besides TLR11, TLR12 was reported to form a heterodimer with TLR11, and was more dominantly expressed in myeloid cells like DCs or macrophages[126,130]. Since both TLR11 and TLR12 require the adaptor molecule MyD88 for activating downstream signaling pathways[130], this hypothesis might explain why MyD88 still plays an essential role in rFlaA:Betv1-mediated mDC- and macrophage-activation while the induced cytokine and metabolic changes were shown to be largely TLR5-independent[78] (chapter 3).

6.4.2 Flagellin:allergen fusion proteins induce a mTOR-dependent IL-10 secretion and metabolic switch towards glycolysis, while Man2 did not activate the mTOR pathway in mDCs

Upon stimulation, dendritic cells show increased rates of glycolysis to quickly generate energy for the induction and maintenance of immune responses[109]. In this study, both Man2 and rFlaA:Betv1 strongly increased glycolysis in mDCs[78] (chapter 1), but the underlying regulatory pathways were shown to be different between both constructs.

In Western Blot analysis, rFlaA:Betv1 induced higher phosphorylation of mammalian target of rapamycin complex 1 (mTORC1) target protein P70 S6 kinase (p70S6K) in mDCs[95]. Furthermore, by pre-treating mDCs with the mTORC1 inhibitor rapamycin, the rFlaA:Betv1-induced Warburg Effect, glucose consumption, and IL-10 secretion were demonstrated to be mTOR-dependent[78,95].

In contrast, Man2-stimulation did not induce p70S6K-phosphorylation, indicating that pathways other than mTOR regulate both glycolysis and IL-10 secretion in Man2-stimulated mDCs (chapter 1). One of the possible pathways is the MAPK cascade, as both Man2- and rFlaA:Betv1-stimulated mDCs showed higher phosphorylation of JNK-, ERK- and p38-MAPK[78] (chapter 1). In line with our results, MAPK pathways were shown to be important for the regulation of glucose metabolism[131], and the JNK-MAPK was previously reported to play a role in regulating MPLA- and rFlaA:Betv1-induced glycolysis[95,132]. Besides, a recent study by Jin and colleagues demonstrated, that the TLR4 agonist LPS could activate the JNK-MAPK pathway, further mediating pyruvate kinase M2 (PKM2) acetylation in DCs, which increased IL-12p35 secretion[133]. This upregulation of IL-12p35 was important for Th1 cell

differentiation, suggesting its importance for type I allergy treatment[133]. MAPK are also important for IL-10 secretion induced by TLR4-ligands, as both ERK- and JNK-MAPK were reported to regulate LPS-induced IL-10 secretion in mDCs[134,135]. Furthermore, our results showed, that Man2-induced mDC activation was dependent on both the TLR4/MD2 receptor complex and the complement receptor C3aR. The available literature indicated that C3aR is located downstream of TLR4 signaling, which can result in further activation of MAPK pathways in several different immune cells including macrophages and mast cells[136,137]. Currently, there are only limited studies available investigating the effects and underlying mechanisms of mannans, more specifically, different sizes and linkages of mannose on the activation of immune cell metabolism or cytokine secretion. Therefore, further research is needed to build on our promising initial results.

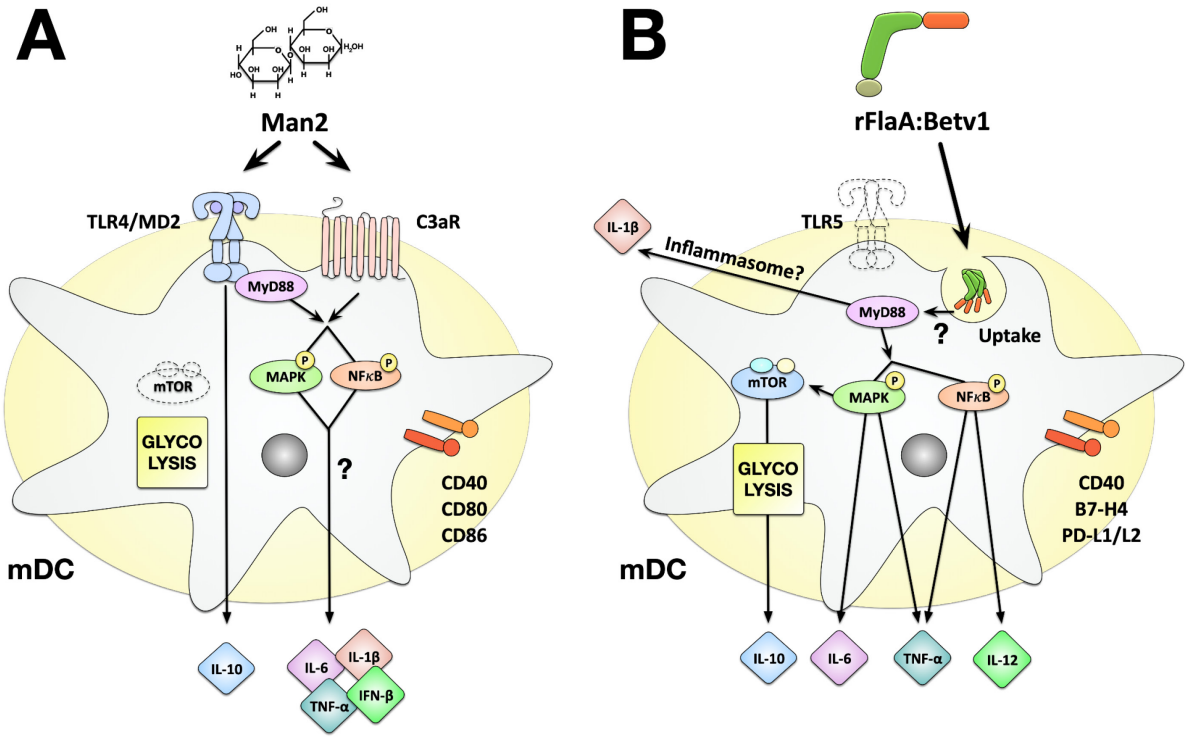


Figure 8: Man2 and Flagellin:allergen fusion activate dendritic cells differently

Stimulation of mDCs with Man2 results in a both TLR4/MD2- and C3aR-dependent secretion of pro-inflammatory cytokines IL-6, IL-1β, TNF-α, and IFN-β as well as the up-regulation of the co-stimulatory surface markers CD40, CD80, and CD86; while anti-inflammatory IL-10 was only dependent on the TLR4/MD2 pathway. Furthermore, Man2 also induced strong phosphorylation of MAPK and a higher Warburg Effect, while only a slight activation of the NFκB signaling pathway was observed (A). The rFlaA:Betv1 fusion protein forms high molecular aggregates and displayed an increased uptake by mDCs. Moreover, rFlaA:Betv1 induced a TLR5-independent, but MyD88-dependent, cytokine production and metabolic switch towards increased glycolysis. In detail, rFlaA:Betv1 was shown to activate the mTOR pathway, which was responsible for both the induced Warburg Effect and IL-10 secretion. Finally, rFlaA:Betv1-induced pro-inflammatory cytokine production was regulated by MAPK- (IL-6 and TNF-α) and NFκB-signaling (TNF-α and IL-12), while IL-1β secretion was potentially induced by inflammasome activation[78,95] (B). Abbreviations: mDCs: myeloid dendritic cells, Man2: β-(1→4)-mannobiose, TLR: Toll-like receptor, MD2: myeloid differentiation factor 2, C3aR: complement C3a receptor, MyD88: myeloid differentiation primary-response protein 88, MAPK: mitogen-activated protein kinase, NFκB: nuclear factor kappa-light-chain-enhancer of activated B cells, mTOR: mammalian target of rapamycin.

