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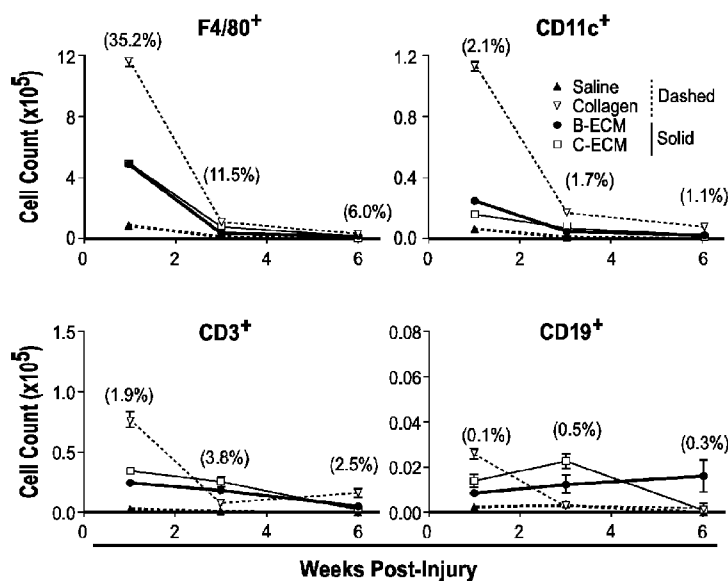
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(54) Title: COMPOSITIONS AND METHODS FOR MODULATING WOUND HEALING AND REGENERATION

(57) Abstract: The present disclosure relates to compositions and methods for modulating wound healing and regeneration. More particularly, the present disclosure relates to immunomodulatory agents that promote wound healing and tissue regeneration, and that may be optionally used in combination with synthetic or bio-material scaffolds.

FIG. 1A





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COMPOSITIONS AND METHODS FOR MODULATING WOUND HEALING AND REGENERATION

RELATED APPLICATIONS

This application is an International Patent Application which claims the benefit of
5 priority under 35 U.S.C. § 119(e) to U.S. Provisional Application No: 62/202,537, filed on
August 7, 2015 entitled, "Compositions and methods for modulating wound healing and
regeneration", which is incorporated herein by reference in its entirety.

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FIELD OF THE DISCLOSURE

The present disclosure relates to compositions and methods for modulating wound
15 healing and regeneration. More particularly, the present disclosure relates to biomaterial
scaffolds that may promote and expedite wound healing and regeneration.

BACKGROUND OF THE DISCLOSURE

Wounds are external or internal injuries caused by, for example, mechanical,
20 chemical, thermal, or pathogenic means that result in the physical disruption of tissue
integrity. For example, a wound may be a type of bodily injury in which the epidermis is
torn, cut, or punctured.

Wound healing (e.g., the restoration of tissue integrity) is orchestrated by various
growth factors and cytokines that regulate cell growth, cell migration, cell differentiation, and
25 cell proliferation, and is generally sub-divided into three broad phases: inflammation,
proliferation, and maturation. Numerous factors are involved in the complex process of
wound healing following injury, and cytokines are considered to play a key role in the
regulation of the entire process.

Wounds may be categorized as acute (e.g., fast healing) or chronic. Chronic wounds
30 are those that fail to close spontaneously or have not healed within approximately six weeks.
These wounds remain in a particular phase of wound healing, such as the inflammatory stage,
for longer than normal. Acute and chronic wounds represent a substantial disease burden to

the United States, affecting nearly ten million people every year at a cost in excess of \$25 billion. Additionally, such wounds can cause patients severe emotional, physical, and financial stress.

Promotion of wound healing and regeneration remains the focus of intensive research and study, and there are currently numerous methods and compositions available to treat wounds and promote wound healing, including a myriad of passive and active dressings and bandages, and topical medicaments, as well as physical and/or chemical debridement of necrotic tissue. Despite this, the results of prior art methods have been inconsistent, and the treatment of chronic or slow healing wounds continues to pose a serious challenge.

Accordingly, there is an urgent need for new compositions and methods for promoting wound healing and regeneration.

SUMMARY OF THE DISCLOSURE

The present disclosure relates to compositions and methods for modulating wound healing and regeneration. More particularly, the present disclosure relates to biomaterial scaffolds that may promote and expedite wound healing and regeneration.

In one aspect, the present disclosure provides a method for treating a wound in a subject that includes administering to the subject an effective amount of a scaffold and an immunomodulatory agent, thereby treating the wound.

In an embodiment, the scaffold comprises a biomaterial or a synthetic material. In some aspects, the biomaterial scaffold is a tissue-derived scaffold or a collagen scaffold.

In an embodiment, the immunomodulatory agent induces an adaptive immune system response.

In an embodiment, the immunomodulatory agent is selected from the group consisting of Mineral salt adjuvants, Tenoactive adjuvants, Bacteria-derived adjuvants, Adjuvant emulsions, Liposome adjuvants, Polymeric microsphere adjuvants, Cytokines as adjuvants, Inulin-derived adjuvants, carbohydrate adjuvants, Adjuvant formulations, Mucosal adjuvants, Cytokines, and Cancer vaccine adjuvants.

In an embodiment, the immunomodulatory agent is a protein or small molecule.

In an embodiment, the small molecule is a peptide.

In an embodiment, a rate of wound healing is increased by 2%, 4%, 6%, 8%, or 10%.

In an embodiment, the scaffold is impregnated with the immunomodulatory agent.

In an embodiment, the wound is an acute wound, a chronic wound, or a surgical wound.

In an aspect, the present disclosure provides a method for treating a wound in a subject that includes administering to the subject an effective amount of an immunomodulatory agent, thereby treating the wound.

In an embodiment, the immunomodulatory agent is administered with a scaffold.

In an embodiment, the scaffold comprises a biomaterial or a synthetic material.

In an embodiment, the biomaterial scaffold is a tissue-derived scaffold or a collagen scaffold.

In an embodiment, the immunomodulatory agent induces an adaptive immune system response.

In an embodiment, the immunomodulatory agent is selected from the group consisting of Mineral salt adjuvants, Tenoactive adjuvants, Bacteria-derived adjuvants, Adjuvant emulsions, Liposome adjuvants, Polymeric microsphere adjuvants, Cytokines as adjuvants, Inulin-derived adjuvants, carbohydrate adjuvants, Adjuvant formulations, Mucosal adjuvants, Cytokines, and Cancer vaccine adjuvants.

In an embodiment, the immunomodulatory agent is a protein or small molecule.

In an embodiment, the small molecule is a peptide.

In an embodiment, a rate of wound healing is increased by 2%, 4%, 6%, 8%, or 10%.

In an embodiment, the wound is an acute wound, a chronic wound, or a surgical wound.

In an aspect, the present disclosure provides a pharmaceutical composition for the treatment of a wound, the composition comprising an effective amount of an immunomodulatory agent.

In an embodiment, the composition is formulated to be impregnated in a scaffold.

In an embodiment, the scaffold comprises a biomaterial or a synthetic material.

In an embodiment, the biomaterial scaffold is a tissue-derived scaffold or a collagen scaffold.

In an embodiment, the composition is formulated for topical administration.

In an embodiment, the composition is formulated for administration with a biomaterial scaffold.

In an embodiment, the immunomodulatory agent induces an adaptive immune system response.

In an embodiment, the immunomodulatory agent is selected from the group consisting of Mineral salt adjuvants, Tenoactive adjuvants, Bacteria-derived adjuvants, Adjuvant emulsions, Liposome adjuvants, Polymeric microsphere adjuvants, Cytokines as adjuvants, Inulin-derived adjuvants, carbohydrate adjuvants, Adjuvant formulations, Mucosal adjuvants, Cytokines, and Cancer vaccine adjuvants.

In an embodiment, the immunomodulatory agent is a protein or small molecule.

In an embodiment, the small molecule is a peptide.

In an embodiment, the composition increases a rate of wound healing by 2%, 4%, 6%, 8%, or 10%.

In an embodiment, the composition treats an acute wound, a chronic wound, or a surgical wound.

In an aspect, the present disclosure provides a method for promoting wound healing or tissue regeneration in a subject, the method comprising administering to said subject an effective amount of a scaffold and an immunomodulatory agent, thereby increasing the rate of at which the wound heals or the tissue regenerates.

In an embodiment, the scaffold comprises a biomaterial or a synthetic material.

In an embodiment, the biomaterial scaffold is a tissue-derived scaffold or a collagen scaffold.

In an embodiment, the immunomodulatory agent induces an adaptive immune system response.

In an embodiment, the immunomodulatory agent is selected from the group consisting of Mineral salt adjuvants, Tenoactive adjuvants, Bacteria-derived adjuvants, Adjuvant emulsions, Liposome adjuvants, Polymeric microsphere adjuvants, Cytokines as adjuvants, Inulin-derived adjuvants, carbohydrate adjuvants, Adjuvant formulations, Mucosal adjuvants, Cytokines, and Cancer vaccine adjuvants.

In an embodiment, the immunomodulatory agent is a protein or small molecule.

In an embodiment, the small molecule is a peptide.

In an embodiment, a rate of wound healing is increased by 2%, 4%, 6%, 8%, or 10%.

In an embodiment, the scaffold is impregnated with the immunomodulatory agent.

In an embodiment, the wound is an acute wound, a chronic wound, or a surgical wound.

In an aspect, the present disclosure provides a kit for the treatment of a wound, the kit comprising an effective amount of an immunomodulatory agent and a biomaterial scaffold, and directions for the use of the kit for the treatment of the wound.

In an embodiment, the biomaterial scaffold is a tissue-derived scaffold or a collagen scaffold.

5 In an embodiment, the immunomodulatory agent is selected from the group consisting of wherein the immunomodulatory agent is selected from the group consisting of Mineral salt adjuvants, Tenoactive adjuvants, Bacteria-derived adjuvants, Adjuvant emulsions, Liposome adjuvants, Polymeric microsphere adjuvants, Cytokines as adjuvants, Inulin-derived adjuvants, carbohydrate adjuvants, Adjuvant formulations, Mucosal adjuvants, Cytokines, and Cancer vaccine adjuvants.

In an embodiment, the immunomodulatory agent is a protein or small molecule.

10 In an embodiment, the small molecule is a peptide.

In an embodiment, the scaffold is modified to enhance immunomodulation.

In an embodiment, the scaffold fills the wound.

In an embodiment, the scaffold partially fills the wound.

In an embodiment, the immunomodulatory agent induces a T cell response.

15 In an embodiment, the immunomodulatory agent induces a T cell response in combination with a scaffold, wherein the scaffold is a biomaterial or a synthetic material.

In an embodiment, the immunomodulatory agent induces a T cell response.

In an aspect, the present disclosure provides a scaffold composition for the treatment of a wound that includes an effective amount of an immunomodulatory agent.

20 In an embodiment, the scaffold composition is formulated to be impregnated with the immunomodulatory agent.

In an embodiment, the scaffold composition comprises a biomaterial or a synthetic material.

In an embodiment, the biomaterial is a tissue-derived scaffold or a collagen scaffold.

25 In an embodiment, the scaffold composition is formulated for topical administration.

In an embodiment, the scaffold composition is formulated for co-administration with the immunomodulatory agent.

In an embodiment, the immunomodulatory agent induces an adaptive immune system response.

30 In an embodiment, the immunomodulatory agent is selected from the group consisting of Mineral salt adjuvants, Tenoactive adjuvants, Bacteria-derived adjuvants, Adjuvant emulsions, Liposome adjuvants, Polymeric microsphere adjuvants, Cytokines as adjuvants,

Inulin-derived adjuvants, carbohydrate adjuvants, Adjuvant formulations, Mucosal adjuvants, Cytokines, and Cancer vaccine adjuvants.

In an embodiment, the immunomodulatory agent is a protein or small molecule.

In an embodiment, the small molecule is a peptide.

5 In an embodiment, the scaffold composition increases a rate of wound healing by 2%, 4%, 6%, 8%, or 10%.

In an embodiment, the scaffold composition treats an acute wound, a chronic wound, or a surgical wound.

10 In an embodiment, the wound is selected from the group consisting of a skin wound, a muscle wound, a cartilage wound, and a nervous system wound.

Other features and advantages of the disclosure will be apparent from the detailed description, and from the claims.

Definitions

15 By "binding to" a molecule is meant having a physicochemical affinity for that molecule.

By "ameliorate" is meant decrease, suppress, attenuate, diminish, arrest, or stabilize the development or progression of a disease.

20 By "compound" is meant any small molecule chemical compound, antibody, nucleic acid molecule, or polypeptide, or fragments thereof.

In this disclosure, "comprises," "comprising," "containing" and "having" and the like can have the meaning ascribed to them in U.S. Patent law and can mean "includes," "including," and the like; "consisting essentially of" or "consists essentially" likewise has the meaning ascribed in U.S. Patent law and the term is open-ended, allowing for the presence of
25 more than that which is recited so long as basic or novel characteristics of that which is recited is not changed by the presence of more than that which is recited, but excludes prior art embodiments.

By "fragment" is meant a portion of a polypeptide or nucleic acid molecule. This portion contains, preferably, at least 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, or 90% of
30 the entire length of the reference nucleic acid molecule or polypeptide. A fragment may contain 10, 20, 30, 40, 50, 60, 70, 80, 90, or 100, 200, 300, 400, 500, 600, 700, 800, 900, or 1000 nucleotides or amino acids.

By "marker" is meant any protein or polynucleotide having an alteration in expression level or activity that is associated with a disease or disorder.

As used herein, "modulating" refers to an increase or decrease in an adaptive immune system response. In a preferred embodiment, this relates to an increased, up-regulated or enhanced adaptive immune system response. An effective amount of an immunomodulatory agent is an amount that when applied or administered in accordance to the techniques herein is sufficient to modulate, preferably up-regulate, an adaptive immune system response.

By "protein" or "polypeptide" or "peptide" is meant any chain of more than two natural or unnatural amino acids, regardless of post-translational modification (e.g., glycosylation or phosphorylation), constituting all or part of a naturally-occurring or non-naturally occurring polypeptide or peptide, as is described herein.

By "subject" is meant a mammal, including, but not limited to, a human or non-human mammal, such as a bovine, equine, canine, ovine, or feline.

By "reference" is meant a standard or control condition. In one embodiment, the effect of an agent on a cell is compared to the effect of the agent on a control cell.

A "reference sequence" is a defined sequence used as a basis for sequence comparison. A reference sequence may be a subset of or the entirety of a specified sequence; for example, a segment of a full-length cDNA or gene sequence, or the complete cDNA or gene sequence. For polypeptides, the length of the reference polypeptide sequence will generally be at least about 16 amino acids, preferably at least about 20 amino acids, more preferably at least about 25 amino acids, and even more preferably about 35 amino acids, about 50 amino acids, or about 100 amino acids. For nucleic acids, the length of the reference nucleic acid sequence will generally be at least about 50 nucleotides, preferably at least about 60 nucleotides, more preferably at least about 75 nucleotides, and even more preferably about 100 nucleotides or about 300 nucleotides or any integer thereabout or therebetween.

By "specifically binds" is meant a compound or antibody that recognizes and binds a polypeptide of the disclosure, but which does not substantially recognize and bind other molecules in a sample.

By "substantially identical" is meant a polypeptide or nucleic acid molecule exhibiting at least 50% identity to a reference amino acid sequence (for example, any one of the amino acid sequences described herein) or nucleic acid sequence (for example, any one of the nucleic acid sequences described herein). Preferably, such a sequence is at least 60%,

more preferably 80% or 85%, and most preferably 90%, 95% or even 99% identical at the amino acid level or nucleic acid to the sequence used for comparison.

Sequence identity is typically measured using sequence analysis software (for example, Sequence Analysis Software Package of the Genetics Computer Group, University of Wisconsin Biotechnology Center, 1710 University Avenue, Madison, Wis. 53705, BLAST, BESTFIT, GAP, or PILEUP/PRETTYBOX programs). Such software matches identical or similar sequences by assigning degrees of homology to various substitutions, deletions, and/or other modifications. Conservative substitutions typically include substitutions within the following groups: glycine, alanine; valine, isoleucine, leucine; aspartic acid, glutamic acid, asparagine, glutamine; serine, threonine; lysine, arginine; and phenylalanine, tyrosine. In an exemplary approach to determining the degree of identity, a BLAST program may be used, with a probability score between e^{-3} and e^{-100} indicating a closely related sequence.

"Therapeutic agent" means a substance that has the potential of affecting the function of an organism. Such a compound may be, for example, a naturally occurring, semi-synthetic, or synthetic agent. For example, an agent may be a drug that targets a specific function of an organism or an antibiotic. A therapeutic agent may decrease, suppress, attenuate, diminish, arrest, or stabilize the development or progression of disease, disorder, or infection in a eukaryotic host organism.

As used herein, a "wound" refers to a physical disruption of the continuity or integrity of a tissue structure. "Wound healing" refers to the process of restoring the integrity of the tissue. The skilled artisan will understand that this may refer to a partial or a full restoration of tissue integrity. Accordingly, treatment of a wound refers to the promotion, improvement, progression, or acceleration of one or more stages or processes associated with the wound healing process. The wound may be acute or chronic. Chronic wounds may simply be described as wounds that fail to heal on a normal timeframe, which may vary depending on the nature of the wound and the specific tissue affected. The wound may also be any internal wound, e.g. where the external structural integrity of the skin is maintained, but the integrity of an underlying tissue/structure is disrupted.

BRIEF DESCRIPTION OF THE DRAWINGS

FIGs. 1A-1D: Biomaterial scaffolds induce a Th2 response in volumetric muscle wounds. C57BL/6 (WT) and B6.129S7-*Rag1*^{tm1Mom}/J (*Rag*^{-/-}) mice received a critical size

quadriceps muscle injury and were treated immediately with 0.05 mL of saline, particulate collagen, bone ECM (B-ECM), or cardiac ECM (C-ECM).

FIG. 1A present line graphs that show proportions of myeloid (F4/80⁺ Macrophages and CD11c⁺ Dendritic Cells) and lymphoid (CD3⁺ T cells and CD19⁺ B cells) cell

5 populations in WT wound environment, determined by flow cytometry (% = mean fraction of live cells across all treatments, bolded at peak level).

FIG. 1B is a bar graph showing the proportion of CD3⁺ T cells that are CD4⁺ Helper T cells or CD8⁺ Cytotoxic T lymphocytes at 1 week post-injury treated with saline, collagen, B-ECM or C-ECM. Saline = white bars, Collagen = Light grey striped bars, Bone ECM =
10 black bars, Cardiac ECM = grey bars.

FIG. 1C is a bar graph showing interleukin 4 gene expression in WT and *Rag*^{-/-} mice at 1 week post-injury.

FIG. 1D is a graph showing 1 week post-injury transcriptome of CD3 cells sorted from wounded muscles treated with saline, collagen, B-ECM or C-ECM. Data are displayed
15 as RQ to Saline-treated wounds. Data are means $\pm$ SEM, $n = 4$, ANOVA $P < 0.0001 = ****$, $P < 0.001 = ***$, $P < 0.01 = **$, $P < 0.05 = *$.

FIGs. 2A-2F: M(IL-4) pro-regenerative myeloid polarization induced by scaffolds is Th2-dependent. Scaffold microenvironment and adaptive immunity shape the wound-associated myeloid compartment. Myeloid cell recruitment and polarization were measured
20 via flow cytometry at 1 and 3 weeks post-operation and analyzed as a function of extracellular matrix (ECM) scaffold application and adaptive immune presence.

FIGs. 2A and 2B are bar graphs that show macrophages in wounded muscle are characterized for CD86 (FIG. 2A) and CD206 (FIG. 2B) expression by flow cytometry at 1 and 3 weeks post-operation in presence of saline or ECM scaffold in WT (Blue bars) and
25 *Rag*^{-/-} (red bars) mice. Mean of fluorescence (MFI).

FIG. 2C is a bar graph that shows CD206 expression at 3 weeks post-injury in C-ECM treated WT, *Il4ra*^{-/-}, *Rag*^{-/-} and *Rag*^{-/-} mice reconstituted with either WT CD4⁺ T cells (T-WT, $n = 2$) or *Rictor*^{-/-} CD4⁺ T cells (T-*Rictor*^{-/-}; Th2-deficient).

FIG. 2D is a graph that shows representative comparison of CD206 expression
30 between WT, *Il4ra*^{-/-}, *Rag*^{-/-} and *Rag*^{-/-} reconstituted with WT and *Rictor*^{-/-} CD4⁺ T cells.

FIG. 2E is a graph that shows gene expression analysis in cell sorted macrophages from wounded muscles 1 week post-injury and treated with collagen (light grey striped bars), B-ECM (black solid bars) and C-ECM (grey solid bars) compared to saline control. RQ to saline = $2^{-\Delta\Delta Ct}$.

FIG. 2F is a graph that shows RQ to saline in WT and *Rag*^{-/-} mice when wounds were treated with C-ECM. The figure shows a loss of scaffold-mediated macrophage polarization in *Rag*^{-/-} mice. WT = blue bars, *Rag*^{-/-} = red bars. Data are means $\pm$ SEM, n = 4; ANOVA (A-B) and Students T-test (D) **** = P < 0.0001, *** = P < 0.001, ** = P < 0.01, * = P < 0.05.

FIG. 2G shows representative FACS histogram showing Bone ECM-treated wounds with heightened CD86 expression in *Rag*^{-/-} mice (red open) over WT mice (blue solid).

FIG. 2H shows a representative FACS histogram showing decreased CD206 expression in Bone ECM-treated wounds at 3 weeks post-operation.

FIG. 2I shows a schematic depicting the role of environment and adaptive immunity in shaping the wound-associated myeloid compartment.

FIGs. 3A and 3B: Systemic immune homeostasis is modified by application of biomaterial scaffolds.

FIG. 3A are images showing inguinal lymph node morphology at 1 week post-operation in saline (left) and C-ECM (right) treated WT type animals. Hematoxylin and eosin staining, scale bars = 500 μ m for composite and 200 μ m for inset.

FIG. 3B are bar graphs showing *Il4* gene expression in local draining lymph nodes (inguinal, top bar graphs) and distal lymph nodes (axillary/brachial, bottom bar graphs) in WT, *Rag*^{-/-}, and *Cd4*^{-/-} mice at 1 and 3 weeks after wound treatment with collagen, B-ECM and C-ECM. RQ to saline is 2^{- $\Delta\Delta$ Ct}. Data are means $\pm$ SEM, n = 4; ANOVA **** = P < 0.0001, ** = P < 0.01, * = P < 0.05.

FIGs. 4A-4F: Th2/M(IL-4) responses to biomaterial-treated muscle wound promote functional tissue regeneration.

FIG. 4A is a bar graph that shows a treadmill exhaustion assay of mice at 6 weeks post-injury to test muscle function in WT (blue bars) and *Rag*^{-/-} (red bars) mice. Normalized to uninjured control (= 100 %). n = 5 mice per condition and genotype.

FIG. 4B is a bar graph that shows treadmill exhaustion at 3 weeks in *Cd4*^{-/-}, and *Rag*^{-/-} mice repopulated with WT (T-WT) or *Rictor*^{-/-} (T-*Rictor*^{-/-}; Th2 deficient) CD4⁺ T cells. n = 4 mice (*Cd4*^{-/-}) or n = 10 mice (T-WT and T-*Rictor*^{-/-}).

FIG. 4C are images showing transverse section of quadriceps muscle at 6 weeks post-operation in collagen and C-ECM treated WT and *Rag*^{-/-} mice. Black arrow = injury/treatment area. A = anterior, P = posterior.

FIG. 4D are images showing C-ECM treated VML at 3 weeks post-injury in WT, *Rag*^{-/-}, and *Cd4*^{-/-} mice stained with hematoxylin and eosin. Small muscle fibers and ectopic adipogenesis are present in *Rag*^{-/-} and *Cd4*^{-/-} wounds. Scale bars = 50 μ m.

FIG. 4E is a bar graph showing gene expression of *Adipoq* (adipose marker) and *Colla1* (collagen I) showing increased adipose gene expression in *Rag*^{-/-} as well as increased collagen gene expression suggesting alterations in connective tissue deposition and possible scarring. *n* = 4 mice.

FIG. 4F is a cartoon showing T cell activation and polarization induce local Th2/M(IL-4) polarization of the SIM, promoting regenerative phenotypes such as wound healing and myotube fusion, and inhibit intramuscular adipose formation and collagen deposition. Data are means $\pm$ SEM; ANOVA (A, D) and Students T-test (E) **** = *P* < 0.0001, *** = *P* < 0.001, ** = *P* < 0.01, * = *P* < 0.05.

FIGs. 5A-E: Materials characterization and selection.

FIG. 5A depicts a schematic showing extracellular matrix (ECM) scaffold preparation.

FIG. 5B depicts histological staining (hematoxylin & eosin) images of tissues pre- and post- processing.

FIG. 5C are images of *in vitro* flow cytometric analysis of iBMM (immortalized bone marrow macrophage; De Palma 2014) cell line cultured on varying ECM substrates identifies Bone (B-ECM) and Cardiac (C-ECM) as strong immunomodulatory scaffolds. CD86 = type-1 inflammatory macrophage, CD206 = type-2 alternative macrophage.

FIG. 5D is a bar graph of *in vitro* flow cytometric analysis of iBMM (immortalized bone marrow macrophage and data are expressed as fold change over TCP control in the corresponding media condition (M1, M2, or M0). Data are means $\pm$ SEM *n* = 3.

FIG. 5E shows the mechanical properties of ECM scaffolds. Data represent means, Collagen *n* = 2, Bone & Cardiac, *n* = 3.

FIGs. 6A-6F Cell recruitment to muscle injury.

FIG. 6A shows gross images of mouse quadriceps muscle at 3 weeks post-operation.

FIG. 6B is a bar graph showing the total number of cells infiltrating saline- and scaffold-treated wounds.

FIG. 6C are bar graphs showing the percent of overall cell population identified as F4/80⁺ macrophages, CD11c⁺ dendritic cells, CD3⁺ T cells or CD19⁺ B cells. Data are means $\pm$ SEM *n* = 4.

FIGs. 6D-6F show bar graphs of type-2 (pro-regenerative) gene expression in wound environment with (WT, blue bars) and without (*Rag*^{-/-}, red bars) adaptive immune cells. Data are displayed as RQ to Saline-treated wounds.

FIG. 6G is a bar graph that shows fraction of myeloid cells positive for MHCII (I-A/I-E) antigen presentation in WT animals.

FIG. 6H depicts percent of overall cell population identified as F4/80⁺ macrophages, CD11c⁺ dendritic cells, CD3⁺ T cells, CD19⁺ B cells, or CD34⁺ vascular progenitor cells.

5 FIG. 6I shows myeloid infiltration at 1 and 3 weeks post-operation in WT and *Rag*^{-/-} mice showing an increased fraction of myeloid cells in *Rag*^{-/-} mice. Data are means $\pm$ SEM *n* = 4.

FIGs. 7A-7D FoxP3⁺ T_{reg} populations at 1 and 3 weeks post-operation.

10 FIG. 7A depicts a bar graph showing the proportion of FoxP3⁺CD4⁺ T cells in the defect at 1, 3, and 6 weeks post-operation.

FIG. 7B depicts ANOVA of FoxP3⁺ cell infiltration results over time. Data are means $\pm$ SEM *n* = 4.

FIG. 7C shows expression of *IL4ra* and CCR5 on CD4⁺ and CD8⁺ T cells at 1, 3, and 6 weeks post-operation.

15 FIG. 7D shows ANOVA data of CCR5 and *IL4ra* expression over time in CD4 and CD8 T cells. Data are means $\pm$ SEM *n* = 4.

FIG. 8 depicts a graph showing data spread of gene expression profiling of CD3⁺ cells sorted from 1 week post-surgery muscle defects. dCt of WT CD3⁺ cells. Saline = Black dots. B-ECM = blue dots, C-ECM = red dots, Collagen = green dots.

20 FIG. 9: M2/M(IL4) Gene expression in scaffold-treated muscle wounds. Biomaterial scaffolds induced the expression of two M2/M(IL4) myeloid genes, *Retnla* (left) encoding Fizz1 and *Arg1* encoding Arginase 1 (right). ANOVA *** = *P* < 0.001, * = *P* < 0.05. Data are means $\pm$ SEM *n* = 4.

FIGs. 10A-10G: Myeloid polarization in WT, *Rag*^{-/-} and *Cd4*^{-/-} mice.

25 FIG. 10A is a bar graph showing confirmation of participation of CD4⁺ T cells in M2-myeloid polarization as determined in *Rag*^{-/-} studies. CD206 mean fluorescence intensity in F4/80⁺ macrophages from *Cd4*^{-/-} mice compared to WT and *Rag*^{-/-} mice at 3wks post-injury.

FIG. 10B depicts statistical analysis of overall effect of genotype and scaffold on expression of CD86 and CD206 at 1 and 3 weeks post-surgery.

30 FIG. 10C depicts two-Way ANOVA data comparing CD86 and CD206 expression in scaffold treatment to Saline control wounds at 1 and 3 weeks post-surgery.

FIG. 10D depicts bar graphs showing mean CD86 fluorescence intensity data at 1 and 3 weeks post-surgery in CD11c⁺F4/80⁻ and CD11c⁺F4/80⁺ dendritic cells.

FIG. 10E depicts bar graphs showing CD206 fluorescence intensity at 1 and 3 weeks post-surgery in CD11c⁺F4/80⁻ and CD11c⁺F4/80⁺ dendritic cells.

FIG. 10F depicts two-way ANOVA data of CD86 and CD206 expression at 1 and 3 weeks post-surgery for CD11c⁺F4/80⁻ and CD11c⁺F4/80⁺ dendritic cells.

5 FIG. 10G depicts a bar graph showing CD19⁺ B cell recruitment, characteristic of Th2 phenotype, dependent on CD4⁺ T cells. Data are means $\pm$ SEM $n = 4$.

FIGs. 11A-11C Adoptive Transfer of CD4⁺ T cells into *Rag*^{-/-} mice.

FIG. 11A depicts a timeline of adoptive transfer studies.

10 FIG. 11B depicts graphs showing purity confirmation of CD4⁺ T cells after isolation from WT and *Rictor*^{F/F} Cd4-Cre mice.

FIG. 11C depicts a graph showing confirmation of adoptive transfer at 11 days post-injection.

FIGs. 12A and 12B depict graphs showing data spread of gene expression profiling of cells sorted from 1 week post-surgery muscle defects. FIG. 12A depicts data of dCt of WT
15 F4/80⁺ cells. FIG. 12B depicts data of dCt of *Rag*^{-/-} F4/80⁺ cells. Saline = black dots, Bone = blue, Cardiac = red, Collagen = green.

FIGs. 13A-13C: Gene ontology analysis of adaptive immune dependent gene expression changes in SIM F4/80⁺ macrophages associated with wound healing and tissue regeneration. Data displayed for genes significant in FIG. 11C (F4/80⁺ Macrophages), input
20 into STRING interaction database (Szklarczyk et. al 2015).

