



**HAL**  
open science

## **Bystander immunotherapy as a strategy to control allergen-driven airway inflammation.**

Séverine Navarro, Anne Lazzari, Akira Kanda, Sébastien Fleury, David Dombrowicz, Nicolas Glaichenhaus, Valérie Julia

► **To cite this version:**

Séverine Navarro, Anne Lazzari, Akira Kanda, Sébastien Fleury, David Dombrowicz, et al.. Bystander immunotherapy as a strategy to control allergen-driven airway inflammation.. *Mucosal Immunology*, 2015, 8 (4), pp.841-851. 10.1038/mi.2014.115 . inserm-01116372

**HAL Id: inserm-01116372**

**<https://inserm.hal.science/inserm-01116372v1>**

Submitted on 13 Feb 2015

**HAL** is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.

**Bystander immunotherapy as a strategy to control allergen-driven airway inflammation**

Severine Navarro, PhD, Anne Lazzari, MSc, Akira Kanda, MD, PhD, Sebastien Fleury, BS,  
David Dombrowicz, PhD, Nicolas Glaichenhaus, PhD and Valerie Julia, PhD

Severine Navarro: IPMC, CNRS UMR7275, INSERM U1080 Université de Nice-Sophia  
Antipolis, route des Lucioles, 06560 Valbonne, France; current address: JCU Cairns Campus,  
Building E4 McGregor Rd, Smithfield, QLD 4878, Australia

Anne Lazzari: IPMC, CNRS UMR7275, INSERM U1080 Université de Nice-Sophia Antipolis,  
route des Lucioles, 06560 Valbonne, France

Akira Kanda: Institut Pasteur de Lille, INSERM U1011, Université de Lille 2, 1 Rue Prof.  
Calmette, 59000 Lille, France

David Dombrowicz: Institut Pasteur de Lille, INSERM U1011, Université de Lille 2, 1 Rue Prof.  
Calmette, 59000 Lille, France

Sébastien Fleury: Institut Pasteur de Lille, INSERM U1011, Université de Lille 2, 1 Rue Prof.  
Calmette, 59000 Lille, France

Nicolas Glaichenhaus: IPMC, CNRS UMR7275, INSERM U1080 Université de Nice-Sophia  
Antipolis, route des Lucioles, 06560 Valbonne, France

Valerie Julia: IPMC, CNRS UMR7275, INSERM U1080 Université de Nice-Sophia Antipolis,  
route des Lucioles, 06560 Valbonne, France

Corresponding authors:

Valerie Julia: IPMC, CNRS UMR7275, INSERM 1080 Université de Nice-Sophia Antipolis,  
route des Lucioles, 06560 Valbonne, FRANCE

TEL: +33 4 93 95 77 82

FAX: + 33 4 93 95 77 08

E-mail: [vjulia@unice.fr](mailto:vjulia@unice.fr)

Severine Navarro: IPMC, CNRS UMR7275, INSERM U1080 Université de Nice-Sophia  
Antipolis, route des Lucioles, 06560 Valbonne, France; current address: JCU Cairns Campus,  
Building E4 McGregor Rd, Smithfield, QLD 4878, Australia

32 The authors declare no conflict of interest.

33 This work was supported by grants from the Fondation pour la recherche Médicale (FRM) and  
34 INSERM.

35 S.N. was supported by a fellowship from the FRM, A.L. was supported by a fellowship from  
36 Vaincre la Mucoviscidose, A.K is supported by a grant from the Agence Nationale de la  
37 Recherche (ANR).

38

39 **Total word count:** which includes the number of words in the body of the manuscript  
40 (Introduction through Discussion only); the abstract should not be included in the count. Figure  
41 and table legends are included in the estimation of the overall space required for figures and  
42 tables, so they should not be counted here.

43

44 **Abstract**

45  
46 **Background:** Allergic asthma is a chronic inflammatory disease that is characterized by airway  
47 hyper responsiveness (AHR), infiltration of Th2 cells in lungs and high levels of circulating IgE.  
48 Allergen-specific immunotherapy (SIT), in which patients are rendered tolerant by exposure to  
49 steadily increasing doses of the allergen, is the only curative treatment to date. Unfortunately, SIT  
50 is not suitable for treating multi-sensitized patients, and some allergens are too immunogenic to  
51 be used in desensitization protocols.

52  
53 **Objective:** To investigate whether, and to understand how, regulatory CD4<sup>+</sup> T cells (T<sub>reg</sub>)  
54 specific for a third-party “drug” antigen could control allergic immune responses and lung  
55 inflammation.

56  
57 **Methods:** Mice were tolerized to ovalbumin (OVA), sensitized to ragweed, and eventually  
58 challenged with aerosols of ragweed alone or ragweed and OVA together. Animals were then  
59 monitored for cardinal features of allergic asthma including AHR and infiltration of Th2 cells in  
60 lungs. In additional experiments aimed at elucidating the mechanisms of OVA-induced  
61 suppression, OVA-tolerized mice were sensitized with the LACK model antigen, challenged with  
62 LACK alone or LACK and OVA together, and LACK-specific T cells were visualized by flow  
63 cytometry.

64  
65 **Results:** In both the ragweed and the LACK model, allergen-induced airway inflammation and  
66 AHR were strongly reduced in mice challenged with both the allergen and OVA compared to  
67 mice challenged with the allergen alone. OVA-induced protection did not result from competition  
68 between OVA and the allergen, was mediated by OVA-specific CD25<sup>+</sup> T<sub>reg</sub>, required both  
69 CTLA-4 and ICOS signaling, and was partially dependent on IL-10. Bystander suppression was  
70 associated with reduced proliferation of allergen-specific Th2 cells and decreased numbers of  
71 airway DC migrating to the lungs.

72  
73 **Conclusion:** Our results demonstrate that T<sub>reg</sub> specific for a third-party drug antigen could  
74 control allergic immune responses and lung inflammation when re-stimulated *in vivo*.

75 **Clinical implications:** This study paves the way for the development of a novel therapeutic  
76 strategy that could control allergen-specific Th2 responses in patients with allergic asthma.

77

78 **Capsule summary:** This study provides the proof of concept that bystander suppression  
79 mediated by T<sub>reg</sub> specific for a third-party drug antigen could be used as an efficient strategy to  
80 control allergen-specific Th2 cells and asthma symptoms in allergic individuals.

