INTERLEUKIN-5 PRODUCING GROUP 2 INNATE LYMPHOID CELLS (ILC2) CONTROL EOSINOPHILIA INDUCED BY INTERLEUKIN-2 THERAPY

by

Frédéric Van Gool¹*, Ari B. Molofsky⁴,⁵*, Malika M. Morar¹, Michelle Rosenzwaig⁶, Hong-Erh Liang², David Klatzmann⁶, Richard M. Locksley²,³,⁴# and Jeffrey A. Bluestone¹,⁴#

¹Diabetes Center, ²Howard Hughes Medical Institute, Departments of ³Medicine, ⁴Microbiology & Immunology, and ⁵Laboratory Medicine, University of California, San Francisco, CA 94143, USA. ⁶AP-HP, Hôpital Pitié-Salpêtrière, Biotherapy (CIC-BTi) and Inflammation-Immunopathology-Biotherapy department (I2B), F-75651, Paris, France

*These authors contributed equally

#Co-Corresponding Authors:

Richard M. Locksley (locksley@medicine.ucsf.edu), 415-476-1559 (phone); 415-502-5081 (fax)
MS 1032B, Box 0795, 513 Parnassus Ave, San Francisco, CA 94143

Jeffrey A. Bluestone (jeff.bluestone@ucsf.edu), 415-476-4451 (phone); 415-476-0816 (fax)
S-115 Box 0400, 513 Parnassus Ave, San Francisco CA 94143
KEY POINTS:

- Tissue resident group 2 innate lymphoid cells are the main cells producing IL-5 and driving eosinophilia in response to low dose IL-2 therapy.
- We described a novel cellular network activated during IL-2 treatment that may lead to a more efficient use of IL-2 in immunotherapy.

ABSTRACT:

Interleukin (IL)-2 promotes regulatory T cell development and function and treatment with IL-2 is being tested as therapy for some autoimmune diseases. However, patients receiving IL-2 treatment also experience eosinophilia due to an unknown mechanism. Here we show that patients receiving low dose IL-2 have elevated levels of serum IL-5 and this correlates with their degree of eosinophilia. In mice, low dose IL-2–anti-IL-2 antibody complexes drove group 2 innate lymphoid cells (ILC2) to produce IL-5 and proliferate. Using genetic approaches in mice, we demonstrate that activation of ILC2 was responsible for the eosinophilia observed with IL-2 therapy. These observations reveal a novel cellular network that is activated during IL-2 treatment. A better understanding of the cross-talk between these cell populations may lead to more effective targeting of IL-2 to treat autoimmune disease.

INTRODUCTION:

Treatment with interleukin-2 (IL-2) has been used for more than two decades to enhance anti-tumor immunity in patients with advanced kidney cancer and melanoma. Unfortunately, this high-dose IL-2 treatment is associated with side effects (capillary leak syndrome, hepatic and
renal dysfunction) limiting its clinical utility. IL-5 induced eosinophilia is one of the most common and unwanted effects observed in cancer patients treated with IL-2-based therapy. Since the discovery of T regulatory cells (Treg), studies in mice have shown that low dose IL-2 therapy actually prevents or ameliorates autoimmune diseases by activating and expanding these cells. These observations were applied in a first series of studies in humans to treat chronic GVHD and hepatitis C virus (HCV)-related vasculitis. These studies showed that low-dose IL-2 treatment could provide clinical benefits for the patient’s disease with minimal side effects. However, in a phase I trial in autoimmune Type 1 diabetes (T1D), low-dose IL-2 plus Sirolimus (an analog of Rapamycin) induced a transient reduction of insulin production, suggesting some residual toxicity, possibly due to toxic effects of the drug on pancreatic beta cells and/or to the activation of non-Treg by IL-2 in this setting.

METHODS:

Mice and Cytokine administration

Red5, YetCre13 and ROSA-DTA mice were described previously and injected with IL-2/anti-IL-2 or PBS. Mice were maintained in the UCSF specific pathogen–free animal facility in accordance with guidelines established by the Institutional Animal Care and Use Committee and Laboratory Animal Resource Center.

Tissue preparation and Flow cytometry

Tissues were processed as previously described and single-cell suspensions were used for flow cytometry analysis with the indicated antibodies.

Clinical studies design and participants
Patient characteristics and studies design for the HCV-related vasculitis and T1D trials have been reported previously\textsuperscript{8,15}.