FIG. 13A depicts a gene interaction network.

FIG. 13B depicts GO processes that are significantly enriched (FDR P -value < 0.05) from genes that alter expression in *Rag*^{-/-} mice related to development and tissue regeneration.

25 FIG. 13C depicts a word map showing common terms in GO processes related to development and tissue regeneration.

FIG. 13D shows a gene interaction network.

FIG. 13E shows GO processes that were significantly enriched (Bonferroni p -value < 0.05) from genes that altered expression in *Rag*^{-/-} mice related to development and tissue
30 regeneration.

FIG. 13F shows a Word map showing common terms in GO processes related to development and tissue regeneration.

FIGs. 14A-14C: Gene expression in draining lymph nodes at 1 and 3 weeks post-operation. Gene expression was measured in local (FIG. 14A, inguinal) and distal (FIG. 14B,

axillary/brachial) lymph nodes at 1 and 3 weeks post-operation to measure type-1 (*Tnfa*, *Il1b*, *Ifng*) and type-2 (*Il4*, *Retnla*) gene changes dependent upon scaffold application. FIG. 14C depicts ANOVA data of WT versus *Rag*^{-/-} effect on gene expression. Data are means $\pm$ SEM. ($n = 4$, Saline, B-ECM, C-ECM; $n = 3$, collagen).

FIG. 15 depicts computed tomography images which reveal irregular muscle density in *Rag*^{-/-} mice. CT imaging of mice at 6 week post-injury shows non-uniform muscle density in Saline treated and C-ECM treated *Rag*^{-/-} mice, but uniform muscle in C-ECM treated WT mice.

FIGs. 16A-16C: Quadriceps muscle at 3 weeks post-operation in WT and *Rag*^{-/-} mice.

FIG. 16A depicts hematoxylin and eosin-stained histological sections of unaffected and affected quadriceps muscle immediately after volumetric muscle loss surgery.

FIG. 16B depicts images showing increased fibrosis and decreased cellularity in collagen treated scaffolds in absence of adaptive immune cells (*Rag*^{-/-}) at 3 weeks post-injury.

FIG. 16C depicts mosaic images of quadriceps muscle at 3 weeks post-operation stained with Masson's trichrome (top) and Hematoxylin and Eosin (bottom). Scale bars = 50 microns in (FIG. 16B) and 500 microns in (FIG. 16C).

FIGs. 17A-17J: Collagen and adipose-related gene expression increases in *Rag*^{-/-} mice.

FIG. 17A depicts bar graphs showing *Colla1* gene expression at 1 and 3 weeks post injury.

FIG. 17B depicts a bar graph showing adipogenesis gene expression shown as a fold change over WT in corresponding scaffold treatment at 3 weeks post-injury

FIGs. 17C-17E show bar graphs of adipogenesis gene expression displayed as a fold change over saline control in (FIG. 17C) collagen, (FIG. 17D) B-ECM and (FIG. 17E) C-ECM treated injuries at 3 weeks post-injury. Data are means $\pm$ SEM $n = 4$. ANOVA *** = $P < 0.001$, ** = $P < 0.01$, * = $P < 0.05$.

FIGs. 17F-17J show that alterations in local and systemic immune phenotype correlated with differences in regenerative outcome of extracellular matrix.

FIG. 17F shows Hematoxylin and Eosin cross-sectional images of wounds at 3 weeks post-operation, specifically showing full cross sections of dissected muscle of saline- and cardiac-treated wounds.

FIG. 17G shows centrally nucleated fibers are larger in WT animals (left; white arrows) compared to *Rag*^{-/-} animals (right; black arrows) with the same scaffold treatment.

FIG. 17H shows increased adiposity in *Rag*^{-/-} animals compared to WT animals.

FIG. 17I shows RT-PCR of *Colla1* (Collagen I) at 3 weeks post-operation reveals decrease in WT animals that is negated in *Rag*^{-/-} animals.

FIG. 17J shows *AdipoQ* (adiponectin) gene expression is increased in *Rag*^{-/-} animals, WT = blue bars, *Rag*^{-/-} = red bars. FIG. 13F shows T cell activation and polarization induce a local Th2/M2 polarization of the SIM, promoting regenerative phenotypes such as wound healing and myotube fusion, and inhibit intramuscular adipose formation and collagen deposition. Data are means $\pm$ SEM, $n = 4$; ANOVA **** = $p < 0.0001$, ** = $p < 0.01$, * = $p < 0.05$ * between scaffold types and Ψ between genotypes.

FIGs. 18A and 18B depict data showing Wilcoxon Rank Sum Test Results on sorted CD3⁺ T cells (FIG. 18A) CD3⁺ Genes analyzed in scaffold treated vs. saline control. (FIG. 18B) CD3⁺ gene group analysis.

FIGs. 19A-19B: Wilcoxon Rank Sum and Linear Regression Test Results on sorted F4/80⁺ macrophages in WT and *Rag*^{-/-} mice.

FIG. 19A depicts Wilcoxon Rank Sum test on F4/80⁺ Genes analyzed in scaffold treated vs. saline control.

FIG. 19B depicts Linear Regression model on material by genotype interaction. WT vs. *Rag*^{-/-} comparison in effect of scaffold on gene expression changes versus saline treated control, p-values displayed for significant comparisons ($P < 0.05$ in at least one scaffold treatments)

FIG. 19C shows a Wilcoxon Rank Sum test on F4/80⁺ Genes analyzed in scaffold treated vs saline control.

FIG. 19D shows a Linear Regression model on material by genotype interaction. WT vs *Rag*^{-/-} comparison in effect of scaffold on gene expression changes versus saline treated control, p-values displayed for significant comparisons ($p < 0.05$ in one or both ECM scaffold treatments).

FIGs. 20A-20E: Gating strategies for flow cytometric analyses. FIG. 20A shows cellular recruitment to volumetric muscle wounds. FIG. 20B shows MHCII expression on macrophages (F4/80⁺CD11b⁺) and Dendritic Cells (CD11c⁺F4/80⁻). FIG. 20C shows gating on Ly6c/Ly6g to identify immature monocytes (CD11b⁺Ly6c⁺) and polymorphonuclear cells (CD11b⁺Ly6g⁺) at 1 week post-surgery. FIG. 20D shows F4/80^{hi} (F4/80⁺) macrophage polarization in WT and *Rag*^{-/-} mice. FIG. 20E shows T cell CD4:CD8 ratio.

FIG. 21 is an image of a FACS histogram that shows that macrophage polarization is diminished in absence of T cells. FACS histogram of F4/80⁺ macrophages shows *Rag*^{-/-} mice with collagen-treated muscle wounds have decreased CD206 expression (M2-polarization).

FIGs. 22A shows schematic depicting immune cell recruitment and polarization in scaffold-treated muscle wounds.

FIG. 22B shows myeloid emigration from local tissue microenvironment accompanies T cell activation in draining lymph nodes leading to increase in type-2 immune phenotype including *Il4* gene expression both locally (wound) and systemically (lymph nodes).

DETAILED DESCRIPTION OF THE DISCLOSURE

The present disclosure relates to compositions and methods for modulating wound healing and regeneration. More particularly, the present disclosure relates to biomaterial scaffolds that may promote and expedite wound healing and regeneration. The disclosure is based, at least in part, on the unexpected discovery that biomaterial scaffolds, tissue-derived and collagen-based, increase the number of immune cells in a site of a volumetric muscle defect. In particular, biomaterial scaffolds trigger a local and systemic type-2 immune response in which an M2 macrophage phenotype was dependent on a Thelper2 adaptive response to create a pro-regenerative environment characterized by high Interleukin 4 (Il4) levels.

Regenerative medicine therapies that primarily target stem cells have achieved limited success. An alternative strategy is to focus on immune cells, the first responders to traumatic wounds, which can interact directly with biomaterial scaffolds. Here, how biomaterial scaffolds shape the immune microenvironment in traumatic muscle wounds and ultimately impact tissue regeneration is investigated. A diverse population of immune cells is recruited into scaffolds and the surrounding area, including macrophages, T lymphocytes and B lymphocytes. The scaffolds induced a pro-regenerative type-2 response, characterized by an mTOR/Rictor-dependent Th2 pathway and IL-4-dependent macrophage polarization, which is critical for functional muscle regeneration. Targeting the adaptive components of the immune system during the process of biomaterials design may support the development of future therapies that efficiently control immune balance in tissues, ultimately stimulating tissue repair.

Wound Healing/Regeneration

Wound healing may be carried out by a variety of growth factors and cytokines that regulate cell growth, cell migration, cell differentiation, and cell proliferation (see e.g., Werner, S. and Grose, R., (2003) *Physiol Rev* 83:835-870). Wound healing may be divided into three general phases: inflammation, proliferation, and maturation (see e.g., Baum, C. L.

and Arpey, C. J., (2005) *Dermatol Surg* 31:674-686). These three phases do not correspond to precisely defined time periods, and may overlap to some extent.

1. Inflammation Stage

The inflammation stage begins immediately following the creation of the wound, and
5 may typically last up to about 6 days. The first stage within the inflammation stage immediately following wound creation is referred to as hemostasis, and typically involves fibrin and platelet mediated vasoconstriction and clotting, mediated by fibrin and platelets, which function to control bleeding by forming clots. The clot may further serve as a temporary matrix for incoming fibroblasts and inflammatory cells, and also as a reservoir for
10 cytokines and growth factors.

Following hemostasis, inflammatory cells may enter the wound and perpetuate the inflammatory stage. For example, inflammatory cells may include polymorphonuclear cells (PMNs), which are attracted by growth factors and cytokines such as platelet derived growth factor (PDGF) and interleukin 8 (IL-8). PMNs begin to clean the wound by removing
15 cellular debris, foreign particles and bacteria that are present in the wound. In turn, PMNs are a major source of cytokines such as IL-1 α , IL-1 β , IL-6, and TNF- α . Approximately three days after wound creation, PMNs are replaced by monocytes, which transform into macrophages that also act as wound cleaners and a further source of IL-1 α , IL-1 β , IL-6 and TNF- α . IL-1 β , IL-6, and TNF- α expression is strongly upregulated during the inflammatory
20 phase (see e.g., Grellner, W. et al., (2000) *Forensic Sci Int* 113:251-264.; Grose, R., et al., (2002) *EMBO Reports* 3:575-582; and Hübner, G et al., (1996) *Cytokine* 8:548-556). IL-1 β and TNF- α regulated fibrocytes play an important role in the inflammatory process and are specifically involved in collagen and cytokine production.

2. Proliferation Stage

25 The proliferation stage may begin as early as about three days after wound creation, and last up to several weeks. Granulation acts as a bridge between the inflammation and proliferation stages. For example, granulation tissue formation usually begins about 3-6 days after injury, and primarily contains fibroblasts and macrophages. Migrating fibroblasts that produce a permanent collagen-based extra-cellular matrix (ECM) and macrophages that
30 produce a variety of growth factors and cytokines such as IL-1 and TNF- α , which in turn stimulate the production of growth factors.

The fibroblast phenotype may have a significant influence on both wound healing responses and clinical outcomes (see e.g., Stephens, P et al. (1996) *J Dent Res*; 75: 1358-1364; Stephens, P et al. (2001) *Br J Dermatol*; 144: 229-237; and Stephens, P et al. (2004), *J Cell Sci*; 117: 3389-3403). For example, fibroblasts from tissues that exhibit preferential wound healing *in vivo* (e.g., oral mucosal tissue) exhibit distinct phenotype responses *in vitro* (al-Khateeb, T et al. (1997) *J Periodontol*; 68: 1063-1069). Furthermore, matrix metalloproteinases and serine proteinases may also play an important role in the regulation of cellular migration and ECM remodelling following injury. For example, decreased ECM reorganization and wound healing (e.g., chronic wounds) is associated with decreased fibroblast MMP production and activation (see e.g., Cook H et al. (2000) *J Invest Dermatol*; 115: 225-33). Chronic wound fibroblasts are often associated with decreased type I collagen lattice reorganization and contraction, as well as with delayed or impaired cellular ECM migration and wound repopulation capabilities *in vitro*, as compared to normal skin fibroblasts (see e.g., Cook et al, 2000).

Re-epithelialization is the next key event in wound healing and is initiated primarily by migrating keratinocytes, which are stimulated by various growth factors and cytokines. Keratinocytes undergo a number of phenotypic changes during migration, expressing proteins associated with the differentiating cellular phenotype. As migration proceeds, keratinocytes acquire a proteolytic phenotype by producing serine proteinases and MMPs. The keratinocytes continue to migrate into the wound space until the mitotically active keratinocytes undergo further phenotypic alteration, such that differentiation and stratification of the epithelium and re-formation of the basement membrane occurs, which completes re-epithelialization.

Cellular ECM attachment, ECM degradation by proteinases, and the overall regulation of these processes by cytokines and growth factors, are key features of wound remodelling and healing that co-ordinate cellular function (e.g., cell migration and wound contraction) via interactions with the ECM. Such interactions regulate cytoskeleton reorganization and new integrin-ECM interactions via, for example, Rho family and actin binding proteins, while proteinases remove existing integrin interactions, thereby allowing rear de-adhesion and cell migration (Martin, P., (1997) *Science*; 276:75-81; Stephens P and Thomas D W (2002) *J Wound Care*; 11:253-261; and Kirfel, G et al. (2004) *Eur J Cell Biol*; 83:717-724). Cellular contractility in the absence of rear de-adhesion results in dermal reorganization, as quantified experimentally by collagen lattice reorganization/contraction.

3. Maturation Stage

The maturation stage may begin anywhere from 4 days to several weeks after wound creation, and may last for weeks, months, or even years. Wound maturation (also known as remodelling) may take as little as days or weeks but the complete process can last up to several years. During this phase contraction, decreased redness, decreased thickness, decreased induration and increased strength of the wound is observed. The wound contracts under the influence of myofibroblasts, collagen production in the granulation tissue decreases and blood vessels diminish. Wound healing is then completed by further re-epithelialization.

Immune cells in wound healing and tissue regeneration

Immune cells are direct participants in wound healing and tissue regeneration and their modulation using biomaterials is a promising approach for regenerative medicine. According to the techniques described herein, biomaterial scaffolds, tissue-derived and collagen-based, increase the number of immune cells in a site of a volumetric muscle defect. Biomaterial scaffolds trigger a local and systemic type-2 immune response in which an M2 macrophage phenotype was dependent on a Thelper2 adaptive response to create a pro-regenerative environment characterized by high Interleukin 4 (IL4) levels. This scaffold-associated immune microenvironment (SIM) included macrophages expressing *Cebpb*, *Arg1* and *Retnla* (*Fizz1*) and T cells expressing increased *Il4* and decreased *Tbx21* and *Ifng*. When implanted in *Rag*^{-/-} mice, deficient of adaptive immune cells, scaffold-associated macrophage (SAM) polarization was skewed towards an M1 phenotype and expression of pro-regenerative signals *Il4*, *Fizz1* and *Arg1*, was lost. *Rag*^{-/-} mice displayed altered regeneration including intramuscular adipogenesis and decreased muscle fiber size. The highly flexible immune system, first responder to trauma and biomaterials, is an attractive target for pro-regenerative therapies aimed at both the innate and adaptive immune systems. Immune homeostasis is indispensable to tissue development, wound healing, and regeneration (see e.g., Wynn, T.A., et al. *Nature*, 2013, 496(7446): p. 445-55; Stefater, J.A., 3rd, et al., *Trends Mol Med*, 2011, 17(12): p. 743-52). For example, in primitive organisms, such as the axolotl, macrophages play a critical role in regenerating entire limbs. In this regard, the cooperation between macrophages and T cells may be important for regenerative medicine. However, traditional tissue engineering and regenerative medicine approaches, employing tools including biomaterials, biological signals such as growth factors, and cells, have demonstrated only limited clinical successes to date.

Trauma initiates a cascade of local and systemic immune events that trigger the mobilization of cells into the damaged site to initiate host defense and tissue repair. The limited success achieved to date in rebuilding human tissues may be due in part to the tendency for therapeutic strategies to target later processes in wound healing and regeneration, such as stem cell proliferation and differentiation. Conversely, the immune system is a highly flexible network that serves as a guardian of tissue integrity, and its early response is adapted to the nature of the local tissue microenvironment (P. Matzinger and T. Kamala *Nature reviews. Immunology* 11, 221-230 (2011)). The immune system participates in tissue repair by scavenging debris and dead cells (Y. F. Peng *et al. Journal of autoimmunity* 29, 303-309 (2007)), recruiting and supporting proliferation of tissue progenitor cells (J. S. Otis *et al. PloS one* 9, e92363 (2014)), and inducing vascularization (S. Frantz, et al. *Circulation research* 96, 15-26 (2005)). Historically, immune interactions with implantable biomaterials were investigated with respect to biocompatibility (J. M. Anderson *Annual Review of Materials Research* 31, 81-110 (2001), J. M. Anderson and K. M. Biomaterials 5, 5-10 (1984), and J. M. Anderson *ASAIO Journal* 34, 101-107 (1988)). However, immune responses initiated with biomaterials may provide an avenue for manipulation of immune-mediated tissue regeneration. Remodeling of tissue-derived biomaterials by cells from the innate immune system in animals and humans now correlates cell phenotypes with successful regenerative outcomes, although the mechanism and dynamics of forming the scaffold immune microenvironment remain unknown (B. M. Sicari et al. *Science translational medicine* 6, 234ra258 (2014), V. J. Mase, Jr. et al. *Orthopedics* 33, 511 (2010), and B. N. Brown et al. *Acta biomaterialia* 8, 978-987 (2012)). Furthermore, the role of the adaptive immune system in response to biomaterials and functional tissue regeneration outcomes is unknown and largely unexplored.

The role of tissue derived biomaterial scaffolds in shaping the regenerative immune microenvironment in a murine model of volumetric muscle loss (VML) and the resulting impact on functional tissue restoration was therefore investigated herein. A critical role for T cells in this process was thereby elucidated.

Biomaterial Scaffolds

Biomaterial scaffolds provide three-dimensional microenvironments that are designed to support cell proliferation, differentiation and new tissue production via specific physical (e.g., mechanical, morphological, and the like) and chemical (e.g., peptide, protein, small molecules, and the like) properties. Both synthetic and biologic materials may serve as

scaffolds to engineer tissues *in vitro* using bioreactors or through direct *in vivo* implantation. More recently, biomaterial scaffolds have been implanted directly into defects to guide and modulate the wound healing response and tissue repair (see e.g., Hubbell, J.A., *Biotechnology* (N Y), 1995, 13(6): p. 565-76).

According to the techniques described herein, the immune system may work in concert with biomaterials in regenerative medicine. Without being bound by theory, it is believed that the limited success of prior art methods of rebuilding human tissues may be due, in part, to the lack of proper targeting of immune system components of the wound healing and regeneration process, such as stem cell proliferation and differentiation. The immune system is highly flexible, and its early response may be adapted to the nature of the local tissue microenvironment (Matzinger, P., *Nat Immunol*, 2007, 8(1): p. 11-3; Matzinger, P. and T. Kamala, *Nat Rev Immunol*, 2011, 11(3): p. 221-30). For example, the immune system may repair tissue by scavenging debris and dead cells (Peng, Y.F., et al., *J Autoimmun*, 2007, 29(4): p. 303-9); recruiting and supporting proliferation of tissue progenitor cells (Otis, J.S., et al., *PLoS One*, 2014, 9(3): p. e92363), and inducing vascularization (Frantz, S., et al., *Circ Res*, 2005, 96(1): p. 15-26). Thus, immune responses initiated by biomaterials according to the techniques herein may provide an avenue for manipulation of immune-mediated tissue regeneration.

Macrophages activate T cells and thereby recruit cytokines, (e.g., cytokine IL4)

As discussed above trauma initiates a cascade of local and systemic immune events, which trigger the mobilization of cells into the damaged site to initiate host defense and tissue repair. After trauma, the first cells to respond to the wound are polymorphonuclear cells, including neutrophils, eosinophils and basophils. Neutrophils are phagocytic cells of the innate immune system that scavenge debris and recognize cues from the environment and in turn produce paracrine factors that modulate the recruitment of other immune cells. One of these recruited cell types, the macrophage, also scavenges debris in the early stages of wound healing and later supports tissue development depending on phenotype.

Historical classification of macrophages defines the M1 phenotype (e.g., CD86⁺ and *Nos2*, *Tnfa* expression) and M2 phenotype (e.g., CD206⁺ and *Arg1*, *Fizz1* expression) as opposite poles governing pro-inflammatory and anti-inflammatory or wound-healing responses, respectively. Recent evidence highlights the heterogeneity of macrophage phenotype and the role of multiple macrophage subtypes in cardiac wound healing (Epelman, S., et al. *Nat Rev Immunol*, 2015, 15(2): p. 117-29), scar formation, and outcomes of certain

cancers (Lewis, C.E. and J.W. Pollard, *Cancer Res*, 2006. 66(2): p. 605-12). Macrophage polarization occurs along a spectrum, and a coordinated timing of the differing phenotypes enables clearance of infection followed by healing of damaged tissue. This polarization is mediated by both environmental factors and further, can be modified by signals from cells of the adaptive immune system, particularly T cells. Macrophages and dendritic cells present antigens and activate T cells, which in turn modulate other immune cells through secretion of cytokines. One such cytokine important for wound healing is interleukin 4 (IL4) (Tidball, J.G. and S.A. Villalta, *Am J Physiol Regul Integr Comp Physiol*, 2010. 298(5): p. R1173-87; Salmon-Ehr, V., et al., *Lab Invest*, 2000. 80(8): p. 1337-43). For example, production of IL4 by innate immune cells is necessary in muscle regeneration (Heredia, J.E., et al., *Cell*, 2013. 153(2): p. 376-88; Badylak, S.F., et al., *Tissue Eng Part A*, 2008. 14(11): p. 1835-42).

According to the techniques herein, biomaterials may induce influx of macrophages with a particularly strong M2 phenotype and that this phenotype may be dependent on the adaptive immune system, which is characterized by a Thelper2 phenotype. The enhanced Th2/M2 response may be associated with a pro-regenerative cytokine environment and enhanced regeneration of damaged muscle. Accordingly, T cells may be implemented as a new cellular target to promote tissue regeneration.

Test Compounds and Extracts

In general, immunomodulators that enhance wound healing/tissue regeneration (*e.g.*, agents that specifically increase the ability of a biomaterial scaffold to recruit immune system molecules that enhance wound healing). Other agents that enhance the efficacy of an agent or molecule described herein are identified from large libraries of natural product or synthetic (or semi-synthetic) extracts, chemical libraries, or from polypeptide or nucleic acid libraries, according to methods known in the art. Those skilled in the field of drug discovery and development will understand that the precise source of test extracts or compounds is not critical to the screening procedure(s) of the disclosure. Agents used in screens may include known those known as therapeutics for the treatment of a neoplasia. Alternatively, virtually any number of unknown chemical extracts or compounds can be screened using the methods described herein. Examples of such extracts or compounds include, but are not limited to, plant-, fungal-, prokaryotic- or animal-based extracts, fermentation broths, and synthetic compounds, as well as the modification of existing polypeptides.

Libraries of natural polypeptides in the form of bacterial, fungal, plant, and animal extracts are commercially available from a number of sources, including Biotics (Sussex,

UK), Xenova (Slough, UK), Harbor Branch Oceanographics Institute (Ft. Pierce, Fla.), and PharmaMar, U.S.A. (Cambridge, Mass.). Such polypeptides can be modified to include a protein transduction domain using methods known in the art and described herein. In addition, natural and synthetically produced libraries are produced, if desired, according to methods known in the art, e.g., by standard extraction and fractionation methods. Examples of methods for the synthesis of molecular libraries can be found in the art, for example in: DeWitt *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* 90:6909, 1993; Erb *et al.*, *Proc. Natl. Acad. Sci. USA* 91:11422, 1994; Zuckermann *et al.*, *J. Med. Chem.* 37:2678, 1994; Cho *et al.*, *Science* 261:1303, 1993; Carrell *et al.*, *Angew. Chem. Int. Ed. Engl.* 33:2059, 1994; Carell *et al.*, *Angew. Chem. Int. Ed. Engl.* 33:2061, 1994; and Gallop *et al.*, *J. Med. Chem.* 37:1233, 1994. Furthermore, if desired, any library or compound may be readily modified using standard chemical, physical, or biochemical methods.

Numerous methods are also available for generating random or directed synthesis (e.g., semi-synthesis or total synthesis) of any number of polypeptides, chemical compounds, including, but not limited to, saccharide-, lipid-, peptide-, and nucleic acid-based compounds. Synthetic compound libraries are commercially available from Brandon Associates (Merrimack, N.H.) and Aldrich Chemical (Milwaukee, Wis.). Alternatively, chemical compounds to be used as candidate compounds can be synthesized from readily available starting materials using standard synthetic techniques and methodologies known to those of ordinary skill in the art. Synthetic chemistry transformations and protecting group methodologies (protection and deprotection) useful in synthesizing the compounds identified by the methods described herein are known in the art and include, for example, those such as described in R. Larock, *Comprehensive Organic Transformations*, VCH Publishers (1989); T. W. Greene and P. G. M. Wuts, *Protective Groups in Organic Synthesis*, 2nd ed., John Wiley and Sons (1991); L. Fieser and M. Fieser, *Fieser and Fieser's Reagents for Organic Synthesis*, John Wiley and Sons (1994); and L. Paquette, ed., *Encyclopedia of Reagents for Organic Synthesis*, John Wiley and Sons (1995), and subsequent editions thereof.

Libraries of compounds may be presented in solution (e.g., Houghten, *Biotechniques* 13:412-421, 1992), or on beads (Lam, *Nature* 354:82-84, 1991), chips (Fodor, *Nature* 364:555-556, 1993), bacteria (Ladner, U.S. Patent No. 5,223,409), spores (Ladner U.S. Patent No. 5,223,409), plasmids (Cull *et al.*, *Proc Natl Acad Sci USA* 89:1865-1869, 1992) or on phage (Scott and Smith, *Science* 249:386-390, 1990; Devlin, *Science* 249:404-406, 1990; Cwirla *et al.* *Proc. Natl. Acad. Sci.* 87:6378-6382, 1990; Felici, *J. Mol. Biol.* 222:301-310, 1991; Ladner *supra.*).

In addition, those skilled in the art of drug discovery and development readily understand that methods for dereplication (e.g., taxonomic dereplication, biological dereplication, and chemical dereplication, or any combination thereof) or the elimination of replicates or repeats of materials already known for their activity should be employed
5 whenever possible.

When a crude extract is found to have immunomodulatory activity that enhances wound healing/tissue regeneration, further fractionation of the positive lead extract may be necessary to isolate molecular constituents responsible for the observed effect. Thus, the goal of the extraction, fractionation, and purification process is the careful characterization and
10 identification of a chemical entity within the crude extract that reduces neoplastic cell proliferation or viability. Methods of fractionation and purification of such heterogenous extracts are known in the art. If desired, compounds shown to be useful as therapeutics are chemically modified according to methods known in the art.

15 **Pharmaceutical Therapeutics**

In other embodiments, agents discovered to have immunomodulatory activity that enhances wound healing/tissue regeneration using the methods described herein are useful as a drug or as information for structural modification of existing compounds, e.g., by rational drug design. Such methods are useful for screening agents having an effect on a neoplasia.

20 For therapeutic uses, the compositions or agents identified using the methods disclosed herein may be administered systemically or locally to a subject to facilitate wound healing/tissue regeneration. Such agents may also be incorporated directly into a biomaterial scaffold of the disclosure to facilitate wound healing upon implantation of the scaffold. Preferable systemic routes of administration include, for example, subcutaneous, intravenous,
25 interperitoneally, intramuscular, or intradermal injections that provide continuous, sustained levels of the drug in the patient. Treatment of human patients or other animals will be carried out using a therapeutically effective amount of a therapeutic wound healing agent identified herein in a physiologically-acceptable carrier. Suitable carriers and their formulation are described, for example, in Remington's Pharmaceutical Sciences by E. W. Martin. The
30 amount of the therapeutic agent to be administered varies depending upon the manner of administration, the age and body weight of the patient, and with the clinical symptoms of the neoplasia. Generally, amounts will be in the range of those used for other agents used in the treatment of other diseases associated with wound healing/tissue regeneration, although in

certain instances lower amounts will be needed because of the increased specificity of the compound.

Formulation of Pharmaceutical Compositions

5 The administration of an agent or compound or a combination of agents/compounds for the treatment of a wound may be by any suitable means that results in a concentration of the therapeutic that, combined with other components, is effective in ameliorating, reducing, or stabilizing a neoplasia. The compound may be contained in any appropriate amount in any suitable carrier substance, and is generally present in an amount of 1-95% by weight of the
10 total weight of the composition. The composition may be provided in a dosage form that is suitable for parenteral (e.g., subcutaneously, intravenously, intramuscularly, or intraperitoneally) administration route. The pharmaceutical compositions may be formulated according to conventional pharmaceutical practice (see, e.g., Remington: The Science and Practice of Pharmacy (20th ed.), ed. A. R. Gennaro, Lippincott Williams & Wilkins, 2000
15 and Encyclopedia of Pharmaceutical Technology, eds. J. Swarbrick and J. C. Boylan, 1988-1999, Marcel Dekker, New York).

 Human dosage amounts can initially be determined by extrapolating from the amount of compound used in mice, as a skilled artisan recognizes it is routine in the art to modify the dosage for humans compared to animal models. In certain embodiments it is envisioned that
20 the dosage may vary from between about 1 µg compound/kg body weight to about 5000 mg compound/ kg body weight; or from about 5 mg/ kg body weight to about 4000 mg/ kg body weight or from about 10 mg/ kg body weight to about 3000 mg/ kg body weight; or from about 50 mg/ kg body weight to about 2000 mg/ kg body weight; or from about 100 mg/ kg body weight to about 1000 mg/ kg body weight; or from about 150 mg/ kg body weight to
25 about 500 mg/ kg body weight. In other embodiments this dose may be about 1, 5, 10, 25, 50, 75, 100, 150, 200, 250, 300, 350, 400, 450, 500, 550, 600, 650, 700, 750, 800, 850, 900, 950, 1000, 1050, 1100, 1150, 1200, 1250, 1300, 1350, 1400, 1450, 1500, 1600, 1700, 1800, 1900, 2000, 2500, 3000, 3500, 4000, 4500, or 5000 mg/ kg body weight. In other embodiments, it is envisaged that doses may be in the range of about 5 mg compound/ kg body to about 20 mg
30 compound/ kg body. In other embodiments the doses may be about 8, 10, 12, 14, 16 or 18 mg/ kg body weight. Of course, this dosage amount may be adjusted upward or downward, as is routinely done in such treatment protocols, depending on the results of the initial clinical trials and the needs of a particular patient.

