Regulatory T cell response to low-dose interleukin-2 in ischemic heart disease

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Abstract

Background

Atherosclerosis is a chronic inflammatory disease of the artery wall. Regulatory T cells (Tregs) limit inflammation and promote tissue healing. Low doses of interleukin (IL)-2 have the potential to increase Tregs, but its use is contraindicated in patients with ischemic heart disease.

Methods

In this randomized, double-blind, placebo-controlled, dose-escalation trial, we tested low-dose subcutaneous aldesleukin (recombinant IL-2), given once daily for five consecutive days. In Part A, the primary endpoint was safety, and patients with stable ischemic heart disease were randomized to placebo or to one of 5 dose groups (range 0.3-3.0 $x10^6$ IU/day). In Part B, patients with acute non-ST elevation myocardial infarction or unstable angina were randomized to placebo or to one of 2 dose groups (1.5 and 2.5 $x10^6$ IU/day). The co-primary endpoints were safety and the dose required to increase circulating Tregs by 75%. Single-cell RNA-sequencing of circulating immune cells was used to provide mechanistic assessment of the effects of aldesleukin.

Results

Forty-four patients were randomized in the study, 26 patients in Part A and 18 patients in Part B. In total, 3 patients withdrew prior to dosing; 27 received active treatment, and 14 received placebo. The majority of adverse events were mild. Two serious adverse events occurred, with one occurring after drug administration. In Parts A and B, there was a dose-dependent increase in Tregs. In Part B, the estimated dose to achieve a 75% increase in Tregs was 1.46 $x10^6$ IU (95%CI 1.06 – 1.87). Single-cell RNA-sequencing demonstrated the engagement of distinct pathways and cell-cell interactions.

Conclusion

In this phase 1b/2a study, low-dose IL-2 expanded Tregs without adverse events of major concern. Larger trials are needed to confirm safety and to further evaluate efficacy of low-dose IL-2 as an anti-inflammatory therapy in patients with ischemic heart disease. (Funded

by the Medical Research Council and the British Heart Foundation; ClinicalTrials.gov number, NCT03113773)

Introduction

Atherosclerosis is a chronic inflammatory condition.[1] Myocardial infarction (MI) triggers an acute immune response which accelerates atherosclerosis[2] and can contribute to heart failure progression.[3] Studies of canakinumab[4] and colchicine[5] have shown that modulating inflammation can improve outcomes in patients with coronary disease; however, despite these advances, there are no targeted immuno-modulatory therapies approved for use in this disease setting.[6]

Regulatory T cells (Tregs) control activation and enforce immune tolerance. Some Tregs reside in non-lymphoid tissues where they maintain tissue homeostasis and control sterile inflammation.[7,8] In pre-clinical models, Treg deficiency accelerated atherosclerosis while Treg supplementation was athero-protective.[9] In pre-clinical models of MI, Tregs were important in promoting myocardial healing.[10] Observational studies have shown a decrease in the number and function of circulating Tregs in patients with MI,[11–13] while expansion and activation of pro-inflammatory effector T cells (Teffs) was positively correlated with the occurrence of ischemic heart disease.[14]

Interleukin-2 (IL-2) at high doses stimulate Teffs and is an approved cancer treatment. Paradoxically, low-dose IL-2 (doses a thousand-fold lower than those used for oncologic indications) can selectively activate Tregs, enabling their expansion without increasing Teffs.[7] Aldesleukin (recombinant IL-2) at low doses has been used in small early phase clinical trials in patients with auto-immune diseases;[15–17] however, its use is contraindicated in patients with a history, or current evidence of, severe cardiac disease, primarily due to the risk of capillary leak syndrome, pulmonary edema and tachyarrhythmias associated with high-dose intravenous regimens.