81

82 **Key words:** asthma, immune tolerance, airway inflammation, Th2, T<sub>reg</sub>, specific immunotherapy

83

84 **Abbreviations:** **Ag**, antigen; **APC**, antigen-presenting cells; **AHR**, airway hyperresponsiveness;  
85 **Alum**, aluminium hydroxide; **BAL**, bronchoalveolar lavages; **BALF**, bronchoalveolar lavage  
86 fluids; **BSA**, bovine serum albumin; **CFSE**, carboxyfluorescence diacetate succinimidly ester;  
87 **DCs**, dendritic cells; **ELISA**, enzyme-Linked immunosorbent assay; **FACS**, fluorescence  
88 activated cell sorter; **FITC**, fluorescein isothiocyanate; **LACK**, *Leishmania* homolog of receptors  
89 for activated c kinase antigen; **LN**, lymph nodes; **Ig**, immunoglobulin; **IFN**, interferon; **IL**,  
90 interleukin ; **i.p.**, intraperitoneal ; **i.n.**, intranasal; **MCH**, methacholine; **MedLN**, mediastinal LN;  
91 **OVA**, ovalbumin; **PCR**, polymerase chain reaction; **PE**, phycoerythrin; **PBS**, phosphate-buffered  
92 saline; **PLN**, popliteal LN; **RNA**, ribonucleic acid; **SIT**, specific immunotherapy; **T<sub>eff</sub>**, effector T  
93 cells; **Tg**, transgenic; **Th**, T helper ; **T<sub>reg</sub>**, regulatory T cells; **WT**, wild type  
94

## 95 **Introduction**

96 Allergic diseases affect up to 30% of the population and their prevalence has steadily  
97 increased in recent decades probably due to numerous changes in the environment. Among  
98 allergic diseases, asthma is a chronic inflammation of the lungs caused by an inappropriate  
99 immune response to a single or multiple airborne allergens. This pathology has a substantial  
100 economic burden for which the only curative and specific method of treatment to this day is  
101 allergen-specific immunotherapy (SIT). SIT involves the administration by either subcutaneous  
102 injection or mucosal application of increasing doses of the allergen to which the patient is allergic  
103 to. Unfortunately, SIT is unsuitable for treating multi-sensitized patients, and some allergens are  
104 too immunogenic to be used in desensitization protocols.

105 SIT induces a state of peripheral tolerance characterized mainly by the generation of  
106 allergen-specific  $T_{reg}$ , suppressed effector cell proliferation and cytokine production against  
107 major allergens <sup>1, 2</sup>. Indeed, IL-10 and/or TGF- $\beta$  producing  $T_{reg}$  are the key factors for specific  
108 immunotherapy in humans, considered as a model of tolerance induction <sup>3-5</sup>. It has been shown  
109 that treating naive (non-sensitized) mice with OVA aerosols leads to IgE-unresponsiveness to  
110 OVA <sup>6</sup>, and induces the development of OVA-specific  $T_{reg}$  which prevented the development of  
111 asthma upon subsequent sensitization and challenge with OVA <sup>7</sup>. Moreover, lung draining lymph  
112 node (LN) dendritic cells (DC) first encountering an inhaled antigen transiently produced IL-10 <sup>7</sup>.  
113 These phenotypically mature DC induce the development of  $CD4^+$   $T_{reg}$  that also produce high  
114 amounts of IL-10 <sup>8</sup>. TGF- $\beta$ -expressing  $T_{reg}$  also play a role for tolerance induction to inhaled  
115 antigens <sup>5, 9</sup>. Interestingly, it has been suggested that tolerance induced by SIT was not only  
116 limited to the administered allergen but also conferred protection against other allergens <sup>10, 11</sup>.  
117 This non-specific and beneficial action of SIT could be explained by the activation of bystander  
118  $T_{reg}$ .

119 Bystander immunosuppression was first described by Bullock *et al.* as a process in which  
120 antigen (Ag)-specific  $T_{reg}$  inhibit T effector cell responses directed to both the targeted Ag and to  
121 a co-localized third-party Ag <sup>12</sup>. Due to the requirement that the tolerogen and the Ag have to be  
122 physically linked, i.e. presented by the same antigen-presenting cell (APCs), in order for  
123 suppression to occur, the terms « linked suppression » or « linked recognition » were coined. In  
124 the field of oral tolerance, the term « bystander suppression » was introduced to describe an  
125 inhibition of a T cell memory response as a result of a regulatory response to an unrelated but

126 colocalized tolerogen<sup>13, 14</sup>. In bystander suppression, the tolerogen and third-party Ag do not  
127 need to be presented by the same APC. In this case, soluble mediators induce suppression of the  
128 response directed to the third-party Ag<sup>13, 15-17</sup>.

129         In the present study, we have sought to investigate whether T<sub>reg</sub> specific for a third-party  
130 “drug” antigen could control allergic immune responses and lung inflammation in mice. To this  
131 aim, mice were tolerized to OVA, sensitized to ragweed, and eventually challenged with aerosols  
132 of ragweed alone or ragweed and OVA together.

133



## 134           **Results**

### 135 136 **Exposure to OVA aerosols inhibits ragweed- and LACK-induced allergic asthma in OVA-** 137 **tolerized mice**

138 To determine whether T<sub>reg</sub> specific for a third-party antigen could control allergic airway  
139 inflammation in mice, mice were tolerized to OVA through intranasal administrations, sensitized  
140 to ragweed and further exposed to aerosols of ragweed alone or ragweed and OVA together (**Fig**  
141 **1, A**). When compared to the group challenged with ragweed only, mice exposed to both ragweed  
142 and OVA showed decreased AHR (**Fig 1, B**) and reduced numbers of total cells, eosinophils and  
143 lymphocytes in broncho alveolar lavage fluids (BALF) (**Fig 1, C**). We further analyzed airway-  
144 infiltrating T cells for expression of the Th2- marker, T1/ST2 (IL-33Ra). Compared to the mice  
145 challenged with ragweed alone, the number of T1/ST2<sup>+</sup> CD4<sup>+</sup> T cells was reduced in the BALF  
146 of mice challenged with ragweed and OVA aerosols (**Fig 1, D**). The amounts of IL-5 and IL-13  
147 in the lungs were also reduced upon challenge with ragweed and OVA while IFN- $\gamma$  levels  
148 remained low and similar in both groups (**Fig 1, E**). This phenomenon was not observed in mice  
149 that have not been tolerized to OVA prior to ragweed sensitization (Supplementary Figure 1)  
150 further supporting a role for OVA-specific T<sub>reg</sub> in this phenomenon and ruling out the possibility  
151 that it could result from competition between OVA and ragweed for antigen presentation.

152 To generalize our findings and to further dissect the mechanisms involved, we switched to  
153 another experimental model in which BALB/c mice were sensitized to the model antigen LACK,  
154 tolerized to OVA through intranasal administration, and further exposed to aerosols of LACK  
155 alone, or LACK and OVA (**Fig 2, A**). As observed with ragweed, mice exposed to both  
156 LACK/OVA showed decreased AHR (**Fig 2, B**), and reduced total numbers of cells, eosinophils  
157 and lymphocytes in BALF (**Fig 2, C**). Compared to control mice challenged with LACK alone,  
158 both the frequency and the number of T1/ST2<sup>+</sup> CD4<sup>+</sup> T cells were reduced in the BALF of mice  
159 challenged with LACK/OVA aerosols (**Fig 2, D**). The amounts of IL-4, and IL-13 in the BALF  
160 were also reduced upon challenge with LACK/OVA aerosols while IFN- $\gamma$  levels remained low  
161 and similar in both groups (**Fig 2, E**). The lungs of LACK/OVA-challenged mice contained less  
162 IL-4-, and IL-5- secreting CD4<sup>+</sup> T cells than those challenged with LACK only as demonstrated  
163 by intracellular cytokine staining of lung cells upon LACK restimulation (**Fig 2, F**). In contrast,  
164 the amounts of LACK-specific -IgE and -IgG1 were not affected by additional OVA exposure

165 **(Fig 2, G)**. Altogether, our results suggested that exposure to OVA aerosols could inhibit allergic  
166 airway inflammation and allergen-specific Th2 immune responses in mice that have been  
167 tolerized with OVA prior to sensitization and challenge with either ragweed or LACK.