Pharmaceutical compositions according to the disclosure may be formulated to release the active compound substantially immediately upon administration or at any predetermined time or time period after administration. The latter types of compositions are generally known as controlled release formulations, which include (i) formulations that create a substantially constant concentration of the drug within the body over an extended period of time; (ii) formulations that after a predetermined lag time create a substantially constant concentration of the drug within the body over an extended period of time; (iii) formulations that sustain action during a predetermined time period by maintaining a relatively, constant, effective level in the body with concomitant minimization of undesirable side effects associated with fluctuations in the plasma level of the active substance (sawtooth kinetic pattern); (iv) formulations that localize action by, e.g., spatial placement of a controlled release composition adjacent to or in contact with the thymus; (v) formulations that allow for convenient dosing, such that doses are administered, for example, once every one or two weeks; and (vi) formulations that target a neoplasia by using carriers or chemical derivatives to deliver the therapeutic agent to a particular cell type (e.g., neoplastic cell). For some applications, controlled release formulations obviate the need for frequent dosing during the day to sustain the plasma level at a therapeutic level.

Any of a number of strategies can be pursued in order to obtain controlled release in which the rate of release outweighs the rate of metabolism of the compound in question. In one example, controlled release is obtained by appropriate selection of various formulation parameters and ingredients, including, e.g., various types of controlled release compositions and coatings. Thus, the therapeutic is formulated with appropriate excipients into a pharmaceutical composition that, upon administration, releases the therapeutic in a controlled manner. Examples include single or multiple unit tablet or capsule compositions, oil solutions, suspensions, emulsions, microcapsules, microspheres, molecular complexes, nanoparticles, patches, and liposomes.

Methods of Treatment

In one embodiment, the present disclosure provides a method of using immunomodulatory activity to enhance wound healing/tissue regeneration in a subject. The methods involve administering to a subject having a wound an effective amount of a therapeutic combination of the disclosure. For example, a composition comprising an effective amount of an immunomodulatory agent that enhances wound healing. Preferably, such agents are administered as part of a composition additionally comprising a

pharmaceutically acceptable carrier. In a further preferable method, such agents may be applied to, or incorporated into, a biomaterial scaffold. Preferably this method is employed to treat a subject suffering from a wound, in particular a large wound. Other embodiments include any of the methods herein wherein the subject is identified as in need of the indicated treatment.

Kits or Pharmaceutical Systems

The present compositions may be assembled into kits or pharmaceutical systems for use in ameliorating a wound. Kits or pharmaceutical systems according to this aspect of the disclosure comprise a carrier means, such as a box, carton, tube or the like, having in close confinement therein one or more container means, such as vials, tubes, ampoules, bottles and the like. The kits or pharmaceutical systems of the disclosure may also comprise associated instructions for using the agents of the disclosure. Kits of the disclosure include at least one or more immunomodulators that enhance wound healing/tissue regeneration. If desired, the kit also includes a reagent to be used as a biomaterial scaffold. The kit may include instructions for administering the immunomodulatory agent in combination with one or more agents that further enhance wound healing.

The practice of the present disclosure employs, unless otherwise indicated, conventional techniques of molecular biology (including recombinant techniques), microbiology, cell biology, biochemistry and immunology, which are well within the purview of the skilled artisan. Such techniques are explained fully in the literature, such as, "Molecular Cloning: A Laboratory Manual", second edition (Sambrook, 1989); "Oligonucleotide Synthesis" (Gait, 1984); "Animal Cell Culture" (Freshney, 1987); "Methods in Enzymology" "Handbook of Experimental Immunology" (Weir, 1996); "Gene Transfer Vectors for Mammalian Cells" (Miller and Calos, 1987); "Current Protocols in Molecular Biology" (Ausubel, 1987); "PCR: The Polymerase Chain Reaction", (Mullis, 1994); "Current Protocols in Immunology" (Coligan, 1991). These techniques are applicable to the production of the polynucleotides and polypeptides of the disclosure, and, as such, may be considered in making and practicing the disclosure. Particularly useful techniques for particular embodiments will be discussed in the sections that follow.

Unless otherwise defined, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. Although methods and materials similar or equivalent to those described herein can

be used in the practice or testing of the present invention, suitable methods and materials are described below. All publications, patent applications, patents, and other references mentioned herein are incorporated by reference in their entirety. In case of conflict, the present specification, including definitions, will control.

5 The following examples are put forth so as to provide those of ordinary skill in the art with a complete disclosure and description of how to make and use the assay, screening, and therapeutic methods of the disclosure, and are not intended to limit the scope of what the inventors regard as their disclosure.

10 **EXAMPLES**

Example 1: Biomaterial scaffolds alter immune cell recruitment in volumetric muscle defects

To model a traumatic wound and create a tissue gap for scaffold implantation, a portion of the quadriceps muscle group in C57BL/6 mice was surgically excised, provoking an irreversible volumetric muscle loss (VML) injury. The VML model was used to determine the role of biomaterial scaffolds on immune cell recruitment, shaping the immune microenvironment, and the Scaffold-associated Immune Microenvironment (SIM). Biological scaffolds have been used to repair muscle defects both in preclinical models and clinical testing (B. M. Sicari, et al. *Sci Transl Med*, 6(234):234ra58. (2014)).

Accordingly, both tissue-derived and collagen biomaterial scaffolds were applied to the VML tissue defects. Tissue ECM scaffolds were generated by mechanical and chemical processing to produce particulates containing the tissue specific extracellular matrix with no intact cells (*see e.g.*, FIGs. 5A, 5B and 5E). Based upon the clinical performance of biological and tissue-based scaffolds, a tissue ECM array was screened *in vitro*; bone- and cardiac muscle-derived tissue ECMs (B-ECM and C-ECM, respectively) were selected for their immunomodulatory properties (FIGs. 5A-5D and (V. Z. Beachley *et al. Nat Meth* advance online publication, (2015))). The tissue ECM scaffolds and a single-component biomaterial control (Type-1 collagen) were injected into muscle wounds, and the resulting immune cell infiltrates into the scaffold and adjacent muscle were characterized at several time points post-injury (FIGs. 1A-1D; FIGs. 6A-6E).

Using flow cytometry and histology, the SIM compared to saline-treated control wounds were analyzed with respect to the magnitude of immune cell recruitment (see e.g., FIG. 6B), types of cells present in the wound environment (see e.g., FIG. 1A and FIGs. 6A-

6I), and the modulation of SIM polarization as determined by surface protein and gene expression analyses.

The presence of biomaterial scaffolds in the damaged tissue significantly increased the number of myeloid cells (F4/80⁺ macrophages, CD11c⁺ dendritic cells, CD11c⁺F4/80⁺ dendritic cells) and lymphocytes (CD3⁺ T cells and CD19⁺ B cells) at the injury site compared to saline control, as shown in FIGS. 6B and 1A and FIGS. 6A-6I), after 1 and 3 weeks (FIG. 1A, FIGS. 6A-6C). At 1 week, collagen treated wounds recruited the highest number of immune cells into the defect region (36.0%, 13.6 million cells) followed by bone (B-ECM) and cardiac (C-ECM) treated wounds (39.3%, 5.32 million and 45.4%, 5.44 million), with saline treated wounds containing the fewest number of cells (36.4%, 0.97 million).

As shown in FIG. 6I, at one-week post-surgery, a diverse myeloid component to the SIM was detected at the site of muscle injury and represented the peak infiltration of cells into the wound microenvironment. The proportion of myeloid cells peaked at 1 week post operation (F4/80⁺ Saline 33.2%, Collagen 30.9%, Bone 35.6%, Cardiac 41.2%). The proportion of myeloid cells in the damaged muscle peaked at 1 week post injury, and the myeloid compartment represented the majority of immune cells at all times. The proportion of myeloid cells in the damaged muscle peaked at 1 week post injury, and the myeloid compartment represented the majority of immune cells at all times. The T cell fraction, consisting of both CD4⁺ and CD8⁺ cells, peaked in all treatment groups at 3 weeks post-injury (CD3⁺ Saline 2.7%, Collagen 1.5%, Bone 5.9%, Cardiac 5.1%), but was present from the earliest times analyzed. The myeloid compartment (F4/80⁺, CD11c⁺) represented the majority of cells present at 1, 3 and 6 weeks post-operation. CD34⁺ vascular progenitor cells were also detected in the wound microenvironment at low levels (< 2%; FIGS. 6C and 6H). In the muscle wound, biomaterial scaffolds generally skewed the ratio of CD4:CD8 T cells towards a higher fraction of CD4⁺ helper T cells (~70% in scaffold treated, versus ~50% in saline treated wounds) at 1 week post injury (FIG. 1B). CD4⁺FoxP3⁺ regulatory T cells were also present in low levels and increased over time (FIGS. 7A and 7B).

Example 2: Scaffold application and adaptive immunity shape the wound-associated immune microenvironment

Macrophages and dendritic cells, which serve as antigen presenting cells (APCs), can interact with CD4⁺ helper T cells and CD8⁺ cytotoxic T cells through presentation of peptide antigens associated with the MHCII and MHCI isoforms of the major histocompatibility

complex, respectively. Bidirectional interactions of APCs with CD4⁺ T cells can alter immune polarization of the environment by inducing secretion of cytokines such as IL-4, a major cytokine associated with both Thelper2 (Th2) and M2 pathways of T cell and macrophage differentiation, respectively.

5 Expression of MHCII (I-A/I-E) was detected at high levels on both F4/80⁺CD11b⁺ macrophages and CD11c⁺F4/80⁻ dendritic cells, though the significant number of MHC^{-/low} CD11c cells signifies a large proportion of immature dendritic cells (see e.g., FIG. 6G). As shown in FIGs. 6D-6F, the expression levels of immune-related genes that support regeneration were also dependent on the presence and type of scaffold.

10 All scaffolds increased expression of *Il4* (*Il4*; a canonical type 2 cytokine important in muscle healing (V. Horsley, et al. *Cell* 113, 483-494 (2003), V. Salmon-Ehr *et al. Laboratory investigation; a journal of technical methods and pathology* 80, 1337-1343 (2000), W. C. Gause, et al. *Nature reviews. Immunology* 13, 607-614 (2013), and J. E. Heredia *et al. Cell* 153, 376-388 (2013))) in the overall wound microenvironment (FIG. 1C),
15 with IL-4 a known modulator of macrophage polarization and muscle regeneration - *Il4* expression was elevated over saline control, with cardiac tissue ECM producing significantly greater levels (see e.g., FIG. 6D; WT blue bars). Biomaterial scaffolds also induced expression of the type-2/M2 macrophage markers *Fizz1* (FIG. 6E) and *Arg1* (FIG. 6F).

As previous studies have focused on the innate component of the immune
20 microenvironment, the participation of adaptive immune cells was evaluated. The detection of CD3⁺ T cells along with M2-type macrophages in the wound microenvironment 1 week post-trauma specifically prompted performance of experiments designed to understand the role of adaptive immunity on the polarization of the wound environment towards a pro-regenerative type-2 immune response. For example, in addition to WT animals, scaffolds
25 were also implanted into B6.129S7-*Rag1*^{tm1Mom}/J (*Rag*^{-/-}) mice, which lacked mature T and B cells. In *Rag*^{-/-} mice, scaffold-mediated *Il4* up-regulation was lost, suggesting a Th2-driven scaffold immune microenvironment. To further characterize the scaffold-associated T cell population in wild type mice, CD3⁺ cells were sorted out of muscle injuries at 1 week post-operation for detailed gene expression analysis (FIG. 1D; FIGs. 11A-11C; FIGs. 18A and
30 18B). Expression levels of *Il4*, *Fizz1* and *Arg1*, respectively markers of Th2 (*Il4*) and M2 (*Fizz1* and *Arg1*) phenotypes, were severely decreased when implants were made in *Rag*^{-/-} mice (FIG. 6D-6F; red bars), which indicated an important role of lymphocytes in type-2 polarization by ECM scaffolds.

Example 3: Adaptive immune system enables local biomaterial-mediated enhancement of type-2 polarization

Macrophages and dendritic cells carry self and foreign antigens to local draining lymph nodes, where they interact with and activate resident T cells. Activated T cells migrate to the site of injury where they may participate in SIM polarization. The strong influx of macrophages and T cells (FIG. 1A) corresponds with the detection of *Arg1* and *Fizz1* only in the wound environment of mice displaying a Th2 immune response (FIG. 6D), suggesting that a dominant Th2 response to the wound in presence of scaffolds is responsible for the recruitment of M2-type macrophages, which are in return beneficial for wound healing and tissue regeneration.

The macrophages recruited into the scaffold microenvironment, in the presence and absence of T and B cells, were characterized by comparing the expression of CD86 (a co-stimulatory molecule expressed at high levels by inflammatory type-1/M1 macrophages) and CD206 (a mannose receptor and type-2/M2 marker) in both wild type and *Rag*^{-/-} mice, respectively (FIGs. 2A-2F; FIGs. 10A-10G). In the presence of adaptive immune cells, biomaterial scaffolds inhibited CD86 up-regulation at 3 weeks post surgery, as shown in (WT; blue bars) (FIGs. 2A-2G). In *Rag*^{-/-} mice (red bars), however, this mitigation of CD86 expression was lost, and ECM scaffold treated wounds returned to a macrophage polarization profile resembling that of saline treated control animals (FIG. 2A). The dampening of CD86 expression, which indicates inhibition of type-1 polarization, displayed the unique nature of scaffold-associated macrophages (SAMs) that differentiates them from other wound-associated myeloid cells.

While all scaffolds produced comparable CD86 expression in wounds of normal mice 3 weeks (see e.g., FIG. 2A), collagen treatment in *Rag*^{-/-} mice significantly upregulated CD86 expression to a greater extent (10x WT levels versus ~2x for scaffold-treated wounds). These results indicated that adaptive immune cells are necessary for reducing M1 polarization in wounds treated with biomaterial scaffolds.

On the other hand, CD206 expression was similar between ECM scaffold-treated and saline-treated mice post-implantation, with increased expression at 3 weeks (FIG. 2B). However, this observed increase in CD206 expression by myeloid cells at 3 weeks was ablated in *Rag*^{-/-} mice in both scaffold and saline treated wounds, suggesting that the adaptive immune system has an additional scaffold-independent role in shaping the wound healing response (see e.g., FIGS. 2B and 2H). Moreover, this CD206 up-regulation was also

impaired in B6.129S2-*Cd4^{tm1Mak}/J* mice (*Cd4^{-/-}*) mice, which maintain B cells and CD8+ T cells but lack CD4+ helper T cells (FIG. 10A). Additionally, in *Cd4^{-/-}* mice, the recruitment of B cells (a Th2-dependent adaptive effector cell) was diminished (Fig. 10G). Collagen treatment severely decreased CD206 expression in *Rag^{-/-}* mice, which confirmed that collagen treatment is a potent natural stimulus for M1 myeloid cell differentiation in the absence of signals from the adaptive immune system, as shown in FIG. 2B. Alterations in CD86 and CD206 expression were also observed on CD11c⁺ dendritic cells, as shown in FIGs. 10A-10F.

Since the expression levels of CD86 and CD206 are not mutually exclusive on macrophages (M1 versus M2), the polarization of the SIM was assessed via cell sorting of F4/80⁺ macrophages and CD3⁺ T cells at 1 week post-injury to compare their gene expression profiles between WT and *Rag^{-/-}* mice. As shown in FIG. 1D, several differences were noted in both T cells and macrophages, as shown in FIGS. 2E and 2F and FIGs. 8, 12A and 12B, 18A and 18B, and 19A and 19B. When comparing bone and cardiac-derived ECM-treated defects to saline-treated defects, both tissue ECMs induced similar patterns of gene expression alteration relative to saline control. Notably, however, cardiac tissue ECM induced a greater magnitude of change.

As shown in FIG. 1D, SIM T cells were highly skewed toward the Th2 phenotype (as demonstrated via increased *Il4* expression), with concomitant decreases in Th1 gene expression (*Ifng* and *Tbx21*). Scaffolds induced a Th2-type gene expression profile as characterized by increased *Il4* expression and decreased expression of *Ifng* and *Tbx21* (Th1 canonical genes). In addition, *Jag2*, which encodes the Notch ligand Jagged 2, was elevated. Jagged 2 helps direct Th differentiation away from Th1 and toward Th2 (D. Amsen, et al., *Immunity*, 27(1):89-99. (2007); T. C. Fang, et al., *Immunity*, 27(1):100-10. (2007)). *Il10*, which encodes a general anti-inflammatory cytokine that is not Th-specific, was also up-regulated. *Spp1* encodes osteopontin, a secreted phosphoprotein involved in bone remodeling and immune cell signaling. Interestingly, *Spp1* was more highly induced in T cells infiltrating cardiac SIM than bone SIM. Other genes that are more selectively expressed by Th1 cells, such as *Fasl* and *Cd28* (the co-stimulatory receptor for CD86), were likewise down-regulated. *Cd8a* is also down-regulated in SIM, concomitant with the decreased proportion of CD8 T cells observed during flow cytometry (see below, FIG. 1B, 3A, 3B and FIG. 22B). Notably, treatment with collagen, but not B-ECM or C-ECM, which was shown to be associated with higher cellularity, (see e.g., FIGS. 6B and 1A) induced the expression of cytokines associated with a Th17 immune signature with elevated expression of *Il17a* and

Il22 (elevated expression of *Il21*, *Cd4*, and T cell activation markers such as *Ctla4* and *Tnfrsf9* (encoding 4-1BB) were also observed). This gene expression profile in the CD3 compartment was coincident with a higher expression of *Il17ra* by macrophages (see e.g., FIG. 2E).

Concomitant with Th2 polarization of sorted T cells, SIM macrophages upregulate genes encoding the canonical M2 molecules *Arg1* and *Retnla* (encoding *Fizz1*) relative to saline, confirming the results of individual qRT-PCR analyses for these genes as shown in FIGs. 6A-6G, and FIG. 1A. Additionally, *Cebpb*, a bZIP transcription factor critical to M2 development and associated with increased muscle strength in mice and humans (J. Blackwell et al. *The journal of physiological sciences: JPS* 65, 145-150 (2015), D. Ruffell et al. *PNAS* 106, 17475-17480 (2009)), was highly upregulated in the presence of the biomaterial scaffolds, (e.g., in SIM), particularly with the cardiac scaffold. M1 associated genes were variably altered – *Nos2* was slightly increased, while *Ifn γ* was decreased. *S100a8* was increased in SIM; while this molecule was originally reported as a DAMP (damage associated molecular pattern), it has more recently been shown to inhibit inflammatory responses induced by pathogen-associated molecular patterns (PAMPs) such as LPS (Y. Sun, et al. *Mol Immunol*, 53(4):443-9. (2013)).

Interestingly, *Timp1* was up-regulated, while *Mmp16* and *Mmp9* were down-regulated. *Timp1* inhibits activation of both *Mmp9* and *Mmp16*; thus, metalloproteinase activity is highly dampened in the scaffold associated immune microenvironment (e.g., SIM). While these MMPs are thought to promote angiogenesis via release of activated pro-angiogenic factors, they have also been reported to block muscle repair (M. E. Davis, et al. *J Appl Physiol* (1985), 115(6):884-91. (2013)). By this reasoning, the *Timp1^{hi}Mmp9/16^{lo}* expression pattern of the scaffold-associated macrophages (e.g., SIM) would enhance muscle regeneration.

In *Rag^{-/-}* mice, scaffold-associated macrophages lost their pro-regenerative transcriptome (FIG. 2F; FIGs. 12A and 12B; FIGs. 19A and 19B). As shown in FIG. 1D, virtually all of the observed variability of macrophage gene expression in SIM vs. saline is absent in *Rag^{-/-}* mice (which cannot mount a Th2 immune response), dramatically demonstrating the critical importance of Th2 adaptive immunity in regulating SIM macrophage M2 polarization. Several genes directly implicated in muscle regeneration such as *Igf1* (insulin-like growth factor-1) (F. Mourkioti and N. Rosenthal *Trends Immunol*, 26(10):535-42. (2005); A. Musaro, et al. *Nat Genet*, 27(2):195-200. (2001); and J. P. Liu, et

al., *Cell*, 75(1):59-72. (1993)) and *Vegfa* (vascular endothelial growth factor) (N. Arsic, et al. *Mol Ther*, 10(5):844-54. (2004)) were significantly decreased in *Rag*^{-/-} mice (see e.g., FIG. 2F and FIGS. 8 and FIG. 12A and 12B and FIGS. 19A and 19B). Genes that were altered in *Rag*^{-/-} mice were associated with several developmental and wound healing processes as shown in FIGS. 13A-13F. Additionally, gene ontology enrichment analysis of genes differentially expressed in *Rag*^{-/-} versus WT macrophages selectively enriches terms associated with morphogenesis and differentiation, suggesting a reliance on the adaptive immune system for up-regulation of developmentally active immune genes (FIGS. 13A-13C).

Example 4: Biomaterial scaffolds impact systemic immune homeostasis

T cell activation and polarization can result in local and systemic alterations of the immune response of an organism. This can lead to immediate effects locally at the injury and implantation site, such as the observed scaffold-mediated CD86 decrease in SAMs (see e.g., FIGS. 2A and 2C). The detection of a local Th2 polarization of the adaptive immune response associated with the presence of M2-type macrophages (see e.g., FIGS. 2A-2I M(IL-4)-type macrophages (FIGS. 2A-2F)) prompted investigation of systemic T cell response in the presence and absence of biomaterial scaffolds (A. J. Allman *et al. Transplantation* 71, 1631-1640 (2001)). As shown in FIGS. 10B-10F, ECM scaffolds skewed the ratio of CD4:CD8 T cells towards a higher fraction of CD4⁺ cells (~70% in scaffold treated, versus ~50% in saline treated wounds) at 1 week post-operation (see e.g., FIG. 10B). As shown in FIGS. 7A-7D, CD4⁺FoxP3⁺ regulatory T cells were present in low levels at 1 week post surgery and increased over time. To probe the systemic impact of locally implanted biomaterial scaffolds in the traumatic defects, local (inguinal) and distal (axillary/brachial) lymph nodes were characterized (FIGS. 3A and 3B; FIGS. 14A-14C). Scaffold treatment induced hypertrophy of local draining lymph nodes (FIG. 3A) FIG. 3A). This size increase accompanied changes in gene expression, most notably a robust increase in *Il4* expression (see e.g., FIG. 3B and FIGS. 14A-14C).

This *Il4* induction was absent at 1 week post-operation in *Rag*^{-/-} mice but present after 3 weeks, suggesting an early adaptive immune-dependent *Il4* up-regulation followed by an innate immune driven *Il4* up-regulation later in the wound healing and regeneration process. Additionally, Cd4^{-/-} mice displayed a significant decrease in scaffold-mediated *Il4* up-regulation in inguinal lymph nodes at 3 weeks post-injury in C-ECM treated animals (FIG. 3B). This *Il4* expression level was higher than that of *Rag*^{-/-} mice, demonstrating an

important role of CD4⁺ helper T cells in scaffold-induced systemic type 2 immunity, but with potential further contributions by B cells or possibly even CD8⁺ T cells.

The pleiotropic nature of immune responses typically results in complex expression profiles beyond stereotypical M1 versus M2 “poles” often defined by one or two canonical markers in the myeloid population (P. J. Murray *et al. Immunity* 41, 14-20 (2014)). Indeed, the expression levels of CD86 and CD206 on macrophages (M1 versus M2) were not mutually exclusive. Concomitant with Th2 polarization of sorted T cells, scaffold-associated macrophages up-regulated the expression of genes encoding the canonical M2 molecules Arg1 and *Retnla* (encoding Fizz1), similar to the results from qRT-PCR analyses of the whole wound (including both the biomaterial and surrounding tissue) (FIG. 2E; FIGs. 11A and 11B).

Example 5: Adaptive immune cells mediate the impact of scaffold environment on the expression of genes associated with increased muscle regeneration

Il4 is regarded as an integral player in wound healing, recruitment of muscle progenitor cells, and development of mature muscle fibers. It was demonstrated that scaffolds have a direct effect in up-regulating T cell mediated production of *Il4* in the wound microenvironment. *Cebpb*, which is up-regulated in macrophages that infiltrate scaffold-treated wounds (see e.g., FIG. 2F; blue bars), has been associated with macrophage-mediated muscle repair, and its expression is associated with an increase in muscle strength (J. Blackwell, et al. *J Physiol Sci*, 65(1):145-50. (2015)).

Histologically, it was observed that cellular infiltration occurred around scaffolds and injured regions. Centrally nucleated muscle fibers, which are indicative of active regeneration or recovery from injury, were present within the biomaterial scaffold and around the defect site as shown in FIG. 4D. Notably, the size and morphology of these fibers differed between genotypes – wild type mice presented with larger, more rounded fibers, whereas *Rag*^{-/-} mice presented with smaller, irregularly shaped fibers, indicating a defect in muscle regeneration. *Rag*^{-/-} mice also produced increased amounts of adipose tissue within the scaffolds and regenerated muscle, which was observed histologically (see e.g., FIG. 4D) and confirmed by RT-PCR analysis of the whole muscle for *AdipoQ* (adiponectin), a gene exclusively present in adipose tissue that is associated with fatty acid and glucose metabolism (see e.g., FIG. 4E and FIG. 2B and FIGs. 17A-17E).

To further characterize the balance of adiposity and fibrosis within the wound region, Type I Collagen gene expression was also measured. Extracellular matrix treatment

decreased the expression of *Colla1* (Collagen I) in the wound environment at 1 and 3 weeks post surgery (see e.g., FIG. 4E and FIG. 17A) and this effect was lost in *Rag*^{-/-} mice, again demonstrating the importance of adaptive immunity. Type I Collagen is a major component of scars; despite reports that type-2 immune polarization can enhance scar formation, the effects of tissue ECM treatment decreased scar formation. Early in the repair process at 1 week post surgery, the collagen scaffold prevents *Colla1* up-regulation even in absence of adaptive immune cells. However, by 3 weeks gene expression increases similarly to the tissue ECM scaffolds. Histologically, a denser fibrous tissue with reduced cellular infiltration developed in *Rag*^{-/-} mice treated with collagen scaffold compared to the WT collagen animals as shown in FIG. 16B. This displays a direct effect of both scaffold treatment and adaptive immune cells on wound healing and regeneration.

Example 6: T cells enable robust M2-macrophage polarization in scaffold-treated muscle wounds.

As discussed above, immune cells are the first responders to tissue damage and biomaterial implantation. The various cells of the immune system interact with the scaffold and surrounding environment to create a regenerative immune microenvironment that can dictate polarization and differentiation of stem cells, ultimately determining the regenerative success of a scaffold that is used.

According to the techniques herein, wild type (WT) C57BL/6 and B6.129S7-*RagI*^{tm1Mom}/J (*Rag*^{-/-}; lack mature T and B cells) mice received bilateral volumetric muscle wounds in their quadriceps femoris muscle. The resulting wounds were treated with saline, particulate extracellular matrix (ECM) scaffolds (bone and cardiac muscle-derived), or particulate bovine collagen. After 1 and 3 weeks, the injuries were analyzed via multicolor flow cytometry to quantify immune cell infiltration and polarization. Proximal (inguinal) and distal (axillary/brachial) lymph nodes were harvested for gene expression analysis.

The largest population of immune cells in muscle wounds was detected at 1 week post-operation, with scaffold-treated wounds recruiting more cells than the control saline treated muscles. In wild type mice, it was observed that infiltration of myeloid (F4/80⁺, CD11c⁺, CD11c⁺F4/80⁺) and lymphoid (CD3⁺CD4⁺, CD3⁺CD8⁺, CD19⁺) immune cells. As shown in FIG. 21, scaffold treated wounds displayed slightly increased CD206 (M2) expression than saline treated animals, and at 3 weeks showed significantly lower CD86 (M1) expression. In *Rag*^{-/-} mice a decrease in CD206 was observed at 1 and 3 weeks post-operation in all wounds, and an increase in CD86 at 3 weeks post operation in scaffold-

treated wounds leading to ablation of the CD86 decrease seen in wild type mice. As shown in FIG. 21, there was also note robust up-regulation of *Il-4* and *Fizz1* in distal (axillary/brachial) local (inguinal) draining lymph nodes of scaffold-treated wounds that is ablated in *Rag*^{-/-} mice.

5 Muscle wounds recruit various myeloid and lymphoid cells such as macrophages, dendritic cells, T cells and B cells. Overall cellular recruitment increases upon treatment with an ECM scaffold, which increases the presence of M2-macrophages. Presence of CD4⁺ and CD8⁺ T cells suggested interaction of these macrophages with the adaptive immune system. In absence of T cells (*Rag*^{-/-}) macrophages to not reach complete M2-polarization as
10 measured by CD206 expression. Accordingly, formation of the local and systemic immune microenvironment of ECM scaffold-treated muscle wounds is mediated by integration of signals from the surrounding environment and cooperative signals from both the innate and adaptive immune responses.

Example 7: Immune Profiles of Particulate ECM Scaffolds in Volumetric Muscle

Wounds

15 As discussed above, the immune system plays a critical role in regeneration, and imbalances in immune polarization can lead to pathologies such as damaging inflammation and scarring. Secreted proteins such as cytokines can also interact with stem cells, altering their growth and differentiation. It was shown that bone-derived and cardiac muscle-derived
20 extracellular matrix (ECM) scaffolds applied to traumatic wounds can alter both local and systemic immune profiles. Overall cellular recruitment to traumatic muscle wounds, which is 45-50 % immune cells, increases if treated with particulate ECM as opposed to saline control. Furthermore, via multicolor flow cytometry, three distinct myeloid cell populations were
25 observed within subcutaneous and traumatic wounds that vary in polarization along the M1-M2 axis. All treatment groups showed adaptive immune cell recruitment, specifically CD4⁺ and CD8⁺ T cells, suggesting communication with myeloid cells within the wound microenvironment. Systemically, it was noted that gene expression changes in both proximal and distal lymph nodes with upregulation of *Il4* in scaffold treated wounds at both 1 and 3
30 weeks post-operation. These phenotypes, both local and systemic, depend upon scaffold tissue source and evolve over time.

The results described herein show that scaffolds recruit immune cells with a complex polarization profile in muscle wounds and a previously undescribed systemic response. These

immune responses can be modulated with application of ECM scaffolds derived from different tissues.

According to the techniques herein, biomaterial scaffolds may be used in regenerative medicine to support the growth and differentiation of cells to create aesthetic and functional replacements for missing or damaged tissue. Upon implantation, biomaterials are generally designed to support the growth of stem and progenitor cells, and to provide biological signals that stimulate tissue growth. Similarly, implanted biomaterials interact with immune cells, and consequently scaffold design is expected to impact the immune environment (i.e. SIM) by providing proteins orchestrating tissue regeneration.