Therefore, we conducted a phase 1b/2a trial to assess the safety of multiple ascending low doses of aldesleukin in patients with stable ischemic heart disease and acute coronary syndromes (ACS), and to determine a dose that selectively and substantially increases Tregs in patients with ACS without altering Teffs. In parallel, we used single-cell RNA-sequencing (scRNAseq) on peripheral blood mononuclear cells derived from patients in the trial to

explore mechanistic underpinnings of the potential therapeutic action of low-dose IL-2 on human immunity more broadly.

Methods

Trial oversight

Low-dose interleukin-2 in patients with stable ischemic heart disease and acute coronary syndromes (LILACS) was an investigator-initiated, randomized, double-blind, placebo-controlled, dose-escalation clinical trial sponsored by Cambridge University Hospitals NHS Foundation Trust. The trial was approved by the Greater Manchester Central Research Ethics Committee, UK, and the UK Medicines and Healthcare Products Regulatory Agency. The trial protocol has been published.[18] The blinded Trial Management Group met after the completion of each dose group to assess safety data prior to dose escalation. After completion of Part A, an unblinded Data Monitoring Committee (DMC) made up of clinical researchers independent from the trial team and not involved with trial design, assessed the unblinded safety data before progression to Part B (Supplement Page 6).

Patients

The study was conducted in two parts. Part A included patients aged 18-75, who had stable ischemic heart disease (defined as having symptoms of angina and a coronary angiogram showing >50% stenosis in at least one vessel; or patients >6 months from an ACS event regardless of their angina status). Upon completion of Part A, Part B recruited hospitalized patients, aged 18-85, admitted with an acute diagnosis of either non-ST elevation MI (NSTEMI) or unstable angina. Dosing commenced within 8 days of the index admission. The full eligibility criteria are described in Supplementary Appendix Pages 4-5.

Trial procedures

In Part A, there were 5 dose groups (range 0.3 - 3.0 $x10^6$ IU daily) and participants were randomly assigned 3:2 within each group to either aldesleukin or placebo. In Part B, 2 doses were explored (1.5 and 2.5 $x10^6$ IU daily based on dose-modeling, Supplement Page 6), and

participants were randomly assigned 3:1 within each group to either aldesleukin or placebo (Figure S1). Investigators and participants were blinded to the allocation. Participants received aldesleukin (Proleukin, Novartis) or placebo over 5 consecutive days (Visit (V)2-6) with a single daily, subcutaneous injection in the abdominal area. Follow-up occurred the day after the final dose (V7) and 7 days later (-3/+11 day window) (V8) (Figure S1).

Endpoints

For Part A, the primary outcome was safety; in Part B, the co-primary endpoints were safety and estimating the dose of aldesleukin to increase Tregs by 75% from baseline to follow-up (V7). For both study phases, safety was assessed by an open query for adverse events (AEs), physical examination, review of concomitant medications, vital signs, and safety blood tests (Supplement Page 6) performed at all visits. Additionally, electrocardiograms (pre-dose and 15, 30, and 60 mins post-dose) and cardiac telemetry were performed at dosing visits, and echocardiography was performed at screening and on final follow-up.

In Part B, the 75% threshold and patient population was chosen based on observational data showing up to a 40% reduction in Tregs in patients with acute NSTEMI,[13] and that a similar reduction of Tregs was associated with increased incidence of MI in a population study.[13,19] Tregs were defined as $CD3^{+}CD4^{+}CD25^{high}$ $CD127^{low}$ and expressed as the percentage of total $CD4^{+}$ T cells in peripheral blood.

Additional information on pre-specified exploratory endpoints, including change in lymphocyte subsets, cardiac and inflammatory biomarkers, and scRNAseq of peripheral blood mononuclear cells can be found in the Supplement Pages 8-11.

Statistical analysis

We analyzed the primary safety endpoint using all participants who were randomized and given at least one dose of aldesleukin or placebo. The dose response was modeled using either a quadratic or Locally Weight Regression fit graphs where the line of best fit is presented with 95% confidence intervals. In Part B, we used a linear dose-response model to estimate the dose required to increase Tregs by 75% and the change per unit increase of

aldesleukin on Treg%. No multiplicity adjustments have been made and the 95% confidence intervals should not be used for inferences. ScRNA-seq statistical analysis is described in the Supplement Pages 9-10.

