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CD25-targeted IL-2 signals promote improved outcomes of influenza infection and boost memory CD4 T cell formation

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Abstract

Interleukin-2 is a pleotropic cytokine with potent pro- and anti-inflammatory effects. These divergent impacts can be directed *in vivo* by forming complexes of recombinant IL-2 and anti-IL-2 monoclonal antibodies (IL-2C) to target IL-2 to distinct subsets of cells based on their expression of subunits of the IL-2 receptor. Herein we show that treatment of mice with a prototypical anti-inflammatory IL-2C, JES6-1-IL-2C, best known to induce $CD25^+$ regulatory CD4 T cell expansion, surprisingly causes robust induction of a suite of inflammatory factors. However, treating mice infected with influenza A virus (IAV) with this IL-2C reduces lung immunopathology. We compare the spectrum of inflammatory proteins upregulated by pro- and anti-inflammatory IL-2C treatment and uncover a pattern of expression that reveals potential beneficial versus detrimental aspects of the influenza-associated cytokine-storm. Moreover, we show that anti-inflammatory IL-2C can deliver survival signals to CD4 T cells responding to IAV that improve their memory fitness, indicating a novel application of IL-2 to boost pathogen-specific T cell memory while simultaneously reducing immunopathology.

Introduction

Interleukin-2 (IL-2) is a critical cytokine for orchestrating optimal immune responses. IL-2 acts as an autocrine T cell growth factor (1, 2) and can signal in a paracrine manner to promote the activation of other leukocyte subsets, most notably NK cells and CD8 T cells (3,

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4). However, IL-2 is also central to the maintenance and function of regulatory CD4 T cells (Tregs) that constrain immune responses and limit immunopathology (5, 6). These divergent activities of IL-2 have been shown in diverse models and have been exploited clinically (6, 7). Many strategies are being developed to specifically engage the pro- versus anti-inflammatory properties of IL-2 in context-dependent situations. For example, exogenously administered IL-2 can be targeted to either the α (CD25) or β (CD122) chain of the IL-2 receptor by using IL-2:anti-IL-2 antibody (Ab) complexes (IL-2C) made with different monoclonal Abs (7–10). In the mouse, the Ab clone S4B6 forms pro-inflammatory IL-2C that preferentially signal cells expressing high CD122, predominantly CD8 T and NK cells, while the anti-inflammatory IL-2C made with Ab clone JES6-1A12 (JES6) targets IL-2 to CD25 expressing cells, most notably Tregs (7) in the steady state.

We recently showed that IL-2 secreted by memory CD4 T cells responding to influenza A virus (IAV) can promote disease symptoms by increasing the production of inflammatory cytokines and chemokines in the lung (11). As part of these studies we treated naive mice or mice infected intranasally with a sublethal 0.2 LD_{50} dose of the mouse-adapted IAV strain A/PuertoRico/8/1934 (A/PR8) for three days with S4B6 IL-2C and found that such treatment induced a remarkably broad inflammatory response that synergizes with IAV infection to exacerbate disease (11). We used this regime of IL-2C treatment as it delivers physiologically relevant IL-2 signals to IL-2 receptor-expressing CD4 T cells that promote memory formation during IAV infection (12), and similar protocols are widely employed in many different murine models.

How JES6 IL-2Cs that target CD25-expressing cells affect inflammatory cytokine and chemokine production systemically as well as in tissues such as the lung is not well-characterized. Here, we determine the impact of JES6 IL-2C on acute inflammation when given to naive mice and to mice challenged with IAV. We confirmed the treatment boosted T reg numbers and innate lymphoid cell populations (ILC) (13–16) in the spleen as well as in the lung. However, JES6 IL-2C treatment drove an acute systemic inflammatory response defined by elevated levels of a diverse suite of cytokines and chemokines detected in serum and in lungs. JES6 IL-2C given to mice also challenged with low dose IAV enhanced levels of IFN-γ paradoxically at the same time as several Th2-associated factors, to levels above those detected in mice receiving either IAV or IL-2C alone. While our previous studies found that treatment of IAV-infected mice with S4B6 IL-2C containing 2 μg of IL-2 results in acute death of all treated mice (11), IAV infected mice treated with JES6 IL-2C all survive infection. Furthermore, JES6 IL-2C treatment reduced the extent of lung immunopathology associated with IAV infection.

Given the differential outcome of JES6 versus S4B6 IL-2C treatment, we directly compared the inflammatory response induced by each in uninfected as well as in IAV infected mice. Our results clearly show shared elements and unique patterns in the inflammatory milieu induced by JES6 IL-2C in the absence and presence of infection, demonstrating a complex governance of cytokine and chemokine expression, especially during IAV infection.

Finally, we asked if JES6 IL-2C could be used to deliver physiological IL-2 signals that are required for memory establishment to conventional CD25-expressing anti-viral CD4 T

effector cells responding to infection. We thus tested if JES6 IL-2C could rescue memory formation by IL-2-deficient (*Il2*$^{-/-}$) CD4 T cells responding to IAV that fail to survive long-term without receipt of IL-2 signals during 5-7 day post-infection (dpi) (12). JES6 IL-2C rescued *Il2*$^{-/-}$ CD4 T cell memory formation to a similar degree as that observed with S4B6 IL-2C (12). Our results thus demonstrate that CD25-targeted IL-2C can deliver physiologically relevant IL-2 signals that promote anti-viral memory CD4 T cell formation while simultaneously promoting tissue integrity during pathogen challenge.

Methods

Ethics Statement

Experimental animal procedures were conducted in accordance with guidelines outlined by the Office of Laboratory Animal Welfare (OLAW), National Institute of Health, USA. Protocols were approved by the Animal Care and Use Committee at Trudeau Institute (Saranac Lake, NY), the Institutional Animal Care and Use Committee of the University of Massachusetts Medical School (UMMS, Worcester, MA), and the University of Central Florida (Orlando, FL).

Mice

BALB/c Thy1.2 or BALB/c Thy1.1 mice were used in experiments when 8 to 12 weeks old. Naïve CD4 T cells were obtained from 5 to 8-week old male or female *Il2*$^{-/-}$ D011.10 Thy1.2 or Thy1.2/Thy1.1 mice originally provided by A. Abbas (UCSF). BALB/c and D011.10 mice were bred in the vivarium of the Trudeau Institute, the University of Massachusetts Medical School, or the University of Central Florida.

Cytokine complex and receptor blockade treatments

Mice were treated for the indicated days with IL-2 complexes (IL-2C) that consisted of 2 μg per day of recombinant IL-2 (ThermoFisher) pre-mixed with 20 μg of anti-mouse IL-2 monoclonal antibody clone JES6-1A12 (JES6) (ThermoFisher), or IL-2C pre-mixed with IL-2 and the anti-IL-2 antibody clone S4B6 (BD Biosciences). In certain experiments, the amount of IL-2 in the complexes was varied, as indicated. Complexes were incubated at room temperature for 20 minutes (min.) before intraperitoneal (i.p.) injection in 200 μL of PBS. Control mice received 200 μL of PBS alone.

For some experiments, mice were treated as indicated with 0.25 mg of anti-CD25 (IL-2Rα) antibody (clone PC-61.5.3, BioXcell) to block IL-2 signaling one day prior to initiation of IL-2C treatment. Antibody was delivered by i.p. injection in 200 μL of PBS.