From the above-discussed section (6.4), we reviewed our findings from chapter 1 and compared the differences between Man2 and flagellin fusion proteins on dendritic cell activation. Both adjuvants displayed potential for improving allergy treatment. However, dendritic cells are not the only cell type contributing to the overall immune responses induced by the different stimuli. Therefore, in the following section, I would like to discuss our findings on rFlaA:Betv1-stimulated epithelial cells and BMDMs, and further compare the different signaling pathways between rFlaA:Betv1-activated BMDMs and mDCs, thereby demonstrating the importance of analyzing different cell types when developing novel adjuvants. Finally, I will summarize what is the potential for Man2 and rFlaA:Betv1 in allergy treatment.

6.5 Why it is important to study different cell types in immunotherapy development: It is not just about DCs!

In chapters 2 and 3, we tested the effects of rFlaA:Betv1 on both epithelial cells and macrophages. Our results demonstrated, that rFlaA:Betv1 could activate epithelial cells inducing the secretion of the chemoattractants CCL2 and CCL20, which can in turn recruit myeloid cells like DCs[138,139]. Moreover, rFlaA:Betv1-stimulated epithelial cells also secreted higher levels of PGE_2, which is known to suppress Th2-inflammation during allergic responses in the lung[140]. Finally, PGE_2 modulated mDC responses by suppressing rFlaA:Betv1-induced TNF-α- and IL-12- while at the same time maintaining IL-10 secretion (chapter 2). These results demonstrated, that intensive communication between epithelial cells and APC shapes the overall immune responses induced by flagellin:allergen fusion proteins.

For macrophages, we found that rFlaA:Betv1 induced both pro- and anti-inflammatory cytokine secretion, a metabolic profile characterized by strongly increased rates of glycolysis, and, most importantly, rFlaA:Betv1-stimulated macrophages suppressed production of Th2 cytokines from Bet v 1-specific T cells (chapter 3). Therefore, our results indicated, that epithelial cells and macrophages are also important target cells that should be considered when developing novel therapeutics for allergen immunotherapy.

Dendritic cells are well-known to be important for linking innate and adaptive immunity. Therefore, when developing vaccine adjuvants, DCs usually are the primary investigated target cells[141]. Not only TLR-ligand based adjuvants, but also Alum- or MF59-adjuvanted vaccines require DCs for inducing proper adaptive immune responses[141–143]. In this project, both Man2 and rFlaA:Betv1 were also first tested for their capacity to activate mDCs. Here, both strongly triggered mDC activation and influenced their T cell priming capacity[76–80,95] (chapter 1).

However, the establishment of the overall immune response is a complex process that requires the interaction of different cell types. Accordingly, increasing evidence has recently emerged showing, that other cell types are also involved in the immune systems response to vaccination[141,144–147]. For example,

the adjuvant MF59 was shown to directly activate muscle fibers at the injection site which displayed transcriptional changes[147]. Besides, in an *in vivo* study Vono *et al.* demonstrated that after intramuscular injection of MF59 as an adjuvant for a trivalent influenza vaccine, mouse muscle cells released higher amounts of ATP at the injection site, which was necessary for MF59's ability to induce both $CD4^+$ T cell activation and IgG antibody production[146]. Upon intranasal administration, studies showed, that flagellin could induce secretion of the chemokine CCL20 from epithelial cells[145]. Furthermore, flagellin mixed with antigens was also shown to induce GM-CSF secretion from local epithelial cells, contributing to antigen presentation by DCs[144] . The involvement of macrophages in the adjuvanticity of antibody responses in the *Corynebacterium parvum* vaccine was already described in 1976[148]. Both lymph node resident and $CD169^+$ subcapsular macrophages were also shown to influence antigen presentation and IgG production upon application of either CpG, Alum, MF59, or QS-21 adjuvants[149,150]. Not surprisingly, antibody-producing B cells were also reported to play a role for the responses induced by TLR-based adjuvants[151]. In summary, these studies indicated that not just DCs should be considered when designing novel therapeutic and prophylactic strategies for allergen immunotherapy.

Currently, most vaccine research is focused on the analysis of DC responses. So far there is little research making detailed comparisons of the effects of different vaccines/adjuvants on different immune cell types. Our research gives both insight and opportunity to compare the mechanisms underlying the immune activating and modulating properties of flagellin:allergen fusion proteins between different cell types. Understanding these differences and similarities between different cell types as well as the underlying molecular mechanisms will allow us establish a more complete picture of the overall immune responses induced by novel vaccines and therapeutics. In the following paragraph, we especially discuss the different mechanisms underlying the activation of two types APCs: dendritic cells and macrophages by rFlaA:Betv1.

6.5.1 rFlaA:Betv1 activates different signal pathways in mDCs and BMDMs

Dendritic cells and macrophages are both APCs; they share certain features while also displaying some differences. For example, DCs are more critical in stimulating T cell responses during disease progression, while macrophages control tissue homeostasis by phagocytosing and degrading dead cells, debris, and foreign material[152]. Besides, macrophages show both higher plasticity and flexibility during stimulation and are well-known to be sub-classified as either the M1 (pro-inflammatory/Th1-inducing) or M2 (anti-inflammatory/Th2-inducing) phenotype[153]. Here, the frequency and activity of M2-macrophages are well known to correlate with the severity of the clinical symptoms of allergy[154].

Currently, studies reporting a direct comparison between macrophages and dendritic cells activated by either different vaccines or adjuvant are limited. Here in chapter 3, we thoroughly analyzed the mechanisms underlying rFlaA:Betv1-induced BMDM activation, which displayed some interesting

differences compared to the results observed for rFlaA:Betv1-stimulated mDCs from our previous research[78,95]. The findings are summarized in Figure 9 and the following paragraph.

6.5.2 mTOR regulates both rFlaA:Betv1-induced IL-10 secretion and glycolysis differently in mDCs and BMDMs

In our group's previous studies, stimulation with rFlaA:Betv1 induced a higher phosphorylation of both PRAS40 and mTOR belonging to the mTOR-complex, as well as the mTOR target protein p70S6K at residue Thr389 in mDCs[78]. In chapter 3, we also demonstrated higher phosphorylation of p70S6K in rFlaA:Betv1-stimulated BMDMs, indicating that rFlaA:Betv1 activated mTOR-signaling in both cell types. However, when analyzing the contribution of mTOR to the rFlaA:Betv1-induced cytokine production and Warburg Effect, we found differences between mDCs and BMDMs: Pre-treatment of the cells with the mTOR-inhibitor rapamycin significantly suppressed both IL-10 secretion and the Warburg Effect in mDCs, and this strong suppression could still be observed 72 hours after treatment[78]. However, in BMDMs, rapamycin only suppressed rFlaA:Betv1-induced IL-10 secretion in the short-term (6-24 hours), and showed no effect on IL-10 secretion at the 96 hour time point, indicating mTOR to only modulate IL-10 secretion in the early phase of BMDM-activation after rFlaA:Betv1 stimulation. Additionally, we found that rapamycin dose-dependently but not significantly suppressed the rFlaA:Betv1-induced Warburg Effect in BMDMs, showing mTOR to only be partially involved in rFlaA:Betv1-induced glycolysis in BMDMs (chapter 3).

