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CD4 T-cells Regulate Angiogenesis and Myogenesis

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Abstract

Ischemic diseases, such as peripheral artery disease, affect millions of people worldwide. While CD4⁺ T-cells regulate angiogenesis and myogenesis, it is not understood how the phenotype of these adaptive immune cells regulate these regenerative processes. The secreted factors from different types of CD4⁺ T-cells (Th1, Th2, Th17, and Treg) were utilized in a series of *in vitro* assays and delivered from an injectable alginate biomaterial into a murine model of ischemia to study their effects on vascular and skeletal muscle regeneration. Conditioned medium from Th2 and Th17 cells enhanced angiogenesis *in vitro* and *in vivo*, in part by directly stimulating endothelial sprouting. Th1 conditioned medium induced vascular regression *in vitro* and provided no benefit to angiogenesis *in vivo*. Th1, Th2, and Th17 conditioned medium, to varying extents, enhanced muscle precursor cell proliferation and inhibited their differentiation *in vitro*, and prolonged early stages of muscle regeneration *in vivo*. Treg conditioned medium had a moderate or no effect on these processes *in vitro* and no discernable effect *in vivo*. These findings suggest that Th2 and Th17 T-cells may enhance angiogenesis and myogenesis in ischemic injuries, which may be useful in the design of immunomodulatory biomaterials to treat these diseases.

Keywords

T-cells; Angiogenesis; Myogenesis; Biomaterial; Regenerative medicine

1. Introduction

Ischemic diseases, such as coronary and peripheral artery disease (PAD), are a leading cause of morbidity and mortality worldwide. PAD, in particular, is estimated to affect over 200 million people worldwide [1]. Due to the chronic loss of blood flow, PAD often leads to loss of skeletal muscle strength [2] and, in severe cases, tissue necrosis and limb auto-amputation. A number of molecular and cellular therapies, including delivery of pro-angiogenic factors or infusion of bone-marrow-derived mononuclear cells, have been

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Data Availability

The raw/processed data required to reproduce these findings are available from the corresponding author on reasonable request.

explored pre-clinically and in the clinic to restore blood flow and function to ischemic limbs. To date, however, these therapies have not passed FDA approval, as they have not demonstrated substantial improvements in randomized clinical trials [3].

It is increasingly appreciated that immune cells play a key role in promoting vascular regeneration and recovery in response to injuries, including those involving ischemia. Macrophages, in particular, have been extensively studied for their ability to promote angiogenesis [4], arteriogenesis [5], and vessel anastomosis [6], with their polarization state regulating their relative contribution to these processes [7]. Other immune cells of the innate immune system are also potent sources of angiogenic factors, including dendritic cells [8], eosinophils [9], mast cells [10], and neutrophils [10]. The adaptive immune system, in particular T-cells, have also been demonstrated to play a role in recovery from ischemia [10, 11]. CD4⁺ T-cells, T-cells responsible for boosting inflammation by secreting cytokines, promote arteriogenesis in animal models of PAD [12] and enhance skeletal muscle regeneration [13]. Furthermore, CD4⁺ T-cells are critical to the activation and polarization of macrophages and may regulate macrophage wound-healing functions. However, it is not fully understood how the various differentiation states of CD4⁺ T-cells, including Th1, Th2, Th17, and Treg, regulate these processes.

We hypothesized that the mixture of secreted factors from different types of CD4⁺ T-cells will differentially regulate angiogenesis and myogenesis. Secreted factors were collected in the form of conditioned media (CM), in which primary CD4⁺ T-cells were first differentiated into Th1, Th2, Th17, and Treg phenotypes, stimulated for factor secretion, and then secreted factors in CM were collected *in vitro*. CM from both human and mouse T-cells were collected, in order to test the effect of these cytokines on different *in vitro* assays that either utilized human or mouse cells. These *in vitro* assays included models of endothelial sprouting, vascular remodeling, and myogenesis. The CM was then delivered from an injectable alginate biomaterial into the ischemic hindlimb muscle of mice, to explore its impact on blood vessel and muscle regeneration *in vivo*. This delivery system was previously developed to provide local and sustained presentation of heparin-binding cell secreted factors [14] and has been shown to allow sustained release of a variety of factors capable of promoting angiogenesis and myogenesis [15].

2. Materials and methods

2.1. T-cell Differentiation and Conditioned Media

Naive mouse CD4⁺ T-cells were isolated from the spleen and lymph nodes of C57BL/6J female mice, ages 6-12 weeks after mice were euthanized in compliance with National Institutes of Health and institutional guidelines. Briefly, isolated spleens and lymph nodes were compressed through a 70 micron cell strainer and depleted of red blood cells (RBC) using a RBC Lysis Buffer (BioLegend #420301). The mouse naïve CD4⁺ T-cell Isolation Kit (Miltenyi Biotech #130-104-453) was used to deplete non-target cells with magnetic-activated cell sorting according to the manufacturer's instructions. Naive T-cells were then activated and cultured in RPMI1640 media, supplemented with 10% heat-inactivated fetal bovine serum (HI-FBS) and 1% penicillin streptomycin (P/S), in the presence of 2 µg/mL anti-CD28 (BioLegend #102112) and 10 µg/mL plate-bound anti-CD3 (BioLegend