Virus stocks and infections

Influenza A/Puerto Rico/8/1934 (PR8) (H1N1) originating from stocks prepared at the Trudeau Institute and in use in experiments since 1997, and A/PR8-OVA$_{II}$ (H1N1) from stock obtained from P. Doherty at St Jude's Children's Hospital (17), were produced in the allantoic cavity of embryonated hen eggs at the Trudeau Institute and the lethal dose (LD_{50}), egg infective dose (EID_{50}) or tissue culture infective dose ($TCID_{50}$) characterized. Mice

were infected intranasally under light isoflurane anesthesia (Webster Veterinary Supply) with a sublethal 0.2 LD_{50} dose of virus in 50 μl PBS and morbidity and mortality monitored.

Tissue preparation and flow cytometry

At different time points after IL-2C treatment and or virus infection, blood and lungs were obtained from euthanized animals for Luminex multiplex analysis. Lungs were harvested and homogenized in RPMI 1640 media supplemented with 2mM L-glutamine, 100 IU penicillin, 100 μg per mL streptomycin (Invitrogen), 10 mM HEPES (Research Organics), 50 μM 2-mercaptoethanol (Sigma-Aldrich) and 7.5% fetal bovine serum (Hyclone) and serum collected from blood.

Alternatively, for flow cytometry, mice were euthanized by cervical dislocation followed by exsanguination by perforation of the abdominal aorta. Lungs were perfused by injecting 10 ml of PBS in the left ventricle of the heart. Lungs and spleen were prepared into single cell suspensions by mechanical disruption of organs and passage through a nylon membrane. Flow cytometry was performed as previously described (18) using fluorochrome-labeled antibodies at manufacturer's recommended dilutions for surface staining including anti-Thy1.1 (OX-7), anti-Thy1.2 (53-2.1), anti-CD4 (RM4.5 and GK1.5), anti-CD8 (53-6.7), anti-CD45.2 (104), anti-γδ TcR (GL3), anti-CD3 (17A2), anti-CD25 (PC61), anti-CD11b (M1/70), anti-Gr-1 (RB6-8C5), anti-CD127 (A7R34), anti-CD49b (DX5), and murine hematopoietic lineage antibody cocktail containing anti-CD3 (17A2), anti-CD45R/B220 (RA3-6B2), anti-CD11b (M1/70), anti-TER-119 (TER-119), anti-Ly-G6/Gr-1 (RB6-8C5). Innate lymphoid cells (ILCs) were identified as $CD45^+$, $Lineage^-$, $CD3^-$, $CD90^+$, $CD127^+$ (IL-7R) lymphocytes.

Intracellular staining for FOXP3 and Ki-67 was performed as per manufacturer's instructions with the FOXP3 Transcription Factor Fixation/Permeabilization Concentrate and Diluent (Life, eBioscience) and fluorochrome-labeled anti-FOXP3 (FJK-16s) and Ki-67 (SolA15) antibodies. Analysis was performed using FACS Canto II and LSRII instruments (BD Biosciences) and FlowJo (Tree Star) analysis software.

Detection of inflammatory cytokines and chemokines

Levels of cytokines and chemokines in lung homogenates or serum were determined using mouse multiplex kits (Invitrogen and Millipore) read on a Bio-Plex Multiplex 200 Luminex reader (Bio-Rad) as per manufactures' instructions. The assay sensitivity for 12 of the 14 the analytes presented is below 1 pg/mL, ranging from 0.03 pg/mL to 0.69 pg/mL, and is 1.16 pg/mL and 3.43 pg/mL for the remaining analytes CXCL2 and CCL2, respectively.

Histology

For assessment of immunopathology following viral infection and IL-2C treatment, lungs lobes were isolated and immediately fixed in 10% neutral buffered formalin. Lung samples were subsequently processed, embedded in paraffin, sectioned, placed on L-lysine-coated slides, and stained with Hematoxylin and Eosin (H&E) using standard histological techniques at the Morphology Core at UMMS. Triplicate non-serial sections were graded

blindly from 0 to 4, for the extent of inflammatory cell infiltration and damage of bronchi, arteries or alveoli by a certified pathologist (S. Sell) as described previously (19).

Measurement of Pulmonary Mechanics

Non-invasive whole-body plethysmography (WBP) (Buxco) was employed to measure respiratory rates (breaths per min.), minute volumes (mL per min.), and enhanced pause PenH, on conscious, unrestrained animals following IL-2C treatment. The minute volume is defined as the volume of air exchanged during a 1-min. interval and is calculated as follows [respiratory rate X tidal volume].

CD4 T cell isolation and *in vitro*-primed memory generation

Naïve $CD4^+$ T cells were obtained from pooled spleen and peripheral lymph nodes as previously described (18). Briefly, cells were purified by nylon wool and percoll density gradient separation. CD4 T cells were isolated by positive CD4 MACS selection (Miltenyi). Resulting CD4 cells routinely expressed a characteristic naive phenotype (small size, $CD62L^{hi}$, $CD44^{lo}$ and $CD25^{lo}$) >97% TcR^+. T_H1-polarized effectors were generated *in vitro* as described (20). Briefly, naïve *Il2*$^{-/-}$ CD4 T cells were cultured with an equal number of irradiated APC ($2x10^5$ per mL) in the presence of exogenous IL-2 (20 ng per mL), 2 ng per mL IL-12 (Peprotech), 10 μg per mL anti-IL-4 antibody (11B11; Bioxcell), and 5 μM OVA_{II} peptide. *In vitro*-primed memory cells were obtained by thoroughly washing effector cultures at 4 days and re-culturing the cells in fresh media for at least 3 days in the absence of Ag and exogenous cytokines. Live cells were isolated by Lympholyte separation (Cedarlane). All donor CD4 T cells were adoptively transferred in 200 μl phosphate buffered saline (PBS) by intravenous (i.v.) injection. A number of donor cells previously determined to be detectable at the memory phase, 2×10^6, was transferred. Donor cell injection and viral infection occurred on the same day.

Statistical analysis

Group sizes of n = 3 to 6 were employed for all experiments. For Unpaired, Students *t*-tests, $\alpha = 0.05$, were used to assess whether the means of two normally distributed groups differed significantly. One-way ANOVA analysis with the appropriate multiple comparison post-test, Bonferroni's or Tukey's, was employed to compare multiple means. All error bars represent the standard deviation. Significance is indicated as * $P < 0.05$, ** $P < 0.005$, *** $P < 0.001$, **** $P < 0.0001$.

Results

JES6 IL-2C induce systemic inflammation when delivered to unprimed mice

We first delivered JES6 IL-2C containing 2 μg of recombinant murine IL-2 to naive mice by intraperitoneal (i.p.) injection for 3 consecutive days. This is the same treatment regime we used to test the impact of S4B6 IL-2C during IAV infection in our previous study (11). The mice were analyzed on the fourth day after initiation of treatment and were compared to control mice receiving PBS. First, we confirmed the expected activity of JES6 IL-2C in dramatically increasing the number of $CD25^+$ $FOXP3^+$ CD4 Tregs in the spleen (Fig 1a **and** b). JES6 IL-2C treatment significantly increased the mean expression of CD25 on $FOXP3^+$

CD4 T cells (Fig 1c). We found the upregulated CD25 expression on FOXP3$^+$ T regs in JES6 IL-2C treated mice to associate with roughly a 2-fold increase in the frequency of FOXP3$^+$ cells high for the proliferation marker Ki-67 (Fig 1d). In JES6 IL-2C treated mice, we also found small but significant increases in total CD4 T cells but not CD8 T cells, a 2-fold increase in NK cells, and a 4-fold increase in γδ T cell numbers (Fig 1e). These results for CD8 T cells and NK cells are not as marked as those observed previously where CD8 T cells and NK cells expand more than 4 -and 16-fold, respectively, following S4B6 IL-2C administration (11). Given published observations of IL-2-dependent expansion of ILC (13–16), we also assessed whether ILC were impacted by IL-2C treatment. JES6 IL-2C administration significantly increased ILCs in a manner consistent with previous findings (13–15). The gating strategies used are shown in Supplemental Fig 1.