Resident immune cells in a tissue can quickly respond and are the key factors that create a Th2 regenerative environment (S. J. Jenkins, et al., *Science*, 332(6035):1284-8. (2011)). Since biomaterial scaffolds essentially create an artificial local microenvironment, they can be readily applied as a tool to increase both the number of immune cells and promote a specific desired functionality in tissues. This SIM can therefore be influenced by the trauma, disease or tissue type in which the material is implanted, and can be manipulated through the biophysical composition and properties of the biomaterial itself. In one of the few examples of biomaterial implant-mediated immune modulation, Mooney et al. leveraged both sponges and hydrogels to educate dendritic cells for cancer treatment (J. Kim and D. J. Mooney, *Nano Today*, 6(5):466-77. (2011) and O. A. Ali, et al., *Cancer Res*, 74(6):1670-81. (2014)). The biomaterials used in this study provided inflammatory signals to mediate anti-tumor responses from the adaptive immune system (O. A. Ali, et al. *Adv Funct Mater*, 23(36):4621-8. (2013)). In addition to biological or chemical cues in the scaffold, biomechanics of the implanted material and even local tissue can influence cell response (D. E. Discher, et al. *Science*, 324(5935):1673-7. (2009) and A. J. Engler, et al. *Cell*, 126(4):677-89. (2006)).

Macrophage polarization along the M1-M2 (pro-inflammatory/anti-inflammatory) axis in response to biomaterials has been appreciated as a contributor to regenerative outcomes (B. N. Brown, et al. *Biomaterials*, 33(15):3792-802. (2012) and B. N. Brown, et al. *Front Immunol*, 5:510. (2014)), and has been studied in the case of extracellular matrix (ECM) scaffolds. ECM scaffolds induce a local type-2 (M2) macrophage polarization in muscle wounds and also diminish the strong type-1 (M1) response to synthetic materials (D. M. Faulk, et al. *Biomaterials*, 35(30):8585-95. (2014) and M. T. Wolf, et al. *J Biomed Mater Res A*, 102(1):234-46. (2014)). This M2 polarization of the immune response is also associated with poor prognosis in cancer progression and fibrotic disease, but positive

outcomes in wound healing and regeneration (W. C. Gause, et al.. *Nat Rev Immunol*, 13(8):607-14. (2013)). Thus, there is a delicate balance of immune polarization that mediates homeostasis and disease, which can potentially be manipulated with biomaterials.

Systemic immune responses may also influence the repair and regeneration process.

5 For example, trauma associated with hip replacement surgery induces significant systemic immunological changes that can impact the recovery time from surgical intervention (B. Gaudilliere, et al. *Sci Transl Med*, 6(255):255ra131. (2014)). Immune signatures (Stat3, Creb and NF-Kb signaling) associated with a CD14⁺ monocyte subset predicted delayed functional impairment and pain. In another clinical example, non-healing bone fractures
10 were associated with terminally differentiated memory CD8⁺ T Cells found in the peripheral blood. These memory cells were not related to the acute trauma of the bone fracture but to lifelong antigen exposure (S. Reinke, et al. *Sci Transl Med*, 5(177):177ra36. (2013)). However in preclinical modeling of the clinical observations, a specific T cell subpopulation (CD8⁺ effector memory cell population) was found in early fracture hematomas where they
15 secreted factors that negatively impacted bone healing. However, as described herein, CD8⁺ cytotoxic T lymphocyte recruitment was decreased upon scaffold implantation in volumetric muscle wounds with a pro-regenerative environment indicating that the presence and absence of certain immune cells are important positive and negative regulators of regenerative success. The results described herein indicate that the local and systemic immune
20 environments cooperate to define the regenerative response to tissue damage.

Immune cells are directly implicated as necessary participants in wound healing and tissue regeneration. Removal of macrophages by clodronate liposome depletion in salamanders completely inhibited limb regrowth after amputation (J. W. Godwin, et al. *Proc Natl Acad Sci U S A*, 110(23):9415-20. (2013)). In mouse models, depletion of *Cebpb* (an
25 important transcription factor mediating M2-macrophage polarization) (D. Ruffell, et al., *Proc Natl Acad Sci U S A*, 106(41):17475-80. (2009)) and *Il4ra* (alpha-chain receptor for IL-4; prevents IL-4/IL-13 signaling) resulted in significant impairment of myogenic recovery of cardiotoxin-injured murine hindlimb muscles. This *Il4ra* knockdown-mediated impairment also correlated with increased adipogenesis, demonstrating the importance of IL-4 in
30 reducing fibro-adipocyte progenitor cells from excessive adipose deposition. The balance of adipogenesis, myogenesis and fibrosis in muscle repair is delicate as there is also evidence for the importance of adipose tissue in wound healing. Surgical transplantation of adipose grafts and lipoaspirate stimulates healing and reduces scarring in burn, skin, and even bone

tissues (G. Rigotti, et al. *Plast Reconstr Surg*, 119(5):1409-22; discussion 1423-4. (2007); M. Nambu, et al., *Ann Plast Surg*, 62(3):317-21. (2009); M. Klinger, et al. *Aesthetic Plast Surg*, 32(3):465-9. (2008); and C. M. Cowan, et al. *Nat Biotechnol*, 22(5):560-7. (2004)). In addition to alterations in adipogenesis, IL-4 is directly related to muscle development. IL-4 is a signal for myotube fusion through an NFAT2c-dependent signaling mechanism in myoblasts (V. Horsley, et al. *Cell*, 113(4):483-94. (2003)). *Rag*^{-/-} mice display smaller regenerating muscle fibers during injury, suggesting an inhibition of myotube fusion due to the decreased presence of IL-4.

According to the techniques herein, adaptive immune cells in local and systemic immune polarization in scaffold-treated muscle wounds may be combined with next generation biomaterial implant immunotherapies. Current cancer immunotherapies are focused on the modulation of T cell responses to direct immune polarization to promote cancer clearance (S. A. Rosenberg, et al. *Nat Med*, 10(9):909-15. (2004)). Just as cancer research has made great strides in T cell therapies, these concepts can be translated to biomaterials design. Currently, through use of nanoparticle therapies, immunologists have been able to control various T cell responses to promote anti-tumor immune responses (D. J. Irvine, et al. *Chem Rev.* (2015) and N. K. Mehta, et al. *Cancer Immunol Res.* (2015)). Combining the fields of cancer immunotherapy with biomaterial scaffolds according to the techniques herein may open a new avenue for promoting tissue repair through T cell manipulation and regenerative immunology.

Example 8: Role of CD4+ T cells (Th2 T cells) on polarization of myeloid cells

To further elucidate the role of CD4+ T cells, and more specifically Th2 T cells on polarization of myeloid cells, myeloid CD206 expression in *Rag*^{-/-} mice that were repopulated with either WT CD4+ T cells or *Rictor*^{-/-} CD4+ T cells (FIGs. 2C-2D; FIGs. 11A-11C) were evaluated. Rictor is a critical component of the mTORC2 complex that integrates signals from the environment and drives the polarization of Th2 cells (G. M. Delgoffe *et al. Nature immunology* 12, 295-303 (2011)). Thus, Rictor deletion in T cells selectively prevents Th2 polarization, while preserving differentiation along Th1 and Th17 pathways. Myeloid cells in *Rag*^{-/-} mice expressed lower levels of CD206 compared to WT mice; however, when repopulated with WT CD4+ T cells (T-WT), this phenotype was rescued. When mice received Th2-deficient T cells (T-*Rictor*^{-/-}), CD206 expression was not rescued, proving that Th2 T cells, dependent upon mTORC2 signaling, are necessary for pro-regenerative myeloid polarization in the scaffold associated immune microenvironment. To confirm the role of IL-

4 in Th2-dependent myeloid polarization, the M1/M2 phenotype was evaluated in BALB/c-
Il4ra^{tm1Sz}/J (*Il4ra*^{-/-}) mice that lack the common receptor chain for IL-4 and IL-13, and thus
 cannot receive signals from IL-4 (FIGs. 2C-2D). Compared to WT controls, myeloid cells in
Il4ra^{-/-} wounds expressed far lower levels of CD206, suggesting that the M2-polarization was
 5 indeed controlled by IL-4, and verifying that the pro-regenerative profile is associated with
 M(IL-4) cells.

Example 9: Gene expression in scaffold-treated muscle wounds

The regenerative outcome of tissue-derived ECM scaffolds in animals and humans is
 correlated with an M2 macrophage phenotype during remodeling (B. M. Sicari et al. *Science*
 10 *translational medicine* 6, 234ra258 (2014), V. J. Mase, Jr. et al. *Orthopedics* 33, 511 (2010),
 and B. N. Brown et al. *Acta biomaterialia* 8, 978-987 (2012)). Biomaterials increased
 expression of key genes associated with a pro-regenerative type 2 immune response including
 hallmark genes of M2 myeloid cells, more specifically macrophages that are stimulated by
 IL-4, known as M(IL-4) macrophages (FIG. 9) (P. J. Murray et al. *Immunity* 41, 14-20
 15 (2014)). Importantly, the expression levels of these genes were seemingly dependent on the
 presence and type of scaffold. As with *Il4* expression, induction of these M(IL-4) markers
 was almost completely lost in *Rag*^{-/-} mice, suggesting that adaptive immune cells play a
 critical role in shaping the pro-regenerative myeloid phenotype (FIG. 9; *Rag*^{-/-} red bars).

20 Example 10: Alterations in the immune phenotype correlated with differential levels of tissue regeneration evaluated using a treadmill exhaustion assay

To determine if alterations in the immune phenotype correlated with differential
 levels of tissue regeneration, the overall muscle function in a treadmill exhaustion assay was
 evaluated. Wildtype animals recovered to run distances similar to healthy uninjured
 counterparts after 6 weeks (FIG. 4A). However, this restoration in running capacity was
 25 ablated in the absence of T and B cells (*Rag*^{-/-}) in ECM scaffold treated wounds. At 3 weeks
 post-injury, repopulation of *Rag*^{-/-} mice with WT T cells rescued their functional capacity and
 the animals could run greater distances compared to mice lacking the CD4 subset (FIG. 4B;
 91.11 ± 3.83 vs 60.06 ± 9.69, *P* = 0.0032). Furthermore, *Rag*^{-/-} mice repopulated with wild
 type CD4 T cells performed better than those repopulated with *Rictor*^{-/-} CD4⁺ T cells (72.31 ±
 30 7.40, *P* = 0.0368), confirming the role of Th2 CD4⁺ T cells in functional muscle regeneration.
 Differences in endurance between the *Cd4*^{-/-} mice and T-*Rictor*^{-/-} mice were not significant (*P*
 = 0.3752).

Muscle structure correlated with the differences in functional capacity. Histologically at 6 weeks post-injury, the quadriceps muscle treated with cardiac- extra cellular matrix (C-ECM) scaffold appeared similar to that of healthy controls, with minimal scaffold visible and repair tissue fully integrated within the surrounding musculature. On the other hand, a large region of fibrous tissue with active inflammation was present in muscles treated with the collagen scaffold (FIG. 4C). In *Rag*^{-/-} mice, there was a greater persistence of all scaffolds, most notably in the collagen-treated animals. Additionally, *Rag*^{-/-} mice displayed increased adipose deposition compared to their wild type counterparts. The presence of scarring and inhomogeneous tissue in *Rag*^{-/-} mice (and collagen-treated WT mice) was also visible with computed tomography (CT) imaging (FIG. 15).

In all cases, the *Rag*^{-/-} mice had substantially greater levels of fibrosis and smaller diameter muscle fibers after 6 weeks (FIG. 4C). To further evaluate treatments during active remodeling and regeneration, the muscle was also characterized after 3 weeks post-injury. Centrally nucleated muscle fibers, which are indicative of active regeneration or recovery from injury, were present within the biomaterial scaffold and around the defect site (FIG. 4D, FIGs. 16A-16C). Notably, the size and morphology of these fibers depended on the presence of an adaptive immune system – wild type mice produced muscle with larger, more rounded fibers, whereas *Rag*^{-/-} mice muscle contained smaller, irregularly shaped fibers, indicating a defect in muscle regeneration. The muscle in the *Rag*^{-/-} mice also appeared to contain larger regions of fibrous and adipose tissue compared to WT mice in all groups, suggesting an imbalance in fibro-adipogenic tissue balance during muscle regeneration (FIG. 4D, FIGs. 16A-16C). In addition, the pathologic *Rag*^{-/-} histo-morphology was recapitulated in *Cd4*^{-/-} mice, confirming the role of CD4⁺ T cells in fibro-adipogenic lineage commitment (FIG. 4D). Increased gene expression of *Adipoq* (adiponectin), a hormone exclusively produced by adipose tissue and involved in the regulation of fatty acid and glucose metabolism, confirmed ectopic adipogenesis in *Rag*^{-/-} whole muscle. Similarly, the expression of *Colla1* (Type I Collagen) increased in *Rag*^{-/-} mice muscle highlighting increased fibrosis (FIG. 4E; FIGs. 17A-17E). While scaffold treatment reduced fibro- and adipogenesis related markers in WT animals, this benefit was lost in *Rag*^{-/-} mice, again demonstrating the importance of adaptive immunity in maintaining lineage fidelity in muscle regeneration.

Thus, biomaterial scaffolds were discovered herein to have enhanced the development of a pro-regenerative immune environment and, for the first time, adaptive immune cells, specifically mTORC2-dependent CD4⁺ Th2 T cells, have been implicated in the process of functional tissue restoration (FIG. 4F). The strong systemic Th2 polarization of the CD4

compartment was critical for M(IL-4) polarization of the myeloid compartment in the scaffolds, since their depletion dramatically reduced the M(IL-4) signature. Furthermore, the ectopic adipogenesis and enhanced fibrosis observed in regenerating muscle in *Rag*^{-/-} mice emphasized the importance of adaptive immunity in maintaining lineage fidelity in the regenerating tissue.

Accordingly, the above studies have defined T cells and the mTORC2 pathway as new targets for immunomodulation with next-generation biomaterials to enhance their efficacy in controlling and balancing the immune microenvironment locally and systemically. Just as cancer research has made great strides in T cell therapies, these concepts can be translated to biomaterials design and strategies to improve tissue repair and regeneration (S. A. Rosenberg, et al. *Nature medicine* 10, 909-915 (2004), O. A. Ali et al. *Cancer research* 74, 1670-1681 (2014), J. Kim, et al. *Nano today* 6, 466-477 (2011), and O. A. Ali, et al. *Advanced functional materials* 23, 4621-4628 (2013)).

MATERIALS AND METHODS

Tissue ECM Preparation

Porcine derived tissues (Wagner Meats, Mt. Airy MD) were decellularized following a standard protocol. Samples were processed into a paste through the use of a knife-mill processor (Retsch, Germany) with particle sizes no larger than 5 mm², and rinsed thoroughly with running distilled water until blood was cleared from samples. Bone samples were pre-treated for decalcification by incubation in 10% formic acid (Sigma) for 3 days, which was verified by a colorimetric calcium test (STANBIO Laboratory). Tissues were then incubated in 3.0% peracetic acid (Sigma) on a shaker at 37°C for 4 hours, with a change to fresh acid solution after 1 hour. The pH was adjusted to 7 with thorough water and PBS rinsing and pH was tested after solution was freshly changed and tissue rested for 20 minutes. Samples were washed once more with distilled water then transferred to a 1% Triton-X100 (Sigma) + 2 mM sodium EDTA (Sigma) solution on a stir plate at 400 rpm, room temperature for 3 days, changing the solution daily. Tissues were rinsed thoroughly with distilled water until no bubbles formed from detergent upon agitation. Finally processed tissues were incubated in 600 U/ml DNase I (Roche Diagnostics) + 10 mM MgCl₂ (J. T. Baker) + 10% Antifungal-Antimycotic (Gibco®) for 24 hours. Tissues were rinsed thoroughly with distilled water, then frozen at -80°C and lyophilized for 3 days. The dry sample was turned into a particulate form using SPEX SamplePrep Freezer/Mill (SPEX CertiPrep). ECM powder was stored between -

20°C and -80°C and UV sterilized prior to use. Collagen from bovine tendon (Sigma) was cryomilled using the SPEX SamplePrep Freezer/Mill to form a particulate similar to the whole tissue ECM samples.

Volumetric Muscle Loss (VML) Surgery

5 Six to eight-week-old female wild type C57BL/6 (Charles River), B6.129S7-*Rag1^{tm1Mom}*/J, BALB/c-*Il4ra^{tm1Sz}*/J, or B6.129S2-Cd4^{tm1Mak}/J (Jackson Laboratories) female mice were anesthetized with 4.0% isoflurane and maintained under 2.5% isoflurane. Hair was removed from the lower extremities with an electric razor (Oster). After ethanol sterilization of the surrounding skin, a 1.5-cm incision was created between the knee and hip joint to
10 access the quadriceps femoris muscle. Through the use of surgical scissors, a 3 mm 3 mm deep defect was created in the quadriceps femoris muscle group. The resulting bilateral defects were filled with 0.05 cc of a 250 - 350 mg/ml biomaterial scaffold (UV-sterilized ECM (manufactured in house) or Collagen (Sigma)) or 0.05 cc of a vehicle (saline) control. Mice were under anesthesia for 10 – 15 minutes during surgical preparation and procedure
15 before return to cage and monitored until ambulatory. Directly after surgery, mice were given subcutaneous carprofen (Rimadyl®, Zoetis) at 5 mg/kg for pain relief and were maintained on Uniprim® antibiotic feed (275 ppm Trimethoprim and 1365 ppm Sulfadiazine, Harlan Laboratories) until the end of study to prevent opportunistic infections. After 1 (7 days), 3 (24 days) and 6 (42 days) weeks, the mice were sacrificed and their entire quadriceps femoris
20 muscle was removed by cutting from the knee joint along the femur to the hip joint. Both inguinal and axillary/brachial lymph nodes and whole muscle samples for RNA isolation were flash frozen in liquid nitrogen and stored at -80°C until RNA extraction. All animal procedures in this study were conducted in accordance with an approved Johns Hopkins University IACUC protocol.

25 T cell Adoptive Transfer

CD4+ T cells were isolated from lymph nodes and spleens of wild type C57BL/6 and B6.*Rictor^{-/-}* mice by crossing B6.*Rictor^{F/F}* with B6.Cd4-cre) using MACS CD4+ T Cell Isolation Kit (Miltenyi Biotec) as per manufacturer's instructions. Purity was confirmed by staining with the following FACS antibody panel: CD3 AlexaFluor488, CD4 PE-Cy7, CD8
30 APC (Biolegend). 3 million CD4+ T cells were injected into B6.129S7-*Rag1^{tm1Mom}*/J. After 12 days post-injection, mice were tested for T cell presence and CD4/CD8 purity in

peripheral blood to confirm repopulation. After 2 weeks, muscle surgery was performed as per previously described.

RT-PCR

In vivo inguinal and axillary/brachial lymph node samples from volumetric muscle loss (VML) studies were homogenized in TRIzol and RNA was extracted using a combination of TRIzol and RNeasy Mini (Qiagen) column-based isolations. cDNA was synthesized through the use of SuperScript Reverse Transcriptase III (Life Technologies) as per manufacturer's instructions. RT-PCR was conducted on an Applied Biosystems Real Time PCR Machine using SYBR Green (Life Technologies) as a reporter and the following primers: *B2m* forward CTC GGT GAC CCT GGT CTT TC, *B2m* reverse GGA TTT CAA TGT GAG GCG GG; *Tnfa* forward GTC CAT TCC TGA GTT CTG, *Tnfa* reverse GAA AGG TCT GAA GGT AGG; *Il1 β* forward GTA TGG GCT GGA CTG TTT C, *Il1 β* reverse GCT GTC TGC TCA TTC ACG; *Retnla* forward CTT TCC TGA GAT TCT GCC CCA G, *Retnla* reverse CAC AAG CAC ACC CAG TAG CA; *Ifny* forward TCA AGT GGC ATA GAT GTG GAA, *Ifny* reverse TGA GGT AGA AAG AGA TAA TCT GG; *Il4* forward ACA GGA GAA GGG ACG CCA T, *Il4* reverse ACC TTG GAA GCC CTA CAG A; *Gata3* forward GAA GGC ATC CAG ACC CGA AAC, *Gata3* reverse ACC CAT GGC GGT GAC CAT GC. Whole muscle samples were processed similarly to lymph nodes to isolate RNA and produce cDNA. Primers used included those previously described and: *Arg1* forward CAG AAG AAT GGA AGA GTC AG, *Arg1* reverse CAG ATA TGC AGG GAG TCA CC; *Colla1* forward CTG GCG GTT CAG GTC CAA T, *Colla1* reverse TTC CAG GCA ATC CAC GAG C; *Fabp4* forward TCA CCT GGA AGA CAG CTC CT, *Fabp4* reverse AAT CCC CAT TTA CGC TGA TG; *AdipoQ* forward TCC TGG AGA GAA GGG AGA GAA AG, *AdipoQ* reverse TCA GCT CCT GTC ATT CCA ACA T; *Lep* forward TTC ACA CAC GCA GTC GGT AT, *Lep* reverse ACA TTT TGG GAA GGC AGG CT; *Actb* forward ATG TGG ATC AGC AAG CAG GA, *Actb* reverse AAG GGT GTA AAA CGC AGC TCA (Integrated DNA Technologies).

F4/80⁺ and CD3⁺ cells from volumetric muscle wounds were sorted directly into RNA lysis buffer; RLT buffer (Qiagen) + β -mercaptoethanol (Sigma). RNA was isolated using an RNeasy Micro Kit (Qiagen) with carrier RNA and on-column DNase treatment. cDNA synthesis was performed with a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems). Isolated RNA underwent preamplification prior to plating in custom 96-well

TaqMan® Array Fast Plates (Life Technologies) and gene expression was detected on an Applied Biosystems StepOne Real-Time PCR System.

Histology

Inguinal lymph nodes were harvested and fixed in formalin overnight before
5 dehydration and paraffin embedding, microtome sectioning, then histological examination via
hematoxylin and eosin staining. Muscle samples were prepared as fresh-frozen samples for
cryosectioning by flash freezing in cold isopentane after mounting in Tragacanth gum (Sigma
Life Sciences). A Microm HM 550 cryostat (Fisher Scientific) was used to obtain 10 µm
10 cryosections from 5-7 different regions of each muscle roughly 300 µm apart. Sections were
stained with a Hematoxylin and Eosin protocol (Sigma Aldrich) or with a Modified Masson's
Trichrome protocol.

Flow Cytometry

Muscle wounds and surrounding area were harvested at 1 (7 days), 3 (24 days) and 6
(42 days) weeks post-surgery by cutting the quadriceps muscle from the hip to the knee and
15 finely diced using a scalpel in 1X PBS. Resultant material was digested for 45 minutes at
37°C in 1.67 Wünsch U/ml Liberase TL (Roche Diagnostics) + 0.2 mg/ml DNase I (Roche
Diagnostics) in serum-free RPMI-1640 medium (Gibco) on a shaker at 400 rpm. Digest was
filtered through a 100 µm cell strainer (Fisher) then washed twice with 1X PBS. Cells were
resuspended in 5 ml 1X PBS and layered atop 5 ml Lympholyte-M (Cedarlane), then spun for
20 20 minutes at 1200 x g. Cellular interphase was washed twice with 1XPBS then transferred to
a 96-well plate for antibody staining. Isolated cells were stained with the following antibody
panel: LIVE/DEAD® Fixable Aqua Dead Cell Stain Kit (Life Technologies), CD19
BrilliantViolet 421 (BioLegend), CD3 AlexaFluor 488 (BioLegend), CD34 PerCP-Cy5.5
(BioLegend), CD11c APC-Cy7 (BD Biosciences), F4/80 PE-Cy7 (BioLegend), CD86
25 AlexaFluor700 (BioLegend), CD206 APC (BioLegend). After staining cells were fixed and
analyzed On a BD LSR Analyser (BD Biosciences). LIVE/DEAD® Fixable Aqua Dead Cell
Stain negative (live) cells were evaluated based upon percent population of T cells (CD3⁺), B
cells (CD19⁺), dendritic cells (CD11c⁺), and macrophages (F4/80⁺). Macrophages were
further analyzed for polarization by mean fluorescence intensity of F4/80⁺, CD11c⁺ and
30 F4/80⁺CD11c⁺ cells in CD86 AlexaFluor700 and CD206 APC channels. All analyses were
performed in FlowJo Flow Cytometry Analysis Software (Treestar). The T cell panel
included LIVE/DEAD® Fixable Aqua Dead Cell Stain Kit (Life Technologies), CD3

AlexaFluor488 (BioLegend), CD4 PE-Cy7 (BioLegend), CD8 AlexaFluor 700 (BioLegend), FoxP3 Pacific Blue (BioLegend), IL4ra PE (BioLegend) and CCR5 APC (BioLegend).

FoxP3 staining followed fixation and permeabilization with BD CytoFix/CytoPerm Kit (BD Biosciences). Myeloid compartment screening at 1 week was done with the following

5 antibody panel: Fixable Viability Dye eFluor®780 (eBioscience), F4/80 PE-Cy7 (BioLegend), CD11b AlexaFluor700 (BioLegend), CD11c APC (BioLegend), Ly6C Per/CP-Cy5.5 (BioLegend), Ly6G PacificBlue (BioLegend), CD86 BrilliantViolet510 (BioLegend), CD206 PE (BioLegend), MHCII I-A/I-E AlexaFluor488 (BioLegend).

10 Samples prepared for sorting of F4/80⁺ and CD3⁺ cells followed the same isolation, then were stained with Fixable Viability Dye eFluor®780 (eBioscience), F4/80 PE-Cy7 (BioLegend), CD11c APC-Cy7 (BD Biosciences) and CD3 AlexaFluor488 (BioLegend). Samples were run on a BD FACS Aria and collected directly into RPE lysis buffer (Qiagen) containing β -mercaptoethanol (Sigma), and stored at -80°C until RNA isolation.

Statistical Analysis

15 All samples are representative of $n = 4$ mice. Data are displayed as mean $\pm$ standard error of the mean. Statistical outliers were removed using Grubbs' outlier test at $\alpha = 0.05$ using GraphPad Prism v6 Software (GraphPad Software Inc., La Jolla, CA), this sample was not included in analysis of percent cell populations at 6 weeks. Two-way ANOVAs were performed (GraphPad Prismv6), with statistical significance designated at $p \leq 0.05$. For

20 multiple comparisons, Tukey or Dunnet post-test corrections were applied. For gene expression analyses of sorted CD3⁺, F4/80⁺ WT, and F4/80⁺ *Rag*^{-/-} cells, scatter plots, heat maps, and correlation matrices of gene expression levels were used to compare across different materials: Saline, Bone, Cardiac, Collagen. To distinguish which groups of genes were differentially expressed based on material vs. saline, a re-sampling based permutation

25 test based on the maximum Wilcoxon Rank Sum statistic within the gene group was used. Individual gene expression was also compared across material and saline using the Wilcoxon Rank Sum test. Expression between F4/80 WT and *Rag*^{-/-} was compared for each material using the Wilcoxon Rank Sum test. Additionally, the difference of each material and saline between F4/80 WT and *Rag*^{-/-} was compared using linear regression models (material by

30 *Rag*^{-/-}-status interaction). Due to the exploratory nature and the small sample size, adjustment for multiple comparisons was not considered. Statistical analyses were performed using the R statistical package (version 2.15.1). Power analysis was not conducted to determine sample size.

Rheology

Rheology was performed with an Ares G2 rheometer (TA Instruments New Castle, DE). Experiments were conducted at physiological temperatures (37 °C) and performed in sequence. Sample was loaded in 400mg (wet) amounts on the rheometer stage (25mm geometry) and gap was set to 1.5mm. The experimental design followed a frequency sweep from .1 rad/s to 15 rad/s, oscillation frequency (15 rad/s constant), creep test (0.5% strain) and axial force until failure (-1.5 mm delta length). 3 samples per group were tested under the same rheological protocol. Samples were kept hydrated during these rheology experiments with PBS.

10 Cell Culture

Murine immortalized bone marrow macrophages (iBMM, Michele de Palma, Ecole Polytechnique Federale Lausanne) were cultured as per developer's protocol in IMDM (Gibco®) media containing 20% FBS (Hyclone, GE Healthcare Life Sciences), 2.5 mM L-glutamine (Gibco®), 1% PenStrep (Life Technologies), and 50 ng/ml M-CSF (Recombinant Mouse, BioLegend). Polarization was compared to primary cells through the use of RT-PCR after 24 hours of polarization in conditioned media (M0, M1, or M2 media) and confirmed to be a reliable in vitro comparison to primary macrophages. iBMM macrophages were cultured on plates coated with ECM powder for 24 hours in growth medium, or medium supplemented with 200 ng/ml *E. coli* lipopolysaccharide (LPS 055:B5, Sigma) and 20 ng/ml interferon gamma (IFN γ , Peprotech) or 20 ng/ml interleukin-4 (IL-4, Peprotech), for M1 and M2 polarizations, respectively.

Flow Cytometry: *In vitro* screening

In vitro samples were harvested using Accutase (Life Technologies) and washed with cold 1X PBS. Then, cells were stained with the following antibody panel: F4/80 PE-Cy7 (BioLegend), CD11b Pacific Blue (BioLegend), CD11c APC-Cy7 (BD Biosciences), CD86 AlexaFluor700 (BioLegend), MHCII (I-A/I-E) AlexaFluor488 (BioLegend), CD206 APC (BioLegend) and LIVE/DEAD® Fixable Aqua Dead Cell Stain Kit (Life Technologies). Samples were fixed using the BD Cytofix/Cytoperm™ kit (BD Biosciences), and run on BD LSRII Cell Analyzer, data was analyzed using FlowJo Flow Cytometry Analysis Software (Treestar). M1/M2 polarization levels were determined by mean fluorescence intensity of CD86 and CD206 in LIVE/DEAD® Fixable Aqua Dead Cell Stain F4/80⁺CD11c⁻ cells.

Micro-CT Imaging

Imaging was conducted using the Sedecal SuperArgus 4R PET/CT system. 720 projection images over 360 degrees in 0.5 degree increments were acquired; the maximum resolution mode was used, which means each acquired projection image was magnified 5.5 times when compared with the object. The x-ray tube was set with 50 kVp and 100 μ A. Exposure time for each projection was 350 ms. Projection images were stored in a matrix with dimensions 1536x972, with 0.15 mm pixel size. CT images were reconstructed using Cobra reconstruction software. Each CT image was stored in a matrix with size 1344x1344x864 with voxel size 0.031 mm. Each image in figure was contrast-enhanced to show defects, the same enhancements were applied for each image.

Treadmill Testing for Muscle Function

48 hours prior to testing mice were trained on treadmill apparatus running at 5 m/min and increased by 1 m/min every minute for a total of 5 minutes. Mice were run to exhaustion starting at 5 m/min and increased by 1 m/min every minute. Exhaustion was defined as when the mouse stayed on the pulsed shock grid for a continuous 30 seconds (Treat NMD: Brunelli et al 2007, Denti et al 2006). Animals were tested at least 48 hours prior to harvesting for analysis via FACS, PCR, or histology.