These differences in regulation of the mTOR pathway between mDCs and BMDMs might be correlated to the differential activation of MAPK. It was shown, that MAPK signaling is important for macrophage-derived IL-10 secretion and changes in metabolic phenotype upon either *Schistosoma mansoni* infection or LPS stimulation[155–157]. Besides, p38-MAPK activation can trigger mTOR-signaling and further block IL-10 secretion upon LPS stimulation in human monocytes and mouse BMDMs[158]. In our study, pre-treatment with MAPK inhibitors (targeting either p38-, JNK-, or ERK-MAPK) dose-dependently reduced rFlaA:Betv1-induced IL-10 production either 72 or 96 hours post-stimulation in both mDCs[95] and BMDMs (chapter 3). Here, mainly JNK-MAPK activation contributed to the induced Warburg Effect in both cell types[95] (chapter 3). Moreover, MAPK was shown to be located up-stream of mTOR in mDCs[95], which is currently unclear in BMDMs. These results indicate, that in mDCs rFlaA:Betv1 first activated MAPK signaling which then further promoted phosphorylation of the mTOR complex and mediated both increased glycolysis and IL-10 secretion. On the contrary, in BMDMs mTOR might be activated independently of MAPK signaling after rFlaA:Betv1-stimulation, while the former mediated short-term IL-10 secretion, and JNK-MAPK regulated both long-term IL-10 production and glycolysis (Figure 9).

6.5.3 TLR5 is partially involved in rFlaA:Betv1-induced TNF-α and IL-10 secretion from BMDMs but not from mDCs

The other interesting difference between rFlaA:Betv1-mediated mDC- and BMDM-activation was TLR5 dependency of the observed effects. Previous studies showed, that the rFlaA:Betv1-induced Warburg Effect, glucose consumption, and cytokine production from mDCs were all TLR5-independent[78]. However, in BMDMs, the induced TNF-α secretion was strongly dependent on TLR5, and IL-10 secretion was partially affected by TLR5-signaling (chapter 3). In contrast, rFlaA:Betv1-induced metabolic effects and other cytokines were unchanged in TLR5-deficient BMDMs compared to wild type controls (chapter 3). In line with our results, Hawn *et al.* also found impaired TNF-α secretion from TLR5-deficient alveolar macrophages infected with *Legionella pneumophila*[159]. Nevertheless, the mechanisms that cause these differences between mDCs and BMDMs are still unclear and need further investigation.

Taken together, our results demonstrated flagellin:allergen fusion proteins to activate different cell types via distinct signaling pathways. Understanding these cell type-specific differences may be helpful for the future design and application of therapeutics for allergy treatment.

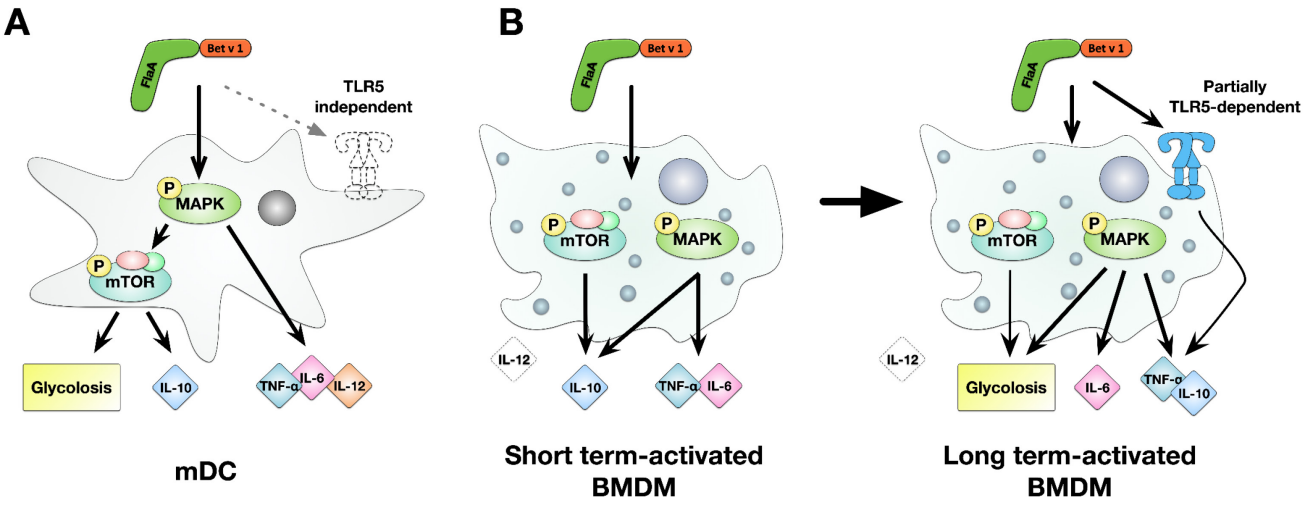


Figure 9: rFlaA:Betv1-induced glycolysis and cytokine secretion is regulated differently in mDCs and BMDMs
Stimulation of mDCs with rFlaA:Betv1 results in a MAPK- and mTOR-dependent, but TLR5-independent long-term secretion of IL-10 and induction of the Warburg Effect[78,95]. At the same time, pro-inflammatory IL-6, TNF-α, and IL-12 production were only dependent on MAPK[95] (A). In contrast, BMDMs showed a more complex activation pattern upon stimulation with rFlaA:Betv1. In short-term, both mTOR and MAPK modulated rFlaA:Betv1-induced IL-10, while TNF-α and IL-6 were only dependent on MAPK in rFlaA:Betv1- stimulated BMDMs (chapter 3) (B). In long-term, MAPK-activation contributed to both rFlaA:Betv1-induced metabolic changes and cytokine production in BMDMs, while mTOR-activity only partially mediated increased rates of glycolysis (chapter 3). Moreover, TLR5 partially contributed to both IL-10- and TNF-α-secretion (chapter 3), which was not observed in mDCs[78]. Finally, in contrast to rFlaA:Betv1-stimulated mDCs[78,95], no IL-12 was detected from rFlaA:Betv1-stimulated BMDMs (chapter 3). Abbreviations: mDC: myeloid dendritic cell, BMDM: bone marrow-derived macrophage, TLR: Toll-like receptor, MAPK: mitogen-activated protein kinase, mTOR: mammalian target of rapamycin.