We next assessed protein levels of a broad array of cytokines and chemokines in the serum on the fourth day following JES6 IL-2C treatment and compared levels to those detected in mice receiving PBS alone. Surprisingly, despite its widespread use as an anti-inflammatory agent, JES6 IL-2C treatment significantly increased levels of a number of prototypical pro-inflammatory factors including TNF, IL-1, IL-6, and IFN-γ (Fig 2a). Treatment also enhanced levels of cytokines typically associated with Th2 and ILC responses including IL-4, IL-5, IL-13, as well as IL-10 (Fig 2b).

JES6 IL-2C induce high levels of lung inflammation

Given that the lung is particularly sensitive to IL-2-driven inflammation (11), we analyzed the impact of JES6 IL-2C treatment on cellular subsets and inflammatory mediators in the lung. As compared to the spleen, which increases in total cellularity following IL-2C treatment by 2–3-fold, significant increases in total cellularity in the lungs were not observed (data not shown). However, similar to the expansion of CD25$^+$ FOXP3$^+$ CD4 T cells (Tregs) in the spleen following JES6 IL-2C treatment, a robust increase in CD25$^+$ FOXP3$^+$ CD4 T cells was observed in the lung (Fig 3a **and** b). A small but significant increase in lung γδ T cells and a 3-fold expansion of ILC were also detected, whereas total CD4 and CD8 T cells, and NK cells numbers were not impacted (Fig 3c). Nevertheless, most of the inflammatory factors that were found to be elevated in the serum in Fig 1 were also detected at higher levels in the lungs of mice treated with JES6 IL-2C versus in control animals (Fig 3d **and** e).

We titrated the amount of recombinant IL-2 delivered during IL-2C treatment and found that while some factors were significantly enhanced when less IL-2 was used, the 2 μg dose was required to see robust, widespread effects in the lung (Supplemental Fig 2). To confirm that the IL-2C promote inflammatory responses by binding to CD25$^+$ cells, we pretreated mice with blocking antibody against CD25 by i.p. injection and the next day administered JES6 IL-2C for 3 consecutive days. Blocking CD25 abrogated the impact of the JES6 IL-2C (Supplemental Fig 2), confirming that IL-2C binding to the CD25 receptor is required and ruling out that contaminants in reagents or unexpected binding of IL-2 to the CD122 component of the IL-2 receptor, or other receptors, is responsible for the proinflammatory impacts observed.

The factors detected in the lungs in Figure 3 could arise from local impacts of JES6 IL-2C or may have originated from systemic cellular sources. We thus delivered the IL-2C to mice by

intranasal (i.n.) administration to analyze the local versus systemic impact. We observed markedly higher levels of inflammation at 4 days post-treatment in the lung versus in the serum following i.n. IL-2C administration, strongly supporting that $CD25^+$ cells in the lungs can respond to IL-2 and promote strong local inflammation in the absence of pathogen infection (Supplemental Fig 3). These results demonstrate the acute induction of pro-inflammatory mediators in the lung by the JES6 IL-2C and reinforce that the lung environment is particularly sensitive to IL-2-induced inflammatory signals (9, 11).

JES6 IL-2C boost inflammation but restrain immunopathology during IAV infection

Given the unexpected ability of JES6 IL-2C to concurrently boost T regs and lung inflammation, we infected mice with a low dose of IAV and on the same day, initiated treatment with IL-2C for three days and assessed infection outcomes. Only one inflammatory cytokine, IFN-γ was detected at markedly higher levels in the lungs of IAV infected mice treated with JES6 IL-2C than in mice infected with IAV or treated with JES6-IL-2C alone (Fig 4a). In contrast, treatment of IAV-infected mice with S4B6 IL-2C i.p. in this model drives heightened lung levels of many inflammatory cytokines and chemokines (IL-6, IFN-γ, IL-17, CCL2, CCL3, and G-CSF) beyond those seen in mice treated with IL-2C alone or in mice only infected with IAV (11). We also observed significant but more restrained increases in the levels of IL-1α, IL-4, IL-5, IL-13, and IL-10 in the lungs of IAV infected mice treated with JES6 IL-2C (Fig 4b).

We thus next tested whether IL-2 targeted to $CD25^+$ cells would impact survival following sublethal IAV challenge. We previously found that S4B6 IL-2C administration for 4 instead of 3 days resulted in the acute death of all mice challenged with a normally sublethal dose of IAV without altering immunopathology in the lungs (11). When the same treatment regime was employed, no mice that received JES6 IL-2C and sublethal IAV infection succumbed to infection (Fig 4c). Remarkably, despite the pro-inflammatory impact of the JES6 IL-2C in terms of induction of soluble factors, there was a striking decrease in the extent of histological changes observed in the lungs of mice treated with JES6 IL-2C and IAV versus mice infected with IAV alone. This was most apparent in terms of reduced bronchial inflammation, which was virtually absent in IAV-challenged mice treated with JES6 IL-2C versus in mice infected with IAV alone (Fig 4d and Supplemental Fig 4). These results reveal a surprising disconnect in terms of levels of soluble factors in the lung typically associated with damaging inflammation and the degree of immunopathology observed during acute pulmonary viral infection.

Even in the absence of IAV infection, S4B6 IL-2C-induced inflammation correlated with acute impairment of respiratory mechanics (11). We therefore tested the extent to which JES6 IL-2C treatment in the absence of IAV infection impacted lung function of mice. Despite the induction of inflammation summarized in Figure 2, JES6 IL-2C did not affect breathing as measured by several parameters (Fig 4e), which we found to be significantly altered by S4B6 IL-2C administration (11).

Distinct and overlapping patterns in JES6 versus S4B6 IL-2C induced inflammation

Our results here and in our previous work (11) demonstrate that S4B6 and JES6 IL-2C both enhance the production of soluble mediators of inflammation, but have dramatically different impacts on respiratory functions and immunopathology. We thus asked if we could identify patterns within the inflammatory responses induced by treatment with these IL-2C. We first compared the relative impact of S4B6 and JES6 IL-2C treatment on cytokines and chemokines detected in the lung in the absence of infection within the same experiment. We present heatmaps based on average protein expression to better visualize and compare the scope of the two distinct inflammatory responses. Fig 5a summarizes the expression patterns of analytes significantly upregulated in the lung by either JES6 or S4B6 IL-2C treatment. A complex pattern of overlapping and unique induction of distinct factors is evident. Both complexes induce the upregulation of the majority of mediators assessed and in general, the upregulation of analytes impacted by both treatments was higher with S4B6 versus JES6 IL-2C. In the absence of infection, JES6 IL-2C uniquely induced higher levels of IL-1α, IL-4, and IL-13. These results support that while S4B6 and JES6-based IL-2C have some overlapping impacts on acute inflammation, each IL-2C also induces a distinct suite as well as different levels of proinflammatory factors.