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What is claimed is:

1. A method for treating a wound in a subject, the method comprising administering to the subject an effective amount of a scaffold and an immunomodulatory agent, thereby treating
5 the wound.
2. The method of claim 1, wherein the scaffold comprises a biomaterial or a synthetic material.
- 10 3. The method of claim 2, wherein the biomaterial scaffold is a tissue-derived scaffold or a collagen scaffold.
4. The method of claim 1, wherein the immunomodulatory agent induces an adaptive immune system response.
15
5. The method of claim 1, wherein the immunomodulatory agent is selected from the group consisting of Mineral salt adjuvants, Tenoactive adjuvants, Bacteria-derived adjuvants, Adjuvant emulsions, Liposome adjuvants, Polymeric microsphere adjuvants, Cytokines as adjuvants, Inulin-derived adjuvants, carbohydrate adjuvants, Adjuvant formulations, Mucosal
20 adjuvants, Cytokines, and Cancer vaccine adjuvants.
6. The method of claim 1, wherein the immunomodulatory agent is a protein or small molecule.
- 25 7. The method of claim 6, wherein the small molecule is a peptide.
8. The method of claim 1, wherein a rate of wound healing is increased by 2%, 4%, 6%, 8%, or 10%.
- 30 9. The method of claim 1, wherein the scaffold is impregnated with the immunomodulatory agent.
10. The method of claim 1, wherein the wound is an acute wound, a chronic wound, or a surgical wound.

11. A method for treating a wound in a subject, the method comprising administering to the subject an effective amount of an immunomodulatory agent, thereby treating the wound.

5 12. The method of claim 11, wherein the immunomodulatory agent is administered with a scaffold.

13. The method of claim 12, wherein the scaffold comprises a biomaterial or a synthetic material.

10

14. The method of claim 13, wherein the biomaterial scaffold is a tissue-derived scaffold or a collagen scaffold.

15 15. The method of claim 11, wherein the immunomodulatory agent induces an adaptive immune system response.

16. The method of claim 11, wherein the immunomodulatory agent is selected from the group consisting of Mineral salt adjuvants, Tenoactive adjuvants, Bacteria-derived adjuvants, Adjuvant emulsions, Liposome adjuvants, Polymeric microsphere adjuvants, Cytokines as adjuvants, Inulin-derived adjuvants, carbohydrate adjuvants, Adjuvant formulations, Mucosal adjuvants, Cytokines, and Cancer vaccine adjuvants.

20

17. The method of claim 11, wherein the immunomodulatory agent is a protein or small molecule.

25

18. The method of claim 17, wherein the small molecule is a peptide.

19. The method of claim 11, wherein a rate of wound healing is increased by 2%, 4%, 6%, 8%, or 10%.

30

20. The method of claim 11, wherein the wound is an acute wound, a chronic wound, or a surgical wound.

21. A pharmaceutical composition for the treatment of a wound, the composition comprising an effective amount of an immunomodulatory agent.

22. The pharmaceutical composition of claim 21, wherein the composition is formulated to be impregnated in a scaffold.

23. The pharmaceutical composition of claim 22, wherein the scaffold comprises a biomaterial or a synthetic material.

24. The pharmaceutical composition of claim 23, wherein the biomaterial scaffold is a tissue-derived scaffold or a collagen scaffold.

25. The pharmaceutical composition of claim 21, wherein the composition is formulated for topical administration.

26. The pharmaceutical composition of claim 21, wherein the composition is formulated for administration with a biomaterial scaffold.

27. The pharmaceutical composition of claim 21, wherein the immunomodulatory agent induces an adaptive immune system response.

28. The pharmaceutical composition of claim 21, wherein the immunomodulatory agent is selected from the group consisting of Mineral salt adjuvants, Tensioactive adjuvants, Bacteria-derived adjuvants, Adjuvant emulsions, Liposome adjuvants, Polymeric microsphere adjuvants, Cytokines as adjuvants, Inulin-derived adjuvants, carbohydrate adjuvants, Adjuvant formulations, Mucosal adjuvants, Cytokines, and Cancer vaccine adjuvants.

29. The pharmaceutical composition of claim 21, wherein the immunomodulatory agent is a protein or small molecule.

30. The pharmaceutical composition of claim 21, wherein the small molecule is a peptide.

31. The pharmaceutical composition of claim 21, wherein the composition increases a rate of wound healing by 2%, 4%, 6%, 8%, or 10%.

32. The pharmaceutical composition of claim 21, wherein the composition treats an acute wound, a chronic wound, or a surgical wound.

5 33. A method for promoting wound healing or tissue regeneration in a subject, the method comprising administering to said subject an effective amount of a scaffold and an immunomodulatory agent, thereby increasing the rate of at which the wound heals or the tissue regenerates.

10 34. The method of claim 33, wherein the scaffold comprises a biomaterial or a synthetic material.

35. The method of claim 33, wherein the biomaterial scaffold is a tissue-derived scaffold or a collagen scaffold.

15

36. The method of claim 33, wherein the immunomodulatory agent induces an adaptive immune system response.

20 37. The method of claim 33, wherein the immunomodulatory agent is selected from the group consisting of Mineral salt adjuvants, Tenoactive adjuvants, Bacteria-derived adjuvants, Adjuvant emulsions, Liposome adjuvants, Polymeric microsphere adjuvants, Cytokines as adjuvants, Inulin-derived adjuvants, carbohydrate adjuvants, Adjuvant formulations, Mucosal adjuvants, Cytokines, and Cancer vaccine adjuvants.

25 38. The method of claim 33, wherein the immunomodulatory agent is a protein or small molecule.

39. The method of claim 38, wherein the small molecule is a peptide.

30 40. The method of claim 33, wherein a rate of wound healing is increased by 2%, 4%, 6%, 8%, or 10%.

41. The method of claim 33, wherein the scaffold is impregnated with the immunomodulatory agent.

42. The method of claim 33, wherein the wound is an acute wound, a chronic wound, or a surgical wound.

5 43. A kit for the treatment of a wound, the kit comprising an effective amount of an immunomodulatory agent and a biomaterial scaffold, and directions for the use of the kit for the treatment of the wound.

10 44. The kit of claim 43, wherein the biomaterial scaffold is a tissue-derived scaffold or a collagen scaffold.

45. The kit of claim 43, wherein the immunomodulatory agent is selected from the group consisting of wherein the immunomodulatory agent is selected from the group consisting of Mineral salt adjuvants, Tensioactive adjuvants, Bacteria-derived adjuvants, Adjuvant
15 emulsions, Liposome adjuvants, Polymeric microsphere adjuvants, Cytokines as adjuvants, Inulin-derived adjuvants, carbohydrate adjuvants, Adjuvant formulations, Mucosal adjuvants, Cytokines, and Cancer vaccine adjuvants.

20 46. The kit of claim 43, wherein the immunomodulatory agent is a protein or small molecule.

47. The kit of claim 43, wherein the small molecule is a peptide.

25 48. The method of claims 1, 12, 22, 33, or 43, wherein the scaffold is modified to enhance immunomodulation.

49. The method of claims 1, 12, 22, 33, or 43, wherein the scaffold fills the wound.

50. The method of claims 1, 12, 22, 33, or 43, wherein the scaffold partially fills the wound.

30 51. The pharmaceutical composition of claims 21-32, wherein the immunomodulatory agent induces a T cell response.

52. The pharmaceutical composition of claims 21-32, wherein the immunomodulatory agent induces a T cell response in combination with a scaffold, wherein the scaffold is a biomaterial or a synthetic material.

5 53. The pharmaceutical composition of claims 21-32, wherein the immunomodulatory agent induces a T cell response.

54. A scaffold composition for the treatment of a wound, the scaffold composition comprising an effective amount of an immunomodulatory agent.

10

55. The scaffold composition of claim 54, wherein the scaffold composition is formulated to be impregnated with the immunomodulatory agent.

56. The scaffold composition of claim 54, wherein the scaffold composition comprises a
15 biomaterial or a synthetic material.

57. The scaffold composition of claim 56, wherein the biomaterial is a tissue-derived scaffold or a collagen scaffold.

20 58. The scaffold composition of claim 54, wherein the scaffold composition is formulated for topical administration.

59. The scaffold pharmaceutical composition of claim 54, wherein the scaffold composition is formulated for co-administration with the immunomodulatory agent.

25

60. The scaffold composition of claim 54, wherein the immunomodulatory agent induces an adaptive immune system response.

61. The scaffold composition of claim 54, wherein the immunomodulatory agent is selected
30 from the group consisting of Mineral salt adjuvants, Tenoactive adjuvants, Bacteria-derived adjuvants, Adjuvant emulsions, Liposome adjuvants, Polymeric microsphere adjuvants, Cytokines as adjuvants, Inulin-derived adjuvants, carbohydrate adjuvants, Adjuvant formulations, Mucosal adjuvants, Cytokines, and Cancer vaccine adjuvants.

62. The scaffold composition of claim 54, wherein the immunomodulatory agent is a protein or small molecule.

63. The scaffold composition of claim 54, wherein the small molecule is a peptide.

5

64. The scaffold composition of claim 54, wherein the scaffold composition increases a rate of wound healing by 2%, 4%, 6%, 8%, or 10%.

65. The scaffold composition of claim 54, wherein the scaffold composition treats an acute wound, a chronic wound, or a surgical wound.

10

66. The method or composition of claims 1-65, wherein the wound is selected from the group consisting of a skin wound, a muscle wound, a cartilage wound, and a nervous system wound.

15

FIG. 1A

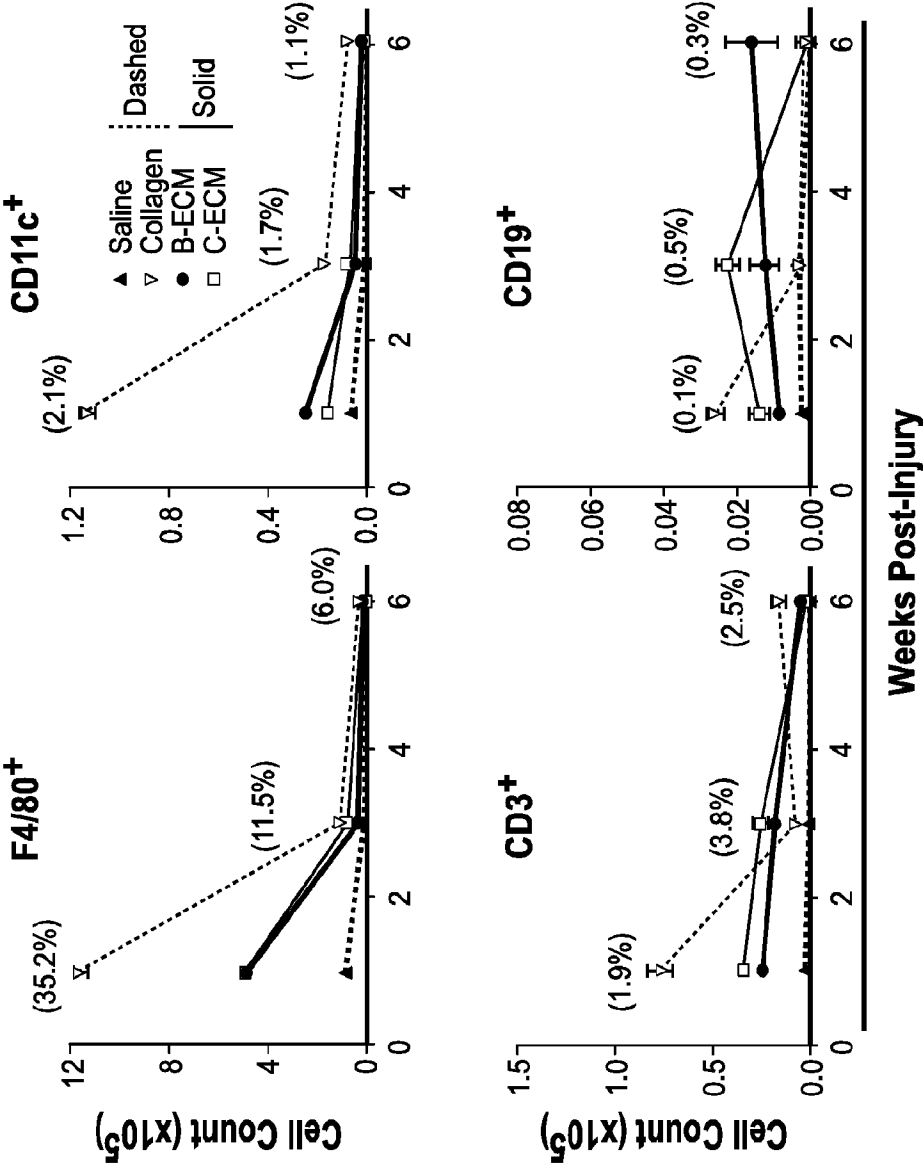


FIG. 1B

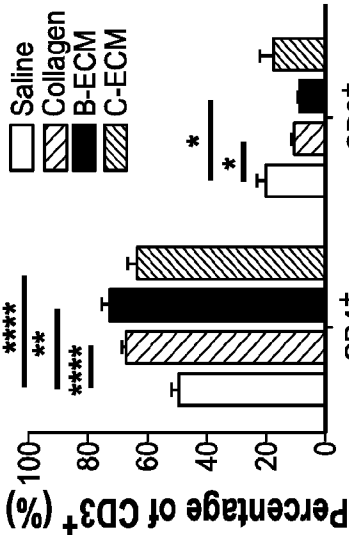


FIG. 1C

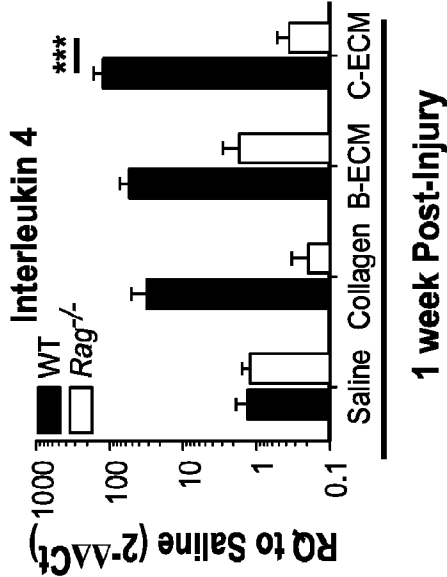
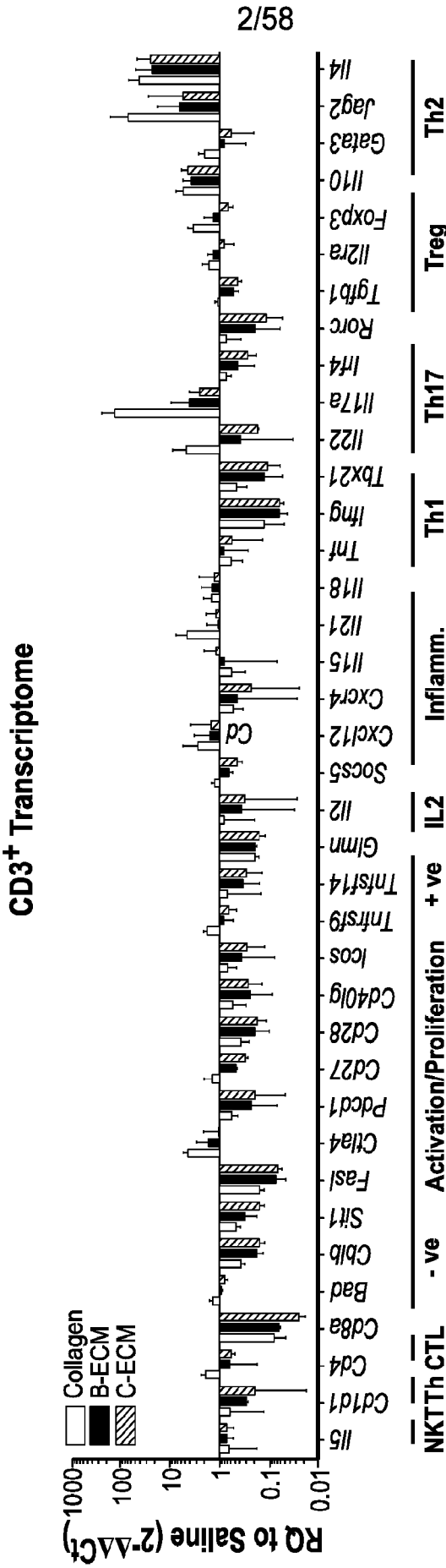
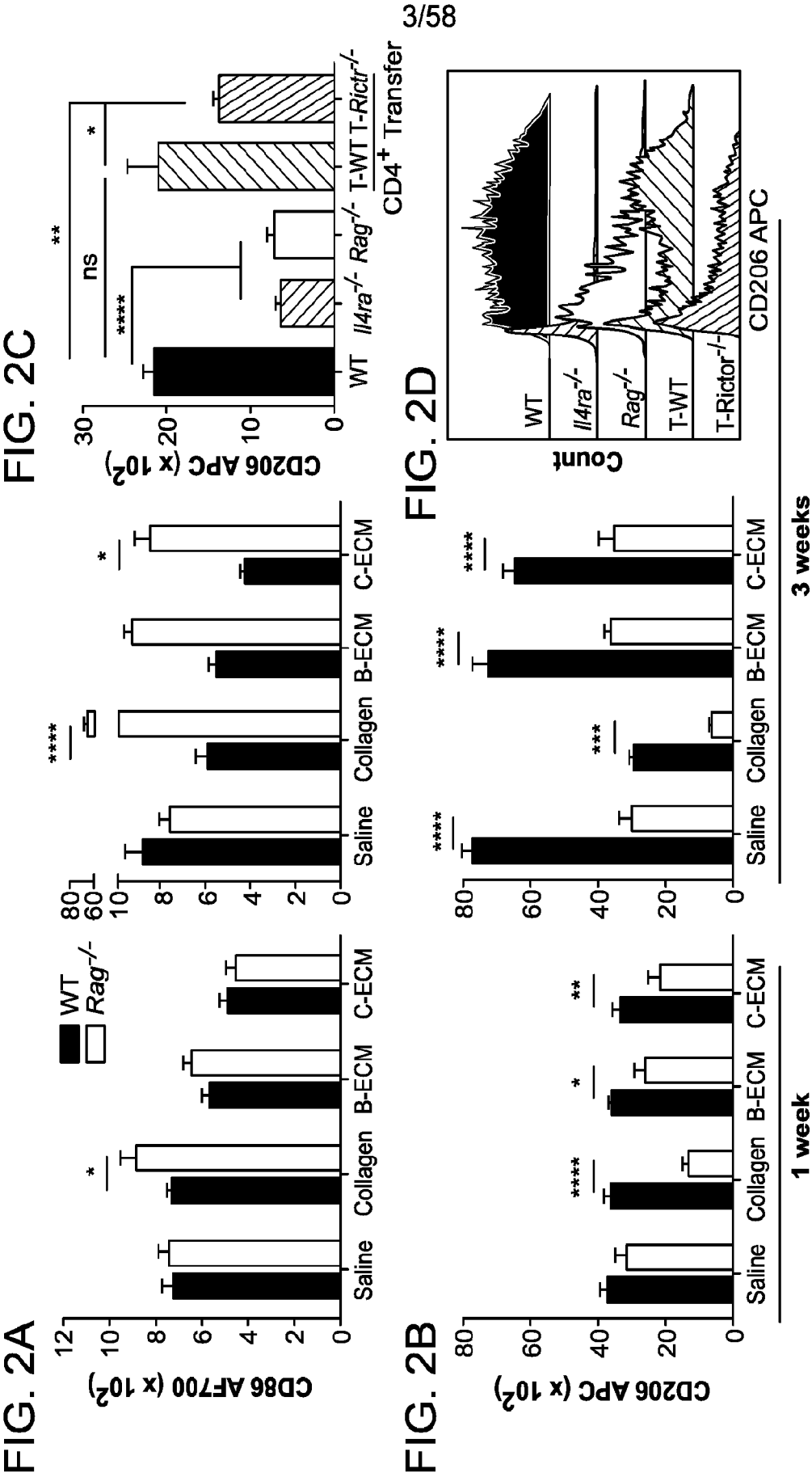
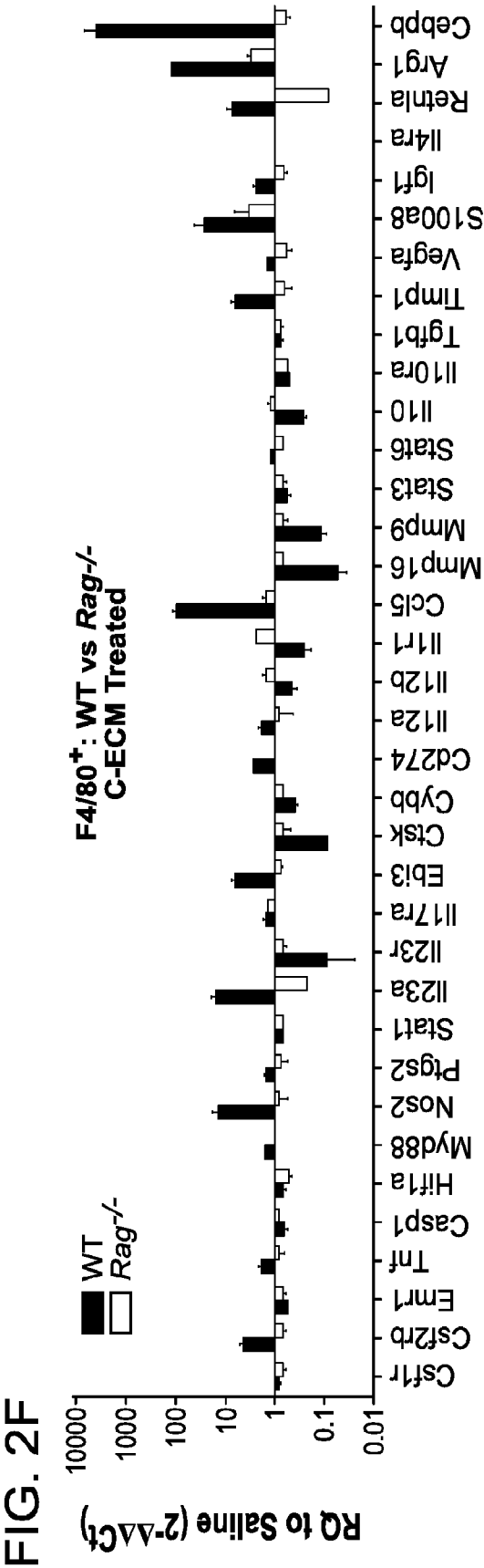
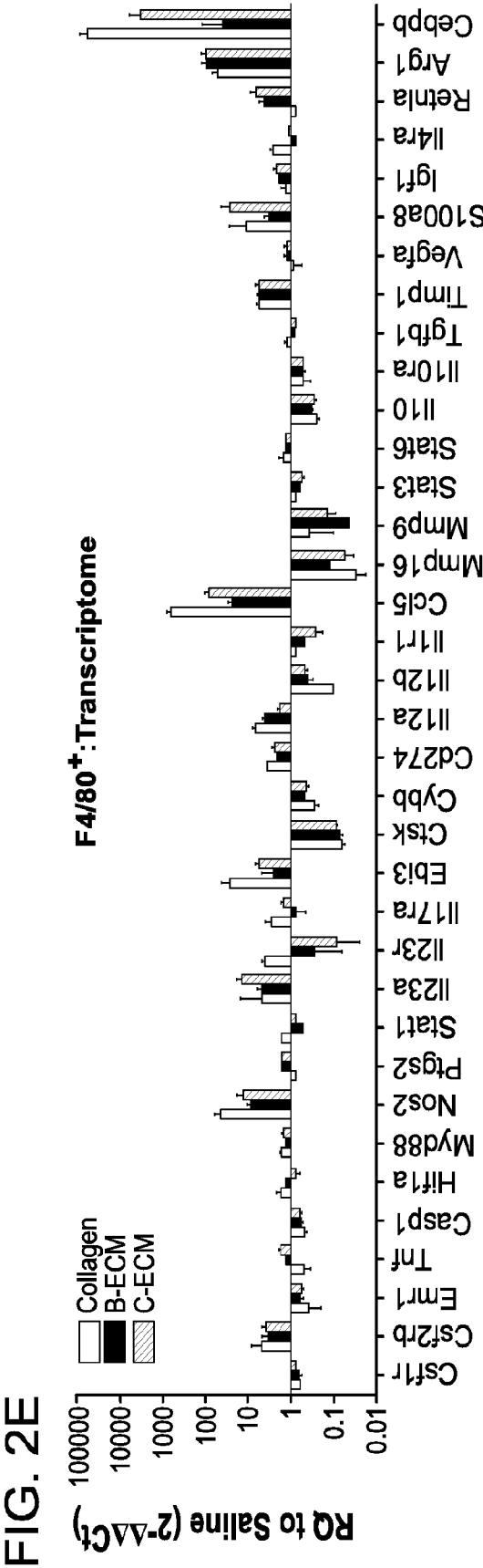


FIG. 1D







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FIG. 2G

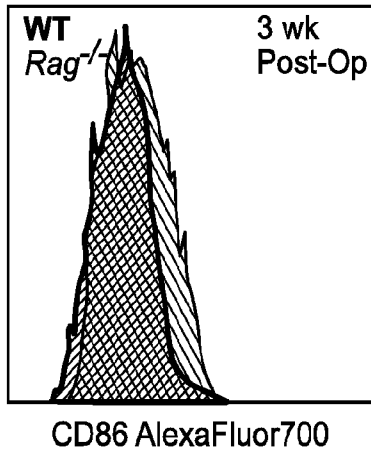


FIG. 2H

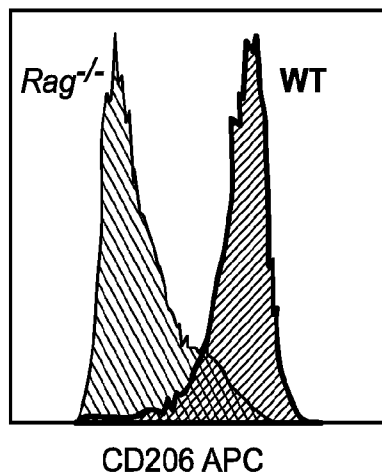


FIG. 2I

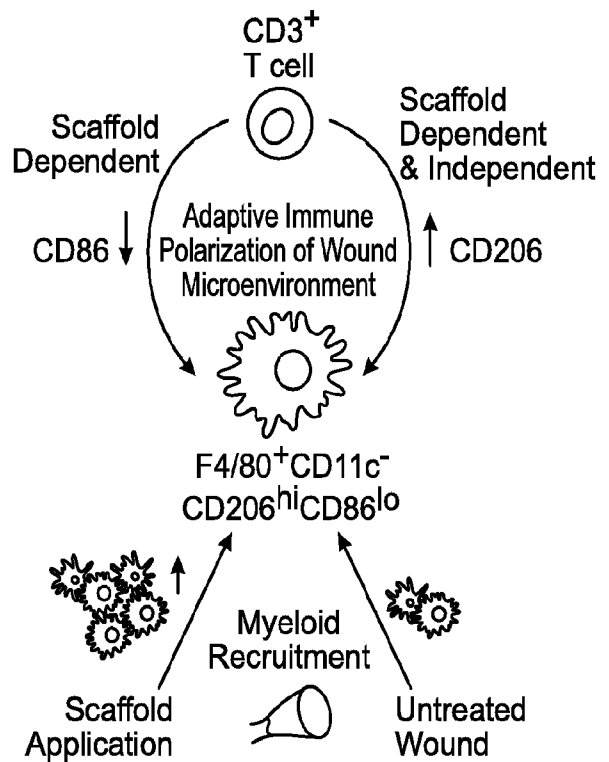
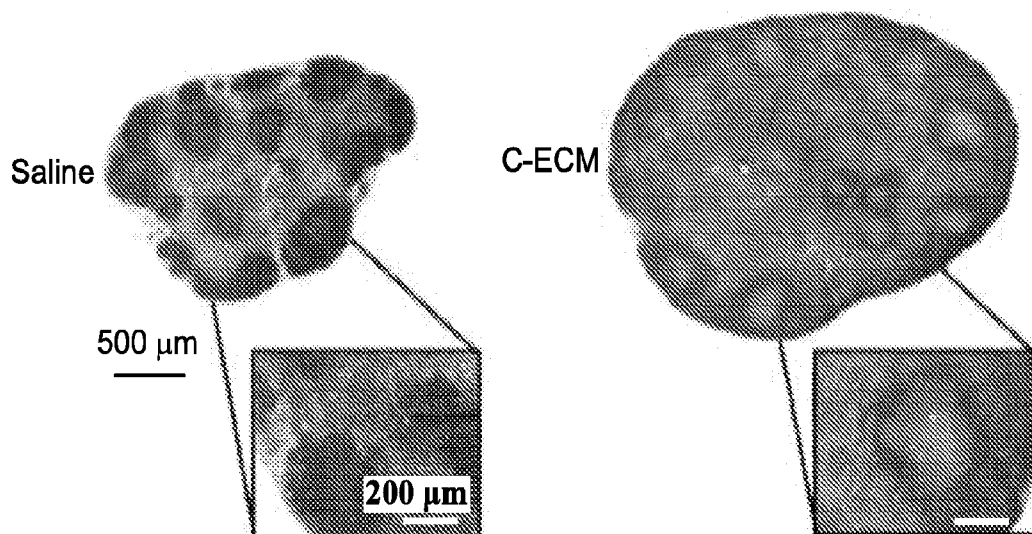
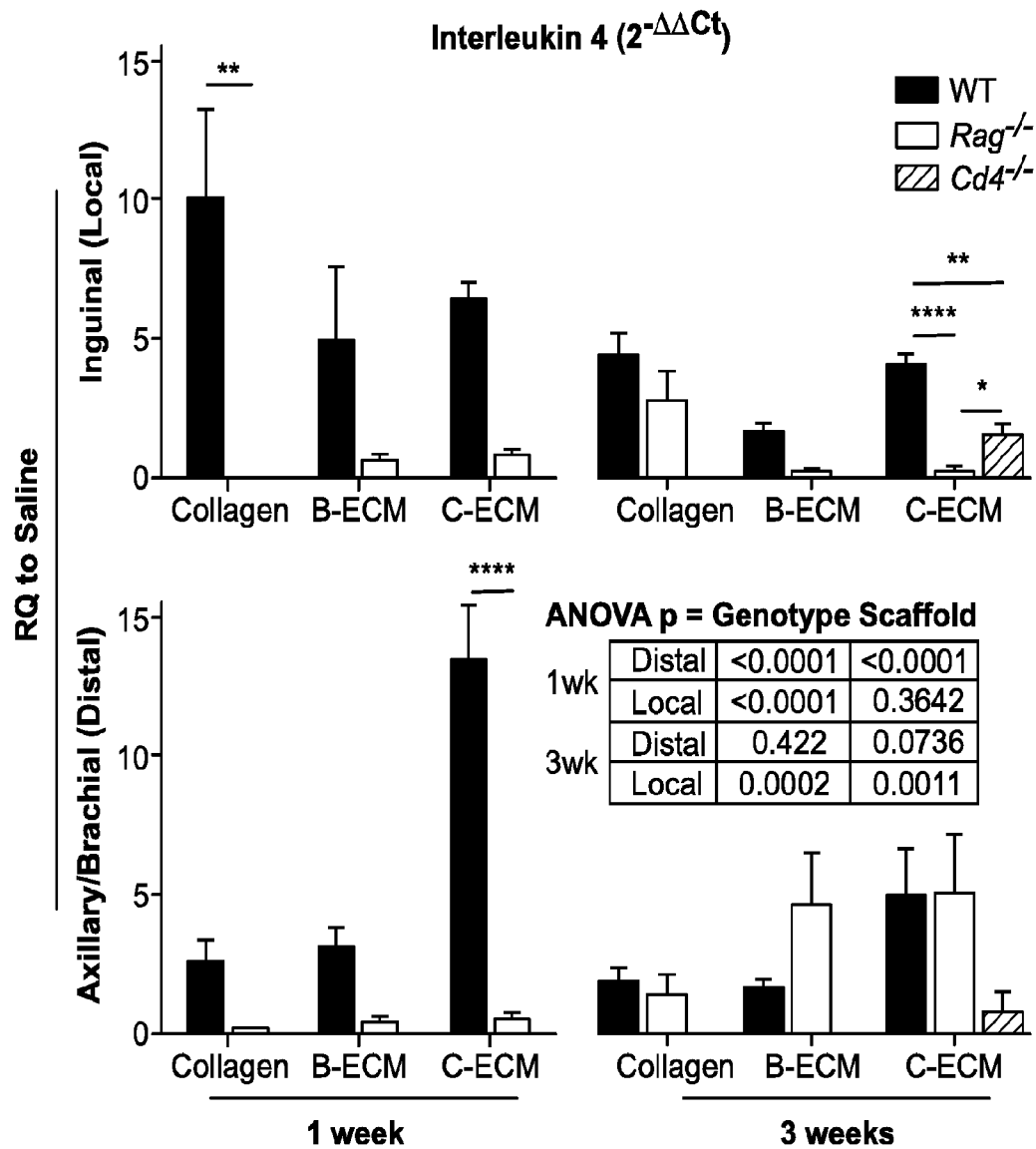


