



Lactic acid bacteria as adjuvants for sublingual allergy vaccines

Laurence Van Overtvelt^a, Helene Moussu^a, Stéphane Horiot^a, Sandrine Samson^b, Vincent Lombardi^{a,1}, Laurent Mascarell^a, Ariane van de Moer^{b,c}, Raphaëlle Bourdet-Sicard^b, Philippe Moingeon^{a,*}

^a Stallergènes SA, Research and Development, 6 rue Alexis de Tocqueville, 92183 Antony, France

^b Danone Research, R.D. 128, 91767 Palaiseau, France

^c Utrecht University, Budapestlaan 6, 3584 CD Utrecht, Netherlands

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ABSTRACT

We compared immunomodulatory properties of 11 strains of lactic acid bacteria as well as their capacity to enhance sublingual immunotherapy efficacy in a murine asthma model. Two types of bacterial strains were identified, including: (i) potent inducers of IL-12p70 and IL-10 in dendritic cells, supporting IFN- γ and IL-10 production in CD4⁺ T cells such as *Lactobacillus helveticus*; (ii) pure Th1 inducers such as *L. casei*. Sublingual administration in ovalbumin-sensitized mice of *L. helveticus*, but not *L. casei*, reduced airways hyperresponsiveness, bronchial inflammation and proliferation of specific T cells in cervical lymph nodes. Thus, probiotics acting as a Th1/possibly Treg, but not Th1 adjuvant, potentiate tolerance induction via the sublingual route.

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1. Introduction

Allergen-specific immunotherapy is the only efficient causal treatment of type I respiratory allergies [1]. Recently, on the basis of multiple double blind placebo controlled studies, sublingual immunotherapy (SLIT) has been established as an effective, safe and convenient alternative to subcutaneous immunotherapy [2–6]. Whereas current SLIT protocols rely upon high doses of unformulated allergens, adjuvants could further improve the efficacy of sublingual vaccines, most particularly as it applies to vaccines based on recombinant allergens [7,8]. In murine models, we recently demonstrated that several IL-10- and IFN- γ -inducing adjuvants enhance the efficacy of sublingual vaccines in establishing allergen-specific tolerance [9–11]. Interestingly, both immune deviation towards Th1 responses and induction of regulatory T (Treg) cells appear to be associated with successful SLIT [12–14]. Noteworthy, whereas allergic patients elicit Th2 responses when exposed to allergens, healthy individuals have rather circulating Th1- and IL-10-producing Treg cells in the blood [15,16]. Collectively, these results support a strategy to induce mixed Th1/Treg CD4⁺ T cell responses to allergens in future immunotherapy protocols.

Increased incidence of allergic disorders in Western countries is associated with a lower incidence of infectious diseases and lim-

ited exposure to microorganisms, due to a more hygienic lifestyle [17,18]. In this context, the use of probiotics appears as a valuable approach for both prevention [19–21] and treatment [21,22] of allergic disorders. Probiotics are defined by WHO as ‘live microorganisms which when ingested in adequate amounts confer a health benefit to the host’ [23]. Such probiotic microorganisms are known to influence dendritic and T cell responses, although their immunomodulatory properties vary considerably depending upon the strains considered [24–37]. Given the capacity of probiotics to modulate immune responses at mucosal surfaces [38,39], the present study was initiated to test various strains as candidate adjuvants for sublingual allergy vaccines. Probiotics were compared for their effect on human dendritic cells (DCs) and CD4⁺ naïve T cells, and further for their capacity to enhance SLIT efficacy in a murine asthma model. We report herein that immunomodulatory properties allow segregating probiotics in two types, skewing CD4⁺ T cells towards a Th1/possibly Treg or Th1 profile, respectively, with only the former enhancing SLIT efficacy.

2. Materials and methods

2.1. Animals, culture media, reagents and bacterial strains

Six-week old female BALB/c mice were purchased from Charles River (L'Arbresle, France) and maintained in a specific pathogen free environment on an ovalbumin (OVA)-free diet. Animal handling was performed by trained personnel. DO11.10 mice, transgenic for a T cell receptor specific for the OVA_{323–339} epitope restricted to the H2d haplotype [40], were bred in the Centre d'Exploration et

* Corresponding author. Tel.: +33 1 55 59 25 20; fax: +33 1 55 59 21 02.

E-mail address: pmoingeon@stallergenes.fr (P. Moingeon).

¹ Current address: University of Southern California, Keck School of Medicine, Department of Molecular Microbiology and Immunology, 1450 Biggy Street, NRT 5509 Los Angeles, CA 90033, United States.

de Recherche Fonctionnelle Expérimentale (Evry, France). International standards for the care and use of animals were applied.