Results

Between May, 2017 and February, 2019, patients at Cambridge University Hospital and Royal Papworth Hospital were recruited (**Figure 1**). Forty-four patients were randomized in the study; 3 patients withdrew before dosing, and 41 patients completed the dosing protocol and follow-up. Baseline demographics are shown in **Table 1**.

Part A

Safety

There were a total of 13 AEs reported from 8 out of the 10 patients treated with placebo and 105 AEs reported from all 15 patients treated with aldesleukin (**Table 2** and Table S1). There were no serious adverse events (SAEs) reported. The most common AEs were administration site reactions (69 AEs in 16 patients), comprised of injection site erythema (86%), nodules (7%), bruising (4%) or pruritus (3%). The second most common AE was a flu-like syndrome.

Effect of aldesleukin on Treg and Teff cells

Treatment with aldesleukin increased plasma levels of IL-2 (Figure S2) and increased Treg percentage (**Figure 2A**) and absolute count after 5 days of treatment in a dose-dependent manner (Figure S3). The increase in Tregs was not accompanied by an increase in Teff cells (**Figure 2B** and Figure S3).

Effect of aldesleukin on exploratory endpoints

Aldesleukin was associated with a decrease (from baseline to V7) in $CD4^+$ central memory T (Tcm) cells, $CD8^+$ T cells, and B cells in a dose-dependent manner; at the same time there

was an increase in circulating eosinophils; the highest dose was associated with an increase in natural killer (NK) cells and monocytes (Figure S4-5). Serum analysis showed transient increases in serum IL-6 levels with an accompanying transient increase in high-sensitivity C-reactive protein (hsCRP) with no effect on levels of Troponin I or brain natriuretic peptide (BNP) (Figure S6-7). Aldesleukin decreased high-density lipoprotein cholesterol (HDL-C) and total cholesterol in a dose-dependent manner. Low-density lipoprotein cholesterol, triglycerides, and non-HDL-C remained stable (Figure S8).

Part B

Safety

There were 6 AEs reported from all 4 patients treated with placebo, 22 AEs reported from all 6 patients treated with $1.5x10^6$ IU/day of aldesleukin, and 51 AEs reported from all 6 patients treated with $2.5x10^6$ IU/day of aldesleukin (**Table 2** and Table S1). Two SAEs were reported in two patients. One SAE occurred before dosing started and the patient was withdrawn from the study. The second SAE was in a patient admitted with a NSTEMI. Diagnostic angiography showed severe triple vessel coronary artery disease, and the patient was awaiting inpatient coronary artery bypass surgery when he was enrolled. Dosing was completed before his scheduled surgery. On the last day of dosing he developed an episode of chest pain with an associated increase in serum levels of Troponin I to a peak of 2,158 ng/L (reference 0-56 ng/L). The patient underwent the planned surgery and immediate post-operative recovery was uneventful. After discharge, symptoms of a cough productive of green sputum developed, and he received treatment with a course of oral amoxicillin from his primary care physician. A second course of oral doxycycline was given on completion of the first course due to ongoing symptoms. A sputum culture showed no growth. On his final trial follow-up, his symptoms had resolved, white blood count, C-reactive protein and chest radiograph were normal.

The most common AEs were administration site reactions (38/79 AEs). In the placebo and $1.5x10^6$ IU dose group, there were no infection-related AEs, while in the $2.5x10^6$ IU dose, there were 4 infection-related AEs in 3 out of 6 patients, all graded mild in severity. This included two respiratory infections (in 1 patient described above), one skin candida

infection, and one arm puncture site hematoma infection (non-trial procedural related). At the end of the trial, all data were reviewed by the independent DMC that did not identify safety concerns in the trial that would preclude further study.