FIG. 3A



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FIG. 3B



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FIG. 4A

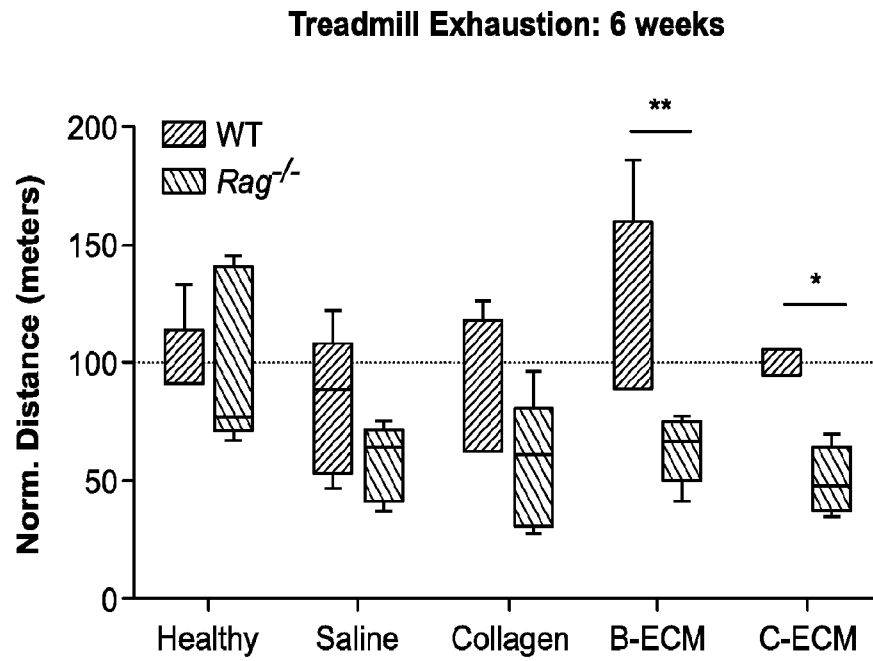
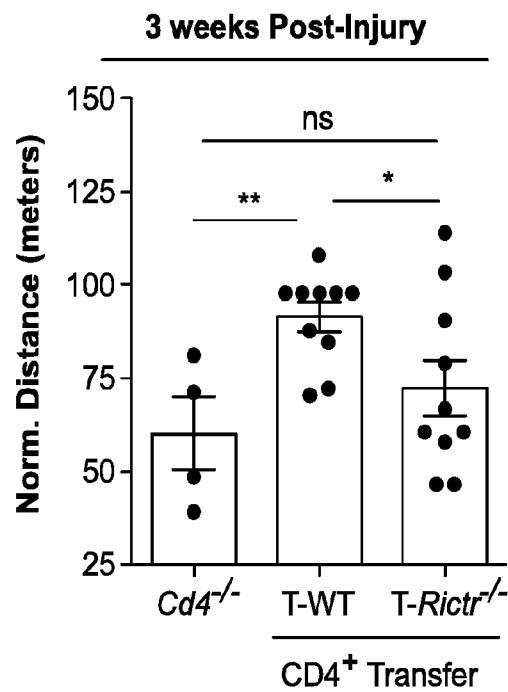


FIG. 4B



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FIG. 4C

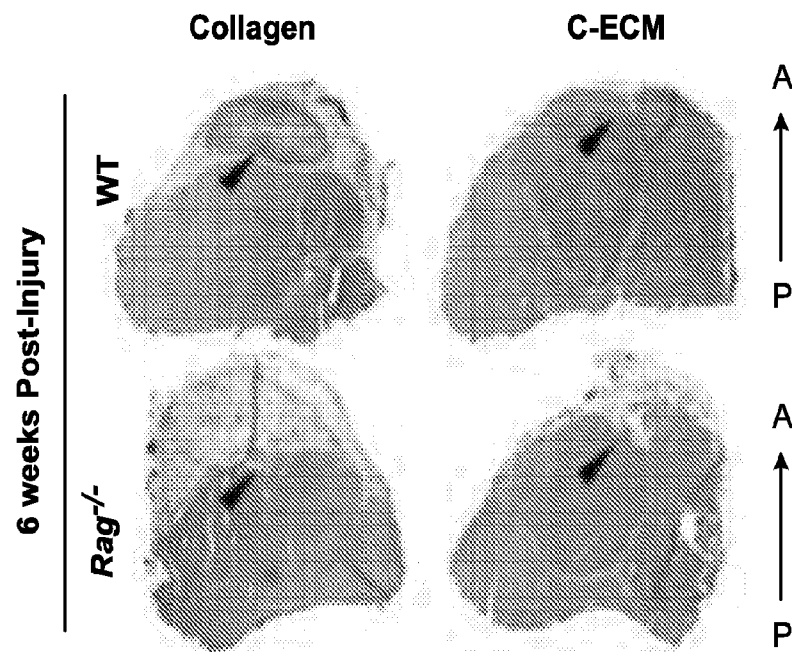
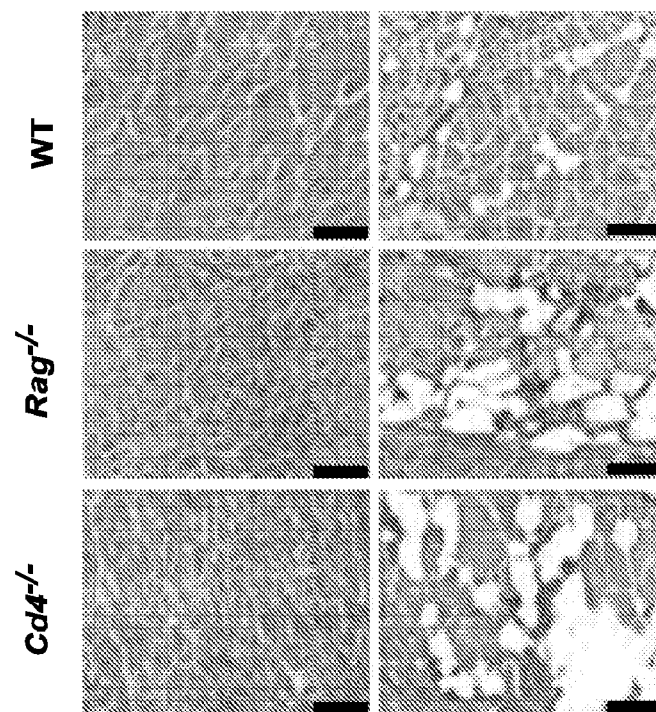


FIG. 4D



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FIG. 4E

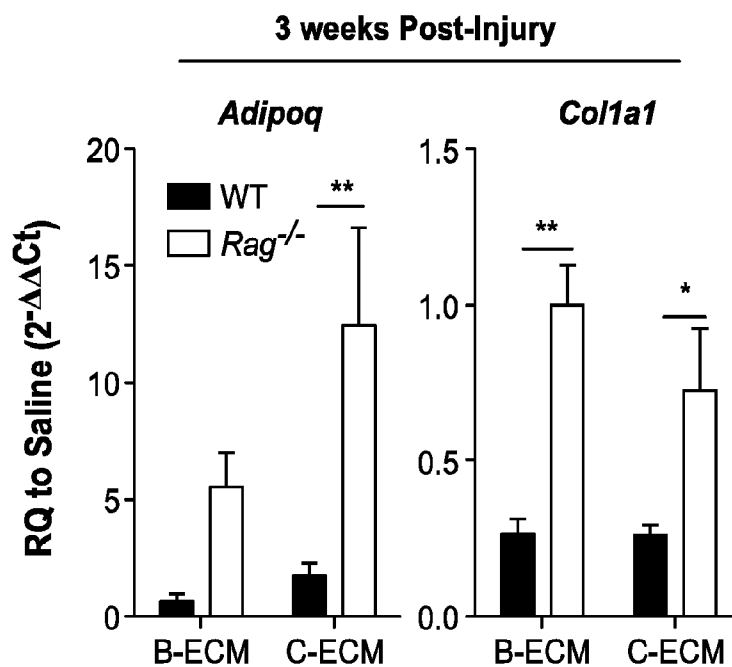


FIG. 4F

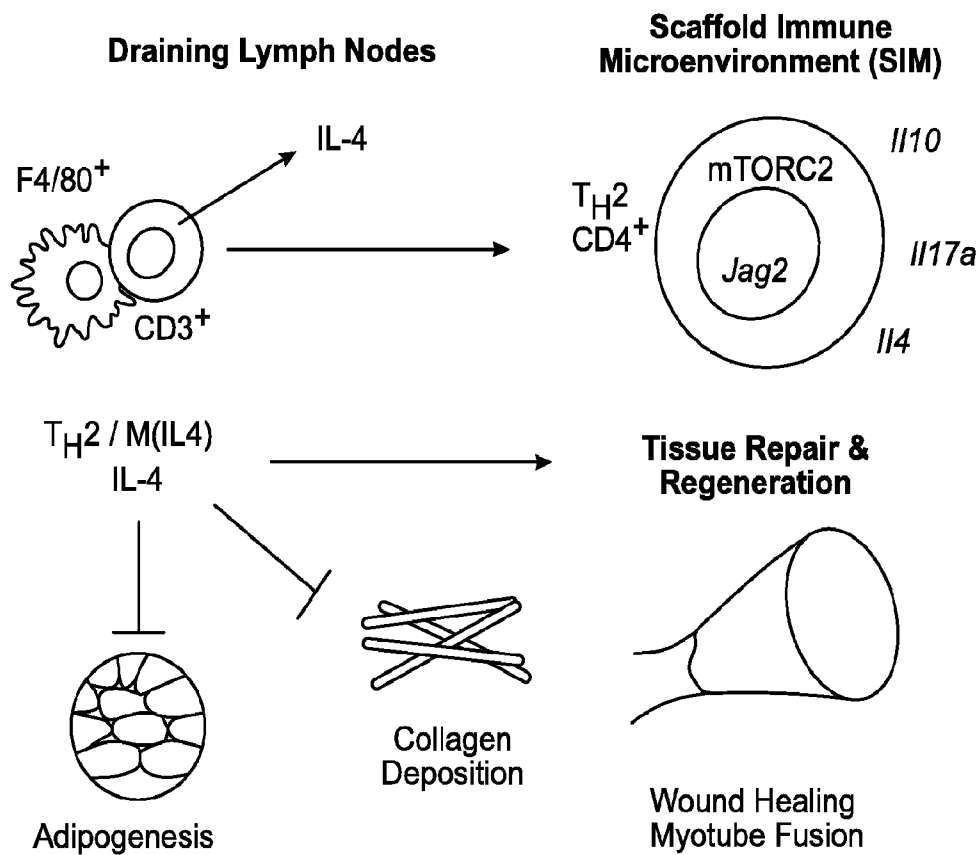


FIG. 5A

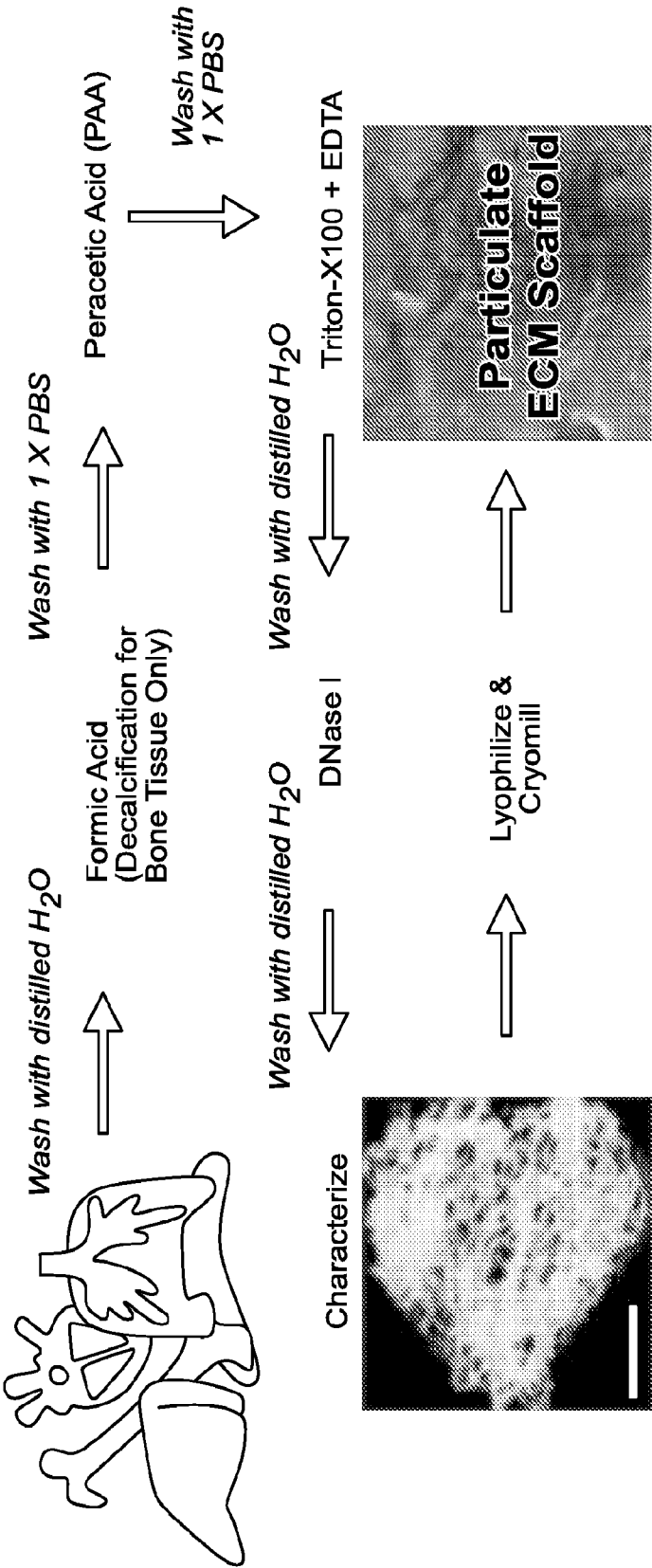


FIG. 5B

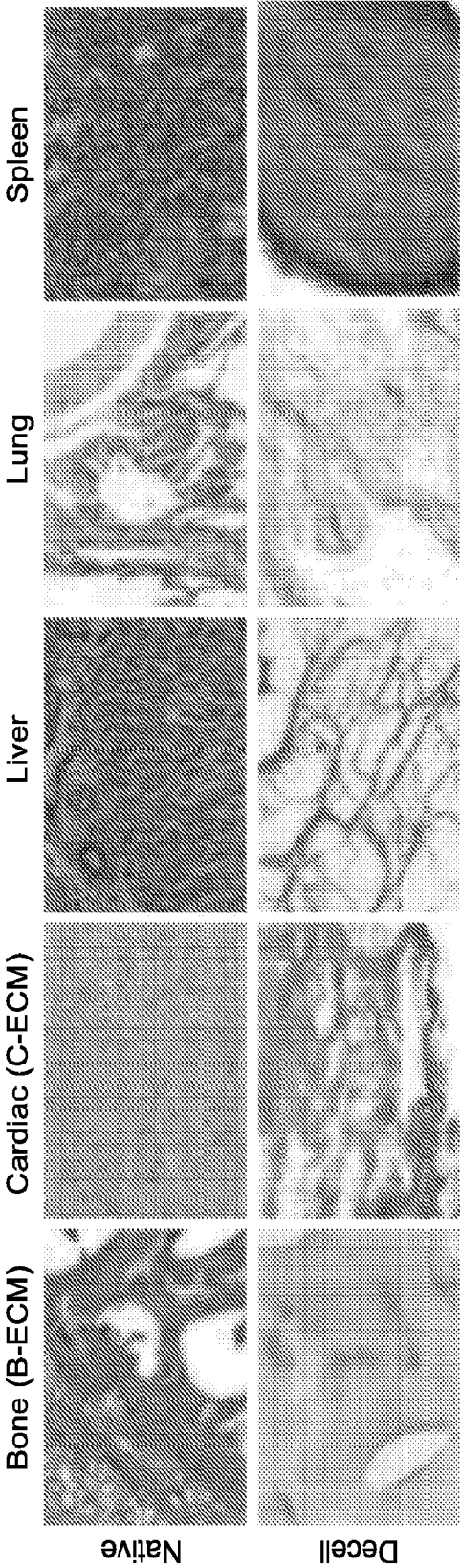


FIG. 5C

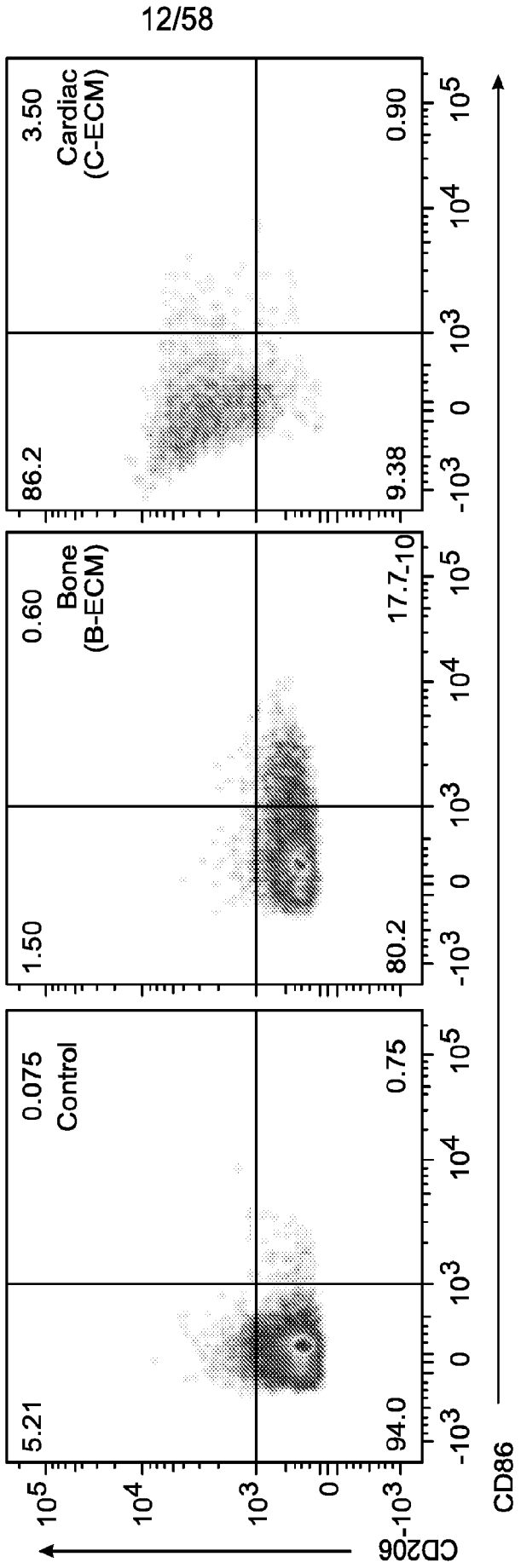
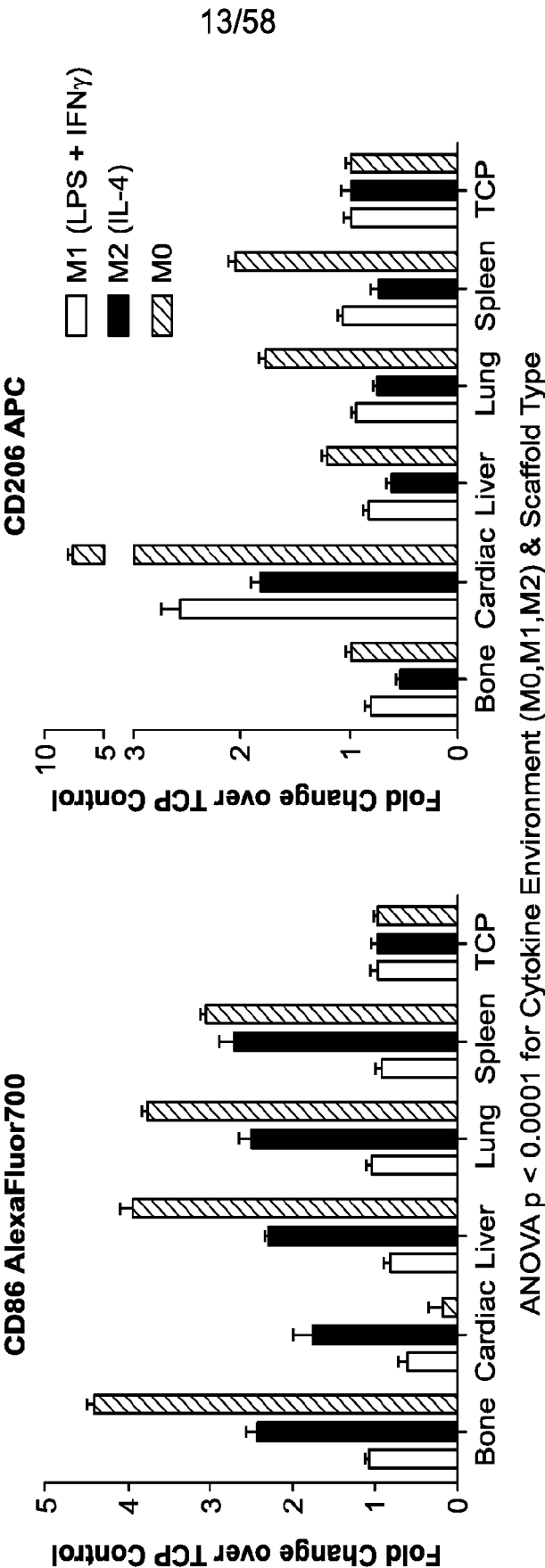
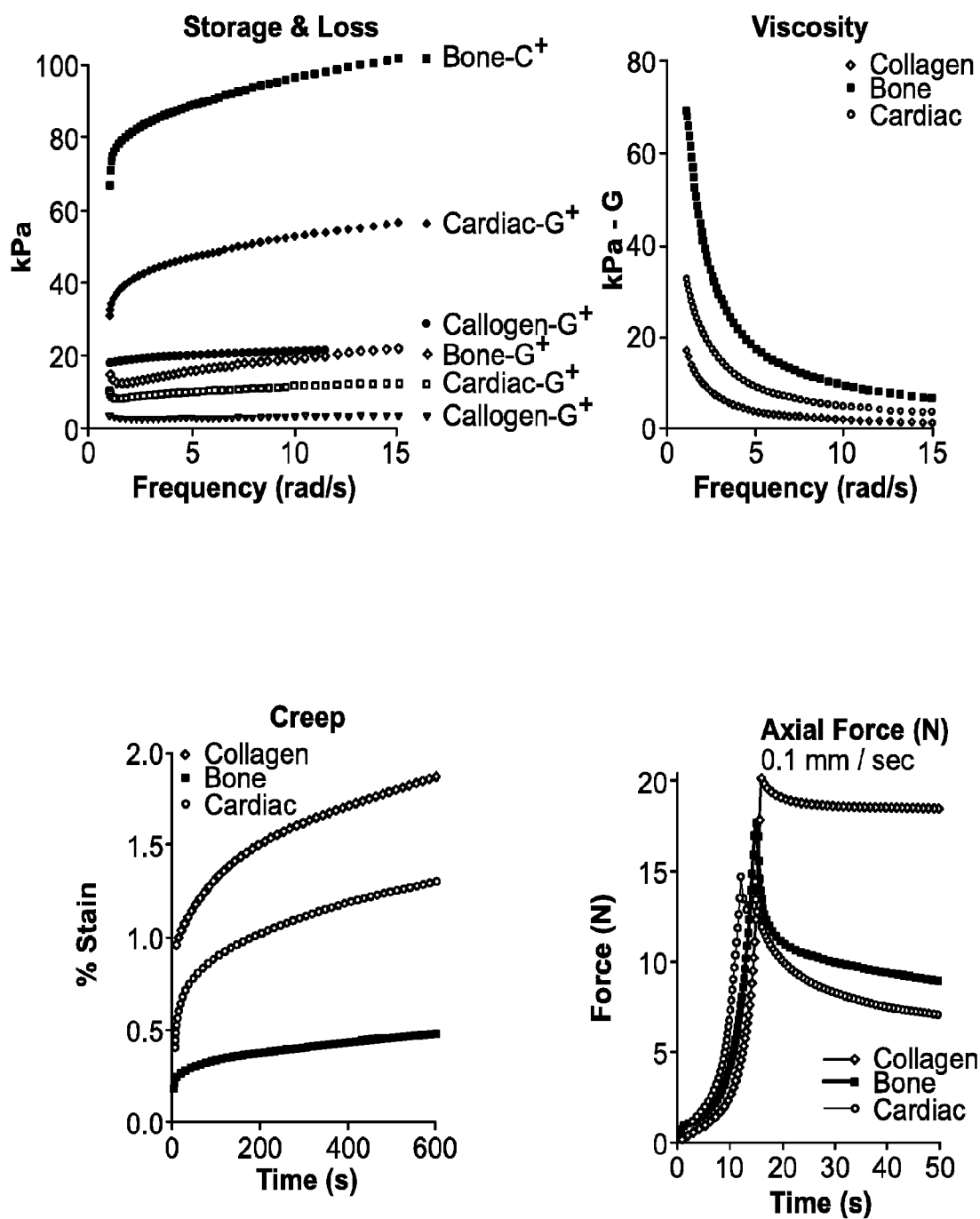