6.6 Investigating the immune metabolism will allow us to better understand allergic pathology and find new targets for allergy treatment

In recent years, it has become more apparent that immune cells change their metabolic state during activation to both fulfill their rapid energy needs and allow for their effector function. This newly emerging research field named “immune metabolism” will likely allow us to better understand disease pathology and find new targets to either combat disease or develop vaccines.

Antigen-presenting cells like DCs and macrophages are reported to increase glycolysis upon stimulation with for example TLR-ligands[160]. In chapters 1 and 3, we tested Man2 on mDCs and rFlaA:Betv1 on BMDMs, and found that both stimuli induced a metabolic phenotype characterized by increased rates of glycolysis. For the novel adjuvant Man2, the upregulation of glycolysis could be correlated to the induced cytokine production of mDCs, which was also shown previously for rFlaA:Betv1-stimulation mDCs[78,95]. In line with our results, research from Oscar Palomares' group in Madrid also showed that allergoids conjugated to non-oxidized mannan enhanced the Warburg Effect and ROS production in human DCs[57]. Interestingly in the same group’s recent publication, in addition to influencing the resident DCs, mannan-allergoids could also reprogram monocytes into tolerogenic DCs by shifting their metabolic profile from glycolysis back to OXPHOS, which enhanced IL-10 expression and further expanded allergen-specific T_{reg} cells *in vitro*[161]. Moreover, in chapter 3, we made a detailed analysis of rFlaA:Betv1-induced glycolysis in BMDMs by using different experimental approaches (Warburg Effect, glucose consumption, metabolic rate measurement, Seahorse Extracellular Flux technology, RNA-Seq analysis, and Western blot). Here, all results suggested that after rFlaA:Betv1 treatment, macrophages increased expression of glycolytic genes, the degree of extracellular acidification (ECAR), glucose consumption, glycolysis-related protein expression (glucose transporter 1 (Glut1) and 6-phosphofructo-2-kinase/fructose-2,6-Biphosphatase 3 (PFKFB3)) paralleled by a decreased expression of the Krebs cycle protein aconitase 2 (ACO2), and the upregulation of the JAK-STAT-HIF-1α pathway. These results are in accordance with studies demonstrating macrophages activated by their TLRs to display an M1-phenotype, which show the activation of transcription factors such as NF-κB, STAT3, and HIF-1α, as well as a glycolytic-biased metabolism[162]. Interestingly, M1-macrophages were shown to improve allergic pathology[163]. Taken together, these results suggest, that by detecting metabolic changes in immune cells, we may be able to monitor either the efficacy or the treatment status during allergen-specific immunotherapy.

Besides better understanding the mechanisms underlying the immune metabolic changes occurring during allergic responses and treatment, the other interesting potential of immune metabolism is to specifically target single molecules or metabolic pathways to improve existing therapeutic strategies or establish new ones. We recently summarized the current understanding of the immune metabolic changes in different cell types which are involved in allergy[107–109]. Currently, there is no treatment for allergic patients based on immune metabolism. However, ongoing research investigates using small

molecules and metabolic intermediates to reshape phenotype and effector functions of immune cells. A recent review by McDermott and O'Neill in 2020 summarizes the clinically tested small molecules that target metabolic processes in immune cells for application in inflammatory and autoimmune diseases[164]. For example, metformin, a widely-used small molecule for controlling type 2 diabetes (T2D), controls glucose metabolism by suppressing gluconeogenesis in the liver, inhibiting mitochondrial respiratory chain complex I, activating AMP-activated protein kinase (AMPK), and suppressing the mTOR complex[165]. Metformin was shown to be effective in models of systemic lupus erythematosus (SLE), colitis, and experimental autoimmune arthritis (EAE) by inducing anti-inflammatory responses[164]. For allergy treatment, a high-fat diet-induced obesity followed with OVA challenge in an asthma mouse model showed, that metformin could reduce the tissue eosinophil infiltration and TNF-α levels in bronchoalveolar lavage[166].

Next, dimethyl fumarate (DMF), currently used for relapse-remitting multiple sclerosis (RRMS) treatment, could achieve anti-inflammatory capacity by repolarizing T cell populations, and upregulating endogenous ROS in DCs, monocytes, and macrophages[164,167]. DMF also showed potential for the treatment of psoriasis, SLE, and colitis[164]. For anti-asthmatic therapy, Jaiswal *et al.* showed that in a HDM-induced murine allergic asthma model, DMF could reduce the migration ability of lung DCs and resulted in attenuation of both allergic sensitization and Th2 immune responses[168].

In addition, methotrexate, another small molecule broadly used as a chemotherapy treatment against a wide range of cancers, has also been successfully used for the treatment of immune-related diseases such as rheumatoid arthritis (RA), Crohn's disease, and psoriasis[164]. Methotrexate regulates inflammatory responses by inducing mitochondrial ROS in different immune cells like monocytes or cytotoxicity T-cells[164,169,170]. A clinical study presented by Patel *et al.* demonstrated that with methotrexate treatment, 25 out of 32 patients (78%) with allergic contact dermatitis had either a partial or complete response to the treatment[171]. However, the underlying mechanisms need to be further investigated.

Finally, rapamycin, a well-known mTORC1 inhibitor that regulates glucose and lipid metabolism, has also been tested in patients with RA, SLE, and MS[164]. Notably, previous studies indicated that after FcεRI stimulation, mTORC1-activation is required for human and mouse mast cell survival as well as IL-8 and TNF-α production[172–174]. Furthermore, several *in vivo* studies also indicated that mTOR could be an ideal target for the treatment of allergic responses since rapamycin decreased mast cell numbers and both antigen-specific Th2 cytokines and IgE production in an OVA-based food challenge model and HDM-induced asthma mouse models[175,176]. In summary, investigating the detailed mechanisms of immune metabolism during either allergic responses or allergy treatment has the potential to improve future therapy development.

6.7 Summary

6.7.1 The potential of using Man2 as a novel adjuvant for treating type I allergy

In the past years, several natural or synthetic carbohydrate structures have been studied as adjuvant candidates[34]. Currently, two carbohydrate-adjuvanted formulations (both containing the TLR4-ligand MPLA) have been approved for clinical use: (1) AS04, which combines alum with MPLA, was approved in 2009 as part of the vaccine Cervarix® for preventing HPV-induced cervical cancer[34]. (2) AS01, a liposomal formulation containing both MPLA and QS-21 (a triterpene glycoside which is purified from the soap bark tree (*Quillaja saponaria*)), has been used in GSK's Mosquirix® (against malaria)[177] and Shingrix® (against shingles) vaccines[178]. Besides these, other carbohydrate-based vaccine adjuvants are still being investigated.

Mannan, a β-1,4-linked mannose polysaccharide derived from the cell walls of plants, fungi, or yeast, is also tested as an adjuvant[34,56–59,179]. Its structure varies considerably with the different mannose residues being connected by various glucosidic linkages, and contains approximately 5% proteins which are naturally linked to mannan[52] (Figure 10A). In our study presented in chapter 1, we were the first to show that different oligomer sizes and residues of β-1,4-mannooligosaccharides could induce differences in cytokine secretion, cluster of differentiation (CD) marker expression and metabolic changes in mDCs, indicating the importance to carefully consider parameters like structure, linkage, and size when designing mannan and other carbohydrate structures as adjuvants.