Effect of aldesleukin on Treg and Teff cells

Percentage change of Tregs from baseline to V7 was increased for both doses tested compared to placebo (median increases: placebo = 6.4% (95%CI -7.7-17.8%); $1.5x10^6$ IU = 95.0% (19.7-122.8%); and $2.5x10^6$ IU = 109.4% (61.8 -173.4%)) (**Figure 2C**). Regression modeling showed the dose required to increase Tregs by 75% was 1.46 $x10^6$ IU/day (95% CI 1.06 to 1.87) (Figure S9I). Comparing baseline to V8, a sustained increase of Tregs was seen only for the 1.5 $x10^6$ IU dose (**Figure 2E-F**). There appeared to be increased Teffs at V8 for the 2.5 $x10^6$ IU dose (**Figure 2F** and Figure S9). Both doses of aldesleukin resulted in similar levels of IL-2 (area under curve analysis).

Effect of aldesleukin on exploratory endpoints

Compared to placebo, aldesleukin resulted in a dose-dependent decrease in B cells at V7, which rebounded by V8 (Figure S10). NK cells increased from baseline to V8 at the 2.5 $x10^6$ IU dose (**Figure 2 E-F**). Aldesleukin increased eosinophil counts, and transiently expanded non-classical and intermediate monocytes (Figure S11). There was a transient dose-dependent increase in IL-6, hsCRP, type 1 (inflammatory) cytokines, and type 2 (anti-inflammatory) cytokine and chemokine responses (Figure S12-14). Troponin I and BNP levels were not affected by aldesleukin (Figure S15).

Single-cell sequencing results

Single-cell RNA- and T cell receptor (TCR)-sequencing on Part B PBMC samples (baseline and V7) revealed 30 cell types (Figure S16A-C). Differential abundance testing using a linear mixed-effect model to account for placebo responses, age, gender, and peak serum troponin levels demonstrated that both aldesleukin doses resulted in an enrichment of Tregs, $CD16^-$ NK cells and C1Q-expressing non-classical monocytes, with a decrease in $CD8^+$

T effector memory (Tem) cells (**Figure 3A**). Data on the comparative increase in $CD4^+$ Tcm in the $2.5x10^6$ IU group versus the $1.5x10^6$ IU group are show in Figure S16D.

Ligand-receptor expression was used to investigate the predicted interactions of Tregs, with a focus on interactions with antigen presenting dendritic cells (DCs). Aldesleukin treatment most notably increased CD28 – CD86 and decreased inhibitory CD52 – sialic acid-binding immunoglobulin-like lectin 10-mediated interactions (Figure S17A). In the $2.5x10^6$ IU group, there was enhanced up-regulation of some inhibitory interactions including via FAS (CD95L) – TNFSF13 (APRIL) (pro-apoptotic activity[20]), HLA-F – LILRB1 (LILRB proteins contain ITIM-domains[20]) and the emergence of a potential tissue recruitment signal via upregulation of SIRPG (Figure S17A).

Analysis of TCRs enables an assessment of whether clones of T cells recognizing antigens via the same TCR were present following MI, and how this was affected by aldesleukin. Clonotype expansion in untreated patients was largely restricted to the central memory compartment, and this was diminished in both 1.5 and 2.5 $x10^6$ IU treatment groups (**Figure 3B**). There was a modest increase in larger clones within the $CD4^+$ Tregs after $1.5x10^6$ IU dose treatment compared to naïve cells, but this effect was more prominent in the $2.5x10^6$ IU treated patients. However, there was also clonal expansion in the $CD4^+$ effector memory compartment at the higher dose (**Figure 3B)**.

