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Effector and central memory T helper 2 cells respond differently to peptide immunotherapy

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Peptide immunotherapy (PIT) offers realistic prospects for the treatment of allergic diseases, including allergic asthma. Much is understood of the behavior of naive T cells in response to PIT. However, treatment of patients with ongoing allergic disease requires detailed understanding of the responses of allergen-experienced T cells. CD62L expression by allergen-experienced T cells corresponds to effector/effector memory (CD62L^{lo}) and central memory (CD62L^{hi}) subsets, which vary with allergen exposure (e.g., during, or out with, pollen season). The efficacy of PIT on different T helper 2 (Th2) cell memory populations is unknown. We developed a murine model of PIT in allergic airway inflammation (AAI) driven by adoptively transferred, traceable ovalbumin-experienced Th2 cells. PIT effectively suppressed AAI driven by unfractionated Th2 cells. Selective transfer of CD62L^{hi} and CD62L^{lo} Th2 cells revealed that these two populations behaved differently from one another and from previously characterized (early deletional) responses of naive CD4⁺ T cells to PIT. Most notably, allergen-reactive CD62L^{lo} Th2 cells were long-lived within the lung after PIT, before allergen challenge, in contrast to CD62L^{hi} Th2 cells. Despite this, PIT was most potent against CD62L^{lo} Th2 cells in protecting from AAI, impairing their ability to produce Th2 cytokines, whereas this capacity was heightened in PIT-treated CD62L^{hi} Th2 cells. We conclude that Th2 cells do not undergo an early deletional form of tolerance after PIT. Moreover, memory Th2 subsets respond differently to PIT. These findings have implications for the clinical translation of PIT in different allergic scenarios.

Specific immunotherapy involves therapeutic delivery of a disease-relevant antigen to induce tolerance (particularly of CD4⁺ T cells) toward that antigen (1, 2). It represents a realistic and potentially disease-modifying therapeutic approach for the treatment of allergic and autoimmune diseases with strong CD4⁺ T-cell components to their pathogenesis, such as allergic asthma (3–5). Traditional immunotherapy, using whole-protein antigens, is associated with the risk of severe allergic reactions, particularly anaphylaxis, in patients harboring allergen-reactive IgE (6, 7). Peptide immunotherapy (PIT) obviates this risk because it uses short synthetic peptides containing known T-cell epitopes, but not conformational antibody epitopes, thereby targeting disease-driving CD4⁺ T cells while avoiding IgE binding (8, 9).

In animal studies, PIT can effectively reduce or prevent CD4⁺ T-cell-driven diseases (10–15). Encouraging findings have also been reported in allergic patients (16–20). However, reduced disease severity is not universal, and limitations in our understanding of the workings of PIT are impeding clinical translation. Mechanistic murine PIT studies have been advanced through the use of traceable populations of T-cell receptor (TCR) transgenic T cells. PIT is highly effective in silencing “naive” T cells whose first encounter with their cognate antigen is at the point of tolerogenic peptide application (21, 22). This is different from the clinical setting where established T-cell-driven pathology, by definition, presents with an increased frequency of antigen-experienced T cells (23). We, and others, have

previously shown that application of tolerogenic peptide induces naive CD4⁺ T cells to enter a brief but abortive phase of proliferation that is followed by their wide-scale apoptotic deletion (21, 22, 24). This is most likely the result of insufficient costimulation from the antigen-presenting cell in the absence of innate immune triggers (21, 22, 24). However, several characteristics of antigen-experienced T cells hint that they may not necessarily respond to PIT in the same way. First, they have lower costimulation requirements (25, 26) that may make them less susceptible to deletion in response to costimulation deprivation in the tolerogenic setting. Antigen-experienced T cells can be phenotypically classified into effector and memory T-cell populations, the latter being subdivided into effector memory T cells (Tem) and central memory T cells (Tcm) (27, 28). Importantly, the phenotype and frequency of allergen-reactive T cells can vary, depending on the presence or absence of allergen exposure (e.g., perennial vs. seasonal allergy) (29–31). In addition, the phenotype of T cells in the end organ (e.g., the lung) may differ from those in peripheral blood (32–34). These complexities could have a major impact upon the clinical response to PIT and have not previously been addressed.

Here, we developed a model to study the effects of PIT upon Th2-polarized TCR transgenic cells driving allergic airway inflammation (AAI). PIT effectively reduced AAI, despite the allergen-experienced nature of the eliciting Th2 cells. Furthermore, PIT was most potent against AAI driven by CD62L^{lo} Th2 cells (a phenotype associated with effector and Tem) compared with CD62L^{hi} Th2 cells (associated with Tcm) (27, 28). Importantly,

Significance

Peptide immunotherapy (PIT) of ongoing allergy must control “memory” T helper 2 (Th2) cells. Memory T cells can be subdivided into effector memory T cells (Tem), which seem to be involved in immediate immune responses, and central memory T cells (Tcm), which are thought to provide long-term memory. We show that PIT can control allergic lung disease more effectively when the disease is driven by Tem Th2 cells, rather than by Tcm Th2 cells. PIT-treated Tcm remained more responsive to allergen, with a greater capacity to produce inflammatory Th2 cytokines in the lung. These were suppressed in PIT-treated Tem. These differences are important for clinical translation of PIT, because Tcm may be particularly dominant in some seasonal allergic conditions, such as hay fever.