To consider how we can apply Man2 as a novel adjuvant for allergy treatment, we checked the currently available literature: Mannan can be conjugated with protein-based antigens via its polymannose backbone in either oxidized or reduced form (Figure 10C&D), both showing increased APC uptake, but leading to differences in induction of either Th1- or Th2-responses[34]. For example, Apostolopoulos *et al.* conjugated the protein mucin 1 (MUC1) with mannan by adding sodium periodate to generate an oxidized mannan-MUC1 fusion protein (ox-M-FP), which induced Th1-responses *in vivo* with high IFN-γ and IgG_{2a} antibody production, paralleled by low IL-4 secretion[179]. In contrast, a mannan-MUC1 fusion protein containing reduced mannan moieties (red-M-FP) was generated by treating mannan with sodium borohydride[179]. In contrast to the oxidized variant, red-M-FP induced stronger IL-4 secretion indicating the predominant induction of Th2-responses[179]. These results indicate, that by careful conjugation with allergens, Man2 might have the potential to shape the immune response towards predominantly Th1-biassed immune responses thereby improving Th2 pathology.

Currently, mannan fused to allergens is investigated as a novel immunotherapy for treating allergic diseases. Weinberger and colleagues were the first to conjugate oxidized mannan with the OVA for testing in allergen-specific immunotherapy[56]. Their results showed, that Mannan-OVA was efficiently

taken up by mDCs *in vitro* and resident DCs *in vivo*, resulting in lower production of OVA-specific IgE while inducing higher OVA-specific IgG production[56].

The oxidation process of mannan can generate reactive aldehydes (−CHO) that form covalent bonds with allergen-derived amino acid residues, resulting in the formation of linkages between allergen and the polymannose backbone (Figure 10C). However, allergoids lack these reactive aldehydes due to their glutaraldehyde treatment[52] (Figure 10B). To solve this problem, the group around Oscar Palomares invented a new method to conjugate allergoids with the native form of mannan[59]. Their strategy was to use a glutaryl-diimine linker, resulting in the polymerization of allergens to allergoids and attachment to protein moieties contained within mannan instead of its mannose backbone[59] (Figure 10E). Treatment with conjugates of grass pollen allergoids and non-oxidized mannan (PM) increased the mannose receptor (CD206)- and myeloid C-type lectin receptor (DC-SIGN and Dectin2)-dependent uptake by human monocyte-derived DCs (hmoDCs), when compared to either native pollen extracts (N) or glutaraldehyde-polymerized pollen allergoids (P)[58]. Moreover, PM induced a stronger expression of PD-L1, and a higher CLR-mediated NFκB-dependent secretion of IL-6 and IL-10, as well as a lower IL-4 levels from hmoDCs[58]. Interestingly, PM also induced metabolic changes in human DCs by shifting the overall metabolism towards glycolysis associated with the detection of higher levels of mitochondrial ROS, which was shown be dependent on the mTOR pathway[57]. Finally, immunization with PM stimulated a higher percentage of $CD4^{+}CD25^{high}FOXP3^{+}$ T_{reg} cells, as well as pollen-specific IgG_{2a}/IgE levels *in vivo* compared to the non-conjugated mixture of P and N[58]. Taken together, mannan-allergen conjugation showed promising potential for allergy treatment.

In our own research, chapter 1 showed that Man2 induced significant mDC activation, but these effects declined with increasing oligomer size (Man3, Man4, and Man5) (chapter 1). Therefore, due to the lack of protein residues on Man2, it is impossible to directly conjugate either allergen or allergoids with native Man2 using the method established by the Palomares' group (see above). A possible strategy in the future to generate Man2-allergen conjugates would be to link the allergen with sodium periodate-pre-incubated oxidized Man2 (Figure 10F). The potential advantages for the Man2-allergen conjugates as a novel treatment for allergy include: (1) higher homogeneity with the possibility to better standardize the ratio of Man2 and allergen. The study from Weinberger *et al.* indicated that conjugation of oligomer-mannan with OVA results in conjugates with a wide range of sizes ranging from 45 kDa up to 120 kDa[56]. This is due to the difficulties in controlling the number of OVA molecules linked to the oligomer-mannan. However, Man2 contains only two mannose units that allow a maximum conjugation of two allergen molecules. Therefore, Man2 may improve batch-to-batch variability. (2) The activation of TLR4- and C3aR-signaling pathways by Man2 demonstrates its potential to induce robust immune cell responses and immunity: In chapter 1 we found that Man2 could activate TLR4- and C3aR-signaling pathways, which was not shown for mannan-allergen/allergoid conjugates in the above referenced studies[56–59]. Besides, we also showed a slight contribution of Dectin-1 and mannan receptors to Man2-induced IL-6 secretion, while the potential of Man2 also activating Dectin-

2 is still unclear and needs further analysis. Furthermore, TLR4 receptor is widely expressed by different cells types, not only APCs but also other immune cells as well as epithelial cells, endothelial cells, endometrial cells, thyroid cells, etc.[180]. During vaccination, it is suggested that, besides APCs, other cell types also contribute to the induction of immune responses, which was also proven by our study for rFlaA:Betv1-activating lung epithelial cells (chapter 2). Thus, Man2 could activate multiple receptors, potentially inducing higher immunogenicity towards either co-applied or fused antigens as an adjuvant. The advantages of Man2 as a novel adjuvant for allergy treatment are summarized in Figure 11.

