SUPPLEMENTAL MATERIAL

Meiler et al., http://www.jem.org/cgi/content/full/jem.20080193/DC1

SUPPLEMENTAL MATERIALS AND METHODS

Frequency calculation of purified allergen-specific T cells.

The minimum number of cells to start antigen-specific cytokine-secreting $CD4^+$ T cell purification is 5×10^7 cells. 2.5×10^7 cells were stimulated with 0.3 μM of antigen in 5 ml of medium in 6-well plates in duplicates for 12 h. The frequency of allergen-stimulated and unstimulated cells was calculated by dividing the number of purified cytokine-secreting $CD4^+$ T cells by the initial number of $CD4^+$ T cells. Cells were counted by using a 9-mm^2 area of a Neubauer counting chamber and Trypan blue exclusion.

The number of $CD4^+$ cells was calculated as follows in an example of PLA-specific IL-10–secreting $CD4^+$ T cells: PLA-stimulated cells, 47.4×10^6 (cell count from culture after 12 h) × 52.8% (percentage of $CD4^+$ T cells determined by flow cytometry) = 25.02×10^6 $CD4^+$ T cells at the start; unstimulated cells, 47.10^6 (cell count from culture after 12 h) × 53.1% (percentage of $CD4^+$ T cells) = 25.01×10^6 $CD4^+$ T cells at the start.

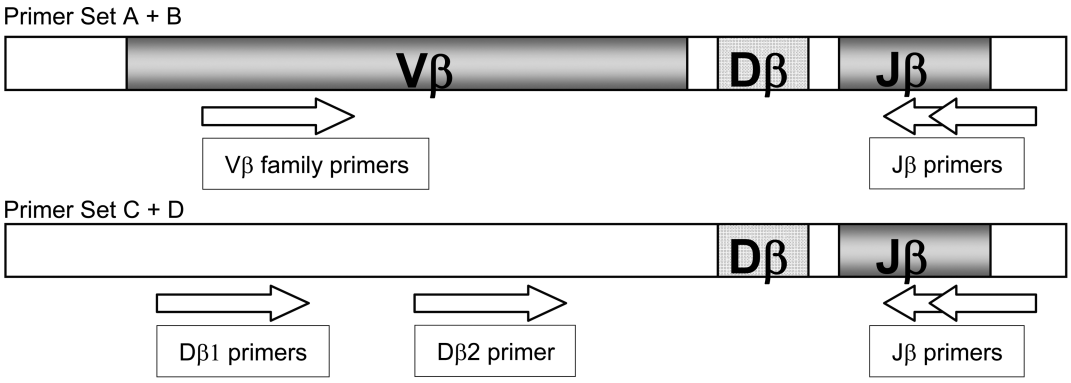
Purified cells were counted and the number of $CD4^+$ IL-10–secreting T cells was calculated as follows: PLA-stimulated cells, 33,000 (cell count after purification) × 92.4% (percentage of purified $CD4^+$ $IL\text{-}10^+$ T cells) = 30,492 purified $CD4^+$ PLA-specific, IL-10–secreting T cells; unstimulated cells, 11,000 (cell count after purification) × 88.4% (percentage of purified $CD4^+$ $IL\text{-}10^+$ T cells) = 9,724 $CD4^+$ and IL-10–secreting T cells without any stimulation.

The $CD4^+$ IL-10–secreting T cell frequency was calculated by dividing the purified $CD4^+$ IL-10–secreting T cell number by $CD4^+$ T cell number at the start of cytokine secretion assay: PLA-stimulated cells, 30,492 divided by 25.02×10^6 = 0.001218 (12.12 cells in 10,000); unstimulated cells, 9,742 divided by 25.01×10^6 = 0.000389 (3.89 cells in 10,000).

The final frequency of PLA-specific IL-10–secreting T cells was calculated by subtracting the unstimulated $CD4^+$ IL-10–secreting cell frequency from the PLA-specific $CD4^+$ IL-10–secreting cell frequency (0.001218 − 0.000389 = 0.000829). That means that 8.29 cells in 10,000 $CD4^+$ cells are PLA-specific, IL-10–secreting, Tr1-like cells.

Specific T cell suppression by IL-10–secreting T cells.

