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

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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⁺ T cells (T_{reg})
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⁺ T_{reg}, 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_{reg} 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_{reg} 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_{reg}, 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_{eff}**, effector T
93 cells; **Tg**, transgenic; **Th**, T helper ; **T_{reg}**, 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 ^{1, 2}. Indeed, IL-10 and/or TGF- β producing T_{reg} are the key factors for specific
108 immunotherapy in humans, considered as a model of tolerance induction ³⁻⁵. It has been shown
109 that treating naive (non-sensitized) mice with OVA aerosols leads to IgE-unresponsiveness to
110 OVA ⁶, and induces the development of OVA-specific T_{reg} which prevented the development of
111 asthma upon subsequent sensitization and challenge with OVA ⁷. Moreover, lung draining lymph
112 node (LN) dendritic cells (DC) first encountering an inhaled antigen transiently produced IL-10 ⁷.
113 These phenotypically mature DC induce the development of $CD4^+$ T_{reg} that also produce high
114 amounts of IL-10 ⁸. TGF- β -expressing T_{reg} also play a role for tolerance induction to inhaled
115 antigens ^{5, 9}. 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 ^{10, 11}.
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 ¹². 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^{13, 14}. 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^{13, 15-17}.

129 In the present study, we have sought to investigate whether T_{reg} 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_{reg} 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⁺ CD4⁺ 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- γ levels
148 remained low and similar in both groups (**Fig 1, E**). This phenomenon was not observed in mice
149 than have not been tolerized to OVA prior to ragweed sensitization (Supplementary Figure 1)
150 further supporting a role for OVA-specific T_{reg} 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⁺ CD4⁺ 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- γ 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⁺ 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⁺ T_{reg}**

171 Previous experiments have shown that the intranasal administration of OVA to BALB/c mice
172 induces OVA-specific T_{reg} 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⁺ 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⁺ T cells purified from BSA-tolerized or PBS-treated
178 donors, mice injected with CD4⁺ 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⁺
180 CD4⁺ T cells in BALF **(Fig 3, D)**, and reduced numbers of IL-4 and IL-5-secreting CD4⁺ 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⁺ T cells from OVA-tolerized
183 mice depleted of CD25⁺ 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⁺ CD4⁺ 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⁺
188 T_{reg}.

189
190 **CTLA-4, ICOS, and IL-10 dependency of OVA-specific T_{reg} in the control of LACK-**
191 **induced asthma**

192 T_{reg} mediate suppression through various mechanisms including the secretion of inhibitory
193 cytokines such as IL-10, the induction of cytolysis, metabolic disruption and the inhibition of
194 antigen presentation by dendritic cells (DCs) through a CTLA-4-dependent mechanism¹⁸.

195 Furthermore, it was recently shown that ICOS mediated the generation and function of CD25⁺
196 CD4⁺ FoxP3⁺ T_{reg} conveying respiratory tolerance¹⁹, and that ICOS expression defined a subset
197 of IL-10 secreting T_{reg}²⁰ and was required for the production of IL-10 by these cells²¹.
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⁺ Th2 cells in BALF, and IL-4- and IL-5-producing LACK-
204 specific CD4⁺ 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_{reg} 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_{reg} inhibited LACK-
216 induced allergic asthma, CD4⁺ T cells from Thy1.1⁺ 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⁺ 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⁺ DCs in MedLN 16 hrs later. While bead⁺ 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⁺
241 DCs was decreased from 4.7 ± 0.7 % in BSA-tolerized mice to 1.4 ± 0.3 % in OVA-tolerized
242 animals (**Fig 5, H**). Altogether, our data suggested that OVA-specific T_{reg} 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_{reg} could inhibit allergic airway
247 inflammation induced by sensitization and challenge with ragweed. OVA-specific T_{reg} 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_{reg} (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^{10, 11, 22}.

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⁺ 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⁺ 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_{reg}.

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_{reg}
267 which constitutively express this molecule. Indeed, CTLA-4 deficiency in T_{reg} impaired both
268 their *in vivo* and *in vitro* suppressive function. In addition, CTLA-4 prevented CD28 signaling in
269 effector T cells (T_{eff}) 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 catabolites²³. 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^{-/-} T_{reg} did not confer protection upon
277 transfer to asthmatic mice demonstrating a crucial role of ICOS in their suppressive function¹⁹.
278 As ICOS was shown to define a subset of IL-10-producing T_{reg}, 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⁺ T_{reg} or by other cells as
282 previously described Kearley *et al.*²⁴. Taken together, these data underline the multiple and
283 complex effects of CD25⁺ T_{reg}. 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 conditions²⁵. 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 changes²⁶⁻²⁹. 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 function³⁰.

291 Adoptive transfer of CFSE-labeled LACK-specific Th2 cells into OVA-tolerized recipient
292 mice demonstrated that OVA-specific T_{reg} 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_{reg} do not directly interact with T_{eff} but rather with DC, altering the latter and
295 diminishing subsequent DC-T_{eff} cells conjugate formation *in vivo*^{31, 32} a phenomenon that could
296 explain our results. In addition, Derks and colleagues have envisioned two hypotheses of APC
297 function in bystander suppression³³: a passive APC model, in which the APCs would present
298 MHC-peptide to the T_{reg}, stimulating them to produce immunosuppressive cytokines that would
299 further binds their cognate receptors on the third-party T_{eff}, or an active APC model, in which the
300 APCs would propagate regulatory effects from the T_{reg} to the T_{eff} through various APC products.
301 These two hypotheses remain to be tested in our model.

