Critical role of mammalian target of rapamycin for IL-10 dendritic cell induction by a flagellin A conjugate in preventing allergic sensitization

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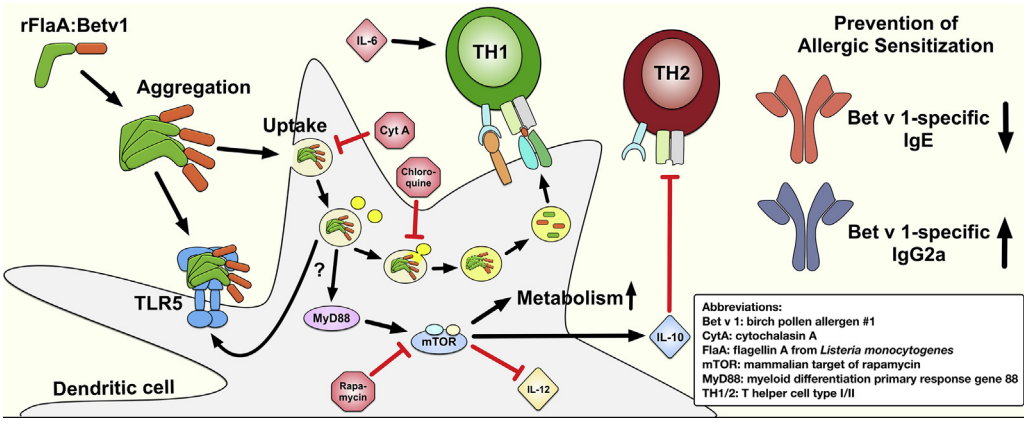
GRAPHICAL ABSTRACT



Background: Fusion proteins incorporating the Toll-like receptor 5 ligand flagellin are currently undergoing clinical trials as vaccine candidates for many diseases.

Objective: We studied the mechanisms of immune modulation by a flagellin:allergen fusion protein containing the Toll-like receptor 5 ligand flagellin A from *Listeria monocytogenes* and the birch pollen allergen Bet v 1 (recombinant flagellin A [rFlaA]:Betv1).

Methods: BALB/c mice were vaccinated with rFlaA:Betv1 in an experimental Bet v 1 sensitization model. Myeloid dendritic cells (mDCs) were differentiated from mouse bone marrow, and PBMCs were isolated from subjects with birch pollen allergy. Cells were stimulated with equimolar amounts of rFlaA, rBet v 1, rFlaA plus rBet v 1, or the rFlaA:Betv1 conjugate and analyzed for cell activation, cytokine secretion, and metabolic state.

Results: rFlaA:Betv1 displayed strong immune-modulating properties both *in vivo* and *in vitro*, as characterized by secretion of both proinflammatory and anti-inflammatory cytokines from murine mDCs and PBMCs from patients with birch allergy. rFlaA:Betv1 suppressed T_H2 responses from Bet v 1–specific $CD4^+$ T cells and prevented allergic sensitization in a mouse allergy model. Aggregation of rFlaA:Betv1 resulted in stronger protein uptake accompanied by an increased resistance to microsomal digestion. Remarkably, rFlaA:Betv1 induced activation of mammalian target of rapamycin, which increased the metabolic activity of the stimulated mDCs.

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rFlaA:Betv1-mediated IL-10 secretion, but not proinflammatory cytokine secretion, was inhibited by rapamycin in mDCs.
Conclusion: These results provide evidence that mammalian target of rapamycin is a key player involved in prevention of T_H2 responses by flagellin A conjugate vaccines. (J Allergy Clin Immunol 2017;■■■:■■■-■■■.)

Key words: *Vaccine, Bet v 1, flagellin, rFlaA, fusion protein, birch pollen allergy, IL-10 dendritic cell, mammalian target of rapamycin, metabolism*

Abbreviations used
alum: Aluminum hydroxide
APC: Antigen-presenting cell
B/A: Bet v 1/alum
DC: Dendritic cell
2-DO: 2-Deoxyglucose
EGFP: Enhanced green fluorescent protein
FACS: Fluorescence-activated cell sorting
FITC: Fluorescein isothiocyanate
FlaA: Flagellin A
mDC: Myeloid dendritic cell
mTOR: Mammalian target of rapamycin
MyD88: Myeloid differentiation primary response gene–88
NADH: Nicotinamide adenine dinucleotide
OVA: Ovalbumin
PE: Phycoerythrin
PI3K: Phosphatidylinositol 3-kinase
PRAS40: Proline-rich Akt substrate of 40 kDa
TLR: Toll-like receptor

Although the introduction of vaccines has drastically improved our prevention options for many different types of diseases, the search for more efficacious vaccines to combat or prevent diseases with a significant effect on public health, such as infections, cancer, or allergies, remains a major task.