PLA-, Bet v 1–, and Der p 1–specific IL-4–, IL-10–, and IFN-γ–secreting T cells were purified from the same beekeeper. As an example of one experiment, 2×10^5 PBMCs were used per well in 96-well flat-bottom plates in triplicates. These cells consisted of 48% $CD4^+$ T cells (determined by flow cytometry). The frequency of PLA-specific, IL-4–secreting T cells was calculated and found to be 1.1 cells per 10,000 $CD4^+$ cells. This means that there were $2 \times 10^5 \times 0.48 = 9.6 \times 10^4$ $CD4^+$ T cells and $1.1 \times 9.6 = 10.6$ PLA-specific IL-4–secreting $CD4^+$ T cells in PBMCs at the starting point. The PBMCs of a beekeeper obtained after multiple bee stings did not show any proliferation. In the next condition, the frequency of the PLA-specific IL-4–secreting $CD4^+$ T cells was increased by 10 times to 96 cells per well, and a visible allergen-stimulated T cell proliferation was determined by $[^3H]$ thymidine incorporation. In the same experimental conditions, the frequency of IL-10–secreting T cells was detected as 11.6 cells per 10,000 $CD4^+$ T cells. When this was increased from 11.6 to 116 cells per 10,000 $CD4^+$ T cells, PLA-induced T cell proliferation was significantly suppressed. This means that the number of IL-10–secreting T cells was increased from 113 cells per well to $11.6 \times 9.6 = 1{,}130$ cells per well. In summary, PLA-induced T cell proliferation, which was abolished after bee stings, was reconstituted by increasing the numbers of PLA-specific IFN-γ– or IL-4– but not IL-10–secreting T cells. PLA-specific IL-10–secreting T cells suppressed PLA-stimulated and proliferating IL-4– and IFN-γ–secreting–specific T cells. For comparison, Bet v 1– and Der p 1–specific IL-10–secreting T cells did not suppress PLA-stimulated T cell proliferation, demonstrating the specificity of the T cell suppression by PLA-specific IL-10–secreting T cells.



Primer Set	Target	Primers used in the primer sets	Product Size in Basepairs
A	Vβ + Jβ1/2	23 Vβ primers + 6 Jβ1 primers and 3 Jβ2 primers	240-285
B	Vβ + Jβ2	23 Vβ primers + 4 Jβ2 primers	240-285
C*	Dβ1 + Jβ1/2	1 Dβ primer + 13 Jβ primers	285-325
D*	Dβ2 + Jβ1/2	1 Dβ primer + 13 Jβ primers	170-210

* Primer set C and D are amplified in the same tube.

Figure S1. Details of TCR clonality analysis. Simplified diagram of a representative rearranged TCRβ gene on chromosome 7 (7q35) showing the approximate placement of primers used in the *TCRB* gene clonality assay. Multiple consensus DNA primers that target conserved genetic regions within the TCRβ gene were used in three different assay tubes. The first assay tube contains primer set A with different Vβ and joining region (J) β1/2 primers, primer set B in the second assay tube consists of Vβ and Jβ2 primers, and the third assay tube contains primer set C with the diversity (D) β1 and Jβ1/2 primers and primer set D with Dβ2 and Jβ1/2 primers. The conserved regions lie on either side of an area within the V-J region, where programmed genetic rearrangements occur during maturation of all T lymphocytes (*InVivo*Scribe Technologies, Inc.).