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CD62L^{lo} and CD62L^{hi} Th2 cells showed markedly distinct behavior in response to PIT, both in comparison with one another and compared with the known behavior of naive T cells. Notably, a sizeable population of PIT-treated CD62L^{lo} cells persisted long-term within the lung following PIT, in contrast to CD62L^{hi} cells. Whereas PIT led to diminished Th2 cytokine production at the time of airway challenge with allergen in CD62L^{lo} cells, this effect of PIT was not seen in CD62L^{hi} cells, which therefore retained pathogenic activity. The composition of Th2 cell subpopulations at the time of PIT is thus an important additional consideration in respect to clinical translation.

Results

A Th2 Cell Transfer Model of Chicken Egg Ovalbumin-Driven Allergic Airway Inflammation. We established a model of AAI driven by traceable (CD45.1⁺) Th2 cells, using OT-II TCR transgenic mice (35) as a source of T cells recognizing the 323–339 peptide of chicken egg ovalbumin (pOVA). In vitro activation of OT-II T cells by pOVA under Th2-polarizing conditions generated a GATA-3-expressing T-cell population that produced high levels of IL-5 and IL-13 upon restimulation with OVA, compared with naive OT-II T cells (Fig. 1A). Transfer of these Th2-polarized OT-II cells, followed by intratracheal (i.t.) challenge with OVA (Fig. 1A), induced AAI, as evidenced by cellular infiltration around airways and blood vessels and goblet cell formation (Fig. 1B). An increase in total cells and eosinophils was evident within the bronchoalveolar lavage (BAL) (Fig. 1C). These pathological changes required OVA in the i.t. challenge and could not be elicited if naive OT-II T cells were transferred (Fig. 1B and C). Flow cytometry identified transferred CD45.1⁺ cells within the lung (Fig. 1D). Of note, low numbers of Th2 cells, but not naive T cells, were evident in the absence of OVA i.t. challenge, but

both the number and the frequency of Th2 cells were significantly increased following OVA challenge (Fig. 1D). This model therefore allowed us to relate numbers, phenotype, and function of preformed Th2 cells in the lung to their pathogenic activity (AAI), in the presence or absence of PIT.

PIT Suppresses AAI Driven by Preexisting Th2 Cells. Naive OT-II cells are sensitive to PIT when pOVA is administered i.v. in PBS (21, 22, 24). We assessed therapeutic efficacy of PIT in AAI by administration of pOVA 2 d after Th2 cell transfer and 4 d before i.t. challenges (Fig. 2A). This PIT regimen led to reductions in cellular infiltration of the lungs, goblet cells in the airways, BAL eosinophilia, and IL-13 in BAL (Fig. 2B–E). The total numbers of OT-II cells in both the lung and the spleen were increased by OVA challenges in the absence of PIT (Fig. 2F). In PIT-treated mice, however, numbers of OT-II cells following OVA i.t. challenge were not significantly different from those seen in PBS-challenged control mice (Fig. 2F). We therefore conclude that PIT is effective at diminishing the AAI-inducing function of allergen-experienced Th2 cells.

PIT Drives Th2 Cell Accumulation in the Lung Before Allergen Challenge. We, and others, have previously demonstrated that the effectiveness of PIT upon naive OT-II cells predominantly involves a deletional effect, which is evident 4 d after PIT (21, 22, 36). The data in Fig. 2F highlighted reduced numbers of transferred Th2 cells after airway challenge in lungs of mice protected from AAI by PIT. The simplest explanation for this was therefore that, as with naive T cells, PIT drives the rapid deletion of transferred Th2 cells. However, assessment of transferred OT-II Th2 cell numbers at the time of first i.t. challenge (4 d after PIT, experimental protocol in Fig. 3A) revealed a widespread increase in number and frequency of OT-II cells in spleen, lymph nodes,