Human cells were cultured in RPMI 1640 supplemented with 2 mM L-glutamine, 20 µg/ml gentamicin, 50 µM 2-mercaptoethanol, 1% nonessential amino acids (all from Invitrogen, Cergy Pontoise, France), and 10% fetal bovine serum (Gentaur, Brussels, Belgium). OVA grade V was purchased from Sigma Chemicals Aldrich (Saint Louis, MO). Contaminant endotoxin (LPS) was removed from all OVA batches used in the present study as previously described [41]. Residual endotoxin levels, determined by Endochrom-K assay (R1708, Charles River), were always less than 0.1 EU/µg protein. Phosphate-buffer saline (PBS) and alum were purchased from Invitrogen and Pierce (Rockford, IL), respectively.

Ten probiotic bacteria were obtained from Danone Research's probiotic collection, including *Lactobacillus casei* DN-114 001 (I-1518), *L. kefirifaciens* DN-138 015 (I-3607), *L. helveticus* DN-119 080 (I-3647), *Streptococcus thermophilus* DN-001 616 (I-3606), *Bifidobacterium longum* DN-164 069 (I-3652), three distinct strains of *B. bifidum*: DN-154 057 (I-3608) called *B. bifidum*1, DN-154 051 (I-3650) called *B. bifidum*2, DN-154 056 (I-3651) called *B. bifidum*3, as well as two of *B. adolescentis*: DN-150 011 (I-3648) called *B. adolescentis*1 and DN-150 012 (I-3649) called *B. adolescentis*2. *L. plantarum* was purchased from the American type culture collection (ATCC no. 8014, Manassas, VA).

2.2. Adjuvant testing in human immune cells

Human DCs were generated from peripheral blood mononuclear cells (PBMCs) in presence of human GM-CSF (80 ng/ml) and IL-4 (25 ng/ml) as previously described [42]. DC purity assessed by morphology and flow cytometry analysis (based on surface expression of CD1a and lack of CD14 expression) was always superior to 90%. Allogeneic CD4⁺ T lymphocytes were purified from PBMCs of healthy volunteers by negative selection using a mix of antibodies directed against CD14, CD16, CD56, CDw123, CD36, CD8, HLA Class II DR/DP, glycoporphin A (Invitrogen) and CD45RO molecules (BD Biosciences, Le Pont de Claix, France), followed by depletion with dynabeads (Invitrogen). Flow cytometry analysis confirmed that CD4⁺ T cells were more than 90% pure, based on CD16, CD20, CD3 and CD4 expression.

To test the effect of candidate probiotic bacteria on human DC maturation and cytokine production, 5×10^5 DCs were incubated in 24-well plates in presence of either medium alone, or with probiotic bacteria in stationary phase unless otherwise specified. Given that γ -irradiation (10 kGy) maintains the bacterial structure integrity [24] and since DC viability was preserved at DC:bacteria ratio of a 1:10 (data not shown), these experimental conditions were selected in the present study. After 24 h, DCs were washed, and tested in terms of viability and surface phenotype. Supernatants were harvested to analyze cytokine levels as described below.

To assess the impact on T cell polarization, adjuvant-pretreated DCs were further cultured in triplicate (1×10^5 DCs/well) in presence of allogeneic CD4⁺ naïve T cells (1×10^6 cells/well) in 48-well flat-bottom plates. Cells and supernatants were collected after either 1 or 5 days of culture. As described below, messenger RNAs were isolated and used in quantitative PCR analysis, whereas supernatants were harvested to analyze cytokine levels using the cytometric bead array (CBA) technology.

2.3. Analysis of surface phenotype and cytokine production

For immunofluorescence staining, cells were harvested, washed in PBS and incubated for 20 min at room temperature with the following mAbs: fluorescein isothiocyanate (FITC) labelled anti-CD80 IgG1, or anti-HLA-DR IgG1, phycoerythrin (PE) labelled anti-CD86

IgG2b or anti-CD40 IgG1, phycoerythrin–cyanin 5.1 (PC5) labelled anti-CD83 IgG2b or anti-CD1a IgG1 or anti-ILT3 IgG1 (Beckman Coulter, Roissy, France), PC7 labelled anti-B7H1 IgG1 (Clinisciences, Montrouge, France). As a control, cells were stained with corresponding isotype-matched mAbs. Samples were analyzed using a FC500 flow cytometer (Beckman Coulter). A relative value, expressed as a percentage fold increase in mean fluorescence intensities (MFI), was calculated as follows: (MFI in presence of probiotic minus MFI in presence of medium alone over MFI in presence of medium alone) $\times 100$.

IL-1 β , IL-6, IL-8, IL-10, IL-12p70 and TNF- α were measured using the human inflammation CBA kit from BD Biosciences as previously described. IL-2, IL-5, IL-10, IFN- γ and TNF- α were measured using the human Th1/Th2 CBA kit. The production of IL-23 was evaluated by ELISA (eBiosciences, San Diego, CA). A relative value, expressed as a percentage fold increase in cytokine production, was obtained as follows: (cytokine level (pg/ml) in presence of probiotic minus cytokine level in presence of medium alone over cytokine level in presence of medium alone) $\times 100$.