We reasoned that this approach may provide insight into the beneficial versus most deleterious elements of the ‘cytokine storm’ associated with detrimental outcomes of severe IAV infection (21). We thus compared levels of proinflammatory factors in lung homogenates of groups of mice on day 4 after either infection with low-dose IAV alone, or infection with IAV and treatment for 3 days with either S4B6 or JES6 IL-2C. A heatmap summarizing those inflammatory factors upregulated by either IL-2C treatment versus levels detected in mice only challenged with IAV is shown in Figure 5b. Improved outcomes associated with reduced immunopathology following treatment with JES6 IL-2C during IAV infection correlate with increased expression of IL-4, GM-CSF, and CXCL2. Conversely, detrimental outcomes in IAV infected and S4B6-treated mice was associated with higher levels of many classic pro-inflammatory factors including TNF, IL-6, CCL2, and CCL3 and IL-1β. Thus, while there is considerable overlap in the inflammatory signatures in IAV-infected mice that are treated with S4B6 and JES6 IL-2C, unique patterns in the response are evident (Fig 5c).

JES-6 IL-2C can signal to CD4 T cells to rescue memory formation

In addition to driving inflammation and modulating diverse lymphocyte populations, IL-2 is a key signal in promoting T cell memory (22, 23). We previously showed that the IL-2-dependent signals required for IAV-specific CD4 T cells responding to IAV to form memory could be delivered to *Il2*$^{-/-}$ CD4 T cells by treating mice with S4B6 IL-2C from 5-7 dpi (12). IL-2 signals promote upregulation of the IL-7 receptor (CD127) on the surface of effector CD4 T cells at 7 dpi, thus increasing their memory fitness versus cells expressing less IL-7 receptor (24, 25). Given that JES6 IL-2C reduce immunopathology associated with IAV infection while S4B6 IL-2C instead promote immunopathology (11), we asked if JES6 IL-2C could be employed to rescue memory formation from *Il2*$^{-/-}$ CD4 T cells responding to IAV. We thus treated recipients of *Il2*$^{-/-}$ DO11.10 cells that were challenged with PR8-

OVA_{II} with JES6 or S4B6 IL-2C between 5-7 dpi and assessed donor cell recovery at 7 dpi, the peak of expansion, and at 28 dpi, a memory timepoint.

S4B6 and JES6 IL-2C both increased CD25 expression on the surface of *Il2*$^{-/-}$ donor CD4 T cells at the effector phase of the response, though the upregulation associated with JES6 IL-2C was not significantly enhanced compared to untreated mice, perhaps due to binding of the JES6 IL-2C to CD25 (Fig 6a **and** b). Nevertheless, S4B6 and JES6 IL-2C similarly upregulated CD127 expression versus expression on donor cells in mice receiving PBS alone (Fig 6c **and** b). At 28 dpi, we observed near identical rescue of memory formation from the *Il2*$^{-/-}$ donor cells in the spleen, dLN, and especially the lung, where the number of donor cells was about 2 logs increased in mice receiving either IL-2C versus in mice not receiving IL-2C (Fig 6c). These results indicate that JES6 IL-2C delivered systemically can efficiently deliver pro-memory IL-2 signals to CD4 T cells responding *in vivo*.

Discussion

Although using IL-2 to promote or inhibit immune responses in clinical settings is gaining momentum, how IL-2 acts to shape complex inflammatory responses is still not well-understood. Furthermore, given that IL-2 available for paracrine consumption during immune responses can signal cells expressing high CD122 and/or high CD25, an analysis of how CD25-targeted IL-2 impacts inflammatory responses is required. This is also an important consideration for the myriad of experimental models that use IL-2C administration as a tool for the targeted expansion of specific subsets of lymphocytes, as this strategy could also have off target effects that impact the results observed. Indeed, we showed recently that IL-2 produced by CD4 T cells responding to IAV, or S4B6 IL-2C given to IAV challenged mice, markedly enhances a broad spectrum of inflammatory cytokines and chemokines both systemically and in the infected lung (11). This IL-2-induced inflammatory response correlated with reduced lung function, less efficient viral clearance, and enhanced weight loss. We show here using the same experimental system that administration of JES6-based IL-2C also drives a strong, acute inflammatory response under steady-state conditions and during viral infection. However, in contrast to results observed with S4B6 IL-2C, JES6 IL-2 administration correlates with improved outcomes after IAV infection.

We provide several novel findings demonstrating that CD25-targeted IL-2 complexes delivered systemically induce a broad range of inflammatory factors systemically and in the lung in otherwise naive mice. An even stronger local response in the lung is generated upon intranasal JES6 IL-2C administration. These observations are surprising for two reasons. The first being that expression of CD25 is most-often tied to lymphocytes in activated states, most notably on T cells in which high CD25 levels are maintained only short-term following antigen stimulation, and relatively few highly activated cells are expected to populate the steady state. The second is the well-known ability of JES6 IL-2C to selectively promote $FOXP3^{+}$ Treg expansion, as this subset constitutively expresses CD25, an outcome commonly associated with anti-inflammatory impacts.

JES6 IL-2C have recently been used to expand innate lymphoid cells *in vivo* (13–15, 26). We speculate that ILC contribute to the 'Th2'-associated cytokines (IL-4, IL-5, and IL-13) induced by JES6 IL-2C administration. In addition, as ILC have been implicated as key players in lung repair following IAV challenge, the activation of ILC by JES6 IL-2C may contribute to the reduced immunopathology seen in our studies (27, 28). Our own results (unpublished) as well as other reports (29) indicate that $FOXP3^+$ T reg cells have a minimal impact on outcomes of primary IAV infection and play a greater role during secondary infection, where stronger T cell responses in the lung have a greater capacity to cause immunopathology. Interestingly, in addition to CD4 T cell-derived IL-2, ILC3-deived IL-2 has recently been shown to promote T reg homeostasis in the small intestine through an inflammatory axis dependent upon the production of the inflammatory cytokine IL-1 (30). Lymphoid tissue inducer-like ILC1s and lung ILC3s are also capable of producing IL-2 (26, 31) and whether they similarly promote T reg homeostasis remains to be determined. JES6 IL-2C administration also induces a marked expansion of γδ T cells in the spleen and a significant but smaller response in the lung. In contrast to the beneficial outcomes reported here, IL-2 stimulation of γδ T cells and the subsequent production of IL-1 has recently been reported to compromise lung integrity (32). The opposing actions of IL-2 signals and the outcome of inflammatory cytokine production are perplexing and further experiments are thus needed to address the precise roles of ILC, T regs, and γδ T cells in the responses summarized here.

Our results highlight an unexpected disconnect between the detection of inflammatory factors at a site of infection and the degree of histological changes observed. This finding prompted us to probe whether patterns could be identified in the inflammatory milieu induced by S4B6 IL-2C that correlate with worsened outcomes (11), and with JES6 IL-2C that correlate with improved outcomes. Indeed, we found striking patterns of inflammation driven by IL-2 that correlated with improved outcomes (higher IL-4, GM-CSF, and CXCL2) or acute death (IL-1β, IL-6, TNF, CCL2, CCL3, and CXCL10). Our analysis may provide a roadmap to begin to determine positive versus negative elements of the 'cytokine storm' associated with severe IAV infection, and thus an approach to develop novel therapeutic interventions to improve clinical outcomes.