#100331). The following cytokines and antibodies were added to the culture to induce the respective phenotypes: 5 µg/mL anti-interleukin-4 (IL-4) (BioLegend #504108), 15 ng/mL mouse interleukin-12 (IL-12) (BioLegend #577002), 30 ng/mL human interleukin-2 (IL-2) (BioLegend #589102) for Th1; 5 µg/mL anti-interferon gamma (IFN-γ) (BioLegend #505812), 5 µg/mL anti-IL12 (BioLegend #505304), 10 ng/mL mouse IL-4 (R&D #404-ML-010), 30 ng/mL human IL-2 for Th2; 5 µg/mL anti-IFN-γ, 5 µg/mL anti-IL-4, 20 ng/mL human transforming growth factor beta-1 (hTGF-β1) (BioLegend #580702), 30 ng/mL human IL-2 for Treg; 5 µg/mL anti-IFN-γ, 5 µg/mL anti-IL-4, 5 ng/mL hTGF-β1, 50 ng/mL mouse interleukin-6 (IL-6) (BioLegend #575702), 50 ng/mL mouse interleukin-23 (IL-23) (BioLegend #589002), 50 ng/mL mouse interleukin-1beta (IL-1β) (BioLegend #575102) for Th17. Every 5-7 days, the T-cells were either reactivated with fresh antibodies and cytokines, or used to collect CM. To collect CM, cells were washed twice with phosphate buffered saline (PBS) and then transferred to dishes with platebound anti-CD28 and anti-CD3 in fresh, FBS-free media at 10⁶ cells/mL; CM was collected after 24 hours of culture in these conditions. Prior to collecting CM, the phenotype of the cells was verified using intracellular flow cytometry analysis of IFN-γ, IL-4, interleukin-17a (IL-17a), T-bet, GATA-3, RORγT, and FoxP3 following activation with eBioscience Cell Stimulation Cocktail (plus protein transport inhibitors) (Invitrogen #00-4975-03). Flow cytometry analysis was run on either the Fortessa or LSR II analyzer, interchangeably. The Proteome Profiler Mouse XL Cytokine Array (R&D ARY028) was used to determine the relative levels of 111 different mouse cytokines and chemokines in CM.

Naive human CD4⁺ T-cells were isolated from apheresis leukoreduction collars obtained from the Brigham & Women's Hospital Clinical Laboratories. Peripheral blood mononuclear cells (PBMCs) were isolated from the blood by density gradient centrifugation with Ficoll-Paque PLUS. The human naive CD4⁺ T-cell Isolation Kit (Miltenyi Biotech #130-049-131) was used to deplete non-target cells with magnetic-activated cell sorting according to the manufacturer's instructions. Naive T-cells were then activated and cultured in either RPMI1640 media supplemented with 10% HI-FBS, or serum-free X-VIVO15 media, in the presence of 2 µg/mL anti-CD28 (BioLegend #302923) and 10 µg/mL plate bound anti-CD3 (BioLegend #317304). The following cytokines and antibodies were added to the culture to induce the respective phenotypes: 5 µg/mL anti-IL-4 (BioLegend #500815), 10 ng/mL human IL-12 (BioLegend #573002), 50 ng/mL human IL-2 for Th1; 5 µg/mL anti-IFN-γ (BioLegend #506513), 25 ng/mL human IL-4 (R&D #204-IL-010), 50 ng/mL human IL-2 for Th2; 5 µg/mL anti-IFN-γ, 5 µg/mL anti-IL-4, 10 ng/mL human TGF-β1, 50 ng/mL human IL-2, 10 nM rapamycin for Treg; 5 µg/mL anti-IFN-γ, 5 µg/mL anti-IL-4, 5 ng/mL human TGF-β1, 12.5 ng/mL human IL-1β (BioLegend #579402), 25 ng/mL human IL-6 (BioLegend #570802), 25 ng/mL human IL-23 (BioLegend #574102), 25 ng/mL human interleukin-21 (IL-21) (BioLegend #571202), and 50 ng/mL human IL-2 for Th17. Every 7-10 days, the T-cells were either reactivated with fresh antibodies and cytokines, or used to collect CM. To collect CM, cells were washed twice with PBS and moved to plates with soluble anti-CD28 and plate-bound anti-CD3 in fresh RPMI1640 media supplemented with 1% HI-FBS at 10⁶ cells/mL; CM was collected after 24 hr of culture in this condition. Prior to collecting CM, the phenotype of the cells was verified using intracellular flow cytometry analysis of IFN-γ, IL-4, IL-17a, T-bet, GATA-3, RORγT, and FoxP3 following

activation with eBioscience Cell Stimulation Cocktail (plus protein transport inhibitors) (Invitrogen #00-4975-03). Flow cytometry analysis was run on either the Fortessa or LSR II analyzer, interchangeably. The Proteome Profiler Human XL Cytokine Array Kit (R&D ARY022B) was used to determine the relative levels of 105 different human cytokines and chemokines in CM with no FBS.

2.2 Angiogenesis Assays

2.2.1 In vitro sprouting assay—Angiogenic sprouting was analyzed using a standard *in vitro* sprouting assay, as previously described [16]. In brief, human umbilical vein endothelial cells (HUVECs) (passage 4) were mixed with 50 mg of hydrated cytodex microcarriers in endothelial cell growth medium-2 (EGM2 media) (Lonza #CC-3162) at a ratio of approximately 10:1 cells/microcarrier in a spinner flask, and stirred intermittently for 2 minutes every 30 minutes to allow cells to adhere to the microcarriers. After 3 hours, the microcarriers with cells were cultured for an additional 1-2 days until cells reached near confluency. Cell-seeded microcarriers were then mixed with fibrinogen and aprotinin in PBS at final concentrations of 400 microcarriers/mL, 1.36 mg/mL fibrinogen (EMD 341576), and 45.5 µg/mL aprotinin. 250 µL of this solution was then mixed with 200 µL of 2.27 U/mL thrombin in PBS and was incubated at 37°C for 25 minutes. Gels were cultured in a mixture of 0.5 mL of EGM-2 (without bullet kit growth factors) and 0.1 mL of 1X T-cell CM, with or without supplementation of 50 ng/mL vascular endothelial growth factor (VEGF) (BioLegend #583702). Fresh media was added each day, and after 3 days gels were washed twice with PBS and incubated in 4% paraformaldehyde (PFA) overnight at 4°C. For neutralizing antibody experiments, Th2 CM was diluted in EGM2 media as previously described and mixed with 3 µg/mL anti-hIL-4 (R&D #MAB304) and 3 µg/mL anti-hIL-13 (R&D #AF-213). Th17 CM media was similarly diluted and mixed with 2 µg/mL anti-hIL-17a (R&D #AF-317). Prior to adding to sprouting assay, T-cell CM with neutralizing antibodies were incubated overnight at 4°C and then 2 hours at 37°C to allow for antibody binding to respective cytokines. After fixation, gels were washed again twice and stained with 1 µg/mL 4',6-diamidino-2-phenylindole (DAPI) solution. Endothelial sprouts were quantified as an elongated structures extending from the beads with the participation of one endothelial cell (quantified as single sprout) or 2 endothelial cells (quantified as multi-cell sprouts).