FIG. 5D



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FIG. 5E



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FIG. 6A

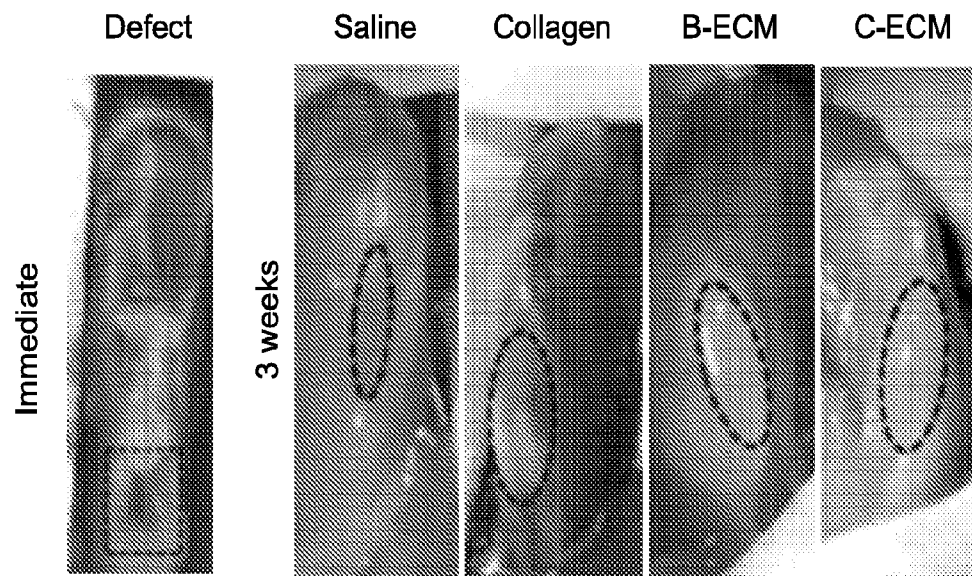


FIG. 6B

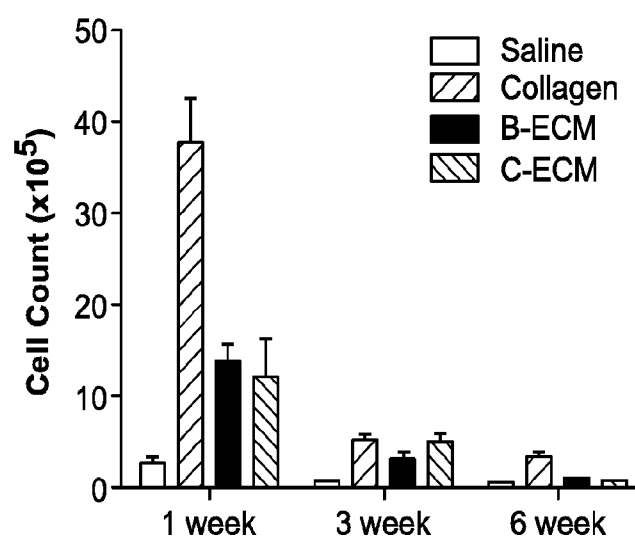
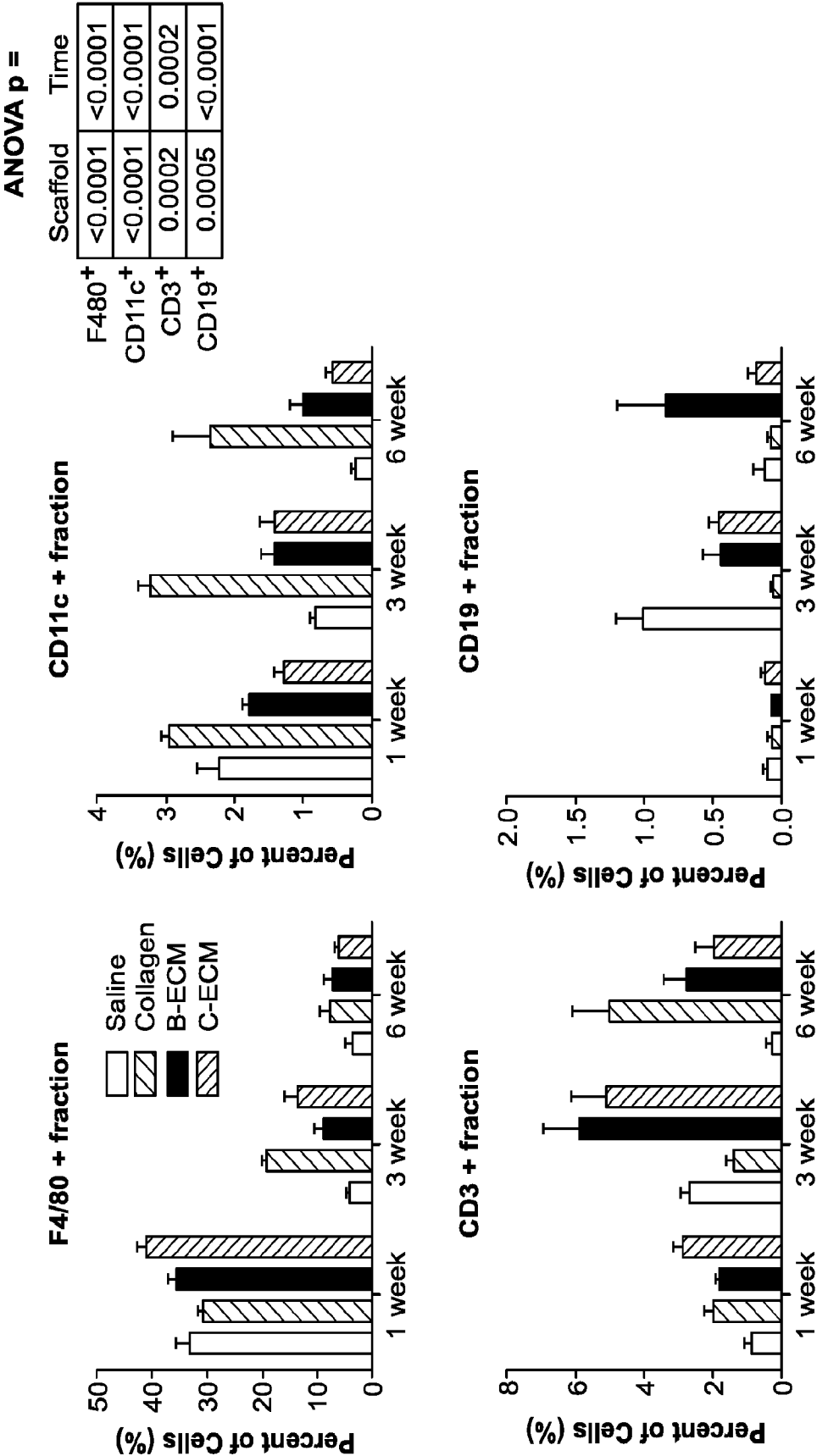


FIG. 6C



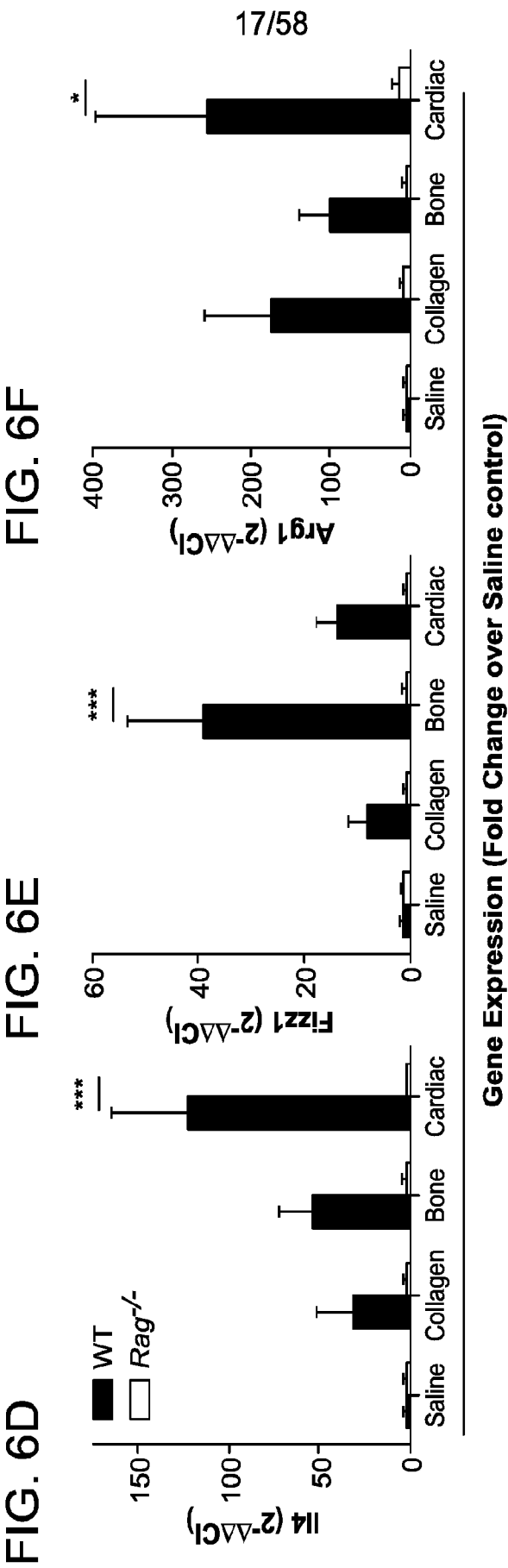


FIG. 6G

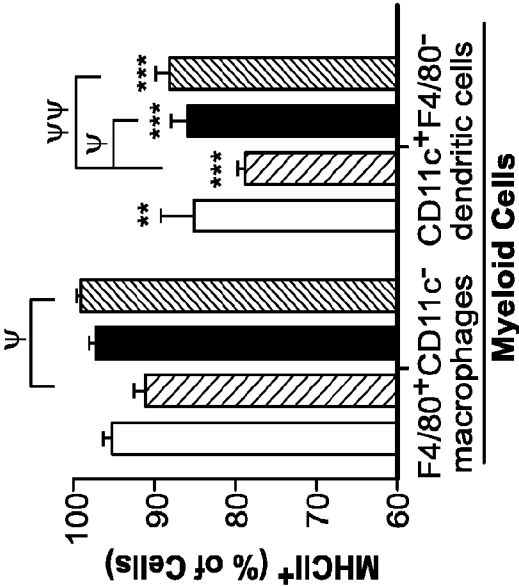
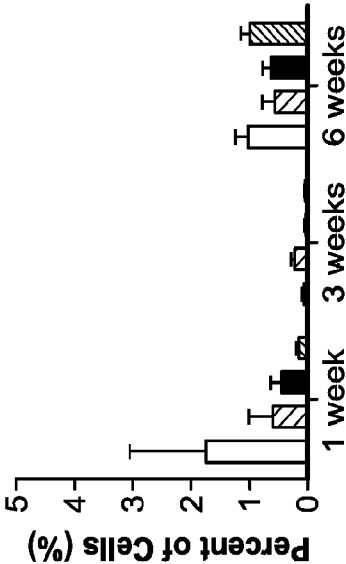
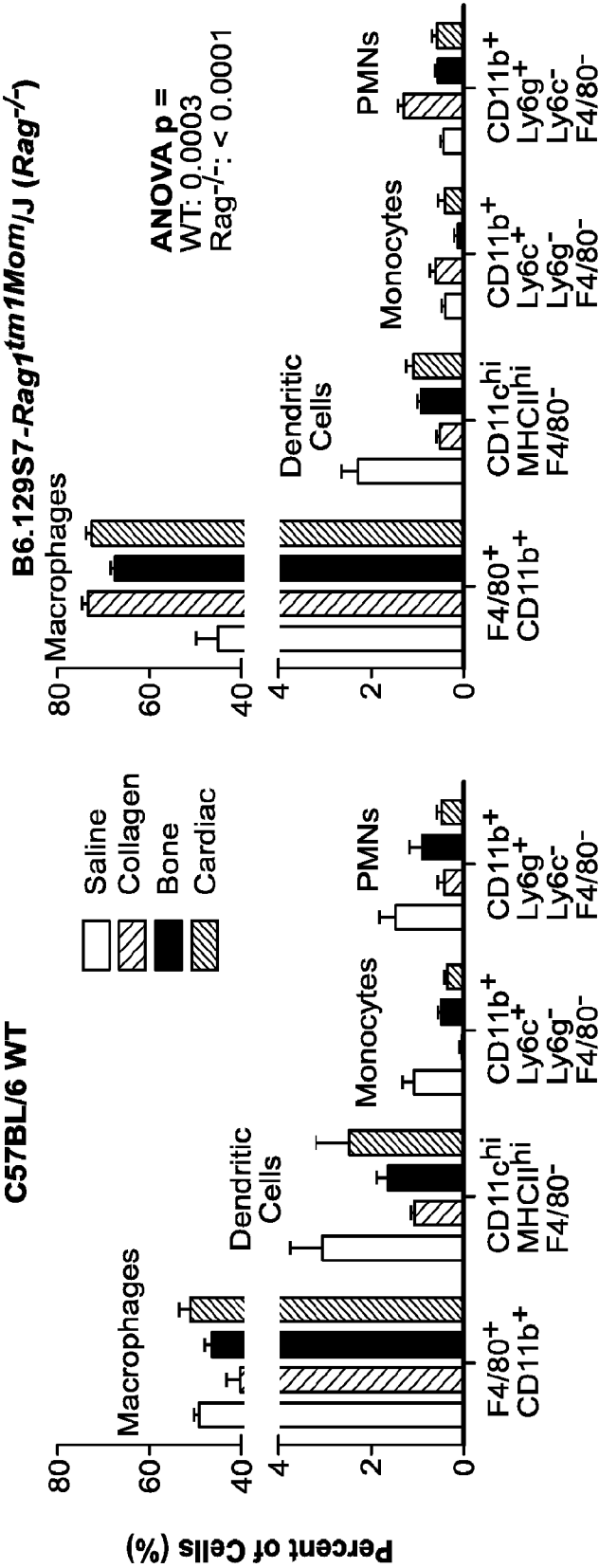


FIG. 6H



ANOVA p =	
Scaffold	Time
F4/80+	< 0.0001
CD11c+	< 0.0001
CD3+	0.0002
CD19+	< 0.0001
CD34+	0.0262

FIG. 6I



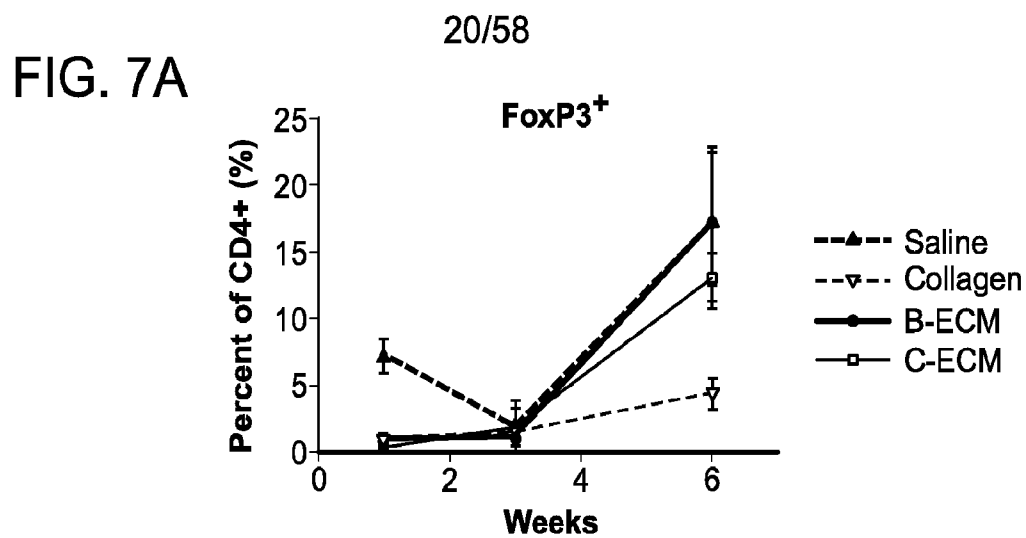


FIG. 7B

Scaffold 0.0185
Time < 0.0001

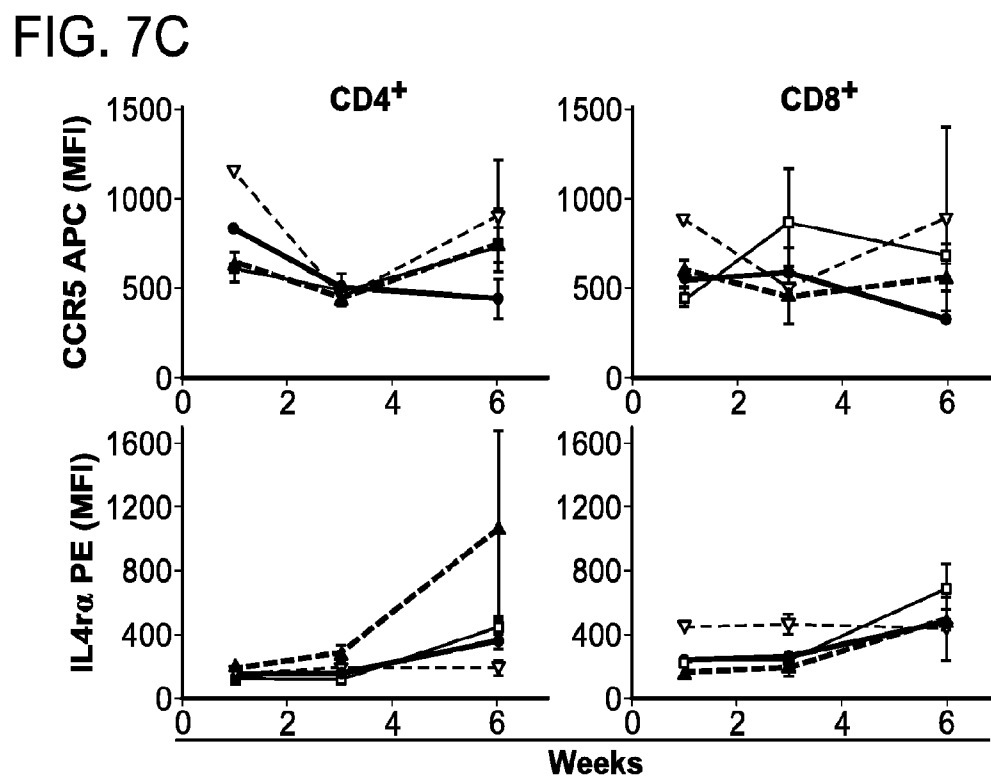
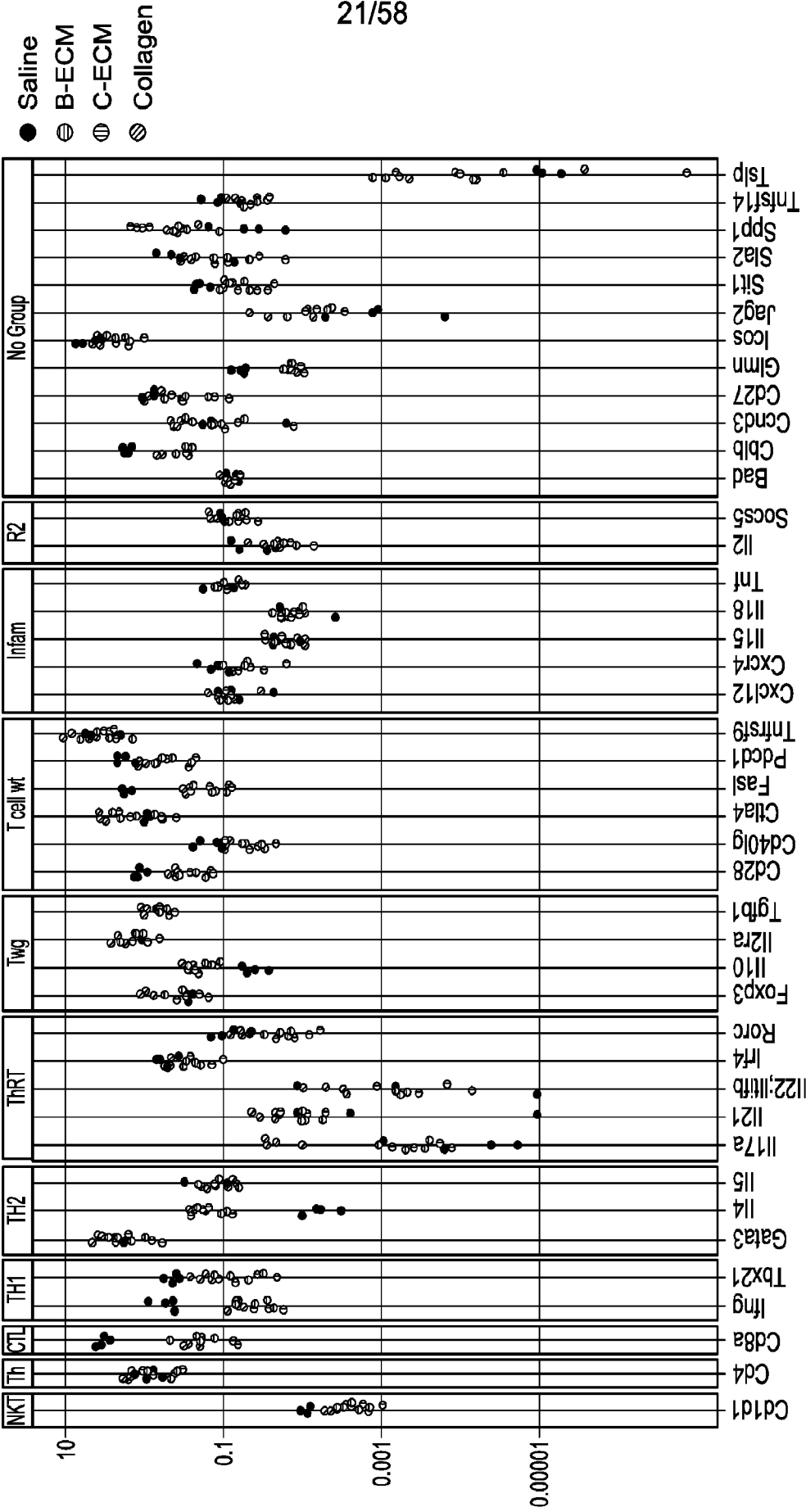


FIG. 7D

	CD4		CD8	
	CCR5	IL4ra	CCR5	IL4ra
Scaffold	0.0567	0.0845	0.3208	0.1045
Time	0.0007	0.0093	0.9914	0.0001

FIG. 8



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FIG. 9

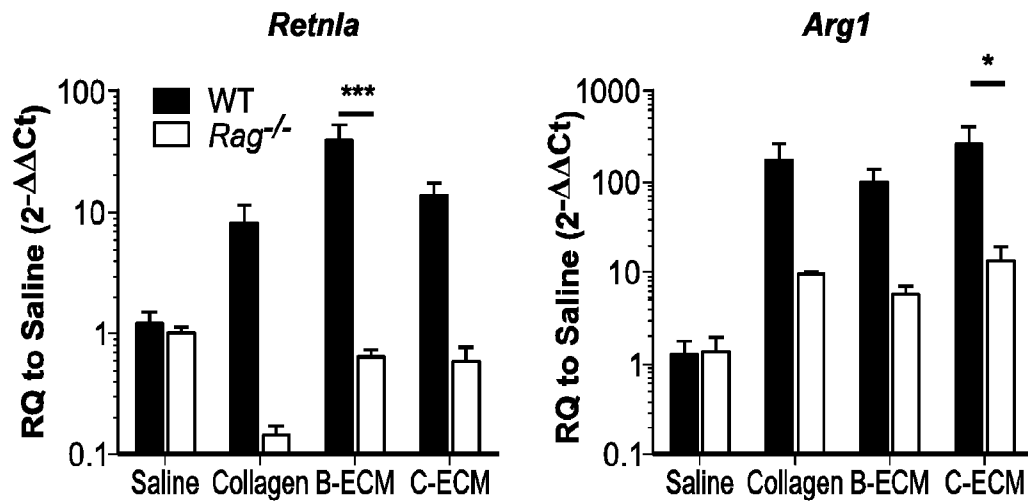


FIG. 10A

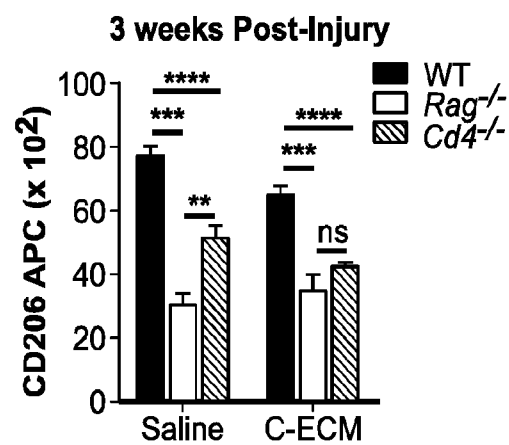


FIG. 10B

	ANOVA	Genotype	Scaffold
1wk	CD86	0.0476	< 0.0001
	CD206	< 0.0001	0.0018
3wk	CD86	< 0.0001	< 0.0001
	CD206	< 0.0001	< 0.0001

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FIG. 10C

ANOVA		1wk		3wk	
to Saline:		CD86	CD206	CD86	CD206
WT	Collagen	ns	ns	ns	****
	B-ECM	*	ns	ns	ns
	C-ECM	**	ns	*	*
Rag ^{-/-}	Collagen	ns	****	****	****
	B-ECM	ns	ns	ns	ns
	C-ECM	***	*	ns	ns

FIG. 10D

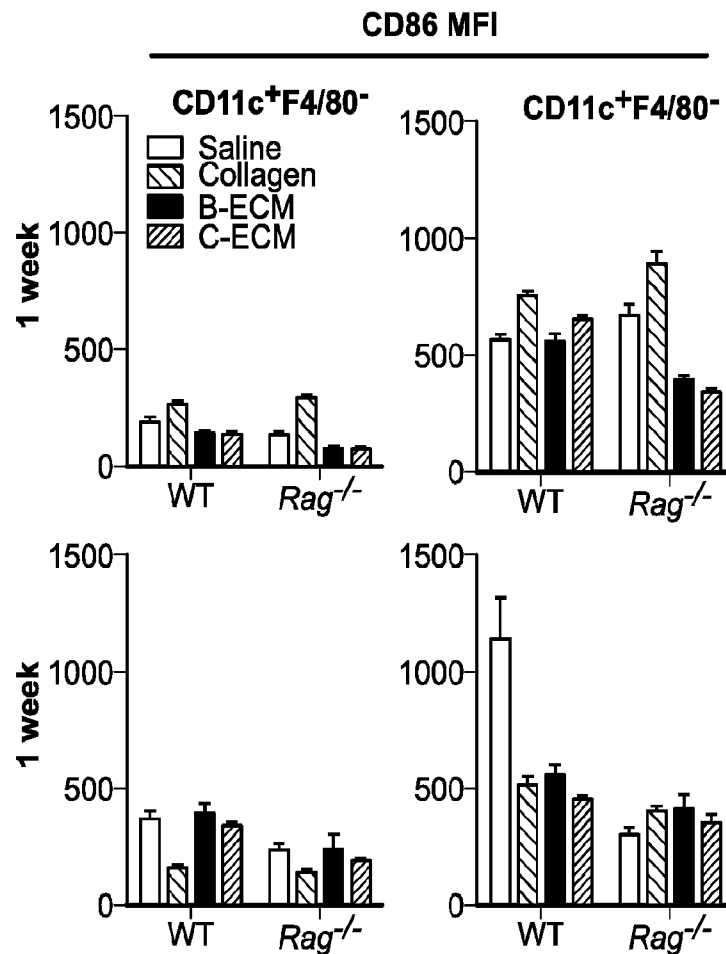


FIG. 10E

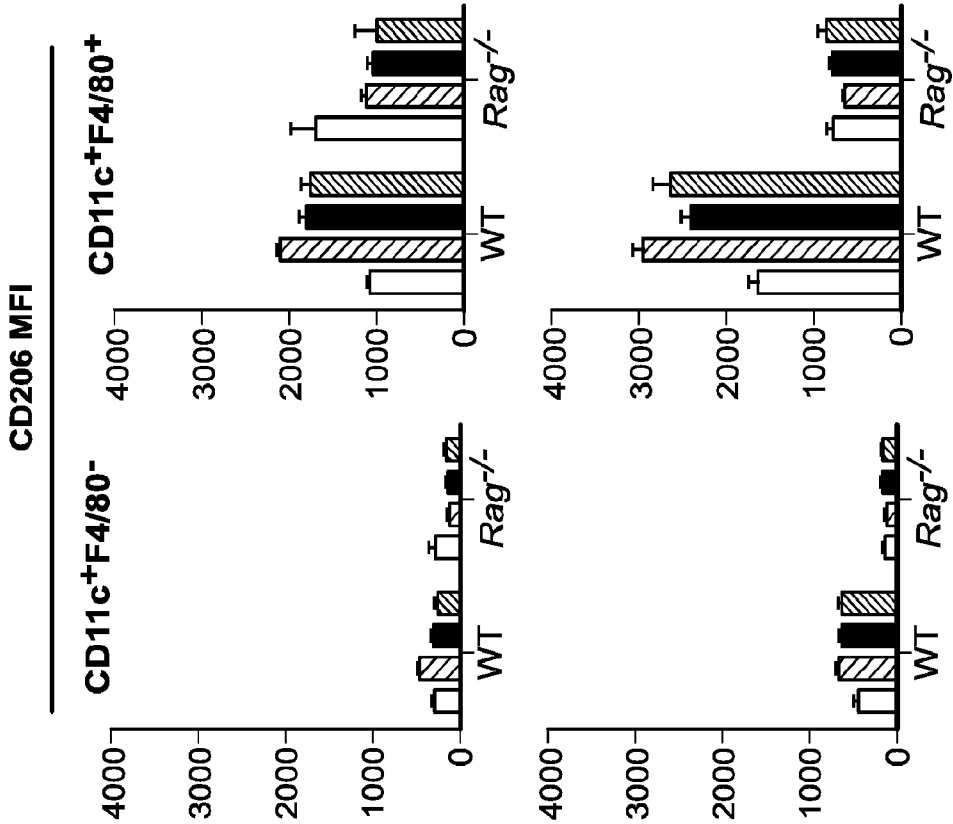
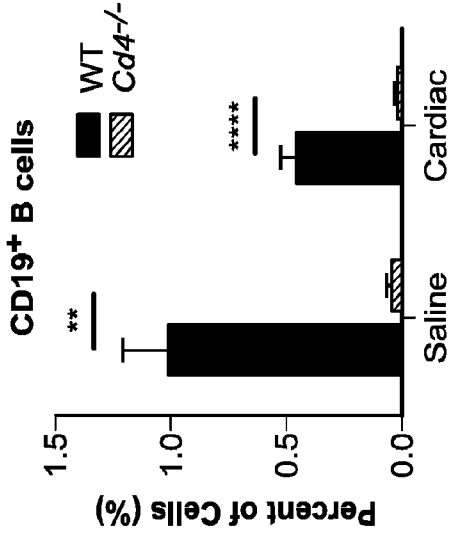
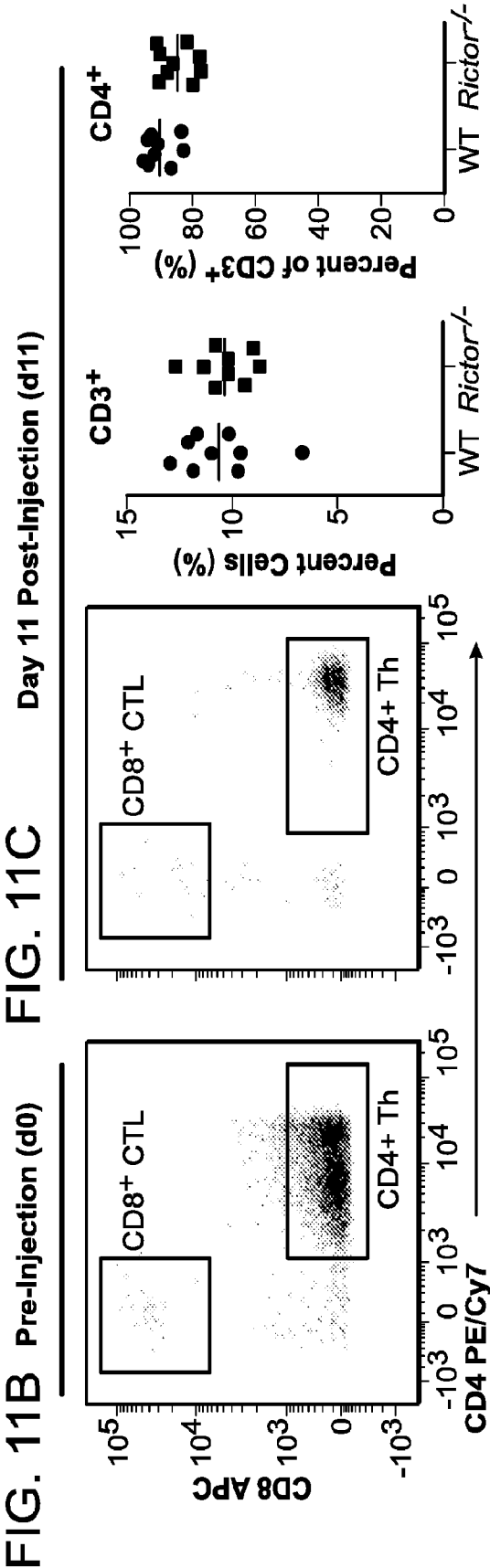
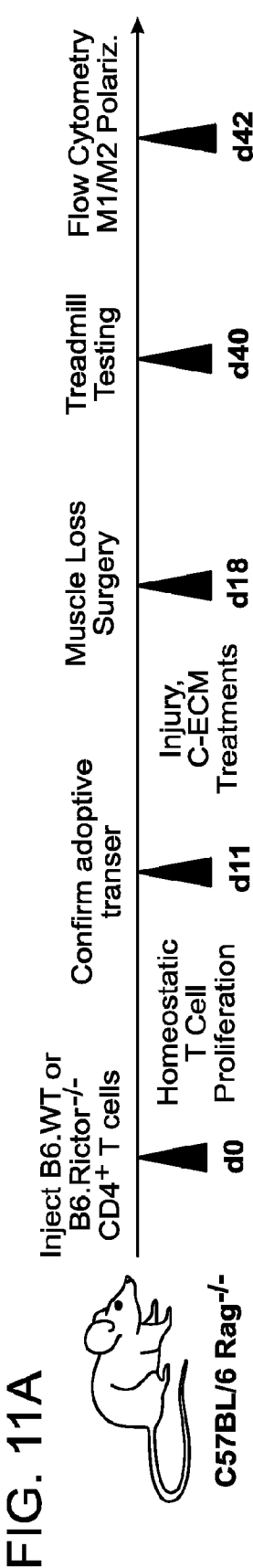