Finally, we show that JES6 IL-2C that target CD25 can be used to deliver pro-memory IL-2 signals to CD4 T cells responding to infection *in vivo*. This may at first glance be surprising given that the CD25 subunit of the IL-2 receptor lacks cytoplasmic signaling capacity. However, given that JES6 IL-2C are well-known to stimulate proliferation of T regs, which is supported here by the observation that the majority of T regs in treated mice are high for the proliferation marker Ki-67, and given that we show that pre-treatment of mice with anti-CD25 antibody abrogates the impacts of JES6 IL-2C, we surmise that the CD25-dependent binding of the IL-2C is able to stimulate similar signaling as S4B6 IL-2C. We stress that induction of different patterns of inflammatory cytokine and chemokine production by JES6 and S4B6 IL-2C but similar rescue of memory formation from $Il2^{-/-}$ CD4 T cells suggests that it is the direct impact of IL-2 signals on the CD4 T cells, and not other aspects of IL-2-induced inflammation that is responsible. This agrees with findings using an adoptive transfer model that show that the inflammatory milieu associated with IAV has a minimal effect in promoting CD4 T cell memory (33). CD25-targeted IL-2 signals may thus be

developed into powerful clinical approaches to simultaneously improve T cell memory while protecting tissue integrity.

In summary, we provide novel data indicating that prototypical anti-inflammatory IL-2C made with recombinant IL-2 and the anti-IL-2 antibody clone JES6-1A12 are capable of driving a robust, acute systemic inflammatory response when administered i.p., and a local inflammatory response in the lungs when delivered i.n.. Surprisingly, this inflammatory response improves rather than worsens immunopathology in the lung during IAV infection. Comparing the patterns of cytokine and chemokine upregulation by JES6- and S4B6-based IL-2C demonstrates remarkably stable hallmarks in both the steady state and during infection. Our analysis provides an avenue for determining the most detrimental elements of the ‘cytokine storm’ induced by influenza versus those aspects that may help to counter tissue-damage, or that correlate with this activity. Our studies also suggest that properly timed IL-2 signals can be used to simultaneously protect against tissue damage and promote robust CD4 T cell memory during IAV infection.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Key points

- Treg boosting IL-2 complexes induce inflammatory factors systemically and in lungs
- Treg boosting IL-2 complexes reduce immunopathology following IAV infection
- Treg boosting IL-2 complexes promote CD4 T cell fitness to survive to form memory

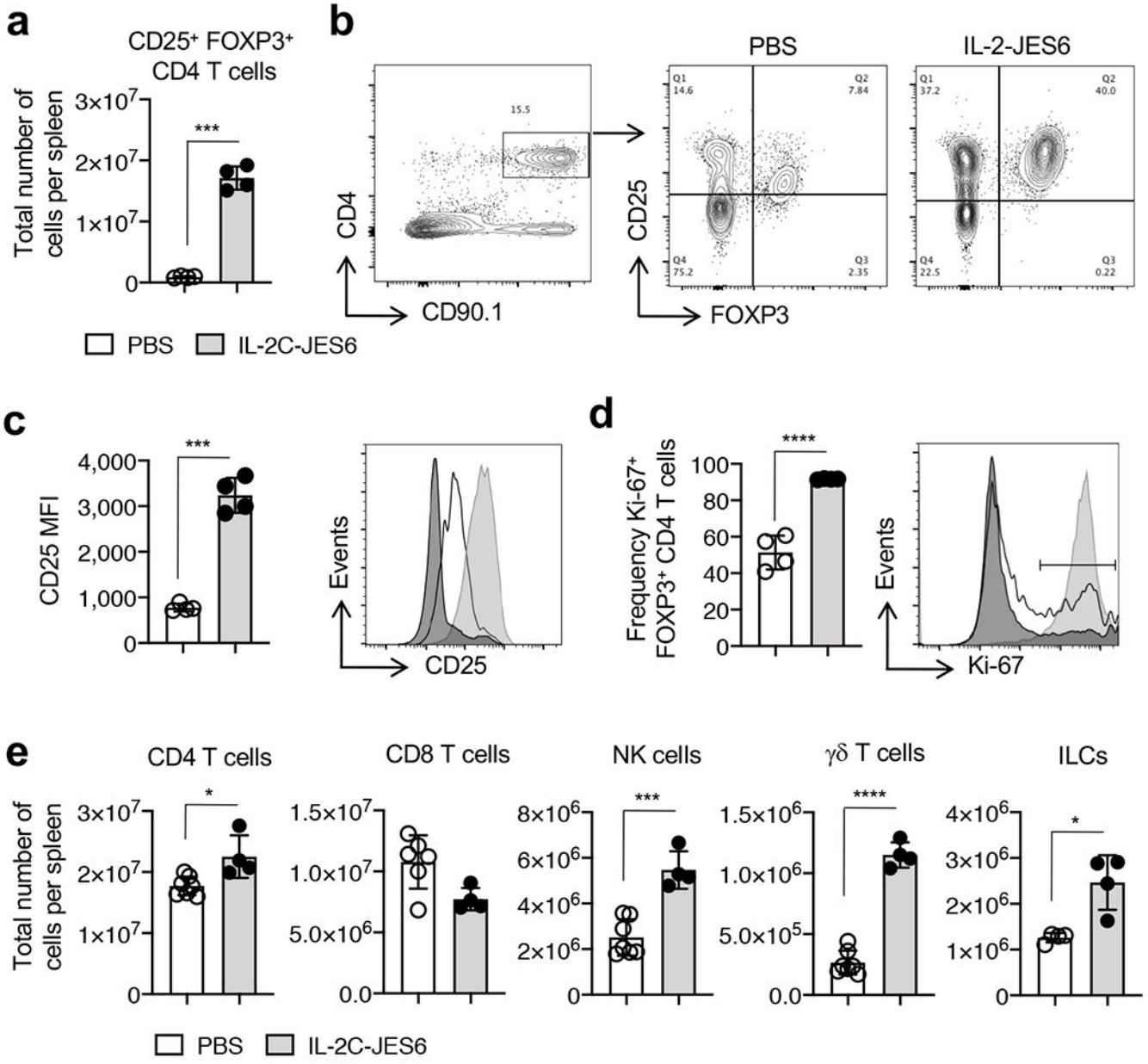

Figure 1:

JES6 IL-2C treatment increases diverse lymphocyte subsets. Mice were treated for 3 days with JES6 IL-2C and on the fourth day spleens were analyzed by flow cytometry. (**a**) The total number of regulatory CD4 T cells ($CD25^+$ $FOXP3^+$) and (**b**) representative staining from untreated and treated mice of CD25 and FOXP3 co-staining. **(c**) CD25 mean fluorescence intensity on $FOXP3^+$ CD4 T cells and **(d)** frequency of Ki-67^+ $FOXP3^+$ cells with representative staining (dark shaded histograms are total CD4 T cells). **(e**) Total numbers of CD4 T cells, CD8 T cells, NK cells, ILCs, and γδ T cells in mice treated only with PBS (white, n = 6) or with JES6-IL-2C (grey, n = 4). Representative results from 1 of 3 replicate experiments and * $P < 0.05$, *** $P < 0.001$, **** $P < 0.0001$ following Students *t*-test analysis.