In both 1.5 and $2.5x10^6$ IU dose groups, gene set enrichment analysis[20] showed an increase in several gene sets related to metabolism in $CD4^+$ Tregs, including '*MTORC1 Signaling*', '*Hypoxia*' and '*Oxidative Phosphorylation*' (notable, given the effects of cell metabolism on T cell differentiation and function[21]), as well in '*IL-2-STAT5 signaling'* pathway genes (**Figure 3C**). In contrast, enrichment of these pathways in $CD4^+$ Tem and $CD4^+$ Tcm cells was only observed following the $2.5x10^6$ IU dose (**Figure 3C**). The most up-regulated pathway after $1.5x10^6$ IU treatment in Tregs was '*Epithelial Mesenchymal Transition*' including for example, *IL32* and *TGFB1*, the latter being a major immune-regulatory cytokine and upregulated in $1.5x10^6$ IU group alone (Figure S17B). Selected metabolic pathway genes were validated in *ex vivo* stimulated human $CD4^+$ Tregs (Figure S17C), and *ex-vivo* Seahorse analysis confirmed increased extracellular acidification (a readout of glycolysis), oxygen consumption rate (a

readout of oxidative phosphorylation), and production of mitochondrial reactive oxygen species in the presence of IL-2 (Figure S17D).

Discussion

Our data showed that administration of IL-2 (aldesleukin) in a small number of patients with stable ischemic heart disease and ACS, conditions where its use is currently contraindicated, was not associated with widespread severe adverse events. The majority of AEs were self-limiting side effects, which were largely, but not completely, of mild intensity. The two most common AEs, administration site reactions and a flu-like syndrome, are both known side effects of aldesleukin. The safety profile we observed in our early phase trial was similar to other studies using low-dose IL-2 in autoimmune disease.[15,16,22] There was a higher rate of infections (3/6 patients) at the 2.5 $x10^6$ IU dose in Part B, which was not observed in Part A at the 2.4 or 3 $x10^6$ IU dose. Although the number of patients within each dose group was small, this safety signal will require further evaluation in a larger trial. Although it is reassuring that infections were not observed in other trials[16,22,23] at similar or higher doses of IL-2 despite concomitant immunosuppression, these trials did not include patients with ACS.

The estimated dose of aldesleukin to increase Tregs by 75% in patients with ACS was 1.5 $x10^6$ IU/day. This dose is further supported by the more sustained increase in %Tregs at the later V8 timepoint without increases in Teff or NK cells associated with the higher 2.5 $x10^6$ IU dose. Using scRNA-seq, we observed an expansion of Tregs with both doses, and showed that the 2.5 $x10^6$ IU dose also increased $CD4^+$ Tcm cells, and was associated with clonal expansion in the $CD4^+$ Tem compartment, representing cells with the potential to increase inflammation following an MI. The ligand-receptor expression analysis offered insight into the possible mechanisms of aldesleukin. The observed CD28-CD86 interaction is an important activating co-stimulatory signal by which dendritic cells might cause Treg expansion. We further speculate that decreases in CD52-SIGLEC10-mediated interactions

will likely promote Treg expansion and activation, while an increase in SIRPG is known to promote T cell transendothelial migration into tissue.[20]

It is increasingly appreciated that the functional profile of Tregs is dependent on their metabolic state, which we explored using scRNA-seq and confirmed using *ex-vivo* Seahorse analysis. The $2.5x10^6$ IU dose was associated with an increase in Treg glycolysis, which is known to promote cell growth and migration at the cost of immune suppressive function, while the lower $1.5x10^6$IU dose was able to more selectively increase oxidation phosphorylation, which has been linked with increased suppressive function.[24] In addition, the $2.5x10^6$ IU dose increased several metabolic pathways associated with activation and proliferation in $CD4^+$ Tem and Tcm cells. Taken together, this shows that while both doses induce $CD4^+$ Treg activation and expansion, the $1.5x10^6$ IU dose may have a more beneficial clinical effect.