168  
169 **The inhibition of LACK-induced airway inflammation in OVA-tolerized mice is mediated**  
170 **by OVA-specific CD25<sup>+</sup> T<sub>reg</sub>**

171 Previous experiments have shown that the intranasal administration of OVA to BALB/c mice  
172 induces OVA-specific T<sub>reg</sub> that express CD25. To investigate whether these cells were  
173 responsible for the inhibition of LACK-induced airway inflammation in OVA-tolerized LACK-  
174 sensitized mice, CD4<sup>+</sup> T cells were purified from the spleen of mice that have been previously  
175 tolerized to OVA or bovine serum albumine (BSA), or treated with PBS. Cells were injected into  
176 LACK-sensitized recipients that were further challenged with both LACK and OVA **(Fig 3, A)**.  
177 Compared to mice injected with CD4<sup>+</sup> T cells purified from BSA-tolerized or PBS-treated  
178 donors, mice injected with CD4<sup>+</sup> T cells from OVA-tolerized mice exhibited decreased AHR  
179 **(Fig 3, B)**, reduced numbers of total cells, eosinophils and lymphocytes **(Fig 3, C)** and T1/ST2<sup>+</sup>  
180 CD4<sup>+</sup> T cells in BALF **(Fig 3, D)**, and reduced numbers of IL-4 and IL-5-secreting CD4<sup>+</sup> T cells  
181 in lungs **(Fig 3, E)**. In striking contrast, no inhibition of LACK-induced airway inflammation or  
182 LACK-specific Th2 immune responses were observed when CD4<sup>+</sup> T cells from OVA-tolerized  
183 mice depleted of CD25<sup>+</sup> cells prior to injection into LACK-sensitized mice. Lastly, both airway  
184 inflammation and LACK-specific Th2 immune responses were inhibited when recipient mice  
185 were injected with CD25<sup>+</sup> CD4<sup>+</sup> T cells purified from the spleen of OVA-tolerized mice **(Fig 3,**  
186 **F-I)**. Therefore, the inhibition of LACK-induced airway inflammation that was observed in  
187 OVA-tolerized mice upon challenge with OVA relied on the activation of OVA-specific CD25<sup>+</sup>  
188 T<sub>reg</sub>.

189  
190 **CTLA-4, ICOS, and IL-10 dependency of OVA-specific T<sub>reg</sub> in the control of LACK-**  
191 **induced asthma**

192 T<sub>reg</sub> mediate suppression through various mechanisms including the secretion of inhibitory  
193 cytokines such as IL-10, the induction of cytotoxicity, metabolic disruption and the inhibition of  
194 antigen presentation by dendritic cells (DCs) through a CTLA-4-dependent mechanism<sup>18</sup>.

195 Furthermore, it was recently shown that ICOS mediated the generation and function of CD25<sup>+</sup>  
196 CD4<sup>+</sup> FoxP3<sup>+</sup> T<sub>reg</sub> conveying respiratory tolerance<sup>19</sup>, and that ICOS expression defined a subset  
197 of IL-10 secreting T<sub>reg</sub><sup>20</sup> and was required for the production of IL-10 by these cells<sup>21</sup>.  
198 Therefore, we sought to elucidate the role of CTLA-4, ICOS and IL-10 in the inhibition of  
199 LACK-induced airway inflammation induced by OVA aerosols. To this aim, LACK-sensitized  
200 OVA-tolerized mice were challenged with both LACK and OVA and treated or not with blocking  
201 mAbs directed to CTLA-4, ICOS or IL-10R (**Fig 4, A**). CTLA-4 blockade prevented the  
202 inhibition of AHR induced by OVA aerosols, as well as the reduction in the number of total cells,  
203 eosinophils, lymphocytes and T1/ST2<sup>+</sup> Th2 cells in BALF, and IL-4- and IL-5-producing LACK-  
204 specific CD4<sup>+</sup> in lungs (**Fig 4, B-D**). Likewise, blocking ICOS/ICOSL interactions abolished the  
205 protection that was induced by OVA aerosols in OVA-tolerized LACK-sensitized mice but did  
206 not have any detectable effect in mice that have not been tolerized to OVA (**Fig 4, F, G**). In  
207 contrast to anti-CTLA-4 and anti-ICOS mAbs that restored both AHR and allergic airway  
208 inflammation in OVA-tolerized mice exposed to OVA, anti-IL-10R mAbs restored AHR but not  
209 allergic airway inflammation (**Fig 4, F, G**). Therefore, the inhibition of AHR and airway  
210 inflammation that was induced by OVA aerosols in OVA-tolerized mice upon sensitization and  
211 challenge with LACK was dependent on both CTLA-4, ICOS and partially on IL-10.

212  
213 **OVA-specific T<sub>reg</sub> inhibited the proliferation of LACK-specific Th2 cells and the subsequent**  
214 **airway inflammation**

215 To further dissect the molecular mechanisms by which OVA-specific T<sub>reg</sub> inhibited LACK-  
216 induced allergic asthma, CD4<sup>+</sup> T cells from Thy1.1<sup>+</sup> LACK-specific WT15 TCR transgenic mice  
217 were differentiated *in vitro* under Th2 polarizing conditions, labeled with CFSE, and injected into  
218 OVA- or BSA- tolerized recipients before being challenged with LACK/OVA (**Fig 5, A**). As  
219 compared to BSA-tolerized mice, OVA-tolerized animals injected with LACK-specific Th2 cells  
220 exhibited decreased AHR (**Fig 5, B**), and reduced numbers of eosinophils and lymphocytes in the  
221 BALF (**Fig 5, C**). In addition, both the frequency and the number of donor Th2 cells were  
222 reduced in the BALF, lung and MLN, but not in the blood of OVA-tolerized mice compared to  
223 BSA-tolerized mice (**Fig 5, D**). Furthermore, LACK-specific Th2 cells had undergone more  
224 divisions in BSA-tolerized mice than in OVA-tolerized mice (**Fig 5, E**) further suggesting that  
225 OVA aerosols inhibited the proliferation of LACK-specific Th2 cells possibly at the level of

226 antigen presentation. To directly test this latter hypothesis, LACK-specific Th2 cells were  
227 injected into either OVA- or BSA-tolerized mice, and further challenged with both LACK and  
228 OVA. Cells were then purified from the mediastinal LN (MedLN) and distal popliteal LN (PLN)  
229 and incubated with CFSE-labeled LACK-specific WT15 CD4<sup>+</sup> T cells to assess their ability to  
230 induce proliferation. As expected, PLN cells did not induce T cell proliferation (**Fig 5, F**..  
231 Furthermore, LACK-specific T cells proliferated more vigorously when incubated with MedLN  
232 cells from BSA-tolerized mice than when incubated with MedLN cells from OVA-tolerized mice  
233 (**Fig 5, F**). To investigate whether this phenomenon resulted from quantitative (i.e. different  
234 number of LACK-loaded DCs in OVA-tolerized and BSA-tolerized mice) or qualitative  
235 differences (i.e. similar number of LACK-loaded DCs in OVA-tolerized and BSA-tolerized mice  
236 but different ability to induce T cell proliferation), we administered FITC-labeled latex beads to  
237 OVA- and BSA-tolerized mice at the time of the challenge with LACK and OVA, and we  
238 measure the frequency of bead<sup>+</sup> DCs in MedLN 16 hrs later. While bead<sup>+</sup> DCs were readily  
239 detected in the MedLN of both OVA- and BSA-tolerized mice and expressed similar surface  
240 levels of CD80, CD86, OX40L and MHC class II molecules (not shown), the frequency of bead<sup>+</sup>  
241 DCs was decreased from  $4.7 \pm 0.7$  % in BSA-tolerized mice to  $1.4 \pm 0.3$  % in OVA-tolerized  
242 animals (**Fig 5, H**). Altogether, our data suggested that OVA-specific T<sub>reg</sub> inhibited the  
243 proliferation of LACK-specific Th2 cells by preventing the migration of airway DCs to MedLN.  
244