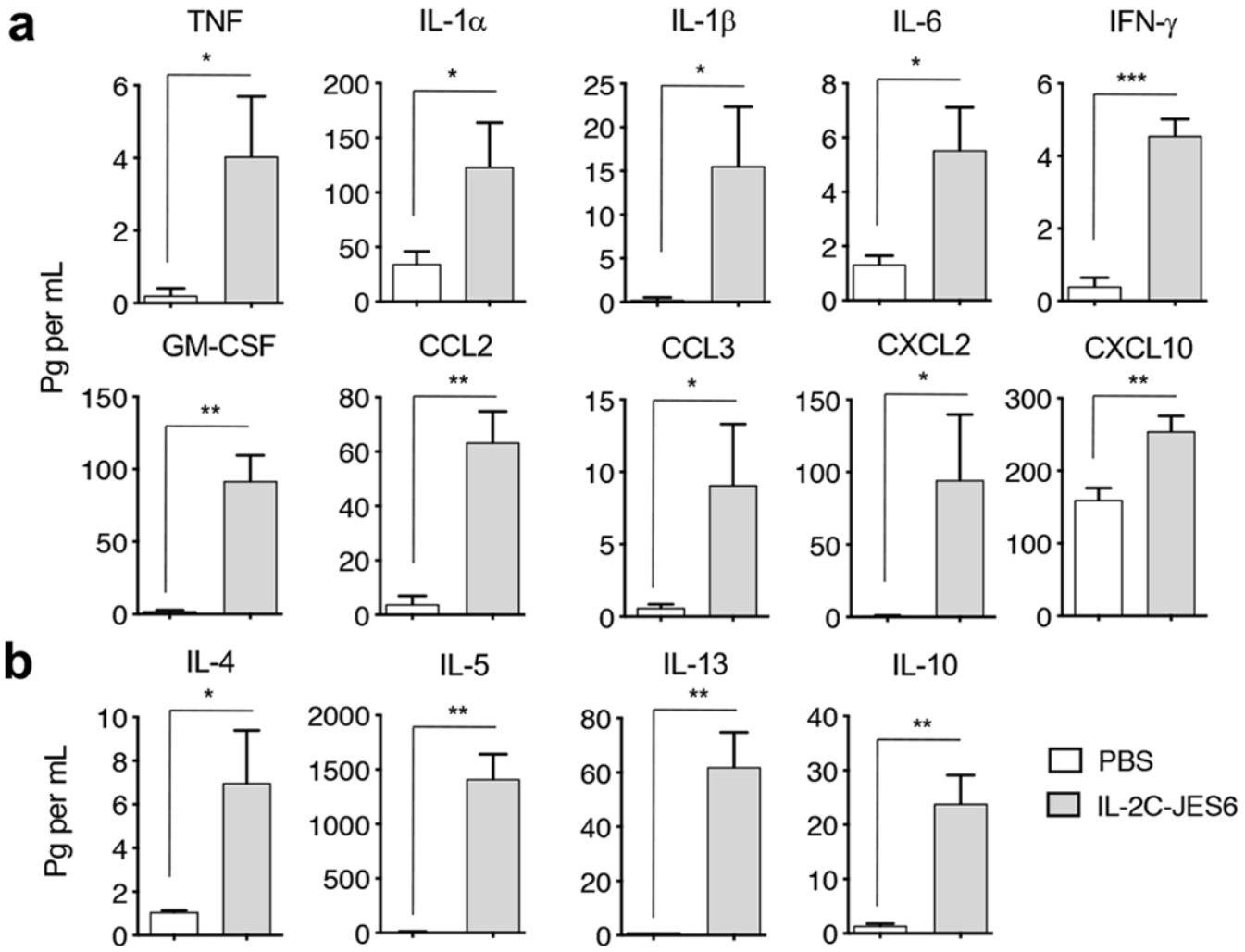

Figure 2:

JES6 IL-2C treatment induces wide-spread systemic expression of inflammatory cytokines and chemokines. Mice were treated with JES6 IL-2C or with PBS alone for 3 consecutive days. On the fourth day, serum was harvested and was analyzed by Luminex for protein levels of (**a**) inflammatory cytokines and chemokines typically associated with Th1 responses and (**b**) cytokines associated with Th2 responses (n = 4 mice per group). Results from 1 of 3 replicate experiments * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$ following Students *t*-test analysis.

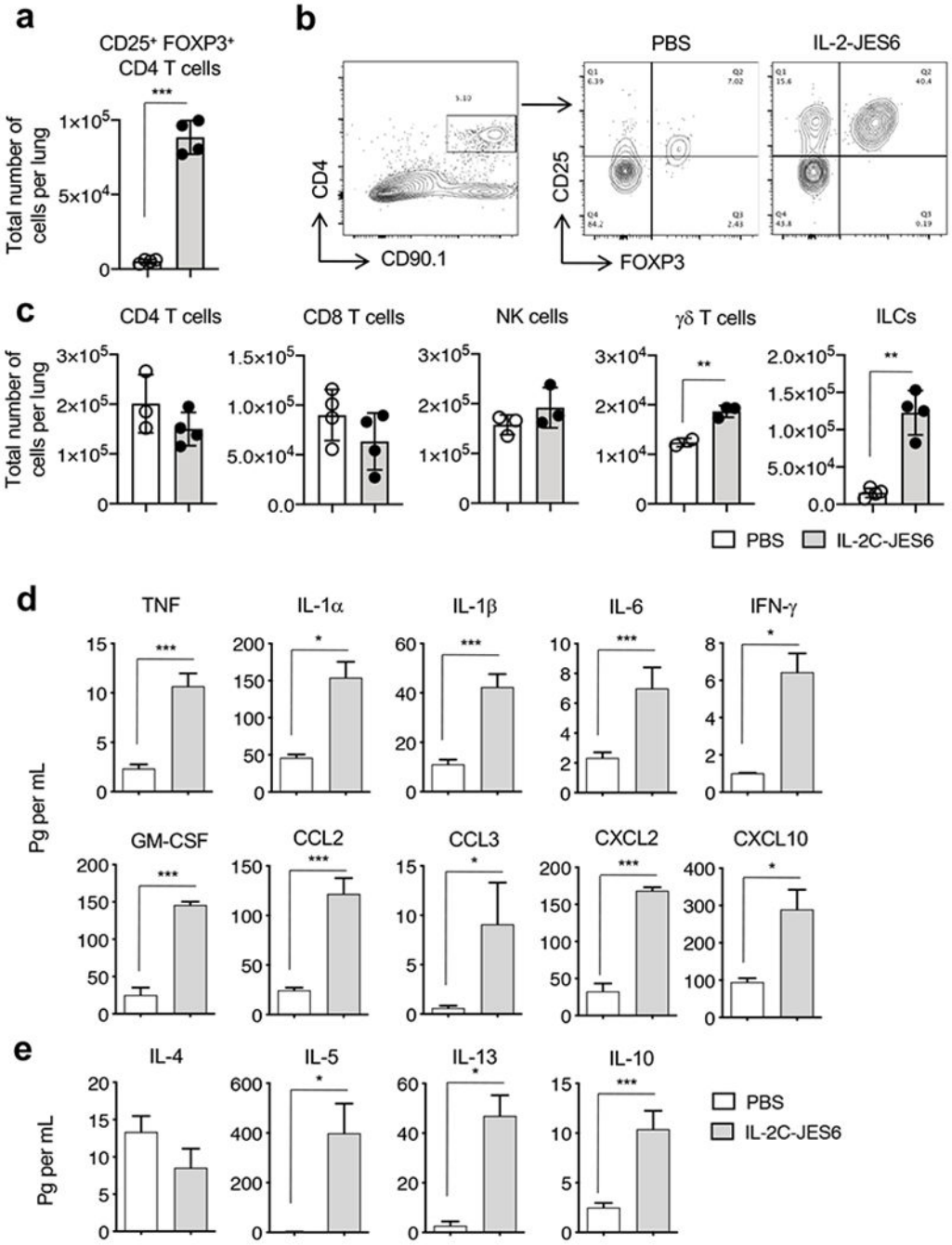

Figure 3:

Systemic JES6 IL-2C treatment induces inflammation in the lung. Mice treated systemically with IL-2C or PBS alone were analyzed for changes in lymphocyte populations and inflammation in the lungs. Shown in (**a**) the number of regulatory T cells with (**b**) representative staining from treated and untreated mice. (**c**) Total numbers of CD4 T cells, CD8 T cells, NK cells, γδ T cells, and ILC (n = 4 mice per group). Lung homogenates from separate groups of JES6 IL-2C treated or control mice (n= 4 per group) were analyzed for protein levels of inflammatory factors associated with (**d**) Th1 or (**e**) Th2 responses. Results

from one of 3 replicate experiments and * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, following Students *t*-test analysis.