Pure antigen preparations, which are often weakly immunogenic, need to be adjuvanted to induce robust immune responses.[1] Here the Toll-like receptor (TLR) 5 ligand flagellin,[2] a bacterial motility protein forming the body of the bacterial flagellum, has demonstrated its potential as mucosal adjuvant to mediate protective immunity.[3-5] One major advantage of flagellin is its protein nature, allowing for the efficient generation of fusion constructs of flagellin and antigen using recombinant DNA technologies. Such fusion proteins combine antigen and adjuvant (flagellin) into a single molecule, allowing for efficient targeting of antigens to and simultaneous activation of $TLR5^+$ antigen-presenting cells (APCs).[5]

A growing body of studies suggest that fusion proteins incorporating flagellin efficiently induce humoral and cell-mediated immunity against a variety of antigens in animal models[6-8] and might have potential as vaccine candidates for many diseases, including influenza,[9-11] poxvirus,[12] West Nile virus,[8] tetanus,[13] *Pseudomonas* species infection,[14] and allergies.[6]

Additionally, flagellin-containing fusion proteins are currently in clinical trials for the prevention of influenza infection, where they were proven to be both safe and efficacious in inducing protective immune responses.[15,16] This vaccine combining flagellin C from *Salmonella* species with peptides derived from the influenza antigen M2e was shown to efficiently induce antibody responses against the otherwise poorly immunogenic M2e[15] without inducing serious adverse events in healthy young adults.[16] Moreover, Kitzmüller et al recently described the enhanced immunogenicity, reduced allergenicity, and intrinsic adjuvanticity of flagellin C:Bet v 1 fusion proteins in human monocyte-derived dendritic cells (DCs) and T cells from allergic patients.[17] The results obtained by Treanor et al,[16] Turley et al,[15] and Kitzmüller et al show that such flagellin:allergen fusion proteins have similar immune-modulating effects on both mouse and human immune cells and therefore a relevance as vaccines for future human application.

However, despite their well-described immune-activating potential, the mechanisms by which such fusion proteins exert their immune-modulating effects are less well understood. The protective immune responses induced upon application of a fusion protein consisting of *Salmonella* flagellin C and *Plasmodium vivid* circumsporozoite protein required TLR5/myeloid differentiation primary response gene–88 (MyD88) signaling.[18] Accordingly, stimulation of $CD11c^+TLR5^+$ cells and signaling through TLR5 was essential for the adjuvant activity of a flagellin-ovalbumin (OVA) fusion protein.[19] Recently, we reported that a fusion protein consisting of flagellin A (FlaA) from *Listeria monocytogenes* and OVA efficiently protected mice against OVA-induced intestinal allergy.[6] The strong immune-modulating capacity of the fusion protein was accompanied by enhanced uptake into $CD11c^+$ myeloid dendritic cells (mDCs), likely facilitated by protein aggregation, resulting in different processing of the fused antigen by mDCs, as well as stronger cell activation.[20] However, the detailed mechanisms by which these potent vaccine candidates modulate immune responses are still unclear.

The mammalian target of rapamycin (mTOR) is a serine/threonine protein kinase belonging to the phosphatidylinositol 3-kinase (PI3K)–related kinase protein family, which forms a multiprotein complex with the proteins mammalian lethal with SEC13 protein 8 (mLST8), proline-rich Akt substrate of 40 kDa (PRAS40), Raptor, *G* protein β-subunit-like protein, pronounced "Gable" (GβL), and DEP domain-containing mTOR-interacting protein (DEPTOR).[21] The mTOR1 complex acts as a master regulator of cellular growth, sensing and integrating diverse nutritional and environmental cues, including growth factors, energy levels, cellular stress, and amino acids.[21] It links these signals to the promotion of cellular growth by phosphorylating substrates; potentiating anabolic processes, such as mRNA translation and lipid synthesis; or limiting catabolic processes, such as autophagy.[21] Recently, mTOR1 activation was implicated to have important functions in immunologic processes, such as the maturation and function of APCs, as well as T-cell differentiation.[22]

Here we investigated the potency and immune-modulating mechanism of a fusion protein consisting of the TLR5 ligand FlaA from *L monocytogenes* and the major birch pollen allergen Bet v 1, causing respiratory allergy in millions of persons. The fusion protein (recombinant flagellin A [rFlaA]:Betv1) displayed strong immune-modulating properties both *in vivo* and *in vitro* characterized by secretion of both proinflammatory and anti-inflammatory cytokines from murine mDCs, as well as PBMCs from patients with birch allergy. Mechanistically, we showed that stimulation with rFlaA:Betv1