**245 Discussion**

246 In his paper, we have demonstrated that OVA-specific T<sub>reg</sub> could inhibit allergic airway  
247 inflammation induced by sensitization and challenge with ragweed. OVA-specific T<sub>reg</sub> were also  
248 efficient to protect mice from allergic airway inflammation induced by the immunodominant  
249 LACK antigen further validating our finding to second allergen. In addition, BSA-tolerized mice  
250 were protected from LACK-induced allergic asthma when challenged with both LACK and BSA  
251 further suggesting that the phenomenon that we have observed was not restricted to OVA-  
252 specific T<sub>reg</sub> (data not shown). Interestingly, the phenomenon that we have described in this paper  
253 may explain the protective effects of SIT against unrelated allergens other than the one primarily  
254 targeted as reported in several epidemiological studies<sup>10, 11, 22</sup>.

255 In the LACK model, we have found that the number of allergen-specific Th2 cells in  
256 BALF, the frequency and number of IL-4 and IL-5 producing CD4<sup>+</sup> T cells as well as the IL-4,  
257 IL-5, and IL-13 amounts in lungs were decreased in mice challenged with both LACK and OVA  
258 compared to mice challenged with LACK alone. OVA-induced protection did not result from a  
259 competition between OVA and LACK for antigen presentation because it was only observed in  
260 mice that have been previously tolerized with OVA. In contrast, experiments in which CD4<sup>+</sup> T  
261 cells were purified from the spleen of PBS-treated, BSA- or OVA-tolerized mice and injected  
262 into LACK-sensitized recipient mice showed that protection was antigen-specific and mediated  
263 by T<sub>reg</sub>.

264 Further experiments showed that CTLA-4 was absolutely required for OVA-induced  
265 suppression of LACK-mediated asthma. While we did not elucidate the mechanisms by which  
266 CTLA-4 acted, CTLA-4 was shown to be essential for the function of naturally occurring T<sub>reg</sub>  
267 which constitutively express this molecule. Indeed, CTLA-4 deficiency in T<sub>reg</sub> impaired both  
268 their *in vivo* and *in vitro* suppressive function. In addition, CTLA-4 prevented CD28 signaling in  
269 effector T cells (T<sub>eff</sub>) by competing with CD80 and CD86, and/or induced the synthesis of the  
270 tryptophan catabolizing enzyme indoleamine 2,3-dioxygenase (IDO) by the APCs leading to T-  
271 cell suppression by both local depletion of tryptophan and induction of apoptosis via tryptophan  
272 catabolites<sup>23</sup>. While we were unable to detect increased death of LACK-specific T cells in OVA-  
273 tolerized mice challenged with both OVA and LACK (not shown), we cannot rule out the  
274 possibility that this phenomenon was partially responsible for OVA-induced protection.

275           Blocking ICOS-ICOSL interactions resulted in the same effect as CTLA-4 inhibition, a  
276 result in agreement with a previous study showing that ICOS<sup>-/-</sup> T<sub>reg</sub> did not confer protection upon  
277 transfer to asthmatic mice demonstrating a crucial role of ICOS in their suppressive function<sup>19</sup>.  
278 As ICOS was shown to define a subset of IL-10-producing T<sub>reg</sub>, we next investigated whether  
279 OVA-induced suppression of the disease required IL-10. IL-10R blockade prevented OVA-  
280 induced suppression of AHR, but did not affect LACK-induced inflammation in our model. It  
281 remains to be determined whether IL-10 is produced by the CD25<sup>+</sup> T<sub>reg</sub> or by other cells as  
282 previously described Kearley *et al.*<sup>24</sup>. Taken together, these data underline the multiple and  
283 complex effects of CD25<sup>+</sup> T<sub>reg</sub>. While these cells have been proposed to act via cell contact-  
284 dependent mechanisms *in vitro*, these cells have been proposed to work through various  
285 mechanisms including inhibitory cytokines and non-cytokine-dependent mechanisms *in vivo*,  
286 depending on the experimental conditions<sup>25</sup>. Our findings also suggest that inflammation and  
287 AHR can be uncoupled and are in agreement with previous studies that demonstrate that effects  
288 on inflammation are not always predictive of AHR changes<sup>26-29</sup>. Indeed, this might also hold true  
289 in human asthma, in which anti-IL-5 mAb treatment reduced blood and lung eosinophilia but did  
290 not affect lung function<sup>30</sup>.

291           Adoptive transfer of CFSE-labeled LACK-specific Th2 cells into OVA-tolerized recipient  
292 mice demonstrated that OVA-specific T<sub>reg</sub> significantly reduced the number of LACK-specific  
293 Th2 cells in the BALF, lung and MLN by affecting their proliferation. Imaging data in mice have  
294 showed that T<sub>reg</sub> do not directly interact with T<sub>eff</sub> but rather with DC, altering the latter and  
295 diminishing subsequent DC-T<sub>eff</sub> cells conjugate formation *in vivo*<sup>31, 32</sup> a phenomenon that could  
296 explain our results. In addition, Derks and colleagues have envisioned two hypotheses of APC  
297 function in bystander suppression<sup>33</sup>: a passive APC model, in which the APCs would present  
298 MHC-peptide to the T<sub>reg</sub>, stimulating them to produce immunosuppressive cytokines that would  
299 further binds their cognate receptors on the third-party T<sub>eff</sub>, or an active APC model, in which the  
300 APCs would propagate regulatory effects from the T<sub>reg</sub> to the T<sub>eff</sub> through various APC products.  
301 These two hypotheses remain to be tested in our model.

302           Our results demonstrate that T<sub>reg</sub> specific for a third-party drug antigen could control  
303 allergic immune responses and lung inflammation when re-stimulated *in vivo*. This study paves  
304 the way for the development of a novel therapeutic strategy that could control allergen-specific

305 Th2 responses in patients with allergic asthma, and more specifically in patients who are  
306 sensitized to multiple allergens.

307

308 **FIGURE LEGENDS**

309 **Figure 1. AHR, airway inflammation and cytokine levels in ragweed-sensitized, OVA-**  
310 **tolerized mice upon ragweed challenge.** (A) Experimental protocol. Mice were treated with  
311 three i.n. injections of OVA, and four i.n. injections of ragweed. Mice were then either  
312 challenged with ragweed or ragweed/OVA, and analyzed one and two days after the last i.n.  
313 injection. (B) AHR. Whole body plethysmography in mice exposed to ragweed (filled squares),  
314 ragweed/OVA (empty circles), or PBS (crosses). (C) Number and phenotype of BALF cells.  
315 BALF cells were analyzed by FACS in mice exposed to ragweed only (black bars), to both  
316 ragweed and OVA aerosols (empty bars) or to PBS (grey bars). Eosinophils, E; neutrophils, N;  
317 lymphocytes, L; macrophages, M. (D) Frequency and number of Th2 cells in the airways. BALF  
318 cells were stained with CD4 and T1/ST2 mAbs and analyzed by FACS. (E) Cytokine levels in  
319 lung cells. IL-4, IL-5 and IFN- $\gamma$  levels were assessed by ELISA after *in vitro* stimulation with  
320 LACK protein (0.1mg/ml), anti-CD28 (1 $\mu$ g/ml) and brefeldin A (5 $\mu$ g/ml). Data are expressed as  
321 mean  $\pm$  s.e.m. of 2 experiments with n=8 mice per group. n.s., non significant; \*  $P<0.05$ ; \*\*  
322  $P<0.01$ .