**RESULTS AND DISCUSSION:**

IL-5 induced eosinophilia is one of the most common unwanted side effects observed with high-dose IL-2 immunotherapy\textsuperscript{4,16,17}. To evaluate if patients treated with low-dose IL-2 also develop eosinophilia, we utilized data from two clinical trials designed to increase Treg cells numbers and induce peripheral tolerance. In the first trial\textsuperscript{8}, 10 individuals with HCV-induced vasculitis received four courses of low-dose IL-2 injections that induced a significant increase in serum IL-5 with a variable change in eosinophil counts, which moderately increased over normal values in 12 of 89 evaluations (Fig1a). However, despite variability and a small number of patients, we observed a strong correlation between increased levels of IL-5 and eosinophils in some patients (Fig1b). Importantly, there was a significant correlation between eosinophil counts and IL-5 plasma levels in those patients that had detectable IL-5 at baseline (Fig.1b, p=0.02). In the second trial\textsuperscript{15}, T1D patients were treated for five days with three different doses of IL-2. The cytokine therapy induced a transient and dose-dependent increase in plasma IL-5 levels, with a cumulative effect after each injection of IL-2 (fig1c). Overall, these data showed that low-dose IL-2 therapy leads to increased blood concentrations of IL-5 and moderate eosinophilia in some patients. However the mechanism(s) involved in this side effect of the IL-2 therapy was unclear.

To determine the mechanism by which IL-2 treatment induced IL-5 and subsequent eosinophilia, we used a newly generated IL-5 reporter mouse\textsuperscript{13}. As in the human studies, analysis of sera showed an increase in IL-5 production following treatment of mice with low-dose
The IL-2/mAb complex (fig1d). The IL-5+ cells were mainly present in non-lymphoid tissues such as lung, visceral adipose tissue (VAT) and pancreas, but not in the spleen, suggesting that the major cells producing IL-5 were not typical circulating lymphocytes (fig1e). After IL-2/mAb treatment, IL-5+ cell number strongly increased, with an average of four to five-fold increase in cell number (fig1e-f). Interestingly, in RAG-/- mice the numbers of IL-5+ cells in the somatic tissues was equivalent or higher to the numbers seen in wild type mice (data not shown). Consistent with the IL-5 data, analysis of eosinophils in IL-2-treated mice showed a pattern with a two to three fold increase of eosinophils in both wild type and RAG-/- animals (fig1g). These results suggest that T- or B-lymphocytes were not necessary for the expansion and/or survival of eosinophils in response to IL-2.

The recent identification of tissue resident group 2 innate lymphoid cells (ILC2), led us to investigate whether these cells were responsible for producing IL-5 in response to IL-2 treatment18. ILC2 lack the expression of lineage-defining markers, express high-level of Gata3, KLRG1, T1/ST2, CD2519,20 and are known to proliferate in response to IL-221,22. After IL-2/mAb therapy, we observed that the majority of cells expressing IL-5 have the characteristics of group 2 ILC (fig1h). At steady state, less than 5% of ILC2 were proliferating based on Ki-67 expression, compared to more than 30% after IL-2/mAb therapy (fig2a). This increased proliferation was associated with a two to five fold increase number of ILC2 (fig2b) and an increased fluorescence intensity of the IL-5 reporter gene (fig1e and 2c). Under these conditions, the CD4+ cells constituted only a minor subset (less than 5%) of the IL-5+ cells (fig2c). Interestingly, the eosinophilia induced by the IL-2 therapy was also observed in RAG-/- mice (fig1g) where the only IL-5 producing cells are ILC2. Under these conditions, we observed an accumulation of tissue ILC2 similar to wild type animals treated with IL-2 (fig2b). These
observations suggested that the eosinophilia generated during this therapy was induced by the activation of ILC2 to produce IL-5.