2.4. RNA isolation and quantitative PCR analysis of T cell polarization

RNA was isolated from T cells stimulated with adjuvant pretreated DCs, using the RNeasy Mini kit (Qiagen, Courtaboeuf, France) as per the manufacturer's instructions. Reverse transcription was performed with multiscribe reverse transcriptase (Applied Biosystems, Courtaboeuf, France) using random hexamers, according to the manufacturer's protocol. The cDNAs thus obtained were quantified on a 7300 Real-time PCR system (Applied Biosystems). Primers and probes were selected in a gene bank from Applied Custom assays and synthesized by Applied Biosystems. As a control, mRNA content was analyzed for β -actin in parallel. The following transcription factors or cytokine genes were monitored as markers for T cell polarization in human cell cultures: T-bet, GATA-3, Foxp3, IFN- γ , TGF- β and IL-10.

2.5. Therapeutic sublingual immunotherapy in BALB/c mice sensitized to OVA

The capacity of candidate probiotic bacteria to enhance SLIT efficacy was tested in a therapeutic *in vivo* model of OVA-induced asthma, as previously described [43]. Briefly, BALB/c mice were sensitized with OVA by two intraperitoneal injections (10 µg OVA and 2 mg Alum) at day 0 and 14. At day 21, a 20 min aerosol challenge was performed with 1% (w/v) OVA on 4 consecutive days using an aerosol delivery system (Buxco Europe Ltd., Winchester, UK). For tolerance induction, 500 µg OVA with or without live probiotic bacteria (2×10^8 colony forming units per mouse) were applied sublingually to groups of 8 mice, twice a week during 2 months in 50 µl PBS. This dose was selected in view of our previous results establishing that *L. plantarum* enhances SLIT efficacy in our murine asthma model with this dosing regimen [11]. Control mice were sensitized with OVA and subsequently treated sublingually with PBS or probiotic bacteria alone. After sublingual immunotherapy, mice were challenged by aerosol during two consecutive days with a 1% (w/v) OVA solution. After 24 h, airway hyperresponsiveness (AHR) was evaluated by measuring enhanced pause (Penh) by whole body plethysmography (Buxco Europe Ltd.). The Penh index was obtained as a ratio between Penh values measured after exposure to either inhaled metacholine (50 mg/ml) or nebulized PBS, as described elsewhere [43].

Table 1

Comparison of immunomodulatory properties mediated on DCs by probiotic bacteria.

Bacterial strains (number of DC donors tested)	Mean fluorescence intensity							Cytokine production					
	CD40	CD80	CD83	CD86	HLA-DR	ILT3	B7H1	IL1 β	IL6	IL8	IL10	IL12p70	TNF α
<i>L. casei</i> (n = 6)	167	81	1405	1201	12	187	405	687	872	178	373	1217	965
<i>L. kefirgranum</i> (n = 2)	235	128	67	473	17	72	59	534	3152	776	2787	4544	1680
<i>L. helveticus</i> (n = 6)	156	98	1544	1648	63	121	415	12,757	78,672	2000	130,208	428,530	202,999
<i>L. plantarum</i> (n = 3)	21	32	65	272	–34	ND	ND	521	39	–9	210	1508	155
<i>S. thermophilus</i> (n = 3)	281	119	2592	1292	75	160	421	2083	11,939	213	657	5569	5402
<i>B. bifidum1</i> (n = 3)	80	77	624	1031	1	ND	ND	3517	411	640	3009	53	2310
<i>B. bifidum2</i> (n = 3)	97	128	3702	1950	93	63	711	5523	39,591	2591	71,261	18,494	247,532
<i>B. bifidum3</i> (n = 3)	104	74	4051	1908	68	56	791	1916	26,104	3421	56,723	29,890	2,074,495
<i>B. adolescentis1</i> (n = 2)	177	132	76	612	16	75	130	1271	1494	330	4010	54	2481
<i>B. adolescentis2</i> (n = 2)	180	126	44	571	17	41	105	6106	2583	222	6723	191	6138
<i>B. longum</i> (n = 3)	56	83	508	770	47	62	332	249	7912	6035	5351	804	15,924

Human immature monocyte-derived DCs were incubated for 24 h with each of the irradiated bacterial strain at a 1/10 (DC/bacteria) ratio. Cell surface phenotypes were analyzed by flow cytometry and cytokine levels were determined in supernatants by cytometric bead array technology or ELISA. Mean relative values obtained from 2 to 6 DC donors for each strain are expressed as % fold increase versus unstimulated cells as described in Section 2. *Bifidobacterium* (B), *Lactobacillus* (L), *Streptococcus* (S), not determined (ND).