Aldesleukin led to dose-dependent decreases in B cells and $CD8^+$ T cells. The mechanism for this change needs further exploration. In the heightened inflammatory state following an MI, pre-clinical models show $CD8^+$ T cells and B cells have a detrimental effect on both atherosclerosis,[25 26] and remodeling,[27,28] and therefore their reduction may represent an additional mechanism of benefit of low-dose aldesleukin. At the higher doses, increases in eosinophils were observed which may contribute to myocardial repair after ischemic injury.[29]

We acknowledge several limitations of our trial design. Early phase clinical trials, by nature, are small and therefore only provide provisional data on safety. In this trial, we treated patients for 5 days; however, larger and longer trials are underway (NCT04241601) in a broader population of patients with ACS. Although we report changes in gene expression profile consistent with increased suppressive function, Treg function was not directly assessed. However, previous evidence shows that low-dose IL-2 not only increased Treg number but also suppressive function.[22] The scRNA-seq data are insightful; its link to clinical outcomes is an unexplored field but has the potential to identify valuable biomarkers and to reveal unappreciated mechanisms of drug activity.

In summary, we present novel safety and mechanistic data for the use of low-dose IL-2 in patients with ACS as a strategy to increase Tregs. We have calculated a dose for therapeutic use in this patient group, which has already been taken into a phase 2b study (NCT04241601). The data from this study provide important insights into the biology and utility of IL-2 in patients with ACS.

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	Part A		Part B	
	Placebo (n=10)	IL-2 (n=15)	Placebo (n=4)	IL-2 (n=12)
Age				
Mean (SD)	62.8 (5.6)	62.1 (8.0)	59.8 (13.0)	60 (8.3)
Sex				
Male	9 (90%)	10 (67%)	3 (75%)	7 (58%)
Female	1 (10%)	5 (33%)	1 (25%)	5 (42%)
Race				
White or Caucasian	10 (100%)	14 (93%)	4 (100%)	12 (100%)
Asian	0	1 (7%)	0	0
BMI				
Mean	32.1	28.1	28.7	28.1
Presentation				
NSTEMI UA	n/a	n/a	3 (75%) 1 (25%)	10 (83%) 2 (17%)
Cardiovascular history				
Myocardial infarction	5 (50%)	8 (53%)	1 (25%)	5 (42%)
Coronary artery revascularization[a]	7 (70%)	13 (86%)	1 (25%)	5 (24%)
Hypertension	6 (60%)	7 (47%)	2 (50%)	6 (50%)
Stroke	2 (20%)	0	1 (25%)	1 (8%)
Peripheral vascular disease	0	1 (7%)	0	1 (8%)
Medications				
Aspirin	10 (100%)	15 (100%)	4 (100%)	12 (100%)
Clopidogrel/ Ticagrelor	4 (40%)	11 (73%)	4 (100%)	12 (100%)
Statin	9 (90%)	14 (93%)	4 (100%)	10 (83%)
β-blocker	9 (90%)	10 (67%)	3 (75%)	7 (58%)
ACEi/ARB	6 (60%)	9 (60%)	3 (75%)	8 (67%)

Table 1. **Summary of demographics.** [a]Coronary artery revascularization includes percutaneous coronary intervention and coronary artery bypass surgery. BMI = body mass index; ACEi = Angiotensin-converting enzyme inhibitors; ARB = Angiotensin II Receptor Blockers.