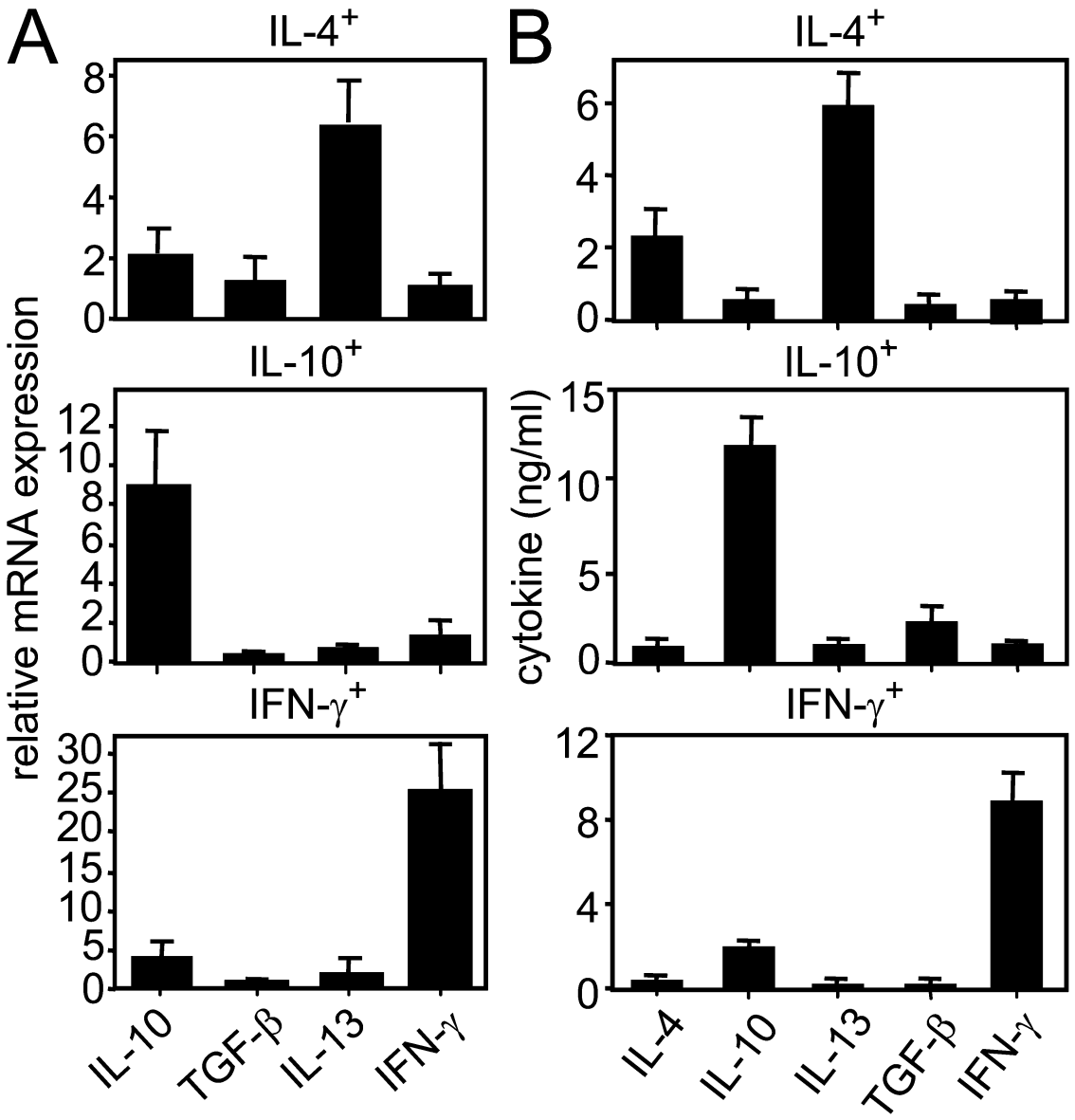


Figure S2. PLA-specific IL-4–, IFN-γ–, and IL-10–secreting T cells represent Th2–, Th1–, and Tr1–like cells, respectively. $CD4^+$ T cells specific to bee venom PLA were isolated according to their IL-4, IFN-γ, and IL-10 secretion profiles. To confirm their cytokine profile, mRNA of IL-10, IL-13, IFN-γ, and TGF-β were quantified immediately after isolation (A). Relative to the housekeeping gene EF-1α, IL-10–secreting T cells expressed significantly high IL-10 mRNA, and IFN-γ–secreting T cells expressed significantly high IFN-γ mRNA. IL-13 mRNA was dominant in IL-4–secreting T cells. The same results were obtained in three independent experiments. Purified allergen-specific cytokine-secreting T cells were expanded for 12 d in the presence of 1-nM doses of growth factors (IL-2 for IFN-γ–secreting T cells, IL-2 and IL-4 for IL-4–secreting T cells, and IL-2 and IL-15 for IL-10–secreting T cells; Novartis). Quantification of the cytokine profile in supernatants by ELISA 72 h after anti-CD2, anti-CD3, and anti-CD28 mAb stimulation demonstrated that these subsets contain Th2-like (IL-4 and IL-13 high), Th1-like (IFN-γ high), and Tr1-like (IL-10 high) cells, respectively (all $P < 0.0001$ compared with other subsets; B). In addition, all three purified subsets consisted of some Th0 cells, which secrete both Th1 and Th2 cytokines, as well as IL-10 and TGF-β. Data are expressed as means + SEM.