2.6. Follow up of immune responses in mice

Broncho-alveolar lavages (BAL) were performed with 1 ml PBS in mice anesthetized by intraperitoneal injection of pentobarbital (50 mg/kg body weight). BAL fluid was centrifuged at 800 $\times$ g for

10 min at 4 °C. Cell pellets were resuspended in PBS, spun onto glass slides by cytocentrifugation, fixed and stained with May-Grünwald Giemsa (Réactifs RAL, Martillac, France). Eosinophils, macrophages and lymphocytes were counted under light microscopy using a 200-fold magnification. T cell responses were assessed in the lung. Cells were isolated and plated in duplicate at 1×10^6 cells per well in 96-well flat-bottomed ELISPOT plates (Millipore Corp., Bedford, MA) with medium alone or OVA (100 μ g/ml). Plates were incubated for 72 h at 37 °C in 5% CO₂/95% air. Spot forming cells (SFCs) were monitored for both IL-10 and IL-5 production using ELISPOT kits (R&D Systems, Minneapolis, MN) as per the manufacturer's instructions and assessed with an ELISPOT reader (Carl Zeiss Microimaging, GmbH, Göttingen, Germany).

To assess the impact of sublingual administration of lactic acid bacteria on T cell responses *in vivo*, CFSE-labelled CD4⁺ T cells (5×10^6) purified from DO11.10 mice were adoptively transferred by retroorbital intravenous injection into BALB/c mice. One day after, groups of three BALB/c mice received 500 μ g OVA sublingually in 50 μ l PBS with or without 2×10^8 colony forming units of live bacteria. Control animals received either sterile PBS or bacteria alone. Seven days later, cervical lymph nodes were removed and pooled within each group of mice. OVA-specific transferred T cells were stained with the anticonotypic PE-KJ1.26 antibody (BD Biosciences). Among those cells, proliferating T cells were further evaluated by FACS analysis as cells with a decreased CFSE-associated fluorescence.

2.7. Statistical analysis

Penh values of mice treated sublingually with OVA with or without bacteria were compared using Student's *t*-test. Levels of cytokines produced by immune cells were compared using the non-parametric Wilcoxon's paired test. Results with a *p* value less than 0.05 are significant.

3. Results

3.1. Identification of two types of lactic acid bacteria, based on immunomodulatory properties on human DCs

To identify the most appropriate bacteria to include as an adjuvant into sublingual allergy vaccines, 11 strains of lactic acid bacteria were compared after γ -irradiation for their impact on the phenotype and cytokine production of human monocyte-derived immature DCs. All bacteria tested induce DC maturation as shown by a substantial induction of cell surface markers (CD40, CD80,

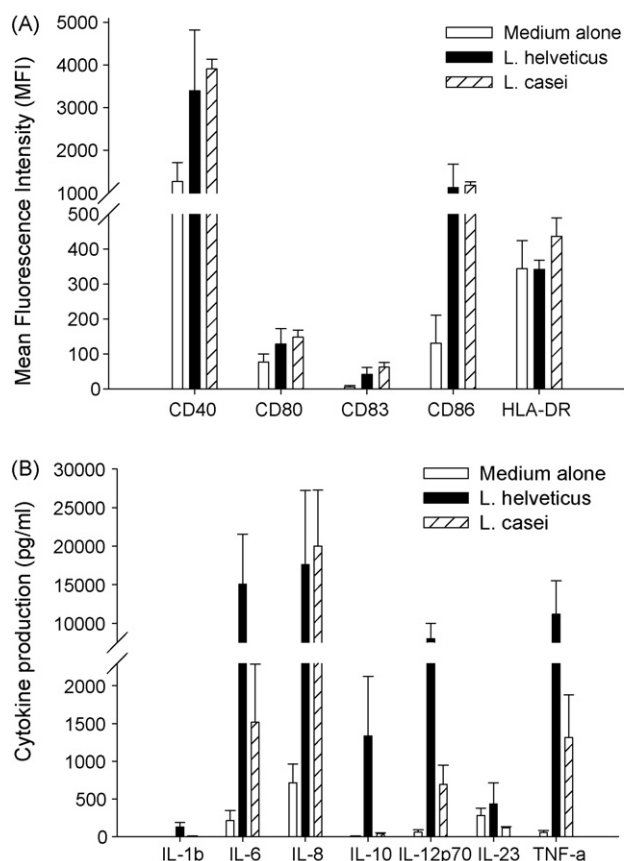


Fig. 1. Impact of *L. helveticus* and *L. casei* on human DC phenotype and cytokine production. Human monocyte-derived immature DCs were incubated for 24 h with either medium alone, *L. helveticus* or *L. casei*. (A) The expression of various surface molecules was assessed by flow cytometry. Results are expressed as mean fluorescence intensity (MFI) $\pm$ SEM (n = 3). (B) Cytokine levels in supernatants were determined by cytometric bead array technology or ELISA. Results are expressed as mean $\pm$ SEM (n = 3–6). MFI and cytokine production were compared with levels in presence of medium alone using the nonparametric Wilcoxon's paired test. A *p* value of 0.06 is obtained for IL-6, IL-8, IL-10, IL-12p70 and TNF- α secretion in presence of *L. helveticus* (when compared with unstimulated cells).