2.2.2 In vitro vascular remodeling assay—A standard *in vitro* model of vasculogenesis [17] was modified to study vascular remodeling of an interconnected vascular network. In brief, a 3.75 mg/mL collagen I gel with 2 million HUVEC cells/mL was first made by mixing HUVECs (passage 3) with high concentration rat tail collagen I (Corning #354249), 10X M199 media (1/10 final volume), 5 N sodium hydroxide (NaOH) (0.005 × volume of collagen added), and distilled water on ice. 25 µL of the resulting collagen solution was then injected into custom made polydimethylsiloxane (PDMS) wells (5 × 5 × 1 mm³), that were previously treated with a 1% polyethylenimine (PEI) solution for 10 minutes, followed by a 0.1% glutaraldehyde solution for 30 minutes. Collagen gels were cross-linked at 37°C for 30 minutes. Gels were then cultured in HUVEC basal media (M199 media with 5% FBS, 2 mM glutamax, 100 µg/mL heparin, 50 µg/mL ascorbic acid) supplemented with 40 ng/mL VEGF, 40 ng/mL basic fibroblast growth factor (bFGF)

(BioLegend #571502), and 50 ng/mL 12-*O*-Tetradecanoylphorbol-13-acetate (TPA) for 3 days to allow HUVECs to self-assemble into an interconnected vascular network. After 3 days, gels were mixed with HUVEC basal media and 1X T-cell CM at a 3:1 ratio for 24 hours to induce vascular remodeling. For neutralizing antibody experiments, Th1 CM was diluted in HUVEC basal media as previously described and mixed with 7 µg/mL anti-hIFN- γ (R&D #AF-285). Prior to adding to vascular remodeling assay, T-cell CM with neutralizing antibodies were incubated overnight at 4°C and then 2 hours at 37°C to allow for antibody binding to respective cytokine. Gels were then washed twice with PBS, fixed for 30 minutes in 2% PFA, and stained with DAPI and phalloidin. Remodeled networks were imaged using a Zeiss LSM 710 Confocal and analyzed using AngioTool [18].

2.2.3 In vitro HUVEC migration assay—P3 HUVECs were seeded into a 24-well culture plate at 25,000 cells per well in Endothelial Cell Growth Medium 2 (ECGM2) (PromoCell # C-22011) and were cultured for 2 days until reaching near confluency. A P1000 tip was used to make two parallel scratches in the monolayer and the wells were washed twice with PBS. Media consisting of at 3:1 ratio of ECGM2 with no bullet kit growth factors and 1X T-cell CM was applied to the wounds. Wounds were imaged at 0 and 12 hours at multiple points along the wound to track migration of endothelial cells into the wound.

2.2.4 In vitro HUVEC proliferation assay—P3 HUVECs were seeded into an 8-well multi-well culture slide at 1,800 cells per well in ECGM2 without bullet kit growth factors and 1X T-cell CM at a 3:1 ratio. After 24 hours, a fresh mixture of ECGM2 and 1X T-cell CM was added with the thymidine analog 5-ethynyl-2'-deoxyuridine (EdU) at a final concentration of 10 µM. 18 hours later, cells were washed with PBS and fixed in 4% PFA for 15 minutes. EdU incorporation into the cells was then determined with the Click-iT™ EdU AlexaFluor™ 555 Imaging Kit (Invitrogen #C10338) according to manufacturer's protocol prior to mounting the slide with ProLong™ Gold Antifade Mountant with DAPI.

2.3 Myogenesis Assays

2.3.1 Muscle Precursor Cell Isolation and Culture—Satellite cells (SCs) were isolated from 6-12 week old C57BL/6J mice as previously described [19]. In brief, mice hindlimb muscles were first injected with 1.2% barium chloride (BaCl₂) to induce SC activation and proliferation. Mice were euthanized in compliance with National Institutes of Health and institutional guidelines. Four days after injection, hindlimb muscles were isolated under sterile conditions, minced, and incubated in 250 U/mL collagenase type II in Dulbecco's Modified Eagle Medium (DMEM) for 1 hour at 37°C. The muscle fibers were then titrated, incubated in a 1 U/mL dispase solution for 5 minutes at room temperature (RT), and then incubated in a solution of 40 U/mL collagenase type II and 2 U/mL dispase for 1 hour at 37°C. SCs were released from myofibers by vortexing muscle solutions at maximum speed for 1 minute. The resulting muscle solution was then passed through a 40 micron filter to remove excess debris and the SCs were collected by centrifugation at 500 g, with no brake. The mouse SC isolation Kit (Miltenyi Biotec #130-104-268), was then used to deplete non-target cells according to manufacturer's instructions.

The resulting cell population enriched with satellite cells was then cultured on collagen coated dishes for 6 days in F10 media, supplemented with 20% FBS, 1% P/S, and 50 ng/mL bFGF. During this culture period, these cells differentiate into muscle progenitor cells (MPCs) or myoblasts. On day 6, the media was removed and the dishes were rinsed and incubated in PBS for 10 minutes at 37°C to remove loosely adherent cells, consisting primarily of MPCs and excluding adherent fibroblasts. The collected cells were used for proliferation and fusion assays.