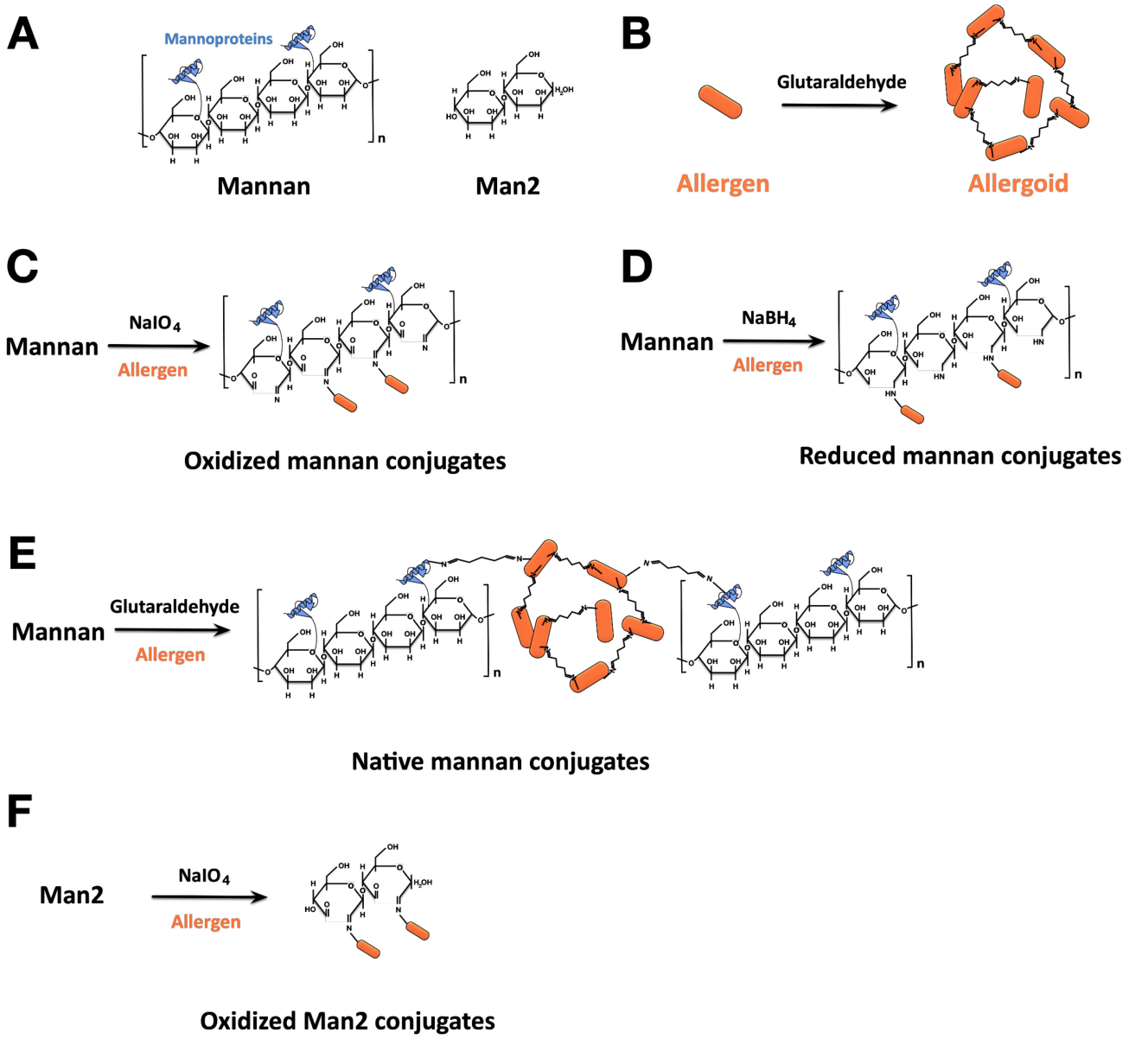


Figure 10: Different methods that can be used for linking either mannan or Man2 to allergens

Mannan is a β-1,4-linked mannose polysaccharide containing naturally-linked mannoproteins[52], while purified Man2 only contains two mannose units without additional mannoproteins (A). Allergens can by polymerized to generate allergoids using a glutaryl-diimine linker after glutaraldehyde treatment[23] (B). Allergens can be conjugated with mannan in either oxidized (C) or reduced (D) form after sodium periodate ($NaIO_4$) or sodium borohydride ($NaBH_4$) treatment, respectively[179]. The research group around Oscar Palomares invented a new method to link allergen and non-oxidized mannoproteins by glutaryl-diimine linker, which retains the native form of mannan resulting in allergoid-mannan conjugates[59] (E). The

proposed method to generate oxidized Man2-allergen conjugate (F): $NaIO_4$ treated Man2 allow allergen-derived amino acid residues to form covalent bonds with mannose backbone, results in maximum two allergens conjugated with Man2. Abbreviations: Man2: β-(1→4)-mannobiose.

6.7.2 The advantages of using flagellin:allergen fusion proteins for treating type I allergy

The advantages of fusing allergens with *Listeria monocytogenes*-derived flagellin (FlaA) as an adjuvant have already been extensively investigated in our previous publications[76–80,95] and this thesis in chapters 2 and 3. To briefly summarize (see also Figure 11): (1) Flagellin being a protein, it can be easily fused to the antigen of choice by recombinant DNA technology (2) FlaA lacks the hypervariable, and immunogenic D2 and D3 domains typically included in other flagellin types[181]. A previous study showed that deletion of D2 and D3 domains of FliC from *Salmonella enterica* impaired flagellin's intrinsic antigenicity but did not affect its capacity to promote innate and adaptive immune responses as an adjuvant[182]. Neutralizing antibodies directed against the hypervariable domains of flagellin molecules are unwanted for vaccines that should be repeatedly administered to patients. Therefore, for flagellin molecules contained in flagellin fusion protein undergoing clinical studies, these hypervariable regions were deleted[72,85]. These results indicate a promising safety profile of FlaA for its application in flagellin:antigen fusion proteins. (3) Flagellin:antigen fusion proteins have already been tested to treat many infectious diseases in *in vivo* animal models[83,183,184] or influenza prevention[72,85] in clinical trials, demonstrating their efficacy and safety. (4) As part of the bacterial flagella, flagellin tends to self-assemble with itself to form the body of the flagellum. Therefore, the flagellin:allergen fusion proteins investigated by us so far also form high molecular aggregates, increasing their uptake by target cells. (5) Upon *in vivo* application of flagellin:antigen fusion proteins, flagellin and allergen are simultaneously delivered to the same target cells (either expressing the target receptor TLR5 or preferentially taking up high molecular aggregates) at a fixed ratio, resulting in the induction of stronger immune responses towards the fused antigens as these are processed and presented in the context of the flagellin-mediated immune cell activation. (6) We have already demonstrated, that FlaA fused with different allergens suppressed allergen-specific Th2 responses while promoting Th1 responses both *in vivo* and *in vitro*[77,78,80] (chapter 3).