To confirm this hypothesis, we used a genetic approach to ablate ILC2. Mice with CRE recombinase engineered under the control of the IL-5 locus were crossed with mice carrying a ROSA-flox-stop-diphtheria toxin, thus ablating all IL-5 producing cells from the mice (termed IL-5-deleter)\textsuperscript{13,14}. Deletion of IL-5\textsuperscript{+} cells decreased the numbers of ILC2 by more than 90% without affecting the total T cell count (fig2d-e and data not shown). More importantly, treatment with IL-2/mAb did not increase the number of eosinophils in the IL-5-deleter mice (fig2f). These results suggested that ILC2, which were the major producers of IL-5 in naïve mice, were needed for the eosinophilia induced by IL-2 therapy. However, deletion of IL-5-producing cells also significantly decreased the basal level of the eosinophils; therefore, we decided to use mice with CRE under the control of the IL-13 locus (termed IL-13-deleter). Indeed, fate-mapping techniques have shown that IL-13 is expressed only in a subset of ILC2\textsuperscript{14,23}, and that IL-13 expression in ILC2 is induced upon activation\textsuperscript{13}. Under steady state conditions, most ILC2 expressed low levels of IL-13, leading to a reduction in ILC2 deletion as compared to IL-5-deleter mice (fig2d-g). Furthermore, the frequency of eosinophils was not significantly affected in IL-13-deleter mice, except for minor reductions in pancreatic and VAT ILC2\textsuperscript{14} (fig2h). During IL-2/mAb treatment, ILC2 expansion was abrogated and the percentage of eosinophils were not significantly increased in the IL-13-deleter mice (fig2g-h). These observations confirm the hypothesis that activated ILC2 were the main cell population involved in the development of eosinophilia with IL-2 therapy.
The clinical use of low dose IL-2 therapy is a promising approach to improve peripheral immune tolerance. However, the early results of IL-2 treatment and clinical outcome has been complex. While clinical improvements have been observed in patients with HCV-induced vasculitis, GVHD, T1D patients treated with low-dose IL-2 in conjunction with sirolimus showed a transient β-cell dysfunction despite an increase in Treg. The absence of clinical benefit was associated with an increase in activated non-Tregs (such as natural killer cells and eosinophils) that might have adversely impacted islet function. These observations suggest that a better understanding of the targets of IL-2 therapy would be important in designing and evaluating future clinical studies. By using reporter and deleter mouse model, we provide the first direct evidence that eosinophilia induced by IL-2/anti-IL-2 mAb treatment is due to the activation of tissue resident ILC2 resulting in the release of IL-5. Interestingly, under the same condition, blood ILC2s did not accumulate (data not shown). Since PBMC were the only clinical samples available from patients treated with low-dose IL-2, we monitored serum IL-5 concentration and eosinophils counts as a surrogate readout to evaluate the activation of ILC2. The IL-2 therapy led to increased IL-5 levels in sera and eosinophils numbers among the PBMCs, suggesting that in addition to targeting Treg, low-dose IL-2 therapy also induces the activation of ILC2 that release IL-5 and drive eosinophilia. However, to directly assess the role of ILC2 in the immunologic outcome and clinical efficacy of IL-2 immunotherapy in humans, more experiments must be conducted where access to tissues is feasible. In conclusion, these observations reveal a novel cellular network activated during IL-2 treatment and provide new information that may lead to a more efficient use of IL-2 in immunotherapy and contribute to a better understanding of the side effect induced by this cytokine in the clinic.
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AUTHORSHIP

A.B.M. and F.V.G. designed experiment, performed research, analyzed data and wrote the manuscript; M.M.M. and M.R. performed research and analyzed data; H.E.L. provided reagent, D.K., R.M.L., J.A.B. analyzed data and wrote the manuscript.

Conflict-of-interest disclosure: M.R. and D.K. are inventors of a patent application claiming the use of low-dose IL-2 for treating autoimmune disease, which is owned by their academic institutions and licensed to ILTOOpharma in which they hold share. The authors declare no other competing financial interests.

REFERENCES


FIGURE LEGENDS

Figure 1. Interleukin-2 promotes IL-5 producing group 2 innate lymphoid cells (ILC2s) and induces eosinophilia.