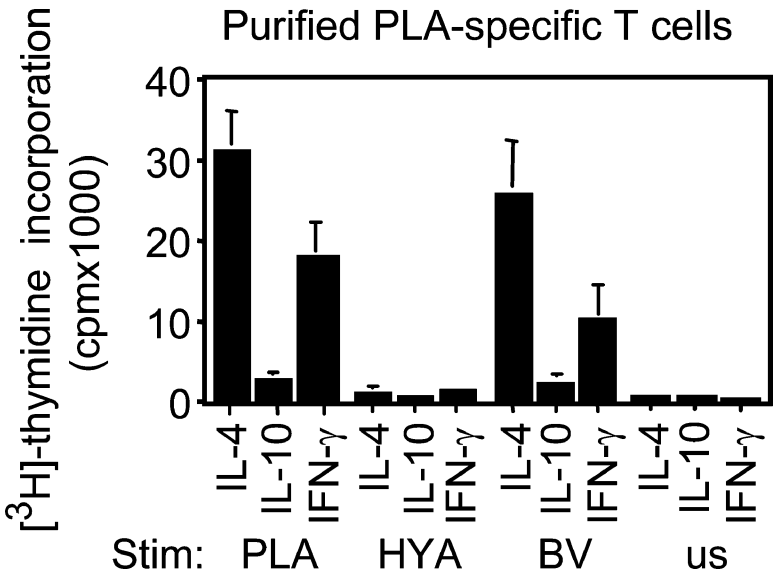


Figure S3. Antigen specificity of purified cytokine-secreting T cells. The antigen specificity of purified cytokine-secreting T cells was determined by stimulation with the allergen that was originally used for stimulation before purification and several control antigens in the presence of autologous APCs. Purified allergen-specific cytokine-secreting T cells were expanded for 12 d in the presence of 1-nM doses of growth factors (IL-2 for IFN-γ–secreting T cells, IL-2 and IL-4 for IL-4–secreting T cells, and IL-2 and IL-15 for IL-10–secreting T cells). T cells purified by certain antigen stimulation did not show any cross-reactivity against control antigens. All three subsets purified by PLA stimulation responded to PLA and whole bee venom but not to hyaluronodise as another bee venom major allergen. [^{3}H]Thymidine incorporation was determined after 5 d. *, $P < 0.001$. One representative of three different donors is shown. Data are expressed as means + SEM.

Table S1. Changes in TCR vβ expression on PLA-specific IL-10-, IFN-γ-, and IL-4-secreting CD4+ T cells before and after bee stings

Beekeeper A
Expanded with PLA

before

TCRvβ	1	2	3	5.1	5.2	5.3	7	8	9	11	12	13.1	13.6	14	16	17	18	20	21.3	22	23
IL-4-secreting cells	1.05	25.80	0.78	3.10	0.16	0.44	1.50	25.30	1.42	0.65	1.85	2.52	0.49	0.81	0.95	3.14	0.37	1.95	0.89	3.00	3.64
IL-10-secreting cells	3.55	3.50	0.18	1.04	0.15	1.19	0.18	1.93	1.40	0.95	0.49	5.86	0.36	0.67	0.00	1.22	0.38	2.74	0.34	0.17	1.18

after

TCRvβ	1	2	3	5.1	5.2	5.3	7	8	9	11	12	13.1	13.6	14	16	17	18	20	21.3	22	23
IL-4-secreting cells	2.00	6.47	2.72	7.71	0.19	0.31	4.09	4.28	7.41	0.56	4.44	3.95	1.31	2.48	1.20	2.99	0.71	2.41	1.11	3.99	0.59
IL-10-secreting cells	6.05	19.50	1.81	4.50	0.22	0.32	0.28	4.99	0.26	0.28	3.18	0.93	4.21	4.39	0.45	0.24	0.16	1.14	1.50	8.97	0.53

Beekeeper A
Expanded with anti-CD3 mAb

before

TCRvβ	1	2	3	5.1	5.2	5.3	7	8	9	11	12	13.1	13.6	14	16	17	18	20	21.3	22	23
IL-4-secreting cells	2.80	10.80	3.03	4.23	0.57	0.97	1.83	13.80	2.46	0.94	3.86	4.40	0.89	0.89	1.99	5.89	1.69	1.69	2.43	3.34	0.77
IL-10-secreting cells	3.14	6.84	0.12	3.60	1.21	0.51	0.74	9.15	2.59	0.62	0.56	9.20	1.59	3.51	0.17	7.30	0.00	6.17	1.11	3.99	0.69
IFN-γ-secreting cells	0.60	3.73	0.07	5.39	0.04	2.16	0.00	0.34	1.52	0.04	9.05	13.60	0.22	4.95	0.30	11.60	5.71	1.74	0.44	0.99	0.37

after

TCRvβ	1	2	3	5.1	5.2	5.3	7	8	9	11	12	13.1	13.6	14	16	17	18	20	21.3	22	23
IL-4-secreting cells	3.46	6.06	1.06	8.46	0.23	0.80	6.06	4.03	1.63	0.31	1.80	4.46	2.37	2.70	0.40	4.09	1.31	1.71	3.09	4.89	0.89
IL-10-secreting cells	8.03	10.30	0.83	2.66	0.09	0.09	0.29	4.77	0.40	0.60	11.90	2.26	7.97	1.43	1.71	0.63	0.26	2.71	0.60	6.86	0.14
IFN-γ-secreting cells	0.49	7.03	0.51	4.69	1.14	0.29	1.34	2.74	0.49	1.34	0.54	4.66	0.49	0.94	0.20	1.17	0.80	1.06	5.20	13.90	0.40