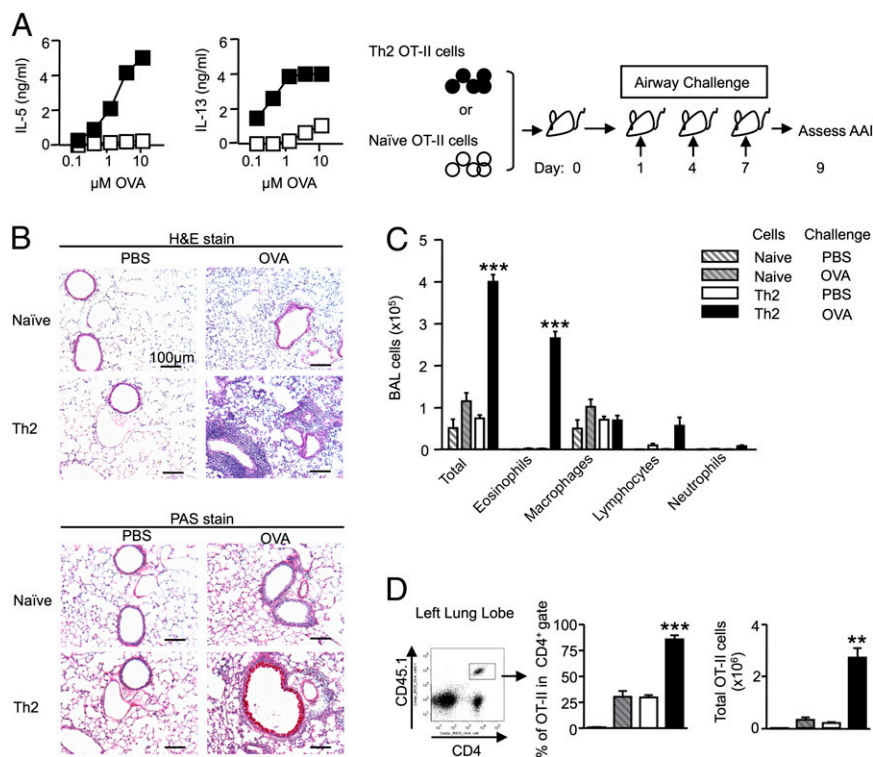


Fig. 1. A Th2 transfer model of OVA-driven allergic airway inflammation. (A) IL-5 and IL-13 production by naive (□) and Th2-polarized (■) CD4⁺ OT-II cells in response to OVA and experimental design for AAI using transfer of 4×10^6 naive or Th2-polarized OT-II cells (see *Materials and Methods* for dose of OVA). (B) Day 9 analysis of H&E and PAS. (C) Differential BAL cell counts. (D) The percentage of OT-II cells in the CD4⁺ population and the total number of OT-II cells in the left lung lobe. Data represent one of two experiments giving consistent results. $n \geq 3$, $**P \leq 0.01$, $***P \leq 0.001$ vs. all other groups.

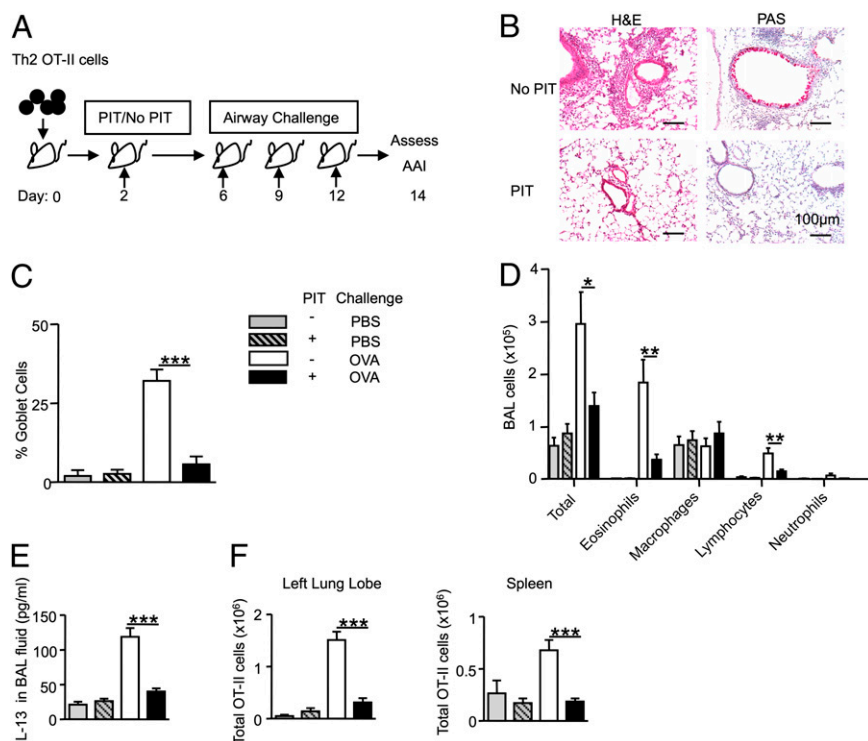


Fig. 2. PIT suppresses AAI driven by preexisting Th2 cells. (A) Experimental design (see *Materials and Methods* for doses of pOVA and OVA). (B) H&E and PAS stains of lung sections. (C) Quantification of goblet cells. (D) Differential BAL cell counts. (E) Concentrations of IL-13 in BAL fluid. (F) Total OT-II numbers in left lung lobes and spleens. "No PIT": mice received PBS alone. Data are pooled from two experiments; consistent data were found in three or more experiments. $n \geq 6$, $*P \leq 0.05$, $**P \leq 0.01$, $***P \leq 0.001$.

and lung (Fig. 3 *B* and *C*). Of particular note was the significant increase in the percentage of OT-II cells in the lung CD4⁺ population, compared with that in distal lymph nodes and spleen (Fig. 3*C*). Importantly, this difference was most pronounced after PIT (Fig. 3*C*).

Our data thus far provided an intriguing scenario in which the protective effects of PIT against Th2-driven AAI (Fig. 2 *B–E*) were associated with ultimately reduced numbers of OT-II cells after allergen challenge (Fig. 2*F*), despite a striking initial enrichment of these cells in the lungs immediately after PIT and before allergen challenge (Fig. 3*C*). Thus, PIT promotes accumulation of allergen-reactive T cells within the lung, but these have substantially impaired pathogenic activity in this relatively compressed model (4 d between exposure to PIT and first airway challenge).

We next sought to understand how long this population of PIT-exposed T cells would persist within the lung. Moreover, given the possible distinct roles for Tem and Tcm in maintaining memory in the presence or absence of allergen exposure, we considered it important to determine whether these two memory populations might behave differently, either (*i*) in their colonization of and persistence in the lung following PIT or (*ii*) in their functional/pathogenic impairment following PIT. Separation of putative Tem and Tcm populations was possible because, although Th2-polarized OT-II cells on the day of adoptive transfer were all CD44^{hi}, they consistently displayed a heterogeneous CD62L profile, including both high- and low-CD62L expressors (Fig. 4*A*). These two profiles are associated with Tem (CD44^{hi}CD62L^{lo}) or Tcm (CD44^{hi}CD62L^{hi}) (27). The presence of antigen-experienced adoptively transferred T cells in nonlymphoid organs is well documented (37, 38), as is the preference for CD62L^{lo} cells to home to nonlymphoid tissues such as the lung, because of the requirement for CD62L expression to facilitate entry into lymph nodes

(32, 33, 39). We therefore predicted that the cells that accumulated in the lung after PIT were derived from the CD62L^{lo} fraction.