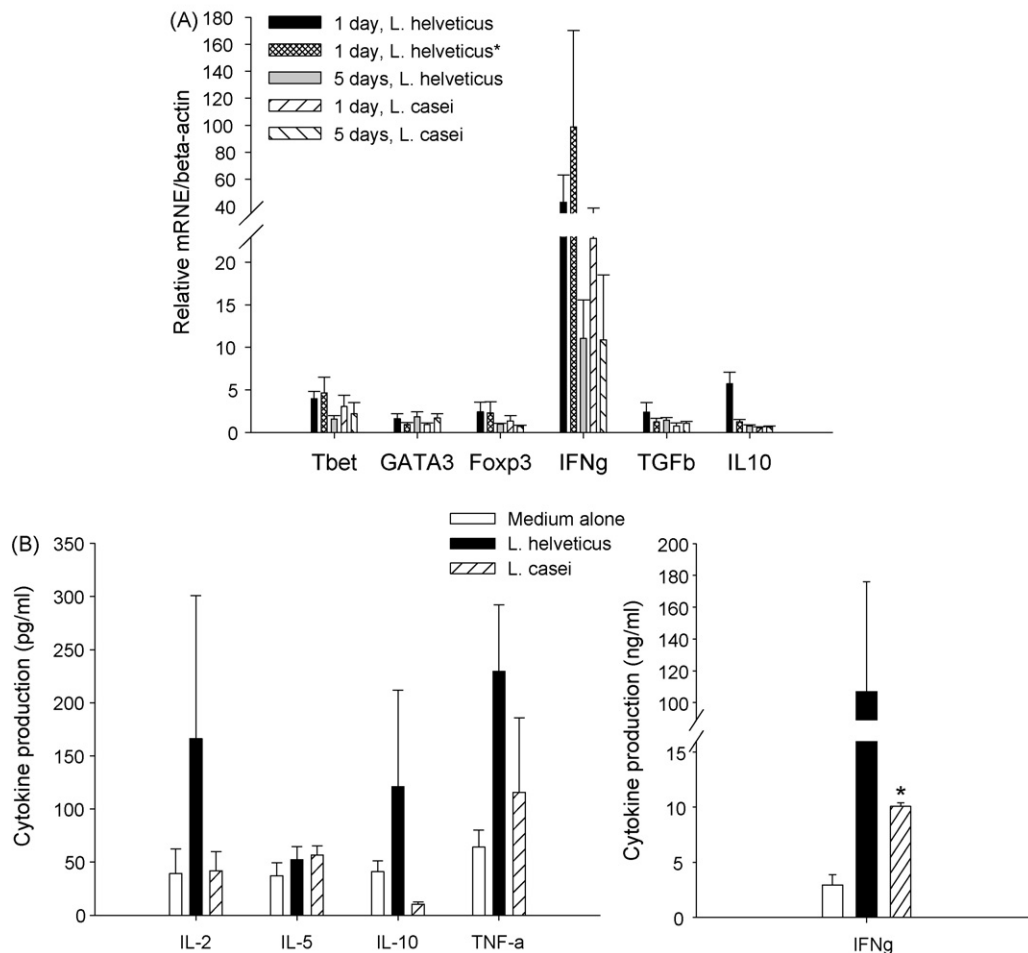


Fig. 2. Impact of *L. helveticus* and *L. casei* on naïve CD4⁺ T cell differentiation. Naïve CD4⁺ T cells were cocultured for 1 or 5 days with allogeneic DCs pretreated with either medium alone, *L. helveticus* harvested in stationary or exponential growth phase (*) or *L. casei* (DC:T ratio of 1:10). (A) Gene expression was analyzed by quantitative PCR. Relative amounts of mRNA in T cells cocultured with either *L. helveticus*-pretreated DC or *L. casei*-pretreated DC are shown as the mean $\pm$ SEM of three independent experiments compared with the negative control (i.e. T cells incubated with DCs in medium alone) arbitrarily set to 1. (B) Cytokine levels in supernatants after 5 days of coculture were determined by cytometric bead array technology. Results are expressed as mean $\pm$ SEM ($n=4-6$). Cytokine levels were compared using the nonparametric Wilcoxon's paired test. * $p < 0.05$ compared with cytokine levels in coculture of T cells with DCs in medium alone.