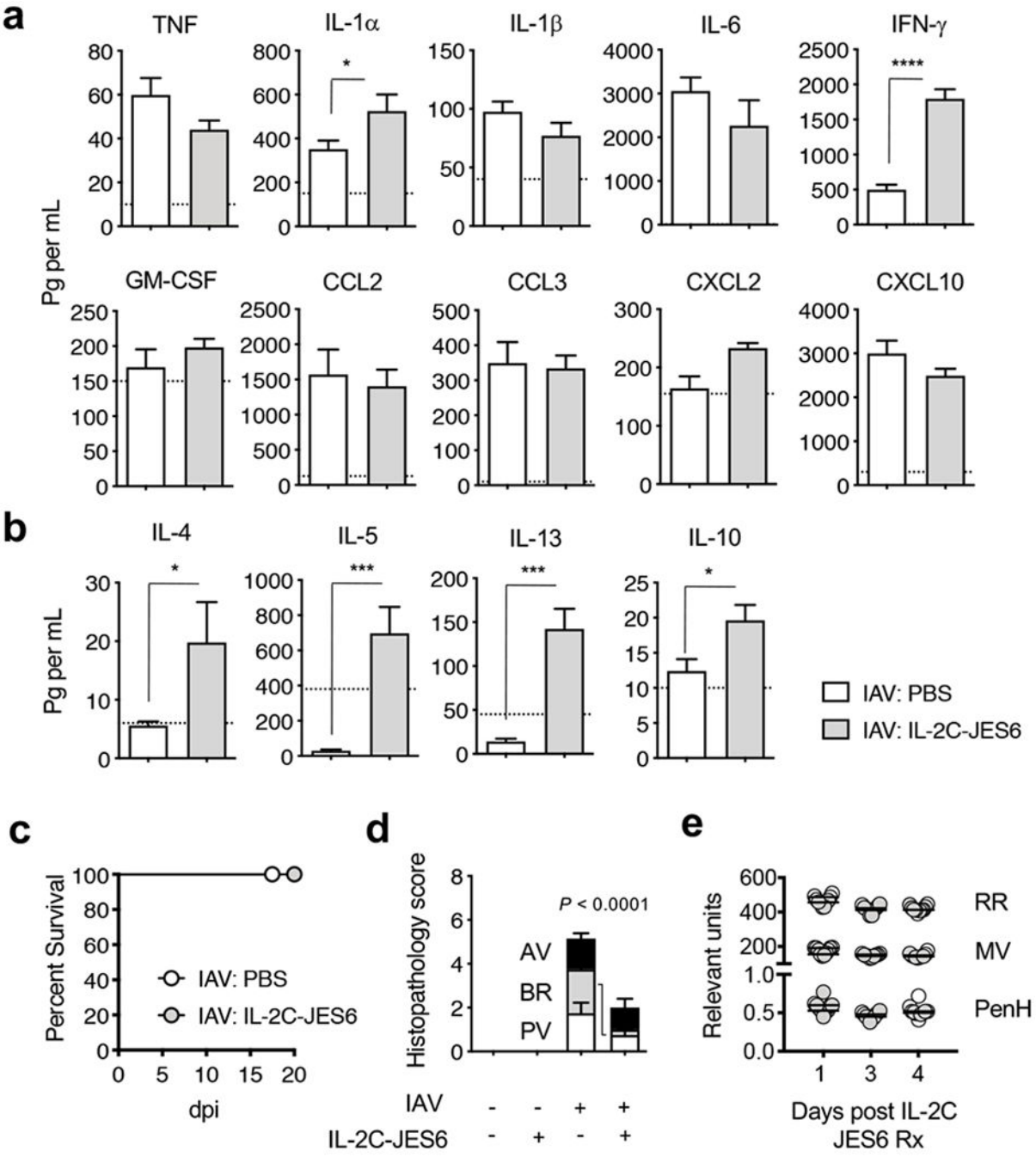

Figure 4:

JES6 IL-2C treatment improves outcomes of IAV infection. Groups of mice were infected with a sublethal 0.2 LD_{50} dose of IAV and treated i.p. with either PBS alone or with JES6 IL-2C. On day 4, levels of stated cytokines and chemokines detected by Luminex from lung homogenates and associated with either (**a**) Th1 or (**b**) Th2 responses from 4 mice per group were determined. The average level of analytes detected following JES6 IL-2C administration alone is depicted as a dashed line in each graph. Separate mice were infected with IAV and treated with either PBS alone (white circle) or with JES6 IL-2C for 3 days. (**c**)

Shown is the survival summarizing 4 mice per group. Mice treated as in (c) were harvested at 7 dpi and assessed for histopathological changes. Shown in (**d**) is the cumulative histopathology score broken down by alveolar inflammation (AV, black), bronchial inflammation (BR, grey), and perivascular inflammation (PV, white). Groups of 5 mice were treated with either PBS or JES6 IL-2C i.p. for 3 consecutive days and (**e**) were assessed on stated days for respiratory rate (RR in breaths per min), minute volume (MV in cm^3 per min), and PenH (Enhanced pause). All results representative of at least 2 replicate experiments and * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$ following Students *t*-test (a and b) or one-way ANOVA analysis (d).