323 **Figure 2. AHR, airway inflammation and cytokine levels in OVA-tolerized mice exposed to**  
324 **both OVA and LACK aerosols.** (A) Experimental protocol. Mice were sensitized with two  
325 intra-peritoneal injections of LACK in Alum, treated with three i.n. injections of OVA, and  
326 challenged daily for 5 days with LACK aerosols or LACK/OVA aerosols. Mice were analyzed  
327 one and two days after the last aerosol. (B) AHR. Whole body plethysmography (right), and  
328 dynamic lung resistance and compliance (left) were monitored in mice exposed to LACK  
329 aerosols (filled squares), LACK/OVA aerosols (empty circles) or PBS (crosses, dashed line). (C)  
330 Number and phenotype of BALF cells. BALF cells were counted and analyzed by FACS in mice  
331 exposed to PBS (dashed bars), LACK (black bars) or LACK/OVA aerosols (empty bars). Data  
332 show the number of eosinophils (E), neutrophils (N), lymphocytes (L), macrophages (M). (D)  
333 Frequency and number of Th2 cells in the airways. BALF cells were stained with anti-CD4, CD3  
334 and T1/ST2 mAbs and analyzed by FACS. Data show representative FACS profiles, numbers  
335 indicate the mean frequency  $\pm$  s.e.m and histograms show the absolute numbers of T1ST2<sup>+</sup> CD4<sup>+</sup>  
336 T cells for the indicated groups. (E) Cytokine levels in BALF. Mice were analyzed for IL-4, IL-5,  
337 IL-13 and IFN- $\gamma$  by cytometric bead array (CBA). (F) Cytokine secretion by lung CD4<sup>+</sup> T cells.



338 IL-4, and IL-5-secreting CD4<sup>+</sup> T cells were assessed by FACS after *in vitro* stimulation with  
339 LACK protein (0.1mg/ml), anti-CD28 (1µg/ml) and brefeldin A (5µg/ml). Data show  
340 representative FACS profiles, numbers indicate the mean frequency ± s.e.m and histograms show  
341 the absolute numbers for the indicated groups. (G) Immunoglobulins. Levels of serum LACK-  
342 specific -IgE and -IgG1 were assessed in mice upon challenge with LACK or LACK/OVA  
343 aerosols. All data show either individual mice with bar indicating the mean, with n = 6 mice per  
344 group pooled from four different experiments. n.s., non significant, \*  $P < 0.05$ , \*\*  $P < 0.01$ , \*\*\*  
345  $P < 0.0001$ .

346 **Figure 3. AHR, airway inflammation and cytokine levels in mice injected with CD4<sup>+</sup> T cells**  
347 **from OVA-tolerized mice.** (A) Experimental protocol. Mice were sensitized with two i.p.  
348 injections of LACK in Alum, and injected 9 d later with  $4 \times 10^6$  CD4<sup>+</sup> T cells (A-E), or  $4 \times 10^6$   
349 CD25<sup>-</sup>CD4<sup>+</sup> T cells (F-I) or  $1.5 \times 10^6$  CD25<sup>+</sup>CD4<sup>+</sup> T cells (F-I) prepared from the spleen of mice  
350 exposed to OVA, BSA, or PBS. Sensitized mice were then challenged with LACK/OVA aerosols  
351 for five days and analyzed one and two days after the last aerosol. (B, F) AHR. Whole body  
352 plethysmography was monitored in the indicated mice challenged to LACK/OVA aerosols in  
353 response to increased doses of inhaled methacholine. Control mice (vehicle) were sensitized with  
354 LACK, non-transferred and challenged with PBS. (C, G) Number and phenotype of BALF cells.  
355 BALF cells were analyzed by FACS for the number of eosinophils (E), neutrophils (N),  
356 lymphocytes (L), macrophages (M). (D, H) Frequency and number of Th2 cells in the airways.  
357 BALF cells were stained with anti-CD3, -CD4 and T1/ST2 mAbs and analyzed by FACS. (E, I)  
358 Cytokine secretion by lung CD4<sup>+</sup> T cells. IL-4, and IL-5-secreting CD4<sup>+</sup> T cells were assessed by  
359 FACS after *in vitro* stimulation with LACK protein (0.1mg/ml), anti-CD28 (1µg/ml) and  
360 brefeldin A (5µg/ml). Data show numbers of IL-4 and IL-5-secreting CD4<sup>+</sup> T cells in lungs. All  
361 data show either individual mice or are expressed as mean ± s.e.m, with n = 5 mice per group  
362 pooled from two experiments. P values have been calculated by comparing OVA-tolerized mice  
363 to BSA-tolerized mice, ns, non significant, \*  $P < 0.05$ , \*\*  $P < 0.01$ , \*\*\*  $P < 0.0001$ .

364  
365 **Figure 4: AHR and airway inflammation in mice treated with anti-CTLA-4, anti-IL-10R, or**  
366 **anti-ICOS.** (A) Experimental protocol. Mice were sensitized with two i.p. injections of LACK in

367 Alum, treated with three i.n. injections of OVA, treated or not with anti- CTLA-4 mAb, -IL-10R,  
368 -ICOS, or IgG1 isotype mAb at the indicated time, and challenged daily for 5 days with LACK or  
369 LACK/OVA aerosols. Mice were analyzed one and two days after the last aerosol. **(B)** and **(F)**  
370 AHR. Whole body plethysmography in response to increasing doses of inhaled methacholine in  
371 the indicated groups of mice. **(C)** and **(G)** Number and phenotype of BALF cells. BALF cells  
372 were analyzed by FACS in the indicated groups of mice. Eosinophils, E; neutrophils, N;  
373 lymphocytes, L; macrophages, M. **(D)**. Number of Th2 cells in the airways. BALF cells were  
374 stained with anti -CD3, -CD4 and T1/ST2 mAbs and analyzed by FACS. **(E)**. IL-4, and IL-5-  
375 secreting CD4<sup>+</sup> T cells were assessed by FACS after *in vitro* stimulation with LACK protein  
376 (0.1mg/ml), anti-CD28 (1µg/ml) and brefeldin A (5µg/ml). Data are expressed as mean ± s.e.m  
377 of 3 experiments with n=6 mice per group. n.s., non significant; \*  $P<0.05$ ; \*\*  $P<0.01$ ; \*\*\*  
378  $P<0.0001$ .