PIT-Treated CD62L^{lo} Th2 Cells Persist Long-Term in the Lung. OT-II Th2 cells were FACS sorted into CD44^{hi}CD62L^{lo} and CD44^{hi}CD62L^{hi} populations and transferred individually before PIT (Fig. 4*A*). To compare the longevity of each population, mice were culled 26 d after cell transfer and frequency and number of transferred OT-II cells determined. Even in the absence of allergen airway challenge, the lungs of mice that had received CD62L^{lo} Th2 cells contained markedly elevated frequencies of transferred cells, to the extent that there were greater numbers of OT-II cells in the lung than in the spleen at 24 d after PIT. This was not the case for CD62L^{hi} transfer, although an OT-II accumulation in the lung was evident following PIT (Fig. 4*B*). There were no differences in the percentage of OT-II cells in the CD4⁺ population or in total OT-II cell number, in spleen or lymph nodes in any of the groups, regardless of PIT treatment (Fig. 4*B*). The greater accumulation of transferred CD62L^{lo} cells in the lungs was not, therefore, because of an inherent improved viability.

We concluded that, unlike the previously reported effects of PIT upon naive OT-II cells, there was no evidence for depletion of transferred CD62L^{hi} or CD62L^{lo} populations from the lymphoid organs. Moreover, the numbers of transferred cells in the lung were enriched following PIT. This effect was particularly pronounced for the transferred CD62L^{lo} population. It therefore became even more important to determine how these differences influenced subsequent susceptibility to AAI following allergen airway challenge.

PIT-Treated CD62L^{lo} Th2 Cells Are Least Pathogenic upon Allergen Airway Challenge. The experimental protocol described in Fig. 4 was extended to include airway challenge with OVA, commencing at day 26 (Fig. 5*A*). Despite having the highest numbers

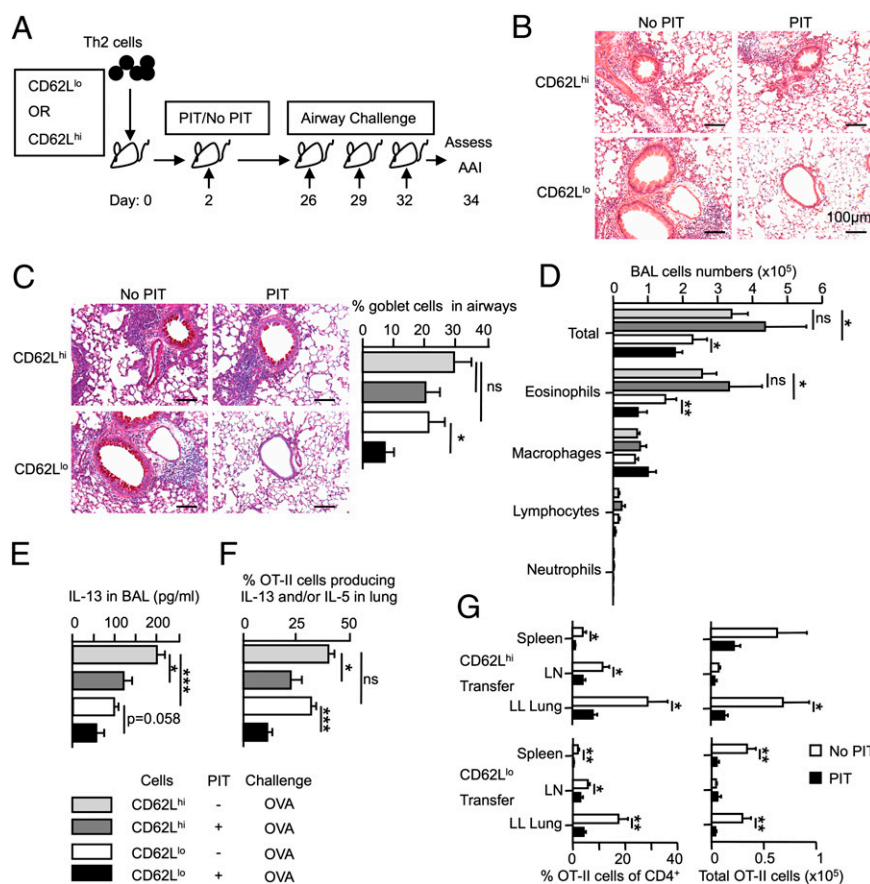


Fig. 5. PIT-treated CD62L^{lo} Th2 cells are least pathogenic upon allergen airway challenge. (A) Experimental design. Mice received 2×10^6 CD62L^{lo} or CD62L^{hi} Th2 cells as in Fig. 4. (B and C) H&E (B) and PAS (C) staining of lung sections and the percentage of goblet cells in the airways. (D) Differential BAL cell counts. (E) Concentrations of IL-13 in BAL fluid. (F) The percentage of IL-5⁺ and/or IL-13⁺ OT-II cells in the left lung lobe after overnight culture in the presence of pOVA. (G) The percentage of OT-II cells in the CD4⁺ population and the total number of OT-II cells in spleen, mediastinal LN (mLN), and left lung lobe. No PIT: mice received PBS alone. Data are pooled from two experiments, giving consistent results. $n \geq 9$, * $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$; ns, not significant.

of PIT-treated CD62L^{lo} cells). This decline in PIT-treated CD62L^{lo} cells continued to the final time point, after the third airway challenge (day 34), but frequencies of OT-II cells in mice that received CD62L^{hi} cells and PIT appeared to stabilize or even increase by this point.