CD83 and CD86) and the production of various levels of pro-inflammatory cytokines (IL-1 β , IL-6, IL-8 and TNF- α) (Table 1). Interestingly, the capacity to boost the production of IL-10 differed considerably between strains; with *L. helveticus* being the most potent inducer (Table 1). For IL-12p70, we also observed significant variations depending upon strains, with high levels produced by DCs incubated with *L. helveticus*, *S. thermophilus*, *B. bifidum*2 and *B. bifidum*3 strains. Moderate amounts of IL-12p70 were produced in presence of *L. casei*, *L. kefirifaciens* or *L. plantarum*. In contrast, *B. bifidum*1, *B. adolescentis*1, *B. adolescentis*2 and *B. longum* strains only induced low amounts of this cytokine (Table 1). Two representative strains inducing the production of either IL-12p70/IL-10 or IL-12p70 alone, namely *L. helveticus* and *L. casei*, respectively (Fig. 1A and B), were selected for further studies.

3.2. Impact of *L. helveticus* and *L. casei* on CD4⁺ T cell differentiation

The impact of *L. helveticus* and *L. casei* on T cell polarization was investigated by monitoring the differentiation of naïve CD4⁺ T cells incubated with DCs pretreated with bacteria. As shown in Fig. 2, DCs stimulated with *L. helveticus* skew CD4⁺ T cells towards IFN- γ and IL-10 gene expression (i.e. a Th1 and possibly Treg profile) (Fig. 2A). Secretion in supernatants of IFN- γ and, to a lower level, of IL-10 was

confirmed (Fig. 2B). In contrast, DCs stimulated with *L. casei* only elicited a Th1 differentiation (Fig. 2A and B). Interestingly, *L. helveticus* harvested during the exponential growth phase only induced IFN- γ gene expression suggesting that immunomodulatory properties vary depending upon the strain, but also for a given strain, upon the growth phase (Fig. 2A).

3.3. Sublingual treatment with *L. helveticus* reduces established AHR and bronchial inflammation

We subsequently compared *L. helveticus* and *L. casei* *in vivo* as adjuvants for their capacity to enhance tolerance induction via the sublingual route using a murine asthma model [10,43]. In this model, OVA-sensitized mice develop a strong AHR associated with elevated Penh values, as well as a severe lung inflammation due to cellular infiltration and mucus hyperproduction [43]. Also, such sensitized animals exhibit OVA-specific Th2 immune responses, including high seric IgE and IgG1 titres plus IL-5 and IL-10-producing splenocytes [43].

Sublingual treatment of those animals with OVA administered together with *L. helveticus* as an adjuvant led to a greater and statistically significant reduction of AHR when compared with mice treated with OVA alone ($p < 0.05$) or PBS ($p < 0.00005$) (Fig. 3A). Interestingly, *L. helveticus* alone also reduced AHR suggesting that

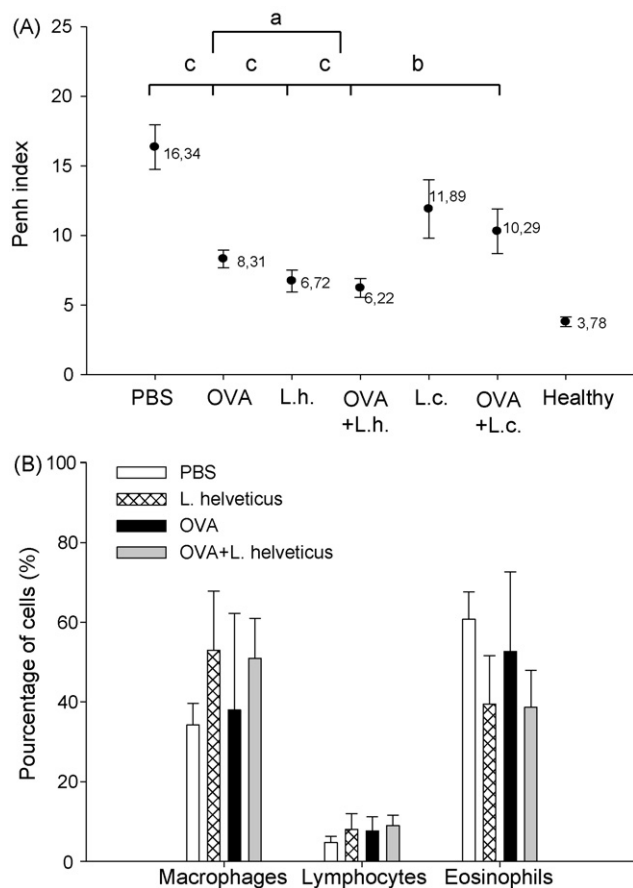


Fig. 3. Sublingual treatment with *L. helveticus* reduces established AHR and bronchial inflammation. (A) AHR was determined by measuring the Penh index in OVA-sensitized mice sublingually treated with either PBS, OVA, probiotic alone or OVA plus probiotic (i.e. *L. helveticus*, L.h. or *L. casei*, L.c.). (B) Inflammatory cell infiltration into the lung was determined by counting cells in BAL fluids. Values are expressed as mean ± SEM from 4 to 8 mice per group. ^a $P < 0.05$ compared with OVA-treated mice; ^b $P < 0.05$ compared with PBS-treated mice; ^c $P < 0.00005$ compared with PBS-treated mice, using Student's *t*-test. Data are representative of two independent experiments.