302 Our results demonstrate that T_{reg} 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- γ levels were assessed by ELISA after *in vitro* stimulation with
320 LACK protein (0.1mg/ml), anti-CD28 (1 μ g/ml) and brefeldin A (5 μ 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⁺ CD4⁺
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- γ by cytometric bead array (CBA). (F) Cytokine secretion by lung CD4⁺ T cells.

338 IL-4, and IL-5-secreting CD4⁺ 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⁺ 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 4×10^6 CD4⁺ T cells **(A-E)**, or 4×10^6
349 CD25⁻CD4⁺ T cells **(F-I)** or 1.5×10^6 CD25⁺CD4⁺ 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⁺ T cells. IL-4, and IL-5-secreting CD4⁺ 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⁺ 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⁺ 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 1.5×10^6 CFSE-labeled Thy1.1⁺ 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⁺ 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⁺CD11c⁺ 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 ³⁴ 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^{-/-} Thy1.1^{+/+} 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 ³⁵, 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 LACK₁₅₆₋₁₇₃ 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 ⁷. 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⁺ 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 described³⁶. 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 described³⁷. 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 H₂O 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⁺ CD3⁻CD19⁻, neutrophils as Gr-1^{high} CD3⁻CD19⁻, lymphocytes as
462 CD3⁺CD19⁺ 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- γ 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⁺ 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⁺ T cells were enriched by negative
480 depletion using CD4 isolation kit (Dynal) and further sorted using a high-speed sorter
481 VANTAGE SETLO⁺ flow cytometer (BD) after staining with anti-CD3 and anti-CD4 mAbs.
482 CD4 purity was > 95%. In some experiments, enriched CD4⁺ T cells were stained with antibodies
483 to CD25, CD4 and CD3, and CD25⁻ and CD25⁺ CD4⁺ T cell populations were sorted by FACS.
484 Sorted cells were then injected i.v. into sensitized mice (4 X 10⁶ or 1.5 X 10⁶ 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

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494 **COMPETING INTEREST STATEMENT**