Proliferative Capacity Is Greater in Transferred CD62L^{hi} Cells than in CD62L^{lo} Cells upon Airway Challenge. As would be expected, both transferred populations showed evidence of maintained proliferation 2 d after transfer (Fig. 7). Ki-67 staining 4 d after PIT was indicative of TCR stimulation, but by day 13 after transfer (11 d after PIT), few cells were in cell cycle, indicating that the more pronounced long-term persistence of CD62L^{lo} cells in the lung was not due to a greater/longer proliferative capacity. Following the second airway challenge (day 30), Ki-67 staining was evident in all transferred populations, but the frequencies of Ki-67⁺ cells were highest in non-PIT-exposed CD62L^{hi} cells (Fig. 7), consistent with these cells showing the most elevated frequencies at days 30 and 34, relative to day 26 (Fig. 6). Frequencies of Ki-67⁺ PIT-treated cells on day 30 were below those seen for non-PIT-treated cells (Fig. 7), with the lowest frequencies seen in the PIT-treated CD62L^{lo} population, consistent with this group having the poorest representation of transferred cells in the lung at day 34 (Figs. 5 and 6).

PIT-Exposed CD62L^{hi} Cells Maintain a Heightened Capacity for Cytokine Production. The transferred CD62L^{hi} population showed robust Th2 cell function in the lung after airway challenge (Fig. 5). To be

of relevance to the increased pathology seen in mice receiving these cells, this feature should be evident earlier, at the time of airway challenge. We therefore assessed Th2 cytokine (IL-13 and/or IL-5) production following PIT and at the point of allergen challenge (Fig. 8A). In PBS-treated controls at day 6, cytokine production was highest among CD62L^{lo} cells, consistent with a predicted strong effector function. In contrast, by the time of airway challenge, cytokine production was at least as evident in the CD62L^{hi} group as in the CD62L^{lo} group in the absence of PIT. At days 6 and 13, PIT-exposed CD62L^{hi} cells showed elevated cytokine production relative to controls. This was not seen in PIT-exposed CD62L^{lo} cells and, by the time of airway challenge, these PIT-exposed cells showed lower frequencies of cytokine-expressing cells than their control group. We did not find any evidence for loss of GATA-3 expression in the transferred CD62L^{lo} cells (whether they had been exposed to PIT or not) that could account for their poorer production of Th2 cytokines (Fig. 8B). Importantly, at the time of first airway challenge (day 26) CD62L^{hi} cells showed strong production of IL-5 and IL-13 whether they had been previously exposed to PIT or not (Fig. 8A). We conclude that, whereas transferred CD62L^{lo} cells that are exposed to PIT have diminished Th2 effector cytokine production in the lung by the time of allergen challenge, this key feature of pathogenic activity is actually prompted, and sustained, in transferred CD62L^{hi} cells responding to PIT.