379 **Figure 5. AHR, airway inflammation, cytokine levels and T cell proliferation in OVA-**  
380 **tolerized mice injected with LACK-specific Th2 cells and exposed to LACK/OVA. (A)**  
381 Experimental protocol. Mice were treated with three i.n. injections of OVA or BSA, injected with  
382  $1.5 \times 10^6$  CFSE-labeled Thy1.1<sup>+</sup> LACK-specific Th2 cells. Mice were injected intranasally 2 d  
383 later with LACK/OVA and analyzed 4 and 5 days later. **(B)** AHR. Whole body plethysmography  
384 was monitored in mice tolerized to OVA (empty circles) or BSA (filled squares, and crosses) and  
385 exposed to aerosols of LACK/OVA (full lines) or to PBS (dashed lines). Data are expressed as  
386 mean ± s.e.m. **(C)** Number and phenotype of BALF cells. BALF cells were analyzed by FACS in  
387 the indicated groups of mice. Eosinophils, E; neutrophils, N; lymphocytes, L; macrophages, M.  
388 Data are expressed as mean ± SEM of 3 experiments with n=6-8 mice per group. **(D)** Numbers of  
389 Thy1.1<sup>+</sup> LACK-specific Th2 cells in BALF, lung, and medLN and frequency in the blood of  
390 BSA- (filled bars) or OVA- (empty bars) tolerized mice challenged with LACK/OVA aerosols.  
391 **(E)** Representative plots of CFSE (left panels) and MFI of CFSE (right panels) of the indicated  
392 mice. **(F)** *In vitro* antigen presentation assay. Whole cell suspensions prepared from the MedLN  
393 and PLN of OVA- or BSA-exposed mice were incubated for 3 days with CFSE-labeled LACK-  
394 specific Th2 cells. Data show representative CFSE plots for the indicated mice with the  
395 frequency of divided cells as mean ± s.e.m of 2 experiments with n=6 mice per group. **(G)** Mice  
396 underwent the same protocol as shown in panel A, but also received fluorescent latex beads

397 during LACK/OVA challenge. Beads<sup>+</sup>CD11c<sup>+</sup> migratory DCs were analyzed by FACS in the  
398 MedLN of BSA- (filled bars) or OVA- (empty bars) tolerized mice challenged with LACK/OVA  
399 aerosols. n.s., non significant; \*  $P < 0.05$ ; \*\*  $P < 0.01$ ; \*\*\*  $P < 0.0001$ .

400  
401 **Supplementary Figure 1. AHR and airway inflammation in LACK-sensitized mice**  
402 **challenged with LACK alone or LACK and OVA aerosols.** (A) Experimental protocol. Mice  
403 were sensitized with two i.p. injections of LACK in Alum, and challenged daily for 5 days with  
404 LACK or LACK/OVA aerosols, or PBS. Mice were analyzed one and two days after the last  
405 aerosol. (B) AHR. Whole body plethysmography in mice exposed to aerosols of LACK (filled  
406 squares), LACK/OVA (empty circles) or PBS (crosses). (C) Number and phenotype of BALF  
407 cells. BALF cells were analyzed by FACS in mice exposed to LACK only (black bars), to both  
408 LACK and OVA aerosols (empty bars) or to PBS (grey bars). Eosinophils, E; neutrophils, N;  
409 lymphocytes, L; macrophages, M. Data are expressed as mean  $\pm$  s.e.m. of 2 experiments with  
410 n=8 mice per group. n.s., non significant.

411

**412 METHODS**

413  
414 **Mice.** 6-week old BALB/c mice were purchased from The Centre d'Élevage Janvier (France) and  
415 housed under SPF conditions. LACK TCR transgenic mice (WT15 RAG-1 KO) on the BALB/c  
416 background as previously described<sup>34</sup> were bred in our animal facility at the Institut de  
417 Pharmacologie Moléculaire et Cellulaire (Valbonne, France). In this study, WT15 transgenic  
418 mice were further crossed onto RAG-1<sup>-/-</sup> Thy1.1<sup>+/+</sup> BALB/c mice. All experimental protocols  
419 were approved by the local animal ethic committee.

420 **Reagents.** LACK recombinant protein was produced in *E. coli*, purified as described<sup>35</sup>, and  
421 detoxified using an Endotrap column (Profos). Lipopolysaccharide contents in LACK protein  
422 were below 5 ng/mg as determined using Limulus Amoebocyte Lysate (LAL) assay (Pierce).  
423 LACK<sub>156-173</sub> peptide was purchased from Mimotopes. T1/ST2 mAbs were purchased from MD  
424 Biosciences. Monoclonal antibodies to CD3, CD4, CD25, Thy1.1, CD11c, IA/IE, CD80, CD86,  
425 IL-4 and IL-5 were purchased from BD Biosciences.

426 **Induction of allergic asthma and tolerization to OVA.** Sensitization was performed by 2  
427 intraperitoneal (i.p.) injections of 10 µg of LACK in 2 mg of Aluminium hydroxide (Alum)  
428 (Pierce) at day 0 and 7. On days 12, 13 and 14, mice were tolerized to OVA by injecting i.n. 100  
429 µg of LPS-free OVA (Profos) as described<sup>7</sup>. From day 23 to day 27, mice were either exposed to  
430 LACK (0.15%) or to LACK plus OVA (0.2%) aerosols (administered 8 hour apart) for 20 min  
431 using an ultrasonic nebulizer (Ultramed, Medicalia). Mice were analyzed on day 28 and 29 for  
432 AHR and airway inflammation, respectively. When indicated, mice were injected with either 0.5  
433 mg of anti-CTLA-4 mAb (9H10), anti-ICOS (17G9), or anti-IL-10R (1B1.3A) every other day  
434 over the challenge period starting one day before the first aerosol. For ragweed-induced asthma,  
435 mice were first tolerized to OVA by receiving i.n. injections of OVA on days 0, 1, and 2 and  
436 further sensitized to ragweed via i.n. administrations of 25 µg ragweed (Greer laboratories) on  
437 days 11, 15, 19, and 23. Mice received a last challenge of ragweed on day 27 or ragweed and  
438 OVA on days 26 and 27. Mice were analyzed on day 28 for AHR and on day 29 for airway  
439 inflammation.

440 **Th2 cell transfers.** In some experiments, mice were first tolerized to OVA, BSA or PBS and  
441 injected i.v on day 11 with LACK-specific CD4<sup>+</sup> Th2 cells. Mice were challenged 24 hours later  
442 with a single i.n. injection of LACK (30 µg) and OVA (100 µg), and assessed for AHR, and  
443 immunological parameters 3 and 4 days later, respectively.

444 **AHR.** For non-invasive measurements, mice were analyzed one day after the last aerosol  
445 challenge using whole body plethysmography as described<sup>36</sup>. Invasive measurements of dynamic  
446 lung resistance and compliance were performed one day after the last aerosol challenge using a  
447 Flexivent apparatus (SCIREQ, Emka Technologies) as previously described<sup>37</sup>. Briefly, mice  
448 were anesthetized (5 ml/kg Dormitor 10 % (Medetomidine, Pfizer) - Imalgene 10% (Ketamine,  
449 Merial) tracheotomized, paralyzed (5 ml/kg Pavulon 1% (Pancuronium bromide, Organon) and  
450 immediately intubated with an 18-G catheter, followed by mechanical ventilation. Respiratory  
451 frequency was set at 150 breaths/min with a tidal volume of 0.2 ml, and a positive-end expiratory  
452 pressure of 2 ml H<sub>2</sub>O was applied. Increasing concentrations of methacholine (0-24 mg/ml) were  
453 administered at the rate of 20 puffs per 10 seconds, with each puff of aerosol delivery lasting 10  
454 ms, via a nebulizer aerosol system with a 2.5-4 µm aerosol particle size generated by a nebulizer  
455 head (Aeroneb, Aerogen). Baseline resistance was restored before administering the subsequent  
456 doses of methacholine.