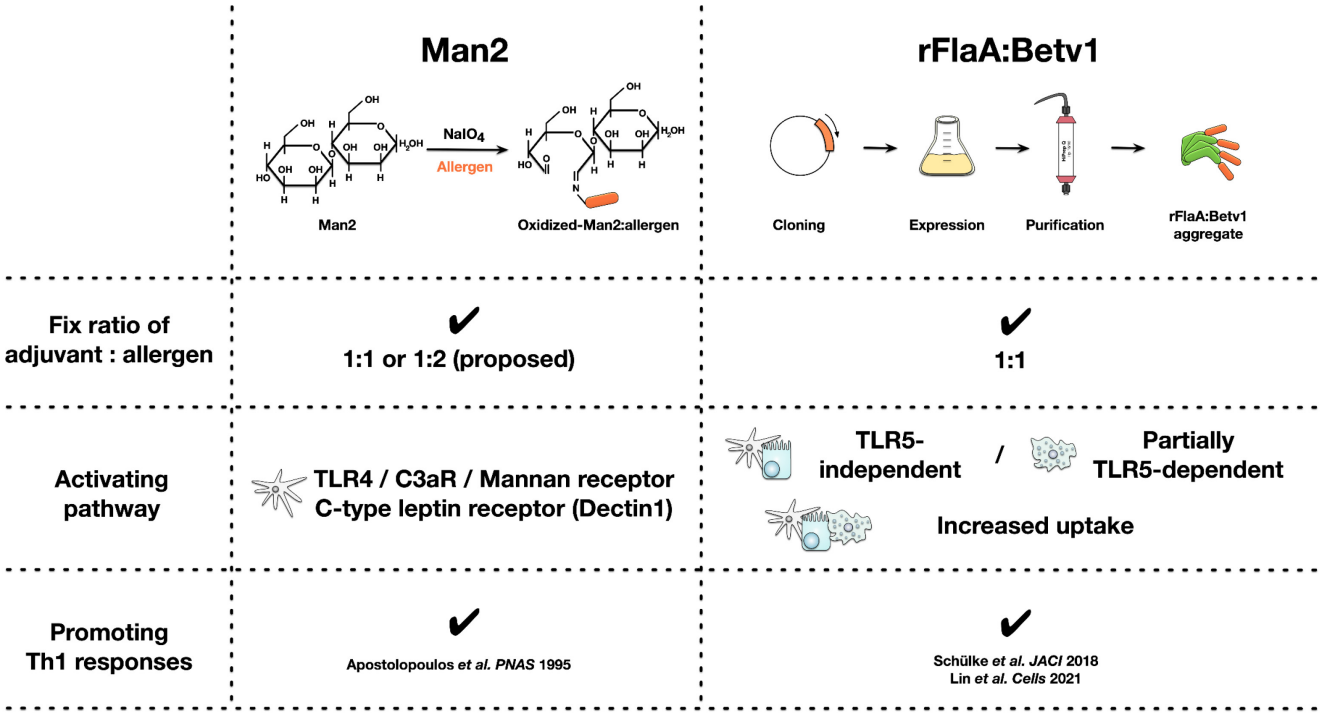


Figure 11: Advantages of Man2 as a novel adjuvant and flagellin:allergen fusion proteins as novel therapeutics for allergy treatment

To generate Man2:allergen conjugates, Man2 could be pre-treated with sodium periodate ($NaIO_4$) to stabilize Man2 in its oxidized form. Flagellin:allergen fusion proteins on the other hand, can be generated by recombinant DNA technology and purified in large amounts using bacterial expression systems. Both strategies have the advantage of defining the adjuvant/allergen ratio while allowing the targeted delivery of the conjugates to the corresponding target cells *in vivo*. Thus, antigen-specific immune responses are induced in the presence of adjuvant-induced immune cell activation, which potentially increase the antigen-specific Th1 response[77,78,80,179]. Moreover, Man2 was shown to induce multiple intracellular signaling pathways in mDCs (chapter 1), while the immunogenicity of rFlaA:allergen fusion proteins was shown to depend on their tendency forming high molecular aggregates, increasing their uptake by target cells and inducing TLR5-independent (mDC[78] and epithelial cell (chapter2)) or TLR5-partially dependent (BMDM) activation (chapter3). For more information see text. Abbreviations: mDC: myeloid dendritic cell, BMDM: bone marrow-derived macrophage, Man2: β-(1→4)-mannobiose, TLR: Toll-like receptor, C3aR: complement C3a receptor.

7 Future prospects

While the present studies have shown that Man2 could be an attractive adjuvant for immunotherapy and rFlaA:Betv1 has the potential to be an effective therapeutic for type I allergy treatment, more questions need to be answered in the future in order to help us understand more about these two candidates.

Man2:

(1) Can Man2 also activate other immune cell types like epithelial cells or macrophages?
(2) What are the intracellular mechanisms underlying Man2-mediated mDCs activation (cytokine secretion and activated metabolic pathways)?
(3) Is oxidized-Man2 also immune-activating and suitable to generate stable Man2-allergen conjugates?
(4) Does conjugation to allergens affect Man2's adjuvanticity?
(5) While Man2 could activate TLR4, what are the advantages and disadvantages of Man2 when compared to currently well-characterized TLR4-ligands like for example MPLA in allergy treatment?
(6) Will Man2-allergen conjugates show the desired capacity to suppress allergen-specific Th2 cytokines from allergen-specific T cells?

rFlaA:Betv1:

(1) The rFlaA:Betv1-stimulated epithelial cell-line LA-4 modulated DC responses. Can comparable results be observed when using primary mouse or human cells?
(2) Could flagellin:allergen fusion proteins also activate other epithelial cell types (for example, intestinal epithelial cells) to improve food allergy treatment?
(3) If and yes how can mDCs modulated by rFlaA:Betv1-stimulated epithelial cells further suppress allergen-specific Th2 responses?
(4) Epithelial cells were reported to show metabolic changes during allergic disease. Therefore, it would be of interest to investigate the metabolic phenotype of rFlaA:Betv1-stimulated epithelial cells.
(5) Can rFlaA:Betv1-stimulated epithelial cells and their soluble factors also modulate macrophage responses?
(6) Inflammasome activation was reported to be involved in immune cell activation during vaccination[185]. In our experiments using both mDCs and BMDMs, in contrast to other cytokines, rFlaA:Betv1-induced IL-1β secretion was neither mediated by mTOR-, MAPK-, or TLR5-pathways. Therefore, it would be highly interesting to investigate, if rFlaA:Betv1-mediated activation of the inflammasome complex might be responsible for the observed IL-1β production.
(7) rFlaA:Betv1 induced upregulation of HIF-1α in BMDMs due to higher glycolysis. Can the same mechanism be observed in rFlaA:Betv1-stimulated mDCs?

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9 Annex

9.1 Abbreviation

ACO2	Aconitase 2
AIT	Allergen-specific immunotherapy
Alum	Aluminum hydroxide
AMPK	AMP-activated protein kinase
APCs	Antigen-presenting cells
AS	Adjuvant System
ASC	Apoptosis-associated speck-like protein
BCR	B-cell receptor
BMDMs	Bone marrow-derived macrophages
C3aR	Complement C3a receptor
CD	Cluster of differentiation
COX2	Cyclooxygenase 2
CpG-ODN	CpG Oligodeoxynucleotides
DAMPs	Damage associated molecular patterns
DCs	Dendritic cells
DMF	Dimethyl fumarate
EAE	Experimental autoimmune arthritis
ECAR	Extracellular acidification rate
EPX	Peroxidase
ERK	Extracellular signal-regulated kinase 1/2
FA	Food allergy
FDA	United States Food & Drug Administration
FliC	*Salmonella typhimurium* flagellin type C
GIT	Gastrointestinal tract
Glut1	Glucose transporter 1
HBV	Hepatitis B virus
HDM	House dust mite
HIF-1α	Transcription factor hypoxia-inducible factor 1 α
HIV-TAT	HIV type 1 trans-activating regulatory protein
hmoDCs	Human monocyte-derived DCs
IFN	Interferon
IKK	IκB kinase
ILC2s	Innate like lymphocytes type II cells
IRAK	Interleukin-1 receptor-associated kinase
IPAF	ICE-protease activating factor
JAK	Janus kinase
JNK	Jun N-terminal kinase
LPS	Lipopolysaccharide
Man	Mannooligosaccharides
Man2	β-(1→4)-mannobiose
MAPK	Mitogen-activated protein kinase
MBP	Major basic protein
MC	Mast cell
MD2	Myeloid differentiation factor 2
mDCs	Myeloid dendritic cells
MPLA	Monophosphoryl lipid A
mTOR	Mammalian target of rapamycin
mTORC1	Mammalian target of rapamycin complex 1
MUC1	Protein mucin 1
MyD88	Myeloid differentiation primary-response protein 88
$NaBH_4$	Sodium borohydride
$NaIO_4$	Sodium periodate
NAIP	NLR family apoptosis inhibitory protein