(a) HCV-induced vasculitis patients received IL-2 at 1.5 MIU/day from day 1-5 (course1, C1), then at 3 MIU/day from day 15-19 (C2), 36-40 (C3) and 57-61 (C4). IL-5 fold increase (pg/ml)
and eosinophil counts in Giga/liter were measured just before and after 5 days of IL-2. Normal eosinophil counts in the local lab are 0-0.7 G/L for men and 0-0.5 G/L for women and are showed as dashed lines. Statistical significance of differences between groups was assessed using the Mann Whitney U test. (b) Correlation between increase in IL-5 and eosinophils for the same patients as in (a) (Correlations between eosinophils and IL-5 concentrations were determined by Spearman’s correlation coefficient, p=0.0218). (c) IL-5 fold increase over the time of T1D patients received a 5-day course of placebo or of IL-2 at the dose of 0.33, 1 and 3 MIU per day. (d) Serum IL-5 concentration in Red5 heterozygous mice treated with PBS or IL-2/anti-IL-2 mAb complex administered every other day for 3 doses. (e) FACS plots of IL-5 (Red5 tdTomato reporter) producing cells in the indicated tissues after PBS or IL-2 treatment. (f) Quantitation of CD45+ IL-5+ cells in tissues from IL-5 reporter mice after PBS or IL-2 treatment (VAT: perigonadal visceral adipose tissue). (g) Quantitation of eosinophils (CD45+ CD11b+ SiglecF+) in wild-type (WT) or Rag-deficient (RAG−/−) animals from the indicated tissues after PBS or IL-2/anti-IL-2 treated mice. (h) FACS plots of the lineage-defining markers negative subset expressing IL-5 (Red5) in the pancreas after PBS or IL-2 complex (top panels). Characterization of the Red5 producing cells in the pancreas, pre-gated on lineage-negative, CD45+ cells (lower panels).

* p<0.05, ** p<0.01, *** p<0.001. Figures display means values ± SDM. All data were analyzed by comparison of means using unpaired two-tailed Student’s t-tests. Data is representative of three or more experiments or pooled from 2-3 experiments.

**Figure 2. IL-5/13 producing ILC2 induce eosinophilia in response to IL-2 therapy.**

(a) Quantitation by flow cytometry of ILC2 Ki-67 expression with and without IL-2 treatment. (b) Quantitation of total ILC2 from the indicated strains and tissues, expressed as a fold change
from PBS treated control animals. (c) FACS gating of CD4+ T cells and ILC2 (lin- CD127+ CD25+) and expression of IL-5 (Red5) in the strains and tissues indicated after PBS or IL-2 treatment. (d) Quantitation of IL-5+ ILC2 in IL-5 reporter (red5 R+) and IL-5 deleter animals in VAT, lung and pancreas (e) FACS gating as in (c) for the lung of IL-5 deleter mice treated with IL-2/anti-IL-2 mAb complex. (f) Quantitation of eosinophils in Red5 reporter heterozygous (Red5 R+) or IL-5 deleter animals. (g) Representative FACS gating (top panels) and quantitation (lower panels) of VAT ILC2 in control IL-13 reporter animals (YetCre13) or IL-13 deleter (YetCre13 x Rosa-DTA) animals after IL-2 complexes. (h) Quantitation of eosinophils in IL-13 reporter controls (YetCre13) or IL-13 deleter animals. Black line (PBS), grey line (IL-2/mAb).

* p<0.05, ** p<0.01, *** p<0.001, ns = not significant. Figures display means values ± SDM. All data were analyzed by comparison of means using unpaired two-tailed Student’s t-tests. Data is representative of three or more experiments or pooled from 2-3 experiments.
FIGURE 1

a. 

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p-values: p = 0.0271, p = 0.0036, p = 0.0015, p = 0.0395

b. 

![Graph](image5.png)

Correlation p-value = 0.0218

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c. 

![Graph](image6.png)

IL-5 Fold Increased/Baseline vs. days

- Placebo
- 0.33 MIU
- 1 MIU
- 3 MIU

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d. 

![Graph](image7.png)

Serum IL-5 pg/mL

- PBS
- IL-2/mAb

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e. 

![Graph](image8.png)

IL-5 (Red5) vs. CD45

- LUNG
- PANCREAS
- SPLEEN

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f. 

![Graph](image9.png)

IL-5 CD45 per g (x10^6)

- VAT
- LUNG
- PANCREAS

- PBS
- IL-2/mAb

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g. 

![Graph](image10.png)

% Eosinophils

- VAT
- LUNG
- SPLEEN

- WT
- Rag^/-

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h. 

![Graph](image11.png)

Lineage

- PBS
- IL-2/mAb

Events (% of max)

- Gata3
- CD25

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Interleukin-5 producing group 2 innate lymphoid cells (ILC2) control eosinophilia induced by interleukin-2 therapy

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