	Part A						Part B		
	Placebo (n=10)	IL-2 0.3x10^{6}IU (n=3)	IL-2 0.6x10^{6}IU (n=3)	IL-2 1.2x10^{6}IU (n=3)	IL-2 2.4x10^{6}IU (n=3)	IL-2 3.0x10^{6}IU (n=3)	Placebo (n=4)	IL-2 1.5x10^{6}IU (n=6)	IL-2 2.5x10^{6}IU (n=6)
Treatments administered[a]	50	15	15	15	15	15	20	30	30
Non-serious adverse events (AEs):									
Number of patients with AEs	8	3	3	3	3	3	4	6	6
Number of AEs	13	17	13	28	27	20	6	22	51
Ratio of AEs: treatments given	0.26	1.13	0.87	1.87	1.80	1.33	0.30	0.73	1.70
Serious adverse events (SAEs):									
Number	0	0	0	0	0	0	0	0	1[b]
Common AEs, % of patients with noted AE (number of patients, number of AEs)									
Administration site reactions[c]	20% (2,3)	100% (3,13)	100% (3,13)	100% (3,18)	100% (3,14)	100% (2,8)	0	83% (5 ,11)	100% (6 ,27)
Flu like syndrome	20% (2,2)	67% (2,3)	0	100% (3,5)	67% (2,8)	67% (2,4)	25% (1,1)	50% (3,5)	33% (2 ,2)
Headache	0	33% (1,1)	0	0	0	66% (2,4)	0	17% (1,1)	0
Chest pain	10% (1,1)	0	0	0	0	0	0	17% (1,1)	17% (1,1)
Any infection[d]	10% (1,1)	0	0	0	33% (1,1)	0	0	0	50% (3 ,4)

Table 2. Adverse events for dosed patients. [a] Treatments administered is the total number of injections that occurred in each group. Each patient received a daily injection of IL-2/placebo for 5 consecutive days. [b] see results section for description of SAE event. [c] administration site reactions included injection site erythema, nodules, bruising and pruritus. [d] any infection included lower respiratory tract infection, hematoma infection, urinary tract infection, common cold, and skin candida infection. IL-2 = Interleukin-2

Figure Legends

Figure 1. Trial profile. Part A included patients who had stable ischemic heart disease; Part B included patients who were hospitalized with either non-ST elevation myocardial infarction or unstable angina. All patients completed dosing and follow-up. *One patient from Part B Group 2 had his final follow-up outside the specified time window due to clinical reasons. SAE = serious adverse event. IL-2 = interleukin-2

Figure 2. The dose effect of low-dose interleukin-2 on T cell subsets and Natural Killer cells. Panels A-D represent individual patient data showing percentage change in the percentage of regulatory T cells (Tregs) or effector T cells (Teff cells) (of $CD4^+$T cells), or their ratio measured at baseline and after 5 days of dosing in patients with either stable angina or acute coronary syndrome. In **panels C-D**, lines and error bars represent median and 95% confidence interval. **Panels E-F** are radar plots showing the percentage change in T cell subsets and natural killer (NK) cells from baseline to the two follow-up timepoints. Tem = T effector memory cells. Tcm = T central memory cells.

Figure 3. **Single-cell RNA-sequencing. Panel A** shows differential abundance testing of single-cell neighborhoods between untreated versus post-IL-2 treatment groups with negative binomial generalized linear mixed-effects model (n=20 samples, 10 patients, 4 placebo and 6 IL-2). Plot shows the beta coefficients for each neighborhood assigned to corresponding cell types where positive and negative coefficient values are interpreted as enriched or depleted after IL-2 treatment, respectively. Differentially abundant neighborhoods are colored according to the beta coefficient value from blue to white to red where white indicates a value of 0 (no change). Non-significant neighborhoods are colored grey. FDR = false discrimination rate. **Panel B** show TCR clonotype size for CD4 T cells visualized on single-cell UMAP for each treatment group. TCR clonotypes were defined based on identical CDR3 amino acid sequence of TCR-alpha-beta pairs between cells. Clonotype sizes are colored with increasing values corresponding to gradients from white to blue for non-Treg cells and white to red for Treg cells. The maximum clonotype size is capped at 5. In all panels, untreated group includes all samples from placebo group (pre and post) and pre-treatment samples from IL-2 dosage groups). **Panel C** shows gene set enrichment analysis (GSEA) of CD4 central memory, effector memory, and regulatory T cells for untreated versus post-IL-2 treatment. Size of circles indicates (absolute) normalized enrichment score (NES). GSEA (permutation) nominal p-value < 0.05 and FDR < 0.25 are considered statistically significant and colors correspond to: 1.5MIU vs untreated (blue); 2.5MIU vs untreated (orange); not significant (grey).