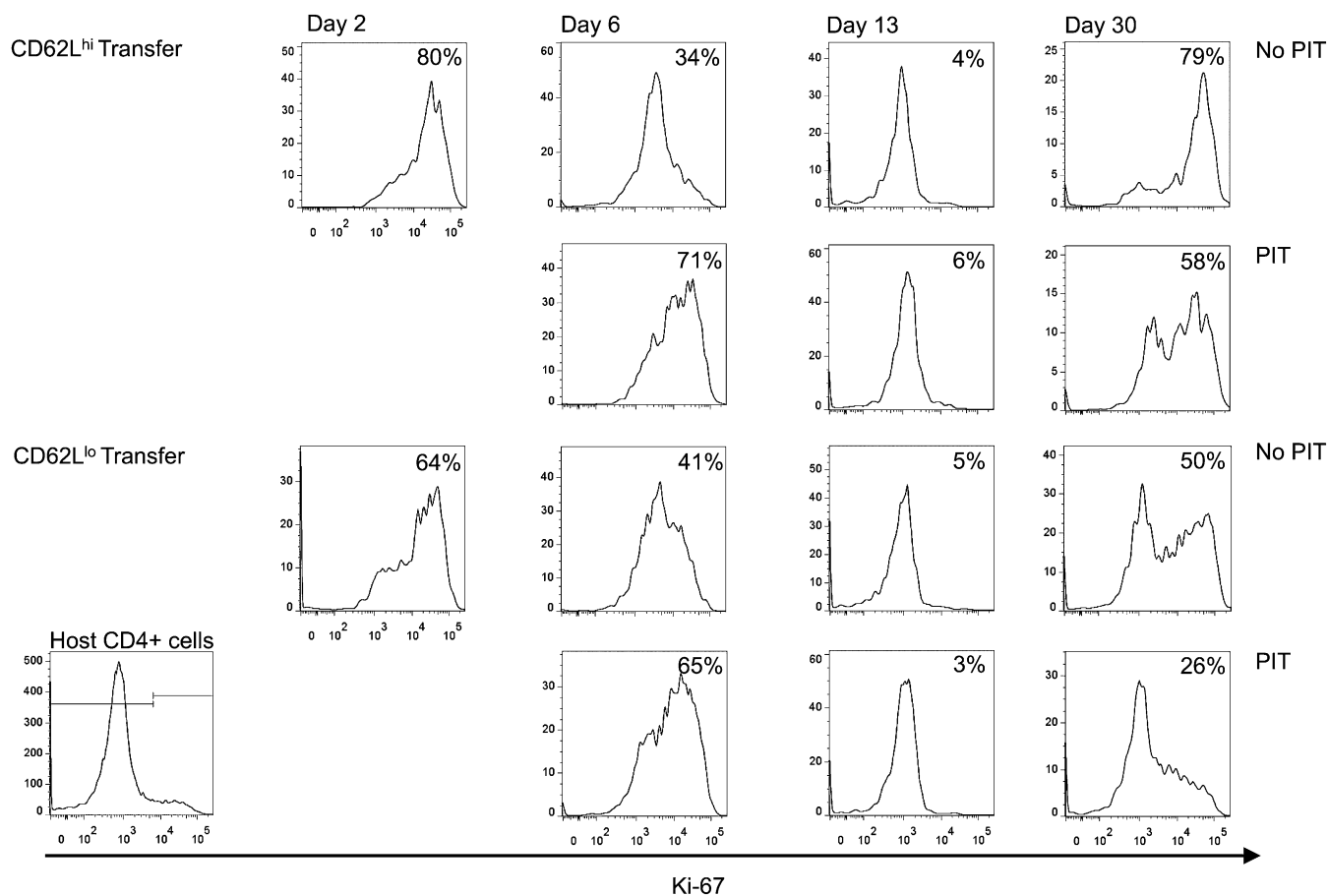


Fig. 7. The effects of PIT upon proliferation of OT-II cells in the lung. Cells were transferred as described in Fig. 6. The percentage of Ki-67⁺ OT-II cells was assessed in lung cell suspensions at each time point. Representative plots are shown with the median percentage for the group shown in each panel. Host CD4⁺ cells were used to set the threshold of positive staining (*Bottom Left* shows an example plot). Data are from three experiments, with three to six mice per group, per time point.

at day 6. The marked difference in effector cytokine capacity in response to PIT—a gradual decline in transferred CD62L^{lo} cells vs. a rapid and sustained gain in transferred CD62L^{hi} cells—is entirely consistent with the different levels of pathology seen following allergen challenge to the lung. A reasonable interpretation of these data is that CD62L^{lo} cells have a more advanced differentiation status and, although PIT does not trigger their rapid deletion through apoptosis (this appeared to occur only at the point of later allergen challenge), PIT does impair their effector function. In contrast, and fitting the Tcm paradigm, CD62L^{hi} cells are less differentiated, allowing the further TCR triggering afforded by PIT to engender more pronounced effector function. The observation that by the point of allergen challenge (day 26) transferred CD62L^{hi} cells had gained effector function in the absence of PIT is of interest. Whether this reflects an enrichment of the minority of transferred cells capable of IL-5 and/or IL-13 production in the lung at day 6 or a gradual gain in this function driven by the lung environment cannot be discerned from these experiments, but warrants further study.

Allergen-reactive CD4⁺ T cells can be found in the blood of both nonallergic and allergic individuals and are predominantly a memory phenotype, but exhibit differences in frequency (often higher in allergic patients) and are Th2 skewed (23, 29, 45, 46). Lymphocytes in BAL from asthmatic patients and controls are overwhelmingly of the memory phenotype (47). The timing of allergen exposure could plausibly be anticipated to influence a patient's allergen-reactive T-cell pool. Indeed, increased frequency of pollen-specific CD4⁺ T cells has been described during pollen

season whereas such fluctuation does not occur for the perennial allergen house dust mite (29–31). Furthermore, a greater frequency of birch-pollen reactive CD4⁺ Tem has been shown in the blood of pollen allergic individuals during pollen season, compared with a greater percentage of Tcm in house dust mite allergic patients (29). Such clinical studies provide valuable information, but can be limited by analysis of peripheral blood (perhaps skewing toward Tcm rather than Tem). The existence of lung resident memory T cells (Trm) may also mean that important changes at the site of inflammation have so far been missed. There is increasing appreciation of the importance of Trm to protective immunity (48, 49) and these are predominantly Tem in both mouse and man (28, 34). Trm in human lungs are cytokine capable (34), and a contribution of such cells to allergic disease is thought likely (28, 50, 51). Our finding that, despite a large and durable accumulation of transferred CD62L^{lo} cells in the lungs of PIT-treated mice, these cells were relatively cytokine incapable and provoked the lowest levels of AAI is therefore reassuring and emphasizes the effectiveness of PIT on these cells.