457 **Analysis of BALF cells.** Mice were bled and a canula was inserted into the trachea. Lungs were  
458 washed 3 times with 1 ml of warmed PBS. For differential BALF cell counts, cells were stained  
459 with mAb anti-CCR3 (R&D), anti-Gr1, anti-CD3 and anti-CD19 mAbs (Becton Dickinson, BD)  
460 and analyzed by FACS using a FACScalibur flow cytometer and Cellquest software. Eosinophils  
461 were defined as CCR3<sup>+</sup> CD3<sup>-</sup>CD19<sup>-</sup>, neutrophils as Gr-1<sup>high</sup> CD3<sup>-</sup>CD19<sup>-</sup>, lymphocytes as  
462 CD3<sup>+</sup>CD19<sup>+</sup> and alveolar macrophages as large autofluorescent cells.

463 **Serum antibody measurements.** Serum LACK-specific IgG1 and IgE were measured by  
464 ELISA. For IgG1 quantification, antigen-coated Maxisorp plates (Nunc) were incubated with  
465 serial dilution of sera and biotinylated anti-IgG1 mAb (BD). For antigen-specific IgE, plates were  
466 first coated with the respective capture mAb (BD), and incubated with serum dilutions.  
467 Biotinylated-LACK antigen was then added. HRP-conjugated streptavidin (BD) and TMB (KPL)  
468 were used for detection.

469 **Tissue processing.** Lungs, LN or spleens were cut to small pieces in HBSS containing 400 U  
470 type I collagenase and 1 mg/ml DNase I and digested for 30 min at 37°C. Cells were strained  
471 through a 70 µm cell strainer. Erythrocytes were lysed with ACK lysis buffer.

472 **Cytokine assays.** Lung samples were homogenized in  $C^{2+}$  and  $Mg^{2+}$  free HBSS. BAL and lung  
473 supernatants were used. Multiplex IL-4, IL-5, IL-13 and IFN- $\gamma$  analysis were performed with  
474 CBA using FACS array (BD Biosciences). For intracellular staining, cells were incubated with  
475 100 µg/ml LACK and 1 µg/ml of anti-CD28 (BD) for 6 h. Brefeldin A (5 µg/ml, Sigma) was  
476 added during the last 4 h. Cells were then stained with anti-CD4 mAb, fixed, permeabilized using  
477 cytofix/cytoperm reagent (BD), stained with anti-IL-4, or IL-5 (BD) and analyzed by FACS.

478 **CD4<sup>+</sup> T cell transfer.** Donor mice were tolerized to OVA, BSA or PBS as described above.  
479 Cells were prepared from spleens 21 days later, and CD4<sup>+</sup> T cells were enriched by negative  
480 depletion using CD4 isolation kit (Dynal) and further sorted using a high-speed sorter  
481 VANTAGE SETLO<sup>+</sup> flow cytometer (BD) after staining with anti-CD3 and anti-CD4 mAbs.  
482 CD4 purity was > 95%. In some experiments, enriched CD4<sup>+</sup> T cells were stained with antibodies  
483 to CD25, CD4 and CD3, and CD25<sup>-</sup> and CD25<sup>+</sup> CD4<sup>+</sup> T cell populations were sorted by FACS.  
484 Sorted cells were then injected i.v. into sensitized mice (4 X 10<sup>6</sup> or 1.5 X 10<sup>6</sup> cells per mouse,  
485 respectively).

486 **Statistic analysis.** ANOVA for repeated measures was used to determine the levels of difference  
487 between groups of mice for plethysmography measurements. Comparisons for all pairs were  
488 performed by Mann-Whitney U test. Significance levels were set at a *P* value of 0.05.

489

490 **ACKNOWLEDGMENTS**

491 We thank Nicolas Guy and the animal facility staff for their excellent animal care, as well as Julie  
492 Cazareth for the cell sorting experiments. This work was supported by a grant from the Fondation  
493 pour la Recherche Médicale.

494 **COMPETING INTEREST STATEMENT**

495 The authors declare no competing financial interests.

496

497 **REFERENCES**

498

- 499 1. Akdis CA, Akdis M, Blesken T, Wymann D, Alkan SS, Muller U, et al. Epitope-specific  
500 T cell tolerance to phospholipase A2 in bee venom immunotherapy and recovery by IL-2  
501 and IL-15 in vitro. *J Clin Invest* 1996; 98:1676-83.
- 502 2. Akdis CA, Blesken T, Wymann D, Akdis M, Blaser K. Differential regulation of human  
503 T cell cytokine patterns and IgE and IgG4 responses by conformational antigen variants.  
504 *Eur J Immunol* 1998; 28:914-25.
- 505 3. Jutel M, Akdis M, Budak F, Aebischer-Casaulta C, Wrzyszczyk M, Blaser K, et al. IL-10  
506 and TGF-beta cooperate in the regulatory T cell response to mucosal allergens in normal  
507 immunity and specific immunotherapy. *Eur J Immunol* 2003; 33:1205-14.
- 508 4. Francis JN, Till SJ, Durham SR. Induction of IL-10+CD4+CD25+ T cells by grass pollen  
509 immunotherapy. *J Allergy Clin Immunol* 2003; 111:1255-61.
- 510 5. Ostroukhova M, Seguin-Devaux C, Oriss TB, Dixon-McCarthy B, Yang L, Ameredes  
511 BT, et al. Tolerance induced by inhaled antigen involves CD4(+) T cells expressing  
512 membrane-bound TGF-beta and FOXP3. *J Clin Invest* 2004; 114:28-38.
- 513 6. Seymour BWP, Gershwin LJ, Coffman RL. Aerosol-induced immunoglobulin (Ig)-E  
514 unresponsiveness to ovalbumin does not require CD8(+) or T cell receptor (TCR)-  
515 gamma/delta(+) T cells or interferon (IFN)-gamma in a murine model of allergen  
516 sensitization. *J Exp Med* 1998; 187:721-31.
- 517 7. Akbari O, DeKruyff RH, Umetsu DT. Pulmonary dendritic cells producing IL-10 mediate  
518 tolerance induced by respiratory exposure to antigen. *Nat Immunol* 2001; 2:725-31.
- 519 8. Akbari O, Freeman GJ, Meyer EH, Greenfield EA, Chang TT, Sharpe AH, et al. Antigen-  
520 specific regulatory T cells develop via the ICOS-ICOS-ligand pathway and inhibit  
521 allergen-induced airway hyperreactivity. *Nat Med* 2002; 8:1024-32.
- 522 9. Chen W, Jin W, Hardegen N, Lei KJ, Li L, Marinos N, et al. Conversion of peripheral  
523 CD4+CD25- naive T cells to CD4+CD25+ regulatory T cells by TGF-beta induction of  
524 transcription factor Foxp3. *J Exp Med* 2003; 198:1875-86.
- 525 10. Des Roches A, Paradis L, Menardo JL, Bouges S, Daures JP, Bousquet J. Immunotherapy  
526 with a standardized *Dermatophagoides pteronyssinus* extract. VI. Specific  
527 immunotherapy prevents the onset of new sensitizations in children. *J Allergy Clin*  
528 *Immunol* 1997; 99:450-3.