495 The authors declare no competing financial interests.

496

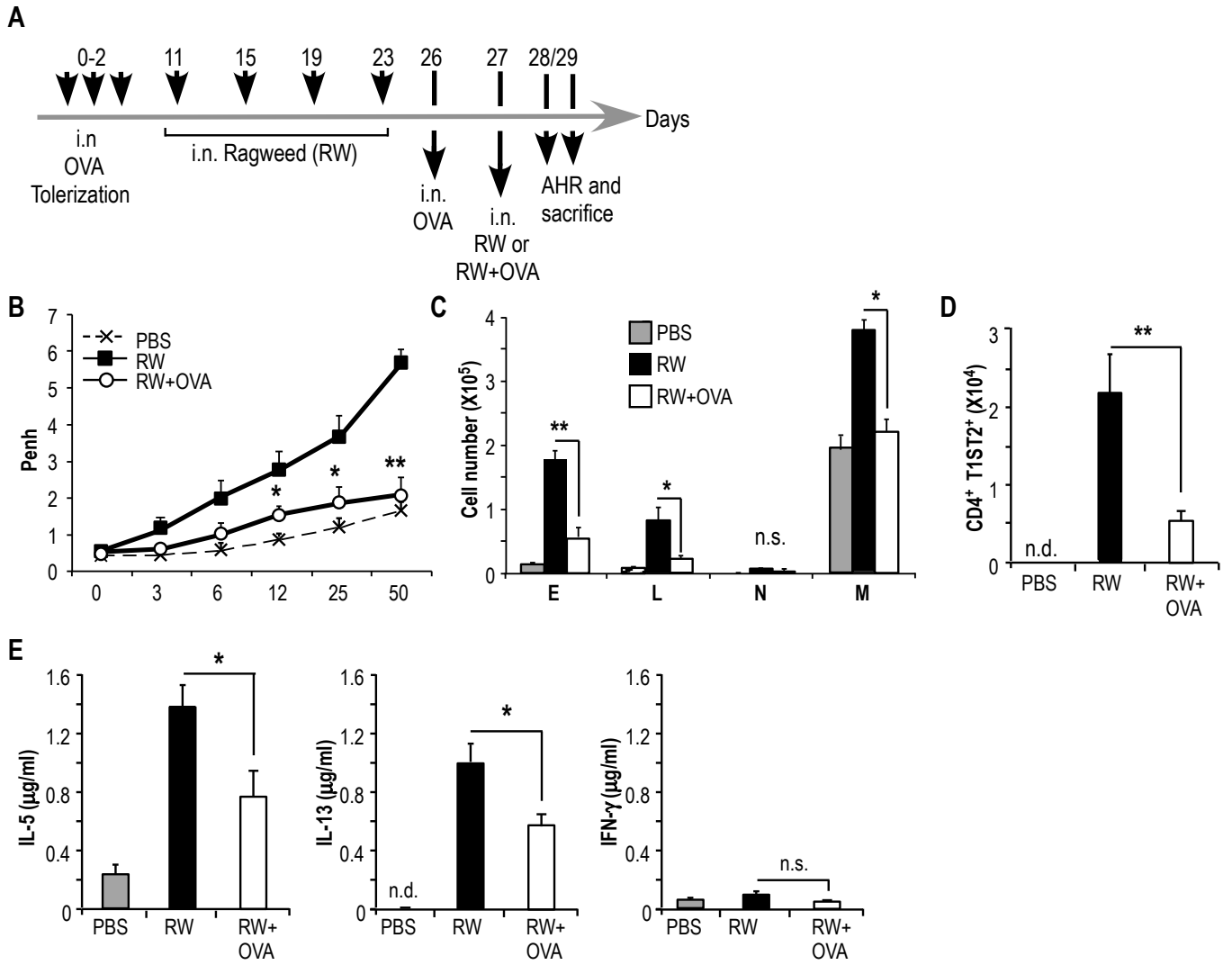
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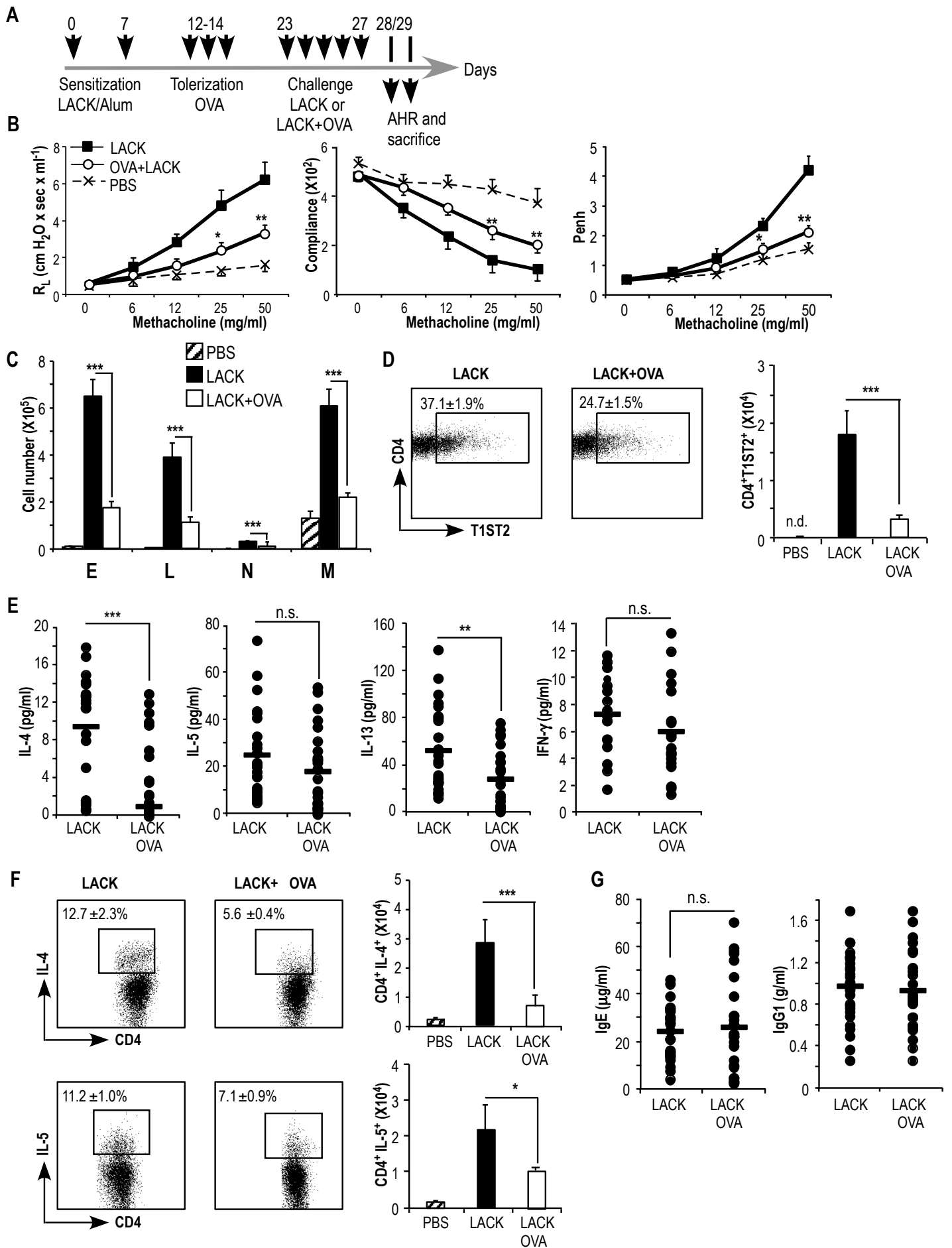
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Navarro, et.al., Figure 2