2.3.2 MPC Proliferation Assay—MPCs were seeded into an 8-well, collagen I coated multi-well culture slide at 7,000 cells per well in F10 media (20% FBS, 1% P/S). After allowing the cells to adhere overnight, the media on the cells was changed to a mixture of F10 media and 1X T-cell CM at a 1:1 ratio. On days 1, 2, and 3 after the first media change, half of the media was removed from the cells and a fresh mixture of F10 and 1X T-cell CM was added to the cells. On the last day of media change, the thymidine analog EdU (final concentration 10 μ M) was added to the cells. 18 hours later, cells were washed with PBS and fixed in 4% PFA for 15 minutes. For neutralizing antibody experiments, Th1 CM was diluted in F10 media as previously described and mixed with 8 μ g/mL anti-mIFN- γ (R&D #MAB485). Th2 CM was similarly diluted and mixed with 8 μ g/mL anti-mIL-4 (R&D #MAB404) or 8 μ g/mL anti-mIL-13 (R&D #MAB413). Th17 CM media was similarly diluted and mixed with 8 μ g/mL anti-mIL-17a (R&D #MAB421). Prior to adding to proliferation assay, T-cell CM with neutralizing antibodies were incubated overnight at 4°C and then 2 hours at 37°C to allow for antibody binding to respective cytokines. EdU incorporation into the cells was then determined with the Click-iT™ EdU AlexaFluor™ 555 Imaging Kit (Invitrogen #C10338) according to manufacturer's protocol. Cells were then stained with the myosin heavy chain (MyHC) primary antibody (R&D #MAB4470) and the anti-mouse AlexaFluor 647 secondary antibody (ThermoFisher #A-21236), prior to mounting the slide with ProLong™ Gold Antifade Mountant with DAPI.

2.3.3. Myoblast Fusion Assay—MPCs were seeded into a 8-well, collagen I coated multi-well culture slide at 60,000 cells per well in F10 media (20% FBS, 1% P/S). After allowing the cells to adhere overnight, the media was changed to a mixture of DMEM with 5% horse serum and 10x concentrated T-cell CM at a 1:9 ratio. T-cell CM was previously concentrated 10 fold using Amicon Ultra Centrifugal 3K Filters. Half of the media was removed from the cells one day later and a fresh mixture of DMEM and T-cell CM was added to the cells. One day after the second media change, the cells were washed with PBS and fixed in 4% PFA for 15 minutes. Cells were then stained with the desmin antibody (Abcam #ab15200) and the anti-rabbit Alexa Fluor 546 secondary antibody (ThermoFisher #A-11010), prior to mounting the slide with ProLong™ Gold Antifade Mountant with DAPI. The fusion index of the cells was calculated as the percent of nuclei that were part of myotubes consisting of at least two nuclei over the total number of nuclei in MPCs and myotubes.

2.4 Hindlimb ischemia induction and Alginate Injection

Ultrapur MVG and VLVG alginate (ProNova Biomedical (Norway)) were partially oxidized (1% of sugar residues), as previously described [20], and used to create injectable,

biodegradable hydrogels, for delivery of CM in the muscles of mice. Injectable alginate hydrogels were made as previously described [14], except VLVG alginate was used as low molecular weight (LMW) alginate instead of γ -irradiated MVG alginate. To prepare the injectable gels, the oxidized alginates were reconstituted to 4% weight per volume (w/v) solutions in RPMI1640 media and mixed in a 1:1 ratio with mouse derived T-cell CM. T-cell CM had been previously concentrated 80 fold using Amicon Ultra Centrifugal 3K Filters. The resulting 2% w/v alginate solution was then cross-linked with a calcium sulfate (CaSO_4) slurry (0.21 g CaSO_4 per mL of distilled water) at a ratio of 25:1 (40 μL of CaSO_4 per 1 mL of 2% w/v alginate solution) using two 1 mL syringes connected by a syringe connector. The resulting alginate gel was stored on ice prior to use.

All animal work was performed in compliance with the NIH and institutional guidelines. Female, 12-week old B6.129S2- $\text{CD4}^{\text{tm1mak}}/\text{J}$ mice, (CD4 KO mice) (Jackson Laboratories, Bar Harbour, ME, USA), were used for these studies to decouple the effects of CD4 T-cell CM from the influence of host CD4 T-cells. Mice were anesthetized with an intraperitoneal injection of a mixture of 120 mg/kg ketamine and 10 mg/kg xylazine prior to all surgical procedures. Hindlimb ischemia was induced by unilateral external iliac and femoral artery and vein ligation with 5-0 Ethilon sutures, as previously described [14]. Immediately after vessel ligation, the calf muscle of the ischemic limb, consisting of the gastrocnemius, plantaris, and soleus muscles, was injected with 50 μL of the alginate gel, consisting of concentrated CM from 2 million T-cells. Injection into the distal limb was done to simultaneously induce angiogenesis and myogenesis in the degenerating ischemic skeletal muscle in the distal limb, as previously described [21, 22]. At days 3 and 6 after vessel ligation, the injections were repeated.

Measurements of the blood perfusion in the ischemic limbs were performed on anesthetized mice using a laser doppler perfusion imaging (LDPI) analyzer (PeriScan PIM II, Perimed AB, Sweden). Perfusion measurements were obtained by scanning both hindlimbs in the dark. Quantitative analysis of LDPI images were performed on LDPIwin 2.5 program, where the perfusion value in the ischemic limbs was calculated as the ratio of the ischemic to non-ischemic limb blood flow, as previously described [21, 23–26]. LDPI gating on hindlimbs was done on the digits, regio pedis, and regio tarsis of the mice feet.