this strain induces both antigen dependent and independent mechanisms to establish tolerance. In contrast, *L. casei* used alone or in combination with OVA, did not improve AHR when compared to OVA alone (Fig. 3A). The decrease of AHR observed after treatment with *L. helveticus* +/- OVA was correlated with a decrease in eosinophil counts and an increase in macrophages in BAL (Fig. 3B). Although not shown, lung histology confirmed that treatment with *L. helveticus* +/- OVA is associated with a reduction in perivascular bronchial inflammation and mucus secretion. No detectable changes in ovalbumin-specific seric IgE, IgG2a or IgG1 antibodies were observed in any of the groups (data not shown).

3.4. Effect of *L. helveticus* and *L. casei* on T cell responses following sublingual administration

We subsequently investigated immunological changes associated with symptomatic improvement in the murine SLIT model. The observed reduction in AHR and inflammation following treatment with *L. helveticus*-OVA was correlated with a decrease in the number of OVA-specific IL-5 and IL-10-producing Th2 cells in the lungs (Fig. 4A) or in the spleen (no shown). In contrast, OVA or *L. helveticus* alone had no or only a limited effect on cytokine production, respectively.

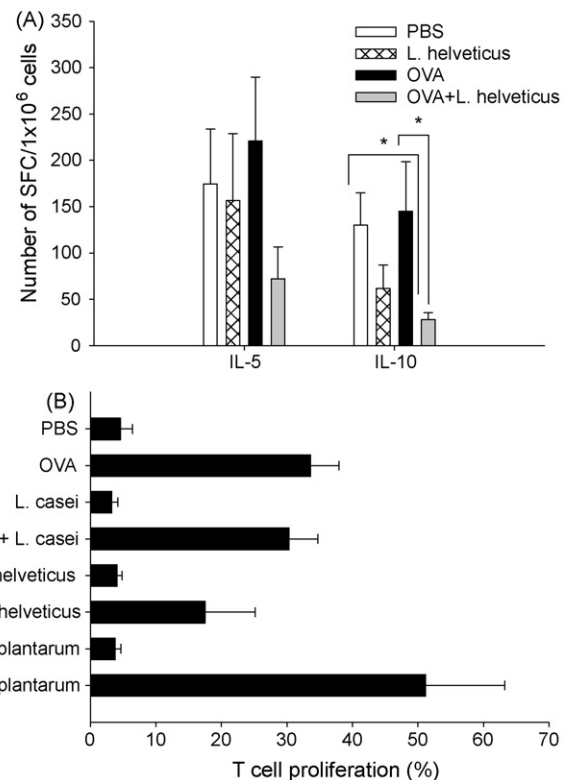


Fig. 4. Impact of *L. helveticus* and *L. casei* on T cell responses after sublingual administration. (A) ELISPOTs were used to measure inflammatory cytokines in the lungs. Values are expressed as mean ± SEM from 8 mice per group. Data are representative of two independent experiments. Cytokine levels were compared using the nonparametric Wilcoxon's paired test (^{*} $p < 0.05$). (B) Purified CFSE-labelled DO11.10 CD4⁺ T cells were adoptively transferred into BALB/c mice. Twenty-four hours later, groups of three mice were treated via the sublingual route with soluble OVA with or without probiotic bacteria (i.e. *L. casei*, *L. helveticus* or *L. plantarum*). Control animals were treated either with sterile PBS or bacteria alone. At day 7, cervical/maxillary LNs were removed and proliferation of transferred cells was assessed by flow cytometry. Data are shown as the mean ± SEM of three independent experiments.

We further compared *L. helveticus* and *L. casei* for their capacity to enhance T cell priming in draining LNs following sublingual administration. To this aim, CFSE-labelled DO11.10 CD4⁺ T cells were adoptively transferred to BALB/c mice before SLIT. After 10 days, cervical/maxillary LNs were harvested and *in vivo* OVA-specific T cell proliferative responses were analyzed by flow cytometry. As expected, T cell proliferation was hardly detectable in cervical/maxillary LNs of mice receiving sublingually PBS or either one of the adjuvants alone (with always less than 5% proliferating T cells, Fig. 4B). In contrast, sublingual administration of OVA induced more than 30% OVA-specific CD4⁺ T cells ($n = 3$, mean ± SEM) to proliferate in cervical/maxillary LNs (Fig. 4B). Treatment with *L. helveticus* reduced T cell proliferation whereas *L. casei* had no effect (with $17.5 \pm 7.6\%$ and $30.3 \pm 4.3\%$ proliferating T cells, respectively). *L. plantarum* used as a control in those experiments rather stimulated T cell proliferation, as previously reported [11].