Clinically, there is limited information on the activation status of T cells before, and after, immunotherapy, and there is no consensus as to the most efficacious means to deliver specific immunotherapy, including PIT, in terms of dose, route, and frequency (40, 52). In light of this study, it is interesting to speculate on how currently favored regimes for delivering allergen immunotherapy, for example multidose regimes (17, 20, 53, 54), affect the dynamics of CD62L expression of Th2 cells and hence their behavior in re-

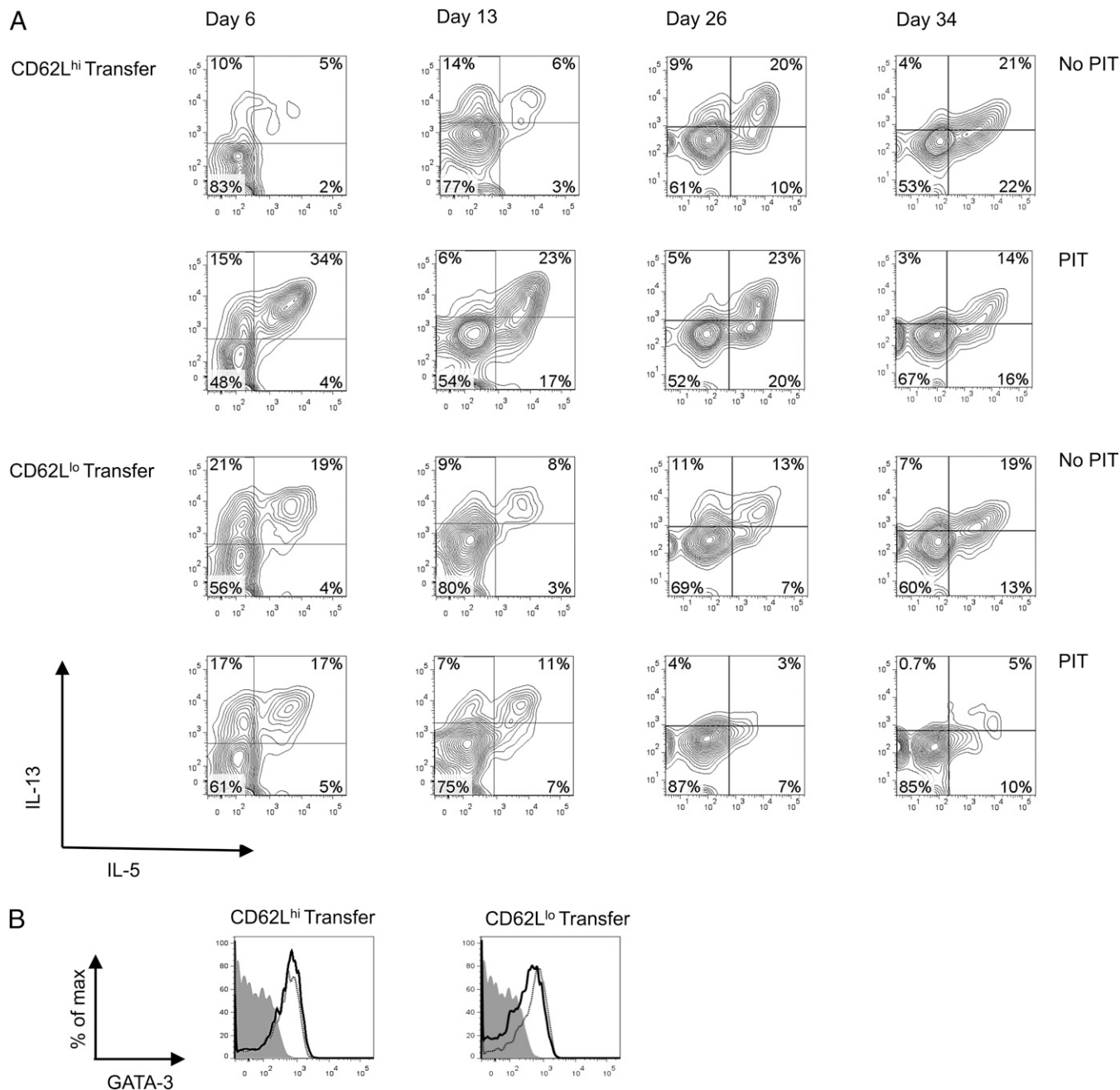


Fig. 8. The effects of PIT upon Th2 cytokine capabilities of transferred CD62L^{lo} or CD62L^{hi} cells in the lung. Cells were transferred as described in Fig. 6. Pooled lung samples isolated on the indicated days were cultured overnight in the presence of pOVA before intracellular staining for cytokines. (A) IL-13 and IL-5 staining of OT-II cells (gated on CD4⁺CD45.1⁺ cells) after culture. (B) GATA-3 expression was assessed by gating on OT-II cells after overnight culture as in A (day 6). Solid line, PIT treated; dotted line, no PIT; shaded histogram, isotype. Data are from three experiments with three to six mice, providing samples for each time point.

sponse to PIT. Here we used a single PIT protocol known to be effective against naive OT-II T cells and found it had limited efficacy in preventing pathogenic activity of Tcm. This therefore provides the impetus, and a tractable system, for further detailed exploration of how different effector and memory T-cell populations might best be controlled by different therapeutic regimens. This should deliver the broadest and most robust protocols for clinical translation.

Materials and Methods

Mice. Congenically identifiable (CD45.1) OT-II transgenic mice with an I-A^b-restricted TCR reactive toward ovalbumin peptide 323–339 (pOVA) (35) were

maintained at the University of Edinburgh. Sex-matched C57BL/6J mice, 6–12 wk old (Charles River), were maintained on chicken egg OVA-free diets. Experiments were conducted under a UK Home Office license and approved by the local ethics review panel.

Antigens. OVA was obtained from Worthington Biochemical Corporation. pOVA was synthesized by Peplogic and reconstituted using sterile water (Sigma-Aldrich).