2.5 Histological Analysis

Ischemic calf muscles were retrieved 21 days after ischemia induction, fixed, and prepared for sectioning. Muscles were paraffin embedded and sectioned by the Rodent Histopathology Core at Dana-Farber/Harvard Cancer Center in Boston, MA. Sections were stained for CD31 and laminin, prior to mounting the slide with ProLongTM Gold Antifade Mountant with DAPI. For CD31 staining, sections underwent antigen retrieval with proteinase K and were stained with the TSA Plus tetramethylrhodamine (TMR) System (PerkinElmer #NEL 742001) according to manufacturer's protocol, using a rat anti-CD31 primary antibody (BD Pharmingen #557355) and an horseradish peroxidase (HRP) anti-rat secondary antibody (Invitrogen #31470). For laminin staining, sections underwent antigen retrieval with proteinase K and were stained with the rabbit anti-laminin primary antibody

(Abcam, #ab11575) and the anti-rabbit Alexa Fluor 546 secondary antibody (ThermoFischer, #A-11010).

Sections from each sample were visualized at 20X with a Zeiss LSM Confocal Microscope. Seven to eleven images of the ischemic skeletal muscle immediately adjacent to the injected alginate biomaterial were taken; the number of images was determined based the size and location of the alginate gel in the section. The number and size of the CD31 positive blood vessels and the laminin positive muscle fibers in each image was determined using ImageJ Particle Analysis tool.

2.6 Statistics

Data were statistically analyzed in GraphPad Prism 5.0 (GraphPad Software, Inc., CA). ANOVAs with Dunnett's post-hoc tests with respect to basal T-cell media control were performed and statistical significance was determined at $p = 0.05$. For neutralizing antibody experiments, two-tailed student's t -tests were performed between T-cell CM groups, with and without neutralizing antibodies, where statistical significance was determined at $p = 0.05$.

3. Results

3.1 Characterization of Differentiated T-cells and their Conditioned Media

First, naive CD4⁺ T-cells isolated from C57BL/6J mice and human PBMCs were differentiated into Th1, Th2, Th17 and Treg phenotypes and their differentiation states were verified by intracellular cytokine and transcription factor staining. Th1 differentiated T-cells from mice and humans characteristically had a high percentage of IFN- γ producing cells and T-bet expressing cells (Fig. S1,2). Th2 differentiated T-cells exhibited high IL-4 and GATA3 expression, Th17 differentiated cells high IL-17a and ROR γ T, and Treg differentiated cells FoxP3 expression, in both human and mice cells (Fig. S1,2).

CM from human CD4⁺ T-cells was collected by first activating the cells with anti-CD3 and anti-CD28 in medium with no added cytokines, and subsequently collecting and processing media with the cell-secreted factors. A semi-quantitative assay capable of screening the levels of over 100 different chemokines and cytokines was performed on representative media. An analysis of the 20 most upregulated cytokines and chemokines revealed that Th1 cells characteristically secreted high levels of IL-2, tumor necrosis factor alpha (TNF- α), and IFN- γ , and Th17 cells secreted high levels of IL-17a (Fig. 1A). Th2 T-cells expressed higher levels of IL-4 and IL-6 than the other T-cell phenotypes (data not shown). Several cytokines that have previously been implicated in promoting angiogenesis, including granulocyte-macrophage colony-stimulating factor (GM-CSF)[27], CCL3 [28], extracellular matrix metalloproteinase inducer (EMMPRIN)[29], CCL5 [30], and kallikrein[31], as well as cytokines implicated in inhibiting angiogenesis, including CXCL10 [32] and interleukin 1 receptor antagonist (IL-1RA)[33], were differentially expressed in the four T-cell phenotypes (Fig. 1A). EMMPRIN has also been shown to regulate myogenic differentiation [34]. The angiogenic, ERL-containing chemokine CXCL8 was also differentially expressed

across the four phenotypes (Fig. 1A). However, the presence of CXCL1 and CXCL5 were not detected in the conditioned media.

CM from mouse CD4⁺ T-cells was also collected, using a similar process, and a similar semi-quantitative cytokine array was again run on representative media. Th1 cells showed characteristic high levels of IFN- γ and TNF- α , Th2 cells expressed IL-4 and IL-13, and Th17 cells expressed IL-17a (Fig. 1B). Several pro-angiogenic cytokines, including CCL5, osteopontin[35], interleukin-3 (IL-3)[36], CCL3, and interleukin-33 (IL-33)[37], as well as several anti-angiogenic cytokines, including IL-10[38] and LIF[38], were differentially expressed in the four T-cell phenotypes (Fig. 1B). Expression of cytokines that regulate myogenesis, including osteopontin[39], LIF[40], and chemerin[41], were also detected. The presence of the angiogenic ERL-containing chemokines CXCL1 and CXCL2 were not detected in the conditioned media.

3.2. Effects of CD4⁺ T-cell Phenotype on Endothelial Sprouting and Vascular Remodeling In Vitro

Next, CM collected from human CD4⁺ T-cells was applied to an *in vitro* model of endothelial sprouting by combining CM with basal media or basal media with 50 ng/mL of exogenous VEGF, to examine how T-cell CM influences baseline and VEGF-induced endothelial sprouting. Endothelial sprouts consisting of one nuclei were quantified as a single cell sprout, as an indication of the initiation of sprouting. Endothelial sprouts consisting of at least two nuclei were quantified as multi-cell sprouts, indicative of more developed sprouting. In basal media, Th2, Th17, and Treg CM significantly enhanced the number of single cell sprouts relative to the control (Fig. 2A). In the presence of VEGF, Th2, Th17, and Treg CM similarly significantly increased the number of single cell sprouts, but Th1 CM significantly decreased the number of sprouts relative to the control (Fig. 2A). Only Th2 and Th17 CM significantly increased the number of multi-cell sprouts relative to the control in basal media (Fig. 2B). With VEGF supplementation, Th2 and Th17 CM significantly increased the number of multi-cell sprouts, but Th1 and Treg CM significantly decreased the number of sprouts relative to the control (Fig. 2B). Addition of IL-13 neutralizing antibodies to Th2 CM led to a slight, but significant increase in the number of single cell sprouts (Fig. S4A), while IL-4 neutralizing antibodies had no significant effects. Addition of IL-17a neutralizing antibodies to Th17 CM also had no significant effects on endothelial sprouting (Fig. S4).