- 529 11. Pajno GB, Barberio G, De Luca F, Morabito L, Parmiani S. Prevention of new  
530 sensitizations in asthmatic children monosensitized to house dust mite by specific  
531 immunotherapy. A six-year follow-up study. *Clin Exp Allergy* 2001; 31:1392-7.
- 532 12. Bullock WW, Katz DH, Benacerraf B. Induction of T-lymphocyte responses to a small  
533 molecular weight antigen. II. specific tolerance induced in azebenzene arsonate (ABA)-  
534 specific T cells in Guinea pigs by administration of low doses of an ABA conjugate of  
535 chloroacetyl tyrosine in incomplete Freund's adjuvant. *J Exp Med* 1975; 142:261-74.
- 536 13. Miller A, Hafler DA, Weiner HL. Tolerance and suppressor mechanisms in experimental  
537 autoimmune encephalomyelitis: implications for immunotherapy of human autoimmune  
538 diseases. *The FASEB Journal* 1991; 5:2560-6.
- 539 14. Miller A, Lider O, al-Sabbagh A, Weiner HL. Suppression of experimental autoimmune  
540 encephalomyelitis by oral administration of myelin basic protein. V. Hierarchy of  
541 suppression by myelin basic protein from different species. *J Neuroimmunol* 1992;  
542 39:243-50.
- 543 15. Homann D, Holz A, Bot A, Coon B, Wolfe T, Petersen J, et al. Autoreactive CD4+ T  
544 cells protect from autoimmune diabetes via bystander suppression using the IL-4/Stat6  
545 pathway. *Immunity* 1999; 11:463-72.
- 546 16. al-Sabbagh A, Miller A, Santos LM, Weiner HL. Antigen-driven tissue-specific  
547 suppression following oral tolerance: orally administered myelin basic protein suppresses  
548 proteolipid protein-induced experimental autoimmune encephalomyelitis in the SJL  
549 mouse. *Eur J Immunol* 1994; 24:2104-9.
- 550 17. Aharoni R, Teitelbaum D, Sela M, Arnon R. Copolymer 1 induces T cells of the T helper  
551 type 2 that crossreact with myelin basic protein and suppress experimental autoimmune  
552 encephalomyelitis. *Proc Natl Acad Sci U S A* 1997; 94:10821-6.
- 553 18. Wing K, Onishi Y, Prieto-Martin P, Yamaguchi T, Miyara M, Fehervari Z, et al. CTLA-4  
554 control over Foxp3+ regulatory T cell function. *Science* 2008; 322:271-5.
- 555 19. Busse M, Krech M, Meyer-Bahlburg A, Hennig C, Hansen G. ICOS mediates the  
556 generation and function of CD4+CD25+Foxp3+ regulatory T cells conveying respiratory  
557 tolerance. *J Immunol* 2012; 189:1975-82.
- 558 20. Ito T, Hanabuchi S, Wang YH, Park WR, Arima K, Bover L, et al. Two functional  
559 subsets of FOXP3+ regulatory T cells in human thymus and periphery. *Immunity* 2008;  
560 28:870-80.
- 561 21. Redpath SA, van der Werf N, Cervera AM, MacDonald AS, Gray D, Maizels RM, et al.  
562 ICOS controls Foxp3(+) regulatory T-cell expansion, maintenance and IL-10 production  
563 during helminth infection. *Eur J Immunol* 2013; 43:705-15.
- 564 22. Inal A, Altintas DU, Yilmaz M, Karakoc GB, Kendirli SG, Sertdemir Y. Prevention of  
565 new sensitizations by specific immunotherapy in children with rhinitis and/or asthma  
566 monosensitized to house dust mite. *J Investig Allergol Clin Immunol* 2007; 17:85-91.
- 567 23. Wing K, Yamaguchi T, Sakaguchi S. Cell-autonomous and -non-autonomous roles of  
568 CTLA-4 in immune regulation. *Trends Immunol* 2011; 32:428-33.
- 569 24. Kearley J, Barker JE, Robinson DS, Lloyd CM. Resolution of airway inflammation and  
570 hyperreactivity after in vivo transfer of CD4+CD25+ regulatory T cells is interleukin 10  
571 dependent. *J Exp Med* 2005; 202:1539-47.
- 572 25. Shevach EM. CD4+ CD25+ suppressor T cells: more questions than answers. *Nat Rev*  
573 *Immunol* 2002; 2:389-400.



- 574 26. Corry DB, Folkesson HG, Warnock ML, Erle DJ, Matthay MA, Wiener-Kronish JP, et al.  
575 Interleukin 4, but not interleukin 5 or eosinophils, is required in a murine model of acute  
576 airway hyperreactivity. *J. Exp. Med.* 1996; 183:109-8.
- 577 27. Humbles AA, Lloyd CM, McMillan SJ, Friend DS, Xanthou G, McKenna EE, et al. A  
578 critical role for eosinophils in allergic airways remodeling. *Science* 2004; 305:1776-9.
- 579 28. Alcorn JF, Rinaldi LM, Jaffe EF, van Loon M, Bates JH, Janssen-Heininger YM, et al.  
580 Transforming growth factor-beta1 suppresses airway hyperresponsiveness in allergic  
581 airway disease. *Am J Respir Crit Care Med* 2007; 176:974-82.
- 582 29. Kearley J, Robinson DS, Lloyd CM. CD4+CD25+ regulatory T cells reverse established  
583 allergic airway inflammation and prevent airway remodeling. *J Allergy Clin Immunol*  
584 2008; 122:617-24 e6.
- 585 30. Pavord ID, Korn S, Howarth P, Bleecker ER, Buhl R, Keene ON, et al. Mepolizumab for  
586 severe eosinophilic asthma (DREAM): a multicentre, double-blind, placebo-controlled  
587 trial. *Lancet* 2012; 380:651-9.
- 588 31. Tang Q, Krummel MF. Imaging the function of regulatory T cells in vivo. *Curr Opin*  
589 *Immunol* 2006; 18:496-502.
- 590 32. Tadokoro CE, Shakhar G, Shen S, Ding Y, Lino AC, Maraver A, et al. Regulatory T cells  
591 inhibit stable contacts between CD4+ T cells and dendritic cells in vivo. *J Exp Med* 2006;  
592 203:505-11.
- 593 33. Derks RA, Jankowska-Gan E, Xu Q, Burlingham WJ. Dendritic cell type determines the  
594 mechanism of bystander suppression by adaptive T regulatory cells specific for the minor  
595 antigen HA-1. *J Immunol* 2007; 179:3443-51.
- 596 34. Wang Q, Malherbe L, Zhang D, Zingler K, Glaichenhaus N, Killeen N. CD4 promotes  
597 breadth in the TCR repertoire. *J Immunol* 2001; 167:4311-20.
- 598 35. Mougneau E, Altare F, Coppola T, Glaichenhaus N, Wakil AE, Reiner S, et al. A  
599 *Leishmania* protective T-cell antigen identified using a new expression cloning strategy.  
600 *Vaccines* 95 1995; CSH eds.:51-6.
- 601 36. Hamelmann E, Schwarze J, Takeda K, Oshiba A, Larsen GL, Irvin CG, et al. Noninvasive  
602 measurement of airway responsiveness in allergic mice using barometric  
603 plethysmography. *Am J Respir Crit Care Med* 1997; 156:766-75.
- 604 37. Mionnet C, Buatois V, Kanda A, Milcent V, Fleury S, Lair D, et al. CX3CR1 is required  
605 for airway inflammation by promoting T helper cell survival and maintenance in inflamed  
606 lung. *Nat Med* 2010; 16:1305-12.
- 607  
608