Th2 Polarization of OT-II Cells. Cells from spleens and lymph nodes of naive OT-II mice were seeded at 4×10^6 cells/mL in RPMI 1640 (Gibco) with 2 mM L-glutamine, 100 units/mL penicillin, 100 μ g/mL streptomycin (all from PAA), 50 μ M β -mercaptoethanol, and 5% (vol/vol) heat-inactivated FCS

(Labtech) with 40 units/mL rIL-2 (PeproTech), 4 ng/mL rIL-4 (PeproTech), 5 μ g/mL anti-IL-12 (BioXcell), 5 μ g/mL anti-IFN- γ (BioXcell), and 10 μ g/mL pOVA. CD4⁺ cells were positively selected after 96 h of culture, or from naive mice, using CD4 (L3T4) MACS Microbeads and an autoMACS Pro Separator (both from Miltenyi Biotec). A total of 4×10^6 cells were adoptively transferred to mice via the tail vein.

FACS Sorting of CD62L^{hi} and CD62L^{lo} Th2 Cell Populations. Th2-polarized, CD4⁺-purified OT-II cells stained with CD4 (Invitrogen; clone RM4.5), CD44 (eBioscience; clone IM7), and CD62L (eBioscience; clone MEL-14) were sorted (BD FACSAria II) into CD4⁺CD44⁺CD62L^{lo} and CD4⁺CD44⁺CD62L^{hi} populations. A total of 2×10^6 cells of either population were transferred i.v. to recipient mice.

Assessing Th2 OT-II Cell Function. A total of 2×10^4 per well Th2 cells [rested for 3 d in 10 units/mL of IL-2 (PeproTech)], or naive CD4⁺ OT-II cells, were cultured with 5×10^5 per well irradiated C57BL/6J splenocytes and OVA (Worthington Biochemical Corporation) in 96-well flat-bottom plates. Cytokines in supernatants were measured by ELISA after 72 h.

Administration of Soluble Peptides. Two days after transfer of OT-II cells, 500 μ g pOVA diluted in sterile PBS (or PBS only as a control) was given i.v. via the tail vein.

Inducing AAI. Mice were anesthetized and 50 μ g of OVA (Worthington Biochemical Corporation), or PBS as a control, was instilled into the trachea. Three i.t. challenges were given 3 d apart. Mice were culled 2 d after final i.t. instillation. In some experiments, a cohort was culled 24 h after the second i.t. instillation to assess OT-II cell location and frequency at this time.

BAL. Lungs were lavaged with 1 mL PBS. BAL fluid was stored at -80°C for cytokine analysis. Cytospins of BAL cells were stained with Quick-Diff (Gamidor Technical Services) and differentially counted at $\times 650$ magnification under blinded conditions (300 cells per slide).

Histological Scoring. Lungs were perfused with PBS to remove blood. In experiments with airway challenge, the left lung lobe was tied off for flow cytometry and removed, and the right lobe was fixed in methacarn and embedded in paraffin. Sections were stained with hematoxylin and eosin (H&E) or Periodic acid-Schiff (PAS). Slides were scored for the presence of goblet cells as previously described (55). Average percentages of goblet cells within 10 consecutive small airways (less than half the width of a field at $\times 200$ magnification) were calculated for each slide, under experimentally blinded conditions.

Isolating Lung Cells. Lung tissue was finely chopped and incubated in collagenase (type I-AS; Sigma-Aldrich) solution for 45 min at 37°C . Tissue was disaggregated via a 20-gauge needle.

Flow Cytometry. Single-cell suspensions from spleens, lymph nodes, and lungs were stained with LIVE/DEAD fixable cell stain (Life Technologies), CD45.1 (eBioscience; clone A20) CD4, CD44, and CD62L (as above). Foxp3 (eBioscience; clone FJK-16a), Ki-67 (eBioscience; clone SolA15), and GATA-3 (BD Biosciences; clone L50-823) staining was carried out using the Foxp3/Transcription Factor staining buffer set (eBioscience). Ki67 and GATA-3 antibodies were applied for 30 min at room temperature whereas Foxp3 antibodies were applied for 30 min at 4°C . GATA-3 was also assessed after overnight culture of lung cells in the presence of 20 μ g/mL pOVA.

Intracellular Cytokine Staining. Lung cells were pooled proportionately for each group and cultured overnight in the presence of 20 μ g/mL pOVA. Intracellular cytokine staining was carried out using the BD Cytofix/Cytoperm kit (BD Biosciences), IL-5 (BD Biosciences; clone TRFK5), and IL-13 (eBioscience; clone ebio13A) antibodies.

Cytokine Detection. Cytokines were detected by ELISA, using IL-5 (BD Biosciences; clone TRFK5) or IL-13 (eBioscience; clone ebio13A) capture antibodies and IL-5 (BD Biosciences; clone TRFK4) or IL-13 (eBioscience; clone ebio1316H) detection antibodies. Binding was visualized using Streptavidin-HRP (R+D Systems) and TMB (Invitrogen). Plates were read at 450 nm, using a BioTek Synergy HT plate reader and Gen5 software (BioTek). IL-13 in BAL fluid was detected using a FlowCytomix analyte detection system (eBioscience).

Flow Cytometric Analysis. Data were collected using an LSR Fortessa with FACS DIVA software (BD Biosciences) and analyzed using FlowJo software (Treestar).

Statistical Analysis. Prism 4 (Microsoft) software was used. Data were analyzed using unpaired *t* tests and *P* < 0.05 was considered significant for all tests.

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