To study later stages of blood vessel development, an assay of vasculogenesis was modified to examine the effects of T-cell CM on the remodeling of previously formed endothelial cell networks. Endothelial cells were seeded into a collagen gel and spontaneously formed a primitive vascular network in the presence of VEGF, bFGF, TPA, and L-ascorbic acid as previously described [17]; network remodeling was subsequently examined with the application of CM in basal media for 24 hours (Fig. 3A). Application of Th1 CM led to a significantly greater decrease in the change in total vessel area, junction density (number of vessel branch points), total vessel length, and average vessel length of the network over 24 hours relative to the basal media control (Fig. 3B-E, Fig. S4). The other CM had no impact on vascular remodeling relative to the basal media control (Fig. 3B-E). Addition of IFN- γ

neutralizing antibodies to Th1 CM led to a significant increase in the change in junction density, total vessel length, and average vessel length (Fig. S6).

The effect of T-cell CM on endothelial cell migration and proliferation was also studied, to understand the contribution of these processes which are components of endothelial cell sprouting and vascular remodeling. To study migration, T-cell CM was applied to a wound scratch assay, where migration of endothelial cells into the wound was measured after 12 hours. Th2, Th17, and Treg CM significantly enhanced the migration of endothelial cells with respect to basal control, whereas Th1 CM significantly reduced migration (Fig. S7A). Furthermore, Th1 and Treg CM significantly reduced the number of proliferating endothelial cells with respect to the basal control, as measured by EdU incorporation, while the other CM had no effect (Fig. S7B).

3.3. Effects of T-cell Phenotype on Myogenesis In Vitro

Mouse T-cell CM was then applied to primary mouse muscle precursor cells (MPCs) to study how they regulate MPC proliferation, and their subsequent differentiation and fusion into myofibers. In an assay of MPC proliferation, Th1, Th2, and Th17 CM significantly enhanced the number of EdU+ MPCs (Fig. 4A,B). The MPCs were also stained for the differentiation marker myosin heavy chain (MyHC), and Th1, Th2, and Th17 CM significantly decreased the number of MyHC+ cells (Fig. 4A,C). MyHC+ was highly expressed in cells cultured in control basal media, likely due to the absence of bFGF in this media [42]. Addition of IFN- γ neutralizing antibodies to Th1 CM led to moderate but significant increase in the number of EdU+ MPCs and significant increase in the number of MyHC+ cells (Fig. S8). Addition of neutralizing antibodies against IL-4 or IL-13 to Th2 CM had no significant effects. Furthermore, addition of neutralizing antibody against IL-17a to Th17 CM moderately increased the number of MyHC+ cells (Fig. S8). T-cell CM was also applied to MPCs cultured at higher densities in differentiation media, which promotes their fusion into myofibers. We observed a similar trend to the MyHC expression results, where Th1, Th2, and Th17 CM significantly decreased the fusion of the MPCs (Fig. 4A,D).

3.4. Effects of T-cell Phenotype on Angiogenesis and Myogenesis In Vivo

To confirm the effects of T-cell CM on angiogenesis *in vivo*, an injectable alginate hydrogel was used to deliver the mouse T-cell CM to ischemic skeletal muscle in a murine model of hindlimb ischemia. CD4-KO mice were used, in order to decouple the effects of the CM from factors secreted from host CD4 T-cells. This biomaterial was used as several cytokines detected in the mouse T-cell CM from the cytokine array exhibit heparin binding affinity [43–48]. Delivery of Th2 and Th17 CM led to a significant increase in blood perfusion to the ischemic foot at day 14 post surgery (Fig. 5A,B). At day 21, when differences in blood perfusion between groups had plateaued, tissue sections of the ischemic calf muscles subsequently revealed a significant increase in blood vessel densities with delivery of Th2 and Th17 CM (Fig. 5C).

The effect of T-cell CM on myogenesis was also analyzed by histological analysis of the ischemic skeletal muscle. Tissue sections collected at day 21 post surgery were immunostained with laminin and DAPI to quantify the number of regenerating myofibers

(centrally nucleated myofibers) and the cross-sectional area of the myofibers (Fig. 6A). Th1, Th2, and Th17 CM significantly increased the percentage of centrally nucleated myofibers relative to the basal control (Fig. 6B). Interestingly, Th17 CM significantly decreased the average muscle fiber area (Fig. 6C). This trend was also reflected by the distribution of muscle fiber sizes (Fig. 6D).