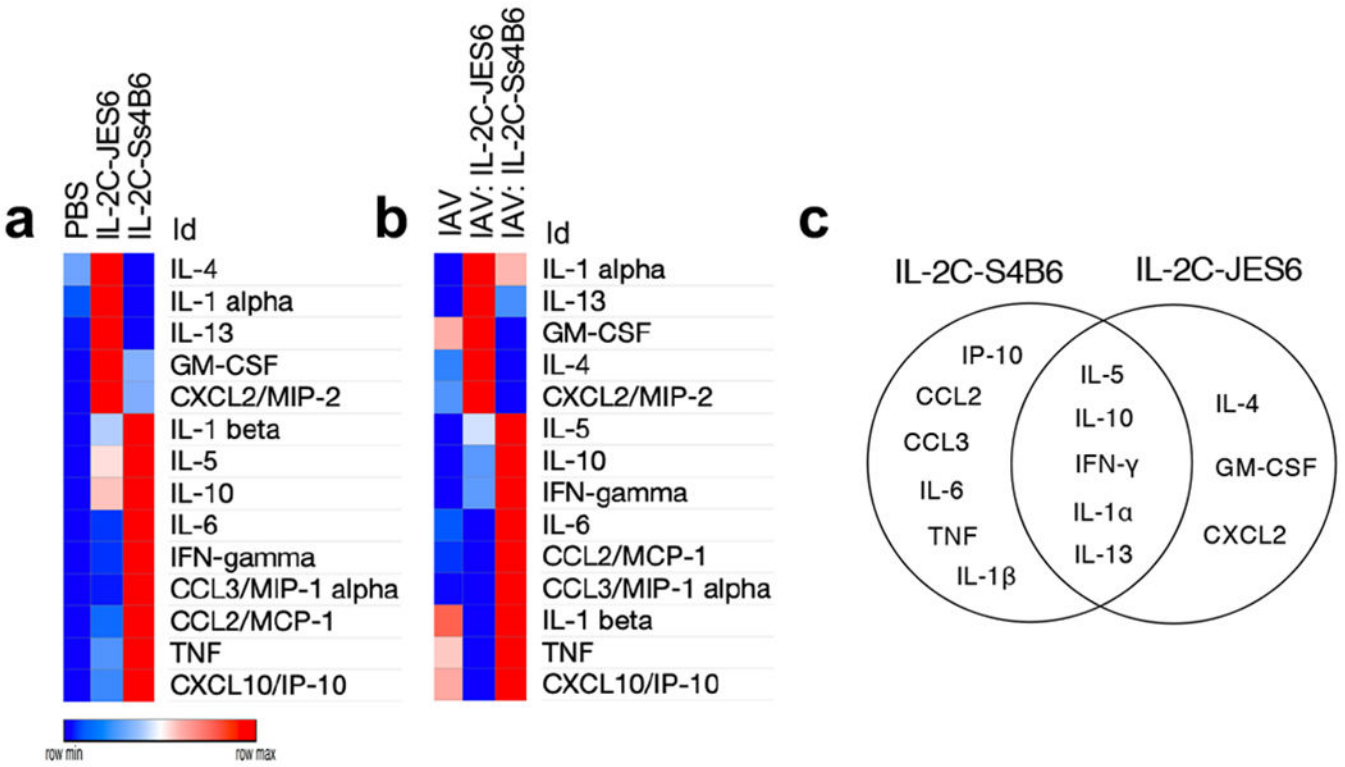

Figure 5:

Distinct and overlapping patterns in IL-2C induced inflammation. Separate groups of uninfected or IAV infected mice were treated with PBS, JES6 IL-2C, or S4B6 IL-2C for 3 consecutive days. On the fourth day, lung homogenates were harvested and analyzed by Luminex for protein levels of inflammatory cytokines and chemokines. A heat map of the average amount of analytes significantly induced with IL-2C complex treatment in (**a**) uninfected mice and (**b**) sublethally 0.2 LD_{50} IAV infected mice (3 mice per group; 1 of 3 experiments). (**c**) Venn diagram depicting analytes significantly induced by both IL-2C or uniquely induced by JES6 IL-2C or S4B6 IL-2C during IAV infection.

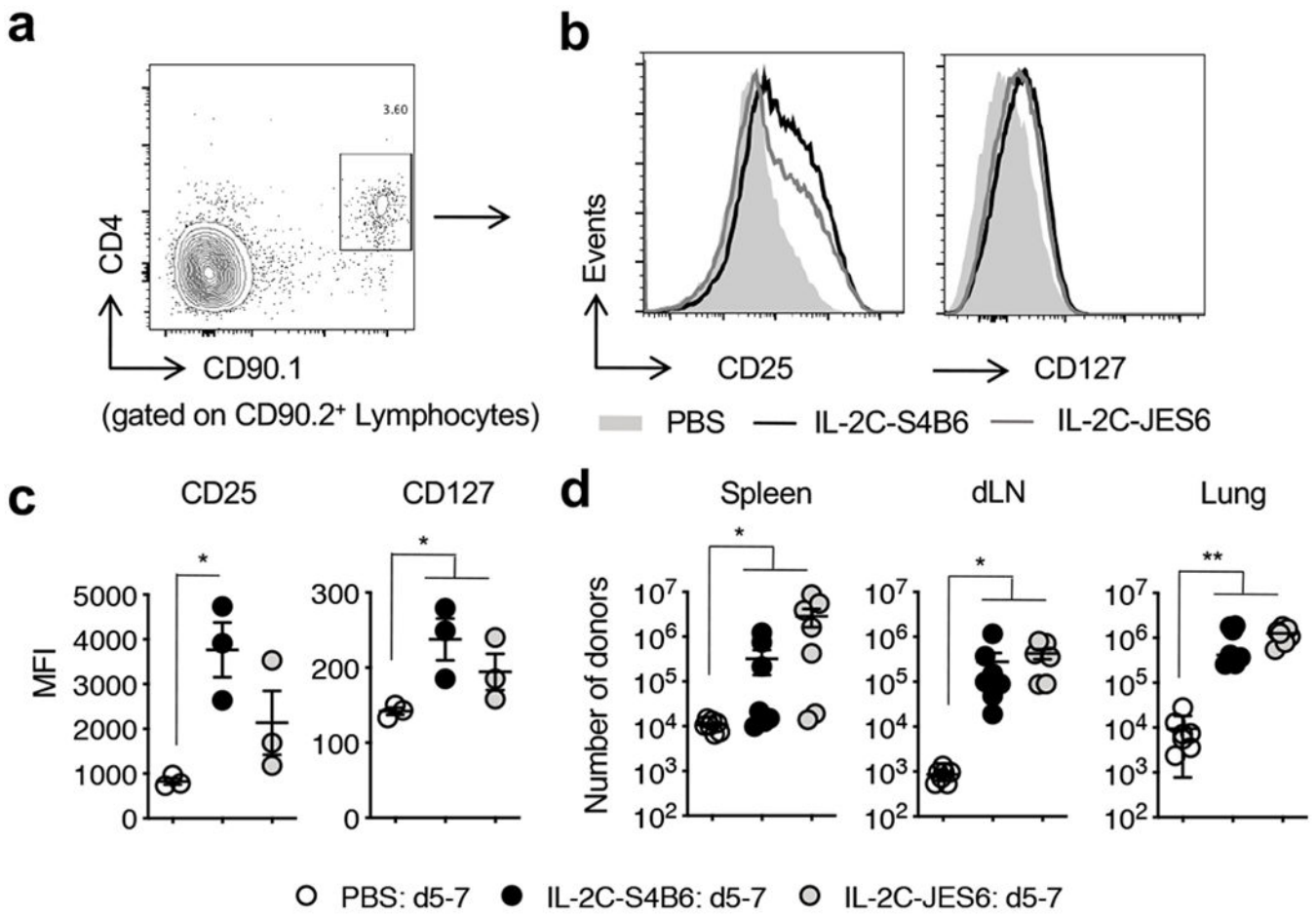

Figure 6:

JES6 IL-2C deliver pro-memory signals to CD4 T cells responding to IAV. BALB/c mice received congenically marked *Il2*$^{-/-}$ DO11.10 CD4 T cells followed by priming with low-dose 0.2 LD_{50} PR8-OVA_{II}. Groups of mice were either treated with S4B6 IL-2C (black line), JES6 IL-2C (grey line), or PBS alone (filled histogram) from 5–7 dpi. At 7 dpi, donor cells gated as in (**a**) were analyzed for expression of CD25 and CD127, with representative staining and summary MFI analysis from 3 mice per group shown in (**b)** and (**c**), respectively. (**d**) At 28 dpi, the total number of donor cells was enumerated in the spleen, dLN, and lung from groups of 3 to 4 mice treated with either IL-2C or with PBS alone. Summary of 2 replicate experiments and * $P < 0.05$, ** $P < 0.01$ determined by one-way ANOVA analysis.