Beekeeper B
Expanded with PLA

before

TCRvβ	1	2	3	5.1	5.2	5.3	7	8	9	11	12	13.1	13.6	14	16	17	18	20	21.3	22	23
IL-4-secreting cells	1.69	5.80	7.74	4.74	0.37	0.26	5.11	1.77	2.06	0.03	5.60	2.11	1.29	1.60	0.57	13.10	0.03	7.03	1.51	4.80	0.77
IL-10-secreting cells	0.18	22.90	19.80	4.31	0.06	0.47	0.95	2.11	3.43	0.00	0.72	6.35	0.00	0.72	0.18	2.06	0.06	0.60	3.14	0.54	0.24
IFN-γ-secreting cells	2.53	6.13	4.74	3.53	0.04	0.39	1.24	11.60	1.00	12.00	2.26	2.21	1.06	3.01	0.55	9.37	0.84	1.25	0.48	1.27	1.25

after

TCRvβ	1	2	3	5.1	5.2	5.3	7	8	9	11	12	13.1	13.6	14	16	17	18	20	21.3	22	23
IL-4-secreting cells	4.09	9.63	3.20	9.03	0.40	0.29	0.31	6.40	2.11	0.03	1.69	3.97	0.51	4.43	0.57	3.40	0.97	0.74	2.46	3.06	0.77
IL-10-secreting cells	6.47	4.57	1.56	3.29	0.32	0.61	0.29	33.50	2.69	0.17	1.18	1.42	2.05	0.09	2.65	1.42	0.41	6.22	1.10	0.78	0.69
IFN-γ-secreting cells	0.16	8.86	3.38	1.18	0.12	0.26	0.26	0.44	0.16	0.00	0.06	1.10	4.32	0.02	0.16	0.44	0.16	0.18	0.38	2.80	0.12

healty controls (n=4)

TCRvβ	1	2	3	5.1	5.2	5.3	7	8	9	11	12	13.1	13.6	14	16	17	18	20	21.3	22	23
mean	3.11	8.85	1.78	4.71	0.63	0.84	1.63	4.94	2.48	0.58	1.83	4.09	2.21	2.06	0.67	5.4	0.78	1.7	2.1	4.63	0.33
Std. Dev.	0.42	1.15	0.89	1.34	0.14	0.08	0.46	0.58	1.54	0.09	0.51	0.33	0.19	0.32	0.23	0.96	0.11	1.18	0.17	3.03	0.03

PLA-specific IL-10–, IFN-γ–, and IL-4–secreting T-cells were purified from two beekeepers before and after multiple bee stings and were stimulated either with PLA (beekeepers A and B) or anti-CD3 mAbs (beekeeper A) in the presence of 3,000 rads of irradiated autologous PBMCs and were expanded with IL-2 for 12 d. Anti-CD3 stimulation was used for beekeeper A, because IFN-γ–secreting T cells did not expand with PLA stimulation. PBMCs of four healthy donors were used for controls. The cells were stained with PE-labeled anti-TCRVβ1, 2, 3, 5.1, 5.2, 5.3, 7, 8, 9, 11, 12, 13.1, 13.6, 14, 16, 17, 18, 20, 21.3, 22, and 23 mAbs; anti-CD3–FITC; and anti-CD4–Pc5 or matching isotype control and analyzed by flow cytometry.

Table S2. Changes in frequency of PLA-specific cytokine-secreting T cells (in 10,000 CD4+ T cells) after bee stings

	IFN-γ before	IFN-γ after	IL-4 before	IL-4 after	IL-10 before	IL-10 after
Beekeeper A	5.4	2.3	2.1	0.9	4.9	14.1
Beekeeper B	4.7	2.2	1.8	0.7	6.2	12.8

These frequencies were used to calculate the frequency of TCRVb-expressing cells in CD4+ T cells demonstrated in Fig. 4 B.