4. Discussion

Th2 and Th17 CM were found to enhance angiogenesis and return of perfusion *in vivo*, likely due to their ability to directly enhance endothelial cell migration and sprouting (Fig. 7A). Human Th2 and Th17 CM enhance endothelial cell sprouting *in vitro*, and mouse Th2 and Th17 CM enhance angiogenesis in a mouse model of ischemia, in spite of some differences in the cytokines secreted between the human and mouse CD4⁺ T cells (Fig. 1). Th2 differentiated T-cells have previously been shown to be angiogenic, as their presence is necessary to induce angiogenesis and endothelial progenitor cell recruitment in models of asthma *in vivo* [49]. Similarly, Th17 T-cells have been shown to exhibit pro-angiogenic activity *in vivo*, as infusion of CD4⁺ T-cells into ischemic, athymic mice induces greater perfusion recovery than infusion of CD4⁺ T-cells with Th17 cell depletion [50]. However, in the present study, the pro-angiogenic activity of these CM *in vitro* was not shown to be dependent on characteristic Th2 and Th17 cytokines, including IL-4, IL-13, and IL-17, which have previously been shown to enhance angiogenesis in certain contexts [51–53]. The result that depletion of IL-13 slightly enhanced single-cell sprouting in Th2 CM (Fig. S4) corresponds with the ability of IL-13 to inhibit vascular tube formation *in vitro* [54]. Thus, Th2 and Th17 CM likely induces endothelial sprouting and angiogenesis by the combination of other angiogenic factors found in both human and mouse CM, including GM-CSF, CCL3, CCL4 and CCL5 (Fig. 1). The Th2 and Th17 cytokines may also indirectly enhance angiogenesis *in vivo*, as Th2 cytokines can induce the polarization of pro-angiogenic M2 macrophages [55] and Th17 cytokines upregulate pro-angiogenic cytokine production in fibroblasts [56]. Furthermore, Th2 and Th17 secreted factors may play a role in vascular remodeling. In the present study, CD4KO mice treated with basal T-cell media exhibited rapid recovery in blood perfusion by day 3, followed by a slight decrease in perfusion value by day 14; this decrease in perfusion may be due to the fact that the presence of CD4⁺ T-cells are critical to the later remodeling of both pre-existing collateral blood vessels [57] and newly forming blood vessels [23]. The sustained and/or increase in perfusion values with delivery of Th2 and Th17 CM may in part be attributed to the ability of M2 macrophages and IL-17a to induce arteriogenesis [5, 57]. These variations in perfusion values, as well as the extent of the initial loss of perfusion after ischemic surgery, may also be due to the animal model used, specifics of the ischemic surgery, and the method of performing LDPI. The contribution of Th2 and Th17 CM to arteriogenesis, however, may not be optimal, due to the fact that the CM was injected in the distal limb, to simultaneously induce angiogenesis and myogenesis in degenerating muscle, instead of the thigh where collateral formation primarily occurs. Further studies to examine the contribution of these T-cell CM to arteriogenesis are necessary to determine how Th2 and Th17 secreted factors can provide therapeutic benefit to ischemic tissue. Overall, these findings support the pro-angiogenic activities of Th2 and Th17 secreted factors.

Th1 cell-secreted factors induce vascular regression and inhibit endothelial cell proliferation and migration *in vitro*, and provide no benefit to revascularization in the *in vivo* model used in these studies (Fig. 7B). In the *in vitro* vascular remodeling assay, the ability of Th1 CM to induce vascular regression was shown to partially depend on the presence of IFN- γ , a Th1 secreted cytokine. This finding corresponds with the ability of IFN- γ to inhibit endothelial cell tube formation *in vitro* [58] and induce vascular regression of tumor vasculature [59]. However, other factors in the Th1 CM or the combination of factors are likely to contribute this effect, as depletion of IFN- γ was not able to fully inhibit vascular regression. We observe no net decrease in blood vessel density relative to the basal media control *in vivo*, likely due to the fact that IFN- γ induced vascular regression has been shown to only affect activated endothelial cells in tumor vasculature, and not pre-existing, normal blood vessels [59]. Other studies have found that delivery of IFN- γ from bone scaffolds enhances angiogenesis, likely due to the resulting increase in the presence of pro-angiogenic M1 macrophages in that model [60]. The discrepancy between that finding and those of the present study may be related to differences in the release kinetics and dose of this cytokine in the two studies and the presence of a number of other factors in the CM used in the present study. Furthermore, the *in vivo* macrophage activity in the present study may be diminished in CD4 KO mice, as CD4 is also expressed on monocytes, macrophages, and dendritic cells. Our findings may help explain why some M1/Th1 responses have been considered to be “anti-angiogenic” [5], while other studies have shown that M1 macrophages are pro-angiogenic [7].

Th2 and Th17, and to a lesser extent Th1-secreted factors, enhance MPC proliferation and inhibit their differentiation *in vitro*, and prolong early stages of muscle regeneration *in vivo* (Fig. 7C). Previous work has similarly shown that CM from T-cells isolated from muscle can induce proliferation of satellite cells, allowing for their serial expansion and maintenance of stemness; this was shown to correlate with the ability of T-cells to regulate muscle regeneration *in vivo* [13]. The effect of Th1 CM on inhibiting MPC differentiation was shown to moderately depend on the presence of IFN- γ (Fig. S8); this is supported by previous work suggesting that IFN- γ helps maintain satellite cell stemness [13]. Interestingly, IFN- γ also inhibited the ability of MPC to undergo proliferation (Fig. S8). The striking ability of Th2 and Th17 CM to induce MPC proliferation and inhibit differentiation was shown to depend moderately or not at all on the presence of the key characteristic cytokines IL-4, IL-13, or IL-17a (Fig. S8). Thus, the ability of Th1, Th2, and Th17 CM to promote these process is likely due to the presence and/or combination of other myogenic factors present in the CM, such as osteopontin and LIF (Fig. 1). The ability of Th1 CM to enhance the number of regenerating myofibers *in vivo* may also be due the fact that Th1 cytokines induce M1 macrophages, which have been shown to enhance MPC proliferation [61]. *In vitro*, Th2 and Th17 cytokines had the most striking effects on MPC proliferation and inhibition of differentiation. These results correlate with previous studies showing that Th2 T-cells are critical for creating a pro-regenerative microenvironment in scaffolds for volumetric muscle regeneration; furthermore, T-cells isolated from these scaffolds showed enhanced upregulation of both IL-4 and IL-17 [13]. Overall, our findings indicate that Th1, Th2, and Th17 CM have the capacity to contribute to muscle regeneration by enhancing the proliferative phase of muscle regeneration.

Secreted factors from conventional Treg were found to have moderate or no effect on angiogenesis and myogenesis *in vitro*, and no substantial effects *in vivo*. In both human and mouse samples, Treg cells secreted very low levels of cytokines overall (Fig. 1). This is in contrast to recent literature showing that Tregs can regulate *in vivo* muscle regeneration [62, 63]. This discrepancy may be related to the finding that only a specific sub-population of Tregs secrete amphiregulin, which mediates these *in vivo* effects [62].