NFκB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NLRC4	NLR family CARD domain containing 4
NLRP3	NOD-, LRR- and pyrin domain-containing protein 3
NLR	NOD-like receptor
NO	Nitric oxide
NOD	Nucleotide oligomerization domain
O/W	Oil-in-water
OVA	Egg allergen ovalbumin
OXPHOS	Oxidative phosphorylation
p70S6K	mTORC1 target protein P70 S6 kinase
PAMPs	Pathogen-associated molecular pattern
pDCs	Plasmacytoid dendritic cell
PFKFB3	6-phosphofructo-2-kinase/fructose-2,6-Bbiphosphatase 3
PGE_2	Prostaglandin E2
PI3K	Phosphatidylinositol 3-kinase
PKM2	Pyruvate kinase M2
PQ	Pollinex® Quattro
PRAS40	Proline-rich Akt substrate of 40 kDa
PRRs	Pattern recognition receptor
RA	Rheumatoid arthritis
rDCs	Regulatory dendritic cell
rFlaA:Betv1	Fusion protein consisting of *Listeria monocytogenes* flagellin A fused to the major birch pollen allergen Bet v 1
ROS	Reactive oxygen species
RRMS	Relapse-remitting multiple sclerosis
SAIT	Subcutaneous allergy immunotherapy
SLE	Systemic lupus erythematosus
ST-AIT	Short-term specific immunotherapy
STAT	Signal transducer and activator of transcription
T2D	Type 2 diabetes
TAK1	TGF-β-activated kinase 1
TC	T cell
TCR	T-cell receptor
TGF-β	Transforming growth factor-β
TLR	"Toll"-like receptor
TRAF	TNF-receptor-associated factor
T_{reg}	Regulatory T cells
TSLP	Thymic stromal lymphopoietin
VLPs	Virus-like particle

9.2 List of figures

9.3 Acknowledgments

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感恩的心，獻給所有在這條路上幫助我，支持我，鼓勵我的人。

9.4 Declaration - Ehrenwörtliche Erklärung

Ich erkläre hiermit ehrenwörtlich, dass ich die vorliegende Arbeit entsprechend den Regeln guter wissenschaftlicher Praxis selbstständig und ohne unzulässige Hilfe Dritter angefertigt habe.

Sämtliche aus fremden Quellen direkt oder indirekt übernommenen Gedanken sowie sämtliche von Anderen direkt oder indirekt übernommenen Daten, Techniken und Materialien sind als solche kenntlich gemacht. Die Arbeit wurde bisher bei keiner anderen Hochschule zu Prüfungszwecken eingereicht. Die eingereichte elektronische Version stimmt mit der schriftlichen Version überein.

Darmstadt, den 2022

02.09.2022

9.5 Curriculum vitae

RESEARCH EXPERIENCE

Paul-Ehrlich-Institut — Langen, Germany
PhD student, Molecular Allergology Lab — *Mar 2019 – Present*

National Taiwan University, Institute of Molecular Medicine — Taipei, Taiwan
Research Assistant, Innate Immunology Lab — *Sep 2017 – Jan 2019*

UBI Pharma Inc. (UBIP) — Hsinchu, Taiwan
Associate Scientist, Biological Testing and Evaluation — *Mar 2015 – Nov 2016*

United Biomedical, Inc. Asia (UBIA) — Hsinchu, Taiwan
Research Associate, Biological Function Testing — *Feb 2013 – Mar 2015*

EDUCATION

National Yang Ming University, School of Medicine — Taipei, Taiwan
Master of Physiology — *Sep 2010 - Aug 2012*

National Chung Hsing University — Taichung, Taiwan
Bachelor of Life Sciences — *Sep 2006 - Jun 2010*

PUBLICATION LIST

- Alexandra Goretzki, Jennifer Zimmermann, **Yen-Ju Lin**, and Stefan Schülke. Immune metabolism -An opportunity to better understand allergic pathology and improve treatment of allergic diseases? *Front. Allergy.* 3:825931, 2022
- **Yen-Ju Lin**, Adam Flaczyk, Sonja Wolfheimer, Alexandra Goretzki, Annette Jamin, Andrea Wangorsch, Stefan Vieths, Stephan Scheurer, and Stefan Schülke. The Fusion Protein rFlaA:Betv1 Modulates DC Responses by a p38-MAPK and COX2-Dependent Secretion of PGE_2 from Epithelial Cells. *Cells.* 10(12): 3415, 2021
- **Yen-Ju Lin**, Alexandra Goretzki, and Stefan Schülke. Immune Metabolism of IL-4-Activated B Cells and Th2 Cells in the Context of Allergic Diseases. *Front. Immunol.* 12:790658, 2021
- **Yen-Ju Lin**[†], Garibald Papp[†], Csaba Miskey, Anna Fiedler, Alexandra Goretzki, Sonja Wolfheimer, Jennifer Zimmermann, Peter Crauwels, Zoltán Ivics, Ger van Zandbergen, Stefan Vieths, Stephan Scheurer, and Stefan Schülke. The Flagellin:Allergen Fusion Protein rFlaA:Betv1 Induces a MyD88− and MAPK-Dependent Activation of Glucose Metabolism in Macrophages. *Cells*. 10(10): 2614, 2021
- Ting-Yu Cheng, **Yen-Ju Lin**, Wataru Saburi, Stefan Vieths, Stephan Scheurer, Stefan Schülke, and Masako Toda. β-(1→4)-Mannobiose Acts as an Immunostimulatory Molecule in Murine Dendritic Cells by Binding the TLR4/MD-2 Complex. *Cells.* 10(7): 1774, 2021
- Alexandra Goretzki[†], **Yen-Ju Lin**[†], and Stefan Schülke. Immune metabolism in allergies, does it matter? - A review of immune metabolic basics and adaptations associated with the activation of innate immune cells in allergy. *Allergy*. 76(11):3314-3331, 2021
- **Yen-Ju Lin**, Martina Anzaghe, and Stefan Schülke. Update on the Pathomechanism, Diagnosis, and Treatment Options for Rheumatoid Arthritis. *Cells.* 9(4): 880, 2020

[†]Equal contribution

CONFERENCES AND PRESENTATIONS

- 20th EAACI Immunology Winter School. *Poster* (Best Poster Presentation Award) — *Jan 27th - Jan 30th 2022*
- World immune Regulation Meeting XV. *Presentation* (EJI-EFIS Registration Grant) — *Jun 30th - Jul 3rd 2021*
- 33. Mainzer Allergie Workshop. *Presentation* — *Mar 26th - Mar 27th 2021*
- 19th EAACI Immunology Winter School. *Presentation* — *Jan 22th - Jan 24th 2021*
- World immune Regulation Meeting XIV. *Presentation* — *Oct 4th - Oct 7th 2020*
- JSA / WAO joint Congress 2020. *Poster* — *Sep 17th - Oct 20th 2020*
- 32. Mainzer Allergie Workshop. *Presentation* — *Sep 24th - Sep 25th 2020*