4. Discussion

SLIT is currently established as a safe and efficacious treatment for type I respiratory allergies in humans [2–6]. However, allergen formulation with adjuvants could further improve sublingual vaccines both in terms of efficacy and simplification of immunization schemes [7]. In this regard, probiotic bacteria represent valid candidates, given their capacity to modulate immune responses at mucosal surfaces, thereby improving allergen-specific tolerance

in mice [44,45] and humans [38,44]. Noteworthy, clinical studies conducted in allergic patients with various probiotic strains have led to variable results, likely due to distinct immunomodulatory properties, depending upon strains.

In this context, the present study was initiated in order to select the most relevant bacteria as potential adjuvants for sublingual allergy vaccines. Eleven strains were compared for their capacity to impact both surface phenotype and cytokine production in human monocyte-derived DCs, as well as CD4⁺ T cell responses. We focused herein on *Bifidobacteria* and *Lactobacilli* as well as *Streptococci* species which are respectively dominant and subdominant representatives of the commensal human flora. We consistently observed an up-regulation of surface expression of CD40, CD80, CD83 and CD86 molecules on human monocyte-derived DCs incubated with all bacteria tested, in agreement with other reports [24,32,36,46]. Nonetheless, strains differed significantly in their capacity to induce cytokine production by DCs. In this regard, two types of strains were identified, including potent inducers of IL-12p70 and IL-10 such as *L. helveticus* DN-119 080, and inducers of IL-12p70 only, such as *L. casei* DN-114 001. Such distinct immunomodulatory properties on DCs are likely to translate into significant differences in terms of T cell responses since IL-12 is a pro-inflammatory cytokine supporting Th1 differentiation, whereas IL-10 production by DCs induces IL-10-producing regulatory T cells (whether inducible Tr1 or natural Foxp3^{high} Treg). In accordance with this hypothesis, *L. helveticus*-treated DCs skewed CD4⁺ T cells towards IFN- γ /IL-10 production whereas *L. casei*-treated DCs rather elicited Th1 cells. Other studies demonstrated that *B. bifidum* W23 drives human neonatal DCs to prime Th1 and Tr1 responses, and may thus be a valid candidate to use in primary prevention of allergic diseases [46]. Although we failed to detect Foxp3 gene induction by any of the strains in the present study, some specific bacterial strains of *L. reuteri*, *L. casei*, *B. adolescentis* or *B. animalis* were shown to convert naïve T cells into Foxp3^{low} hypo-responsive T cells displaying suppressive function [24,35].

We subsequently tested each of those two strains for their capacity to enhance allergen-specific tolerance via the sublingual route in a murine asthma model. SLIT with *L. helveticus*, but not *L. casei*, reduced both AHR, eosinophil numbers in BAL, as well as Th2 responses in the lungs. These results are in agreement with our previous observations that various adjuvants promoting IFN- γ and/or IL-10 production by naïve CD4⁺ T cells dramatically enhance tolerance induction in the course of sublingual immunotherapy [9–11]. Interestingly, sublingual administration of *L. helveticus* decreased antigen-specific T cell proliferation in cervical lymph nodes. In a previous study, we noticed that *L. plantarum* (ATCC no. 8014), which also enhances tolerance via the sublingual route, rather increases T cell proliferation in draining lymph nodes. Herein, we hypothesise that probiotics enhance tolerance by inducing Foxp3⁺ IL-10⁺ regulatory T cells (e.g. Tr1 cells) as observed previously in another murine SLIT model [41]. In addition, we cannot exclude the involvement of suppressive B cells and/or increased IgA production, as raised by Sun et al. [47,48] and Smith et al. [49], respectively. In addition, as suggested by our *in vivo* experiments in mice, probiotics may as well elicit tolerance through non antigen-specific mechanisms.

Probiotics can stimulate DC regulatory functions by targeting specific pathogen recognition receptors (PRR) or TLRs [28,31,35]. Noteworthy, CD11b⁺ DCs within buccal and lingual tissues from BALB/c mice express high levels of TLR2 and 4, suggesting that probiotics could enhance tolerance induction via those receptors. Alternatively, probiotics could also facilitate tolerance induction following interaction with lectins expressed by APCs. Consistent with this, the capacity of selected probiotic strains to induce IL-10 production by immune cells has been related to the structure of

lipoteichoic acid in the bacterial wall, thus impacting its affinity for DC SIGN3 [50].

Collectively, our results confirm that multiple types of lactic acid bacteria can be defined based on their distinct capacities to influence T cell polarization. With respect to sublingual allergy vaccines, we conclude that strains acting as Th1/possibly Treg inducers represent valid candidate adjuvants.

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