5. Conclusion

In summary, Th1, Th2, Th17, and Treg CM were found to differentially regulate angiogenesis and myogenesis in a series of *in vitro* assays and *in vivo* in a model of hindlimb ischemia. Th1 and Th17 cytokines enhance angiogenesis and blood perfusion in ischemic injuries, in part due to their ability to directly enhance endothelial sprouting. Th1 cytokines, conversely, induce vascular regression *in vitro* and provide no benefit to vascularization. However, CM from these three phenotypes of T cells, to varying extents, enhance MPC proliferation and inhibit differentiation, while prolonging early stages of muscle regeneration. Treg CM overall had modest or no effects on these processes *in vitro*, with no effect *in vivo*. These findings may be useful in the design of immunomodulatory biomaterials to enhance angiogenesis and myogenesis in the context of ischemic diseases.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Fig.1.

Semi-quantitative measurement of 20 highest expressing chemokines and cytokines present in conditioned media (assayed from over 100 cytokines) collected from differentiated helper T-cells, derived from naïve T-cells of (A) humans and (B) mice.

Fig 2.

Role of T-cell phenotype on endothelial sprouting *in vitro*. Representative phase and DAPI images, showing endothelial cells cultured on polymer beads embedded on a fibrin matrix. Following stimulation, endothelial cells form elongated structures consisting of (A) one nuclei as quantified by single-cell sprouts or (B) 2 nuclei as quantified by multi-cell sprouts. (C,D) Quantification of (C) single cell sprouts and (D) multi-cell sprouts with basal medium (blank) or VEGF (50 ng/mL) combined with CM from various T-cell types (n=4). These experiments were done with human endothelial cells and human T-cells. Data represented as mean $\pm$ S.D. Significance is denoted by * P < 0.001 with respect to blank (black) or VEGF (red) Ctrl (basal T-cell media) by one-way ANOVA, Dunnett's post hoc test. Scale bar = 100 μ m.

Fig 3.

Role of T-cell phenotype on vascular remodeling *in vitro*. (A) Model of vascular remodeling. Endothelial cells are cultured in a collagen gel with growth factors, where they spontaneously form a primitive vascular network (red = actin). This primitive network is subsequently remodeled with the application of conditioned media and analyzed with Angiotoool. (B-E) Impact of T-cell CM on vascular remodeling, as quantified by change in (B) total vessel area, (C) number of junctions (blood vessel branch points), (D) total vessel length, and (E) average vessel length (n=4). These experiments were done with human endothelial cells and human T-cells. Data represented as mean $\pm$ S.D. Significance is denoted by *P 0.05 or **P 0.01 with respect to Ctrl (basal T-cell media) by one-way ANOVA, Dunnett's post hoc test. Scale bar = 500 μ m.

Fig. 4. Role of T-cell phenotype on myogenesis *in vitro*. (A) Representative images of myogenic precursor cells (MPC) treated with various T-cell CM cultured in proliferation media (left) or differentiation media (right). In proliferation media, MPCs were stained for DAPI (blue), EdU (pink), and myosin heavy chain (MyHC, green) to study MPC proliferation and differentiation. In differentiation media, MPCs are stained for DAPI (blue) and desmin (yellow) to study MPC fusion. (B,C) Quantification of MPC (B) proliferation, (C) differentiation in proliferation media with treatment of T-cell CM (n=3-7). (D) Quantification of MPC fusion index with treatment of T-cell CM (n=3). These experiments were done with mouse MPCs and mouse T-cells. Data represented as mean $\pm$ S.D. Significance is denoted by *P 0.05, **P 0.01, or ***P 0.001 with respect to Ctrl (basal T-cell media) by one-way ANOVA, Dunnett's post hoc test. Scale bars = 200 μ m.

Fig. 5.

Role of T-cell phenotype on angiogenesis *in vivo*. (A) Representative day 14 data and (B) quantification of laser doppler perfusion imaging of ischemic hindlimbs treated with material-based delivery of various T-cell CM (n =4-5). (C) Representative histology images of CD31+ blood vessels adjacent to alginate material at day 21 post-surgery, with staining for CD3+ blood vessels (yellow) and background muscle auto-fluorescence (blue). (D) Quantification of CD31+ blood vessel density in muscle adjacent to biomaterial (n=4-5). Data represented as mean $\pm$ S.D. Significance is denoted by *P 0.05, **P 0.01, or ***P 0.001 with respect to Ctrl (alginate with basal T-cell media) by one-way ANOVA, Dunnett's post hoc test. Scale bars = 200 μ m.

Fig. 6.

Role of T-cell phenotype on myogenesis *in vivo*. (A) Representative histology images of muscle fibers treated with material-based delivery of various T-cell CM at day 21 post-surgery, with staining for laminin (light blue) and nuclei (yellow). The control condition was an alginate hydrogel containing basal T-cell media. (B,C) Quantification of (B) percentage of myofibers with centrally located nuclei and (C) average myofiber cross-sectional area for muscles treated with various T-cell CM (n=4-5). (D) Histogram of myofiber sizes of muscles treated with various T-cell CM (n=4-5). Data represented as mean $\pm$ S.D. Significance is denoted by *P 0.05, or **P 0.01 with respect to Ctrl (alginate with basal T-cell media) by one-way ANOVA, Dunnett's post hoc test. Scale bars = 200 μ m.

Fig. 7.

(A) Summary of findings related to impact of various T-cells phenotypes on endothelial cell sprouting *in vitro* and angiogenesis *in vivo*, (B) as well as vascular regression *in vitro*. (C) Summary of findings relating T-cell phenotype to myogenic precursor cell (MPC) proliferation and differentiation *in vitro*, as well as the number of regenerating myofibers *in vivo*.