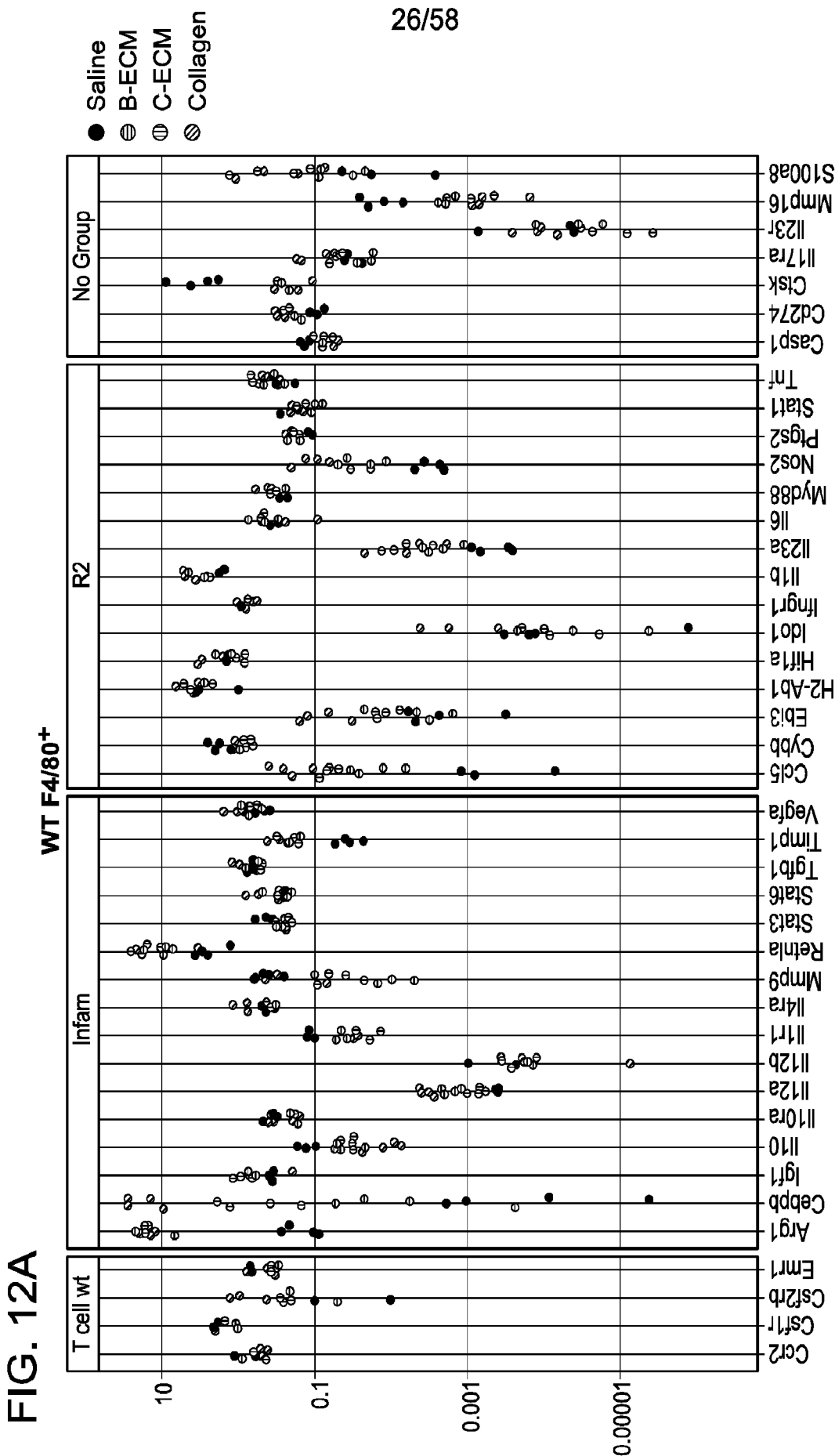
FIG. 10F

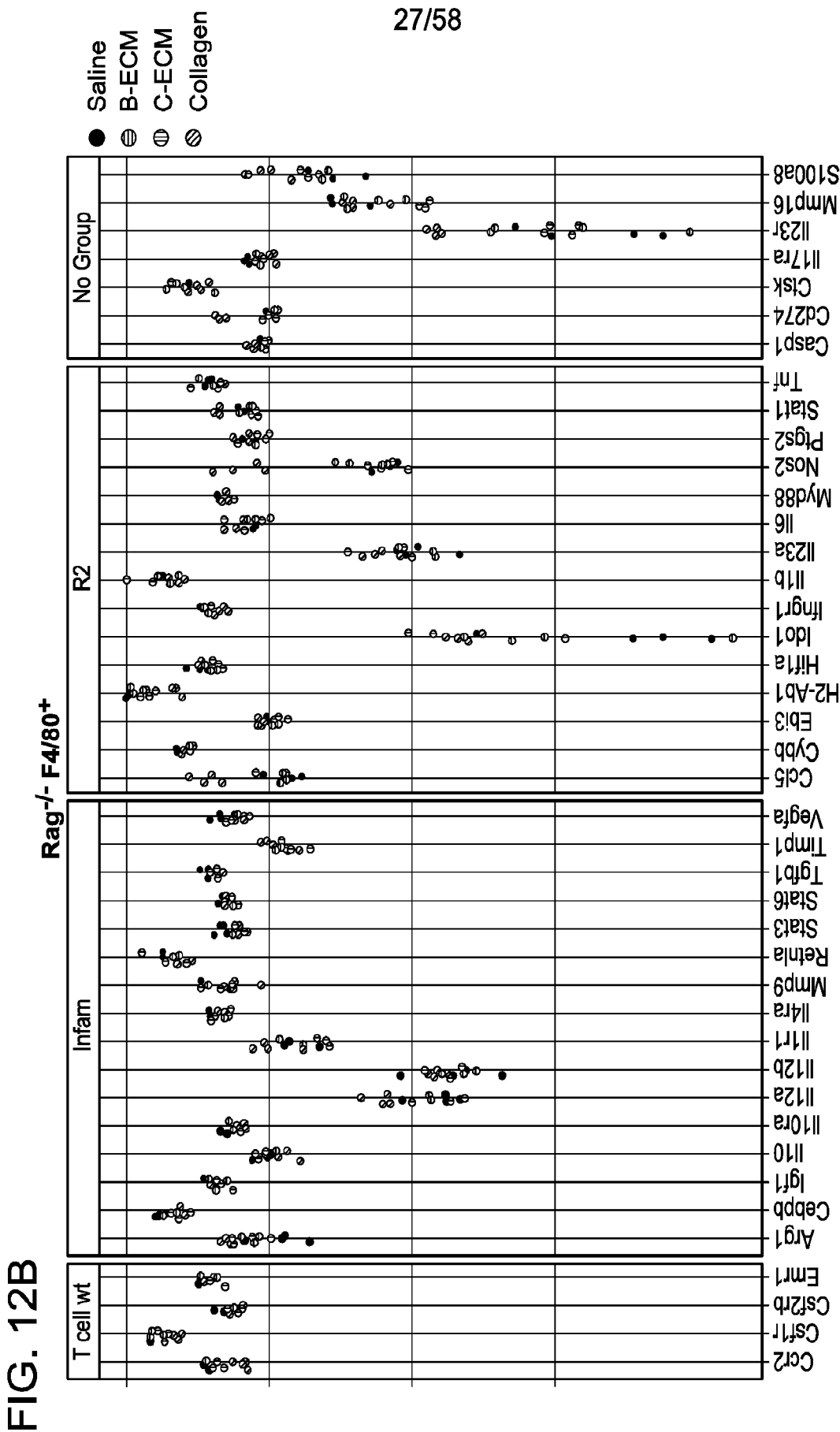
ANOVA	CD86				CD206			
	CD11c +F4/80 ⁻	CD11c +F4/80 ⁺	CD11c +F4/80 ⁻	CD11c +F4/80 ⁺	CD11c +F4/80 ⁻	CD11c +F4/80 ⁺	CD11c +F4/80 ⁻	CD11c +F4/80 ⁺
1 week								
Scaffold	< 0.0001	< 0.0001	< 0.0001	0.3113				
Genotype	< 0.0001	0.0102	< 0.0001	< 0.0001				
3 weeks								
Scaffold	< 0.0001	0.0007	< 0.0001	< 0.0001				
Genotype	< 0.0001	< 0.0001	< 0.0001	< 0.0001				

FIG. 10G



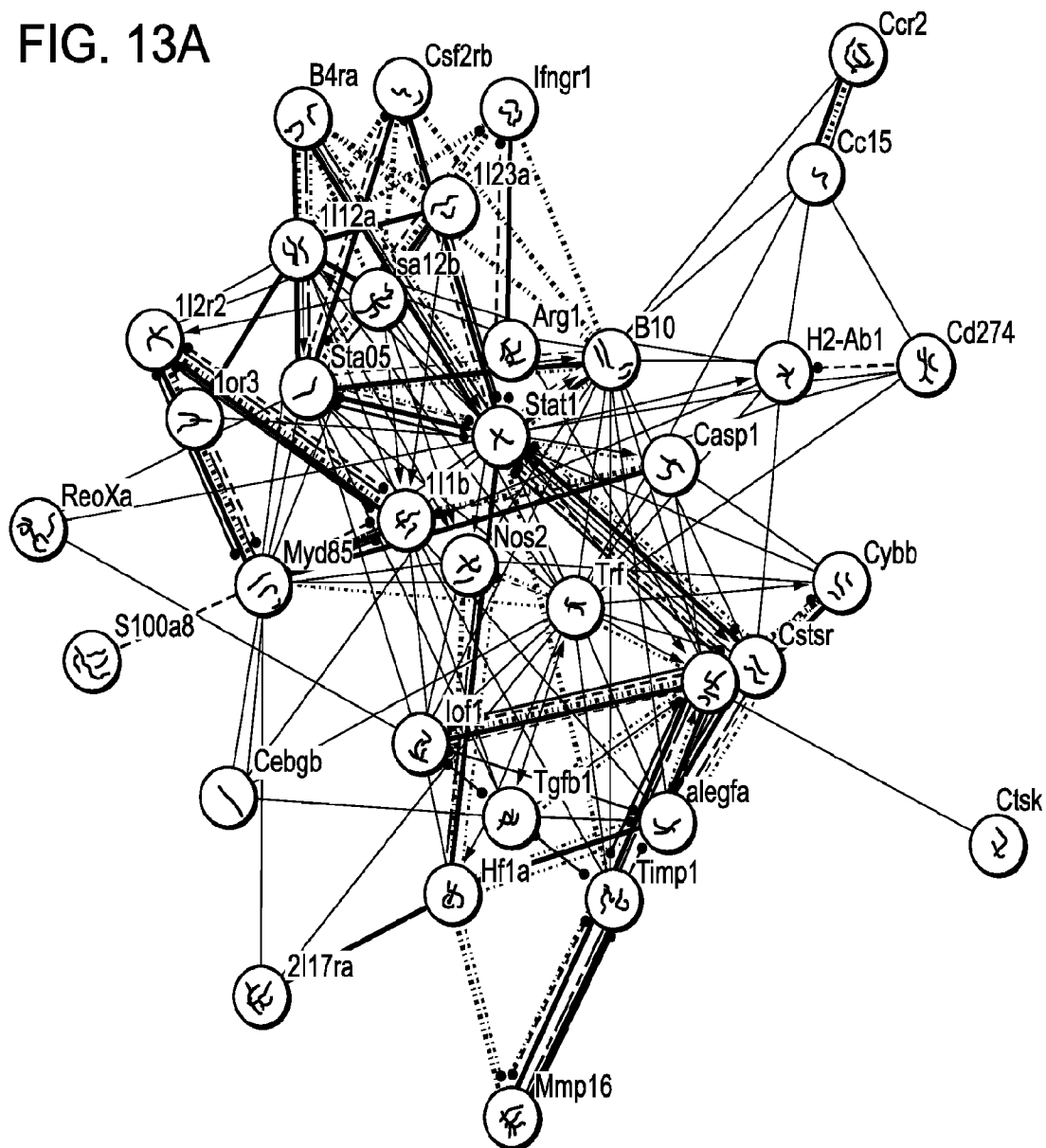






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FIG. 13A



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FIG. 13B-1

GO_id	Term	# Of Genes	P-value	FDR	Bonferroni
GO:0048660	regulation of smooth muscle cell proliferation	9	4.04E-14	1.09E-11	5.44E-10
GO:0051094	positive regulation of developmental process	16	1.59E-12	2.91E-10	2.14E-08
GO:1903034	regulation of response to wounding	10	5.42E-11	6.23E-09	7.29E-07
GO:0043771	tissue remodeling	7	1.38E-10	1.40E-03	1.86E-06
GO:0050703	regulation of developmental process	18	1.49E-10	1.47E-03	2.00E-06
GO:0032963	collagen metabolic process	5	2.37E-09	1.82E-07	3.19E-05
GO:0045595	regulation of cell differentiation	14	1.07E-08	7.14E-07	1.44E-04
GO:0030879	mammary gland development	6	1.56E-08	1.01E-06	2.10E-04
GO:1903035	positive regulation of response to wounding	6	4.91E-08	2.90E-06	6.61E-04
GO:2000177	regulation of neural precursor cell proliferation	5	2.07E-07	1.06E-06	2.79E-03
GO:0022603	regulation of anatomical structure morphogenesis	10	2.39E-07	1.20E-05	3.21E-03
GO:0045765	regulation of angiogenesis	6	4.52E-07	2.12E-05	6.07E-03
GO:0022612	gland morphogenesis	5	6.48E-07	2.90E-05	8.71E-03
GO:1901342	regulation of vasculature development	6	7.02E-07	3.11E-05	9.44E-03
GO:0048856	anatomical structure development	19	7.64E-07	3.34E-05	1.03E-02
GO:0048732	gland development	7	8.30E-07	3.60E-05	1.12E-02
GO:0061180	mammary gland epithelium development	4	1.65E-06	6.38E-05	2.22E-02
GO:0032502	developmental process	19	1.95E-06	7.44E-05	2.62E-02
GO:0060688	regulation of morphogenesis of a branching structure	4	2.04E-06	7.68E-05	2.74E-02
GO:0050679	positive regulation of epithelial cell proliferation	5	3.07E-06	1.11E-04	4.13E-02
GO:1901514	regulation of neuron death	6	3.08E-06	1.11E-04	4.14E-02
GO:0030674	collagen catabolic process	3	5.09E-06	1.75E-04	6.84E-02
GO:0060429	epithelium development	9	5.99E-06	2.01E-04	8.05E-02
GO:0007275	multicellular organismal development	17	6.26E-06	2.09E-04	8.41E-02
GO:0010634	positive regulation of epithelial cell migration	4	1.01E-05	3.18E-04	1.36E-01
GO:0010574	regulation of vascular endothelial growth factor production	3	1.08E-05	3.36E-04	1.45E-01

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FIG. 13B-2

GO:1902692	regulation of neuroblast proliferation	3	2.13E-05	6.16E-04	2.87E-01
GO:0001974	blood vessel remodeling	3	2.53E-05	7.17E-04	3.40E-01
GO:0045766	positive regulation of angiogenesis	4	2.82E-05	7.89E-04	3.79E-01
GO:0003006	developmental process involved in reproduction	7	3.41E-05	9.31E-04	4.59E-01
GO:0009887	organ morphogenesis	8	3.44E-05	9.31E-04	4.62E-01
GO:1904018	positive regulation of vasculature development	4	3.68E-05	9.86E-04	4.94E-01
GO:0048513	organ development	13	4.44E-05	1.17E-03	5.97E-01
GO:0090594	inflammatory response to wounding	2	4.84E-05	1.25E-03	6.51E-01
GO:0002245	wound healing involved in inflammatory response	2	4.84E-05	1.25E-03	6.51E-01
GO:0030855	epithelial cell differentiation	6	5.14E-05	1.31E-03	6.91E-01
GO:0010660	regulation of muscle cell apoptotic process	3	5.92E-05	1.50E-00	7.96E-01
GO:0034393	positive regulation of smooth muscle cell apoptotic process	2	6.45E-05	1.62E-03	8.67E-01
GO:2000179	positive regulation of neural precursor cell proliferation	3	6.67E-05	1.66E-03	8.97E-01
GO:0010632	regulation of epithelial cell migration	4	7.21E-05	1.79E-03	9.69E-01
GO:0060426	lung vasculature development	2	8.28E-05	2.01E-03	1.00E+00
GO:0009653	anatomical structure morphogenesis	11	8.68E-05	2.10E-03	1.00E+00
GO:0031099	regeneration	3	1.20E-04	2.77E-03	1.00E+00
GO:0030198	extracellular matrix organization	4	1.62E-04	3.60E-03	1.00E+00
GO:0043062	extracellular structure organization	4	1.66E-04	3.67E-03	1.00E+00
GO:0060644	mammary gland epithelial cell differentiation	2	1.79E-04	3.87E-03	1.00E+00
GO:0061138	morphogenesis of a branching epithelium	4	1.84E-04	3.98E-03	1.00E+00
GO:0033598	mammary gland epithelial cell proliferation	2	2.08E-04	4.42E-03	1.00E+00
GO:0001763	morphogenesis of a branching structure	4	2.21E-04	4.63E-03	1.00E+00
GO:0010661	positive regulation of muscle cell apoptotic process	2	2.40E-04	4.98E-03	1.00E+00
GO:0048608	reproductive structure development	5	2.48E-04	5.13E-03	1.00E+00
GO:0009888	tissue development	9	2.57E-04	5.30E-03	1.00E+00
GO:0061458	reproductive system development	5	2.64E-04	5.40E-03	1.00E+00

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FIG. 13B-3

GO:0031100	organ regeneration	2	2.74E-04	5.55E-03	1.00E+00
GO:0050673	epithelial cell proliferation	3	2.95E-04	5.92E-03	1.00E+00
GO:0001936	regulation of endothelial cell proliferation	3	3.27E-04	6.43E-03	1.00E+00
GO:0042060	wound healing	4	9.95E-04	7.61E-03	1.00E+00
GO:0001501	skeletal system development	5	4.72E-04	8.90E-03	1.00E+00
GO:0048568	embryonic organ development	5	5.03E-04	9.42E-03	1.00E+00
GO:0001892	embryonic placenta development	3	5.07E-04	9.42E-03	1.00E+00
GO:0060749	mammary gland alveolus development	2	5.25E-04	9.60E-03	1.00E+00
GO:0061377	mammary gland lobule development	2	5.25E-04	9.60E-03	1.00E+00
GO:0002009	morphogenesis of an epithelium	5	5.30E-04	9.68E-03	1.00E+00

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FIG. 13C

developmental morphogenesis
 process epithelial
 reproduction remodeling factor
 organismal vessel regeneration tissue metabolic
 vascular death anatomical system
 lung embryonic regulation angiogenesis
 organization organ involved gland neuroblast
 muscle growth extracellular collagen
 wound skeletal migration neural wounding
 precursor production matrix inflammatory endothelial
 multicellular blood differentiation neuron structure
 vasculature response smooth healing
 development epithelium
 proliferation branching apoptotic

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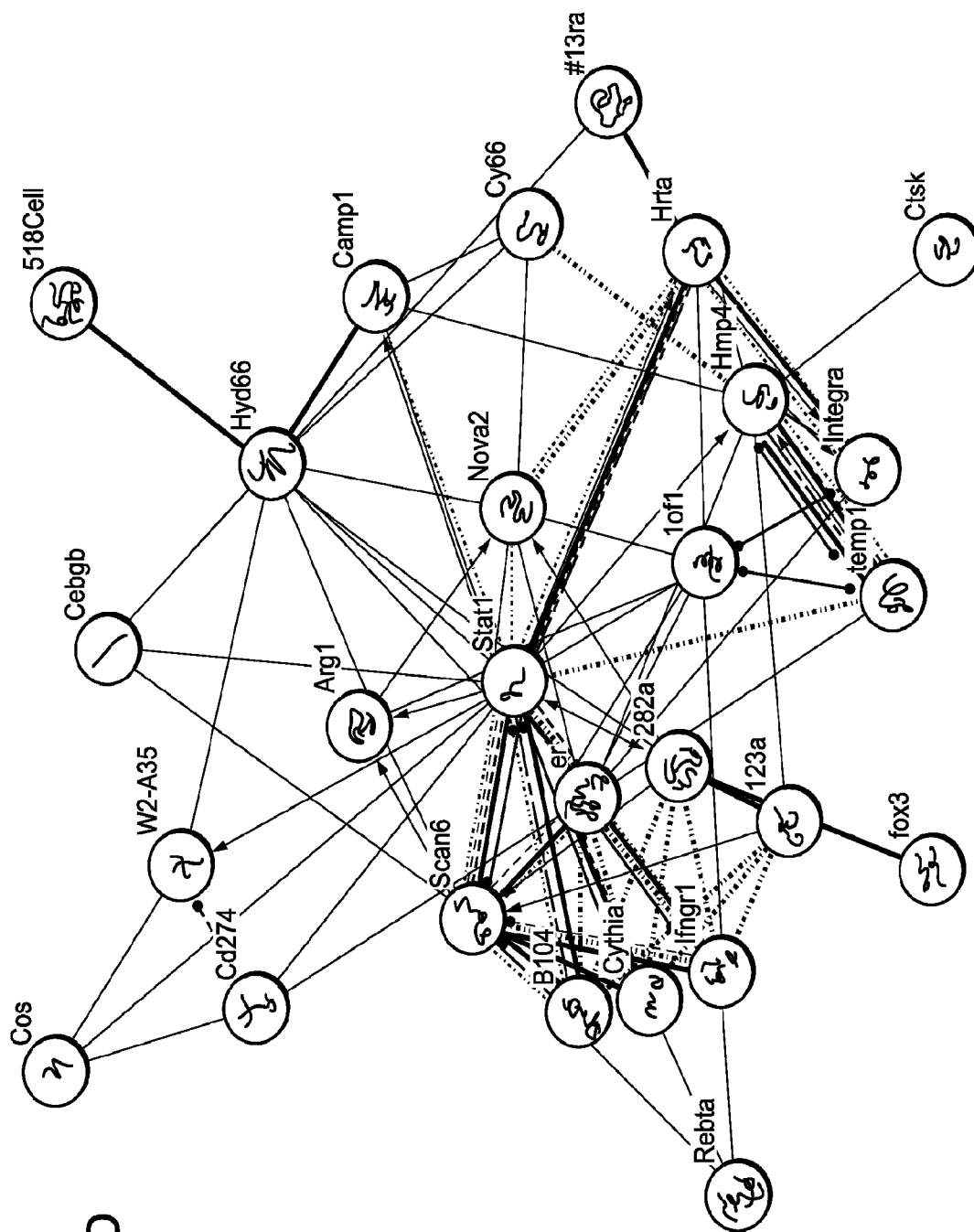


FIG. 13D

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FIG. 13E

GO_id	Term	Number Of Genes	p-value	p-value bonferroni
GO:0048660	regulation of smooth muscle cell proliferation	7	2.87E-11	1.61E-08
GO:0048661	positive regulation of smooth muscle cell proliferation	6	1.36E-10	6.07E-08
GO:0048771	tissue remodeling	6	1.54E-09	4.22E-07
GO:0032963	collagen metabolic process	4	9.16E-08	1.47E-05
GO:0045597	positive regulation of cell differentiation	8	2.89E-06	2.32E-04
GO:0060429	epithelium development	8	6.25E-06	4.35E-04
GO:1903034	regulation of response to wounding	5	4.11E-05	2.19E-03
GO:0045765	regulation of angiogenesis	4	7.52E-05	3.64E-03
GO:1901342	regulation of vasculature development	4	1.01E-04	4.54E-03
GO:0071542	dopaminergic neuron differentiation	2	1.62E-04	6.55E-03
GO:0009888	tissue development	8	1.94E-04	7.57E-03
GO:0045595	regulation of cell differentiation	8	1.94E-04	7.57E-03
GO:0045766	positive regulation of angiogenesis	3	3.32E-04	1.17E-02
GO:0002052	positive regulation of neuroblast proliferation	2	4.03E-04	1.39E-02
GO:1903036	positive regulation of response to wounding	3	5.10E-04	1.65E-02
GO:0043524	negative regulation of neuron apoptotic process	3	7.11E-04	2.20E-02
GO:0014911	positive regulation of smooth muscle cell migration	2	7.49E-04	2.29E-02
GO:1902692	regulation of neuroblast proliferation	2	7.93E-04	2.42E-02
GO:0001974	blood vessel remodeling	2	8.87E-04	2.69E-02
GO:0048856	anatomical structure development	12	9.40E-04	2.78E-02
GO:0051146	striated muscle cell differentiation	3	1.14E-03	3.30E-02
GO:0001701	in utero embryonic development	4	1.33E-03	3.78E-02
GO:0061138	morphogenesis of a branching epithelium	3	1.35E-03	3.83E-02
GO:0045600	positive regulation of fat cell differentiation	2	1.43E-03	4.01E-02
GO:0001763	morphogenesis of a branching structure	3	1.55E-03	4.29E-02
GO:0010660	regulation of muscle cell apoptotic process	2	1.55E-03	4.29E-02
GO:0032502	developmental process	12	1.62E-03	4.41E-02
GO:0048568	embryonic organ development	4	1.63E-03	4.43E-02
GO:0002009	morphogenesis of an epithelium	4	1.70E-03	4.60E-02
GO:0051962	positive regulation of nervous system development	4	1.81E-03	4.82E-02
GO:0014910	regulation of smooth muscle cell migration	2	1.81E-03	4.82E-02
GO:0060249	anatomical structure homeostasis	3	1.87E-03	4.95E-02

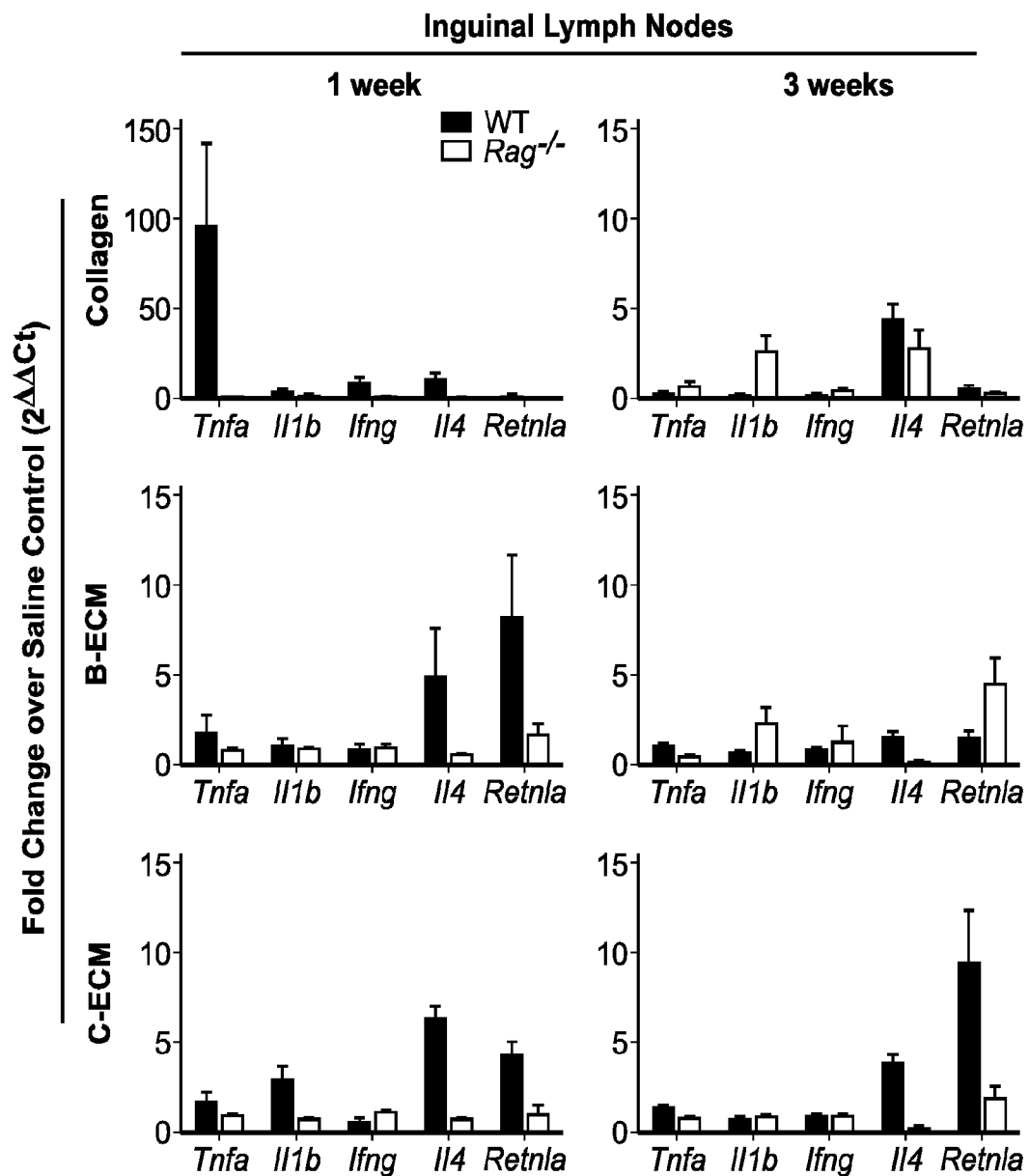
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FIG. 13F

smooth process positive
 developmental angiogenesis
 structure migration
 response morphogenesis remodeling
 differentiation embryonic
 fat striated organ
 branching vessel neuron metabolic collagen dopaminergic neuroblast
 negative homeostasis
 apoptotic nervous
 wounding blood
 muscle
 development tissue epithelium
 vasculature anatomical
 utero proliferation
 cell regulation

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FIG. 14A



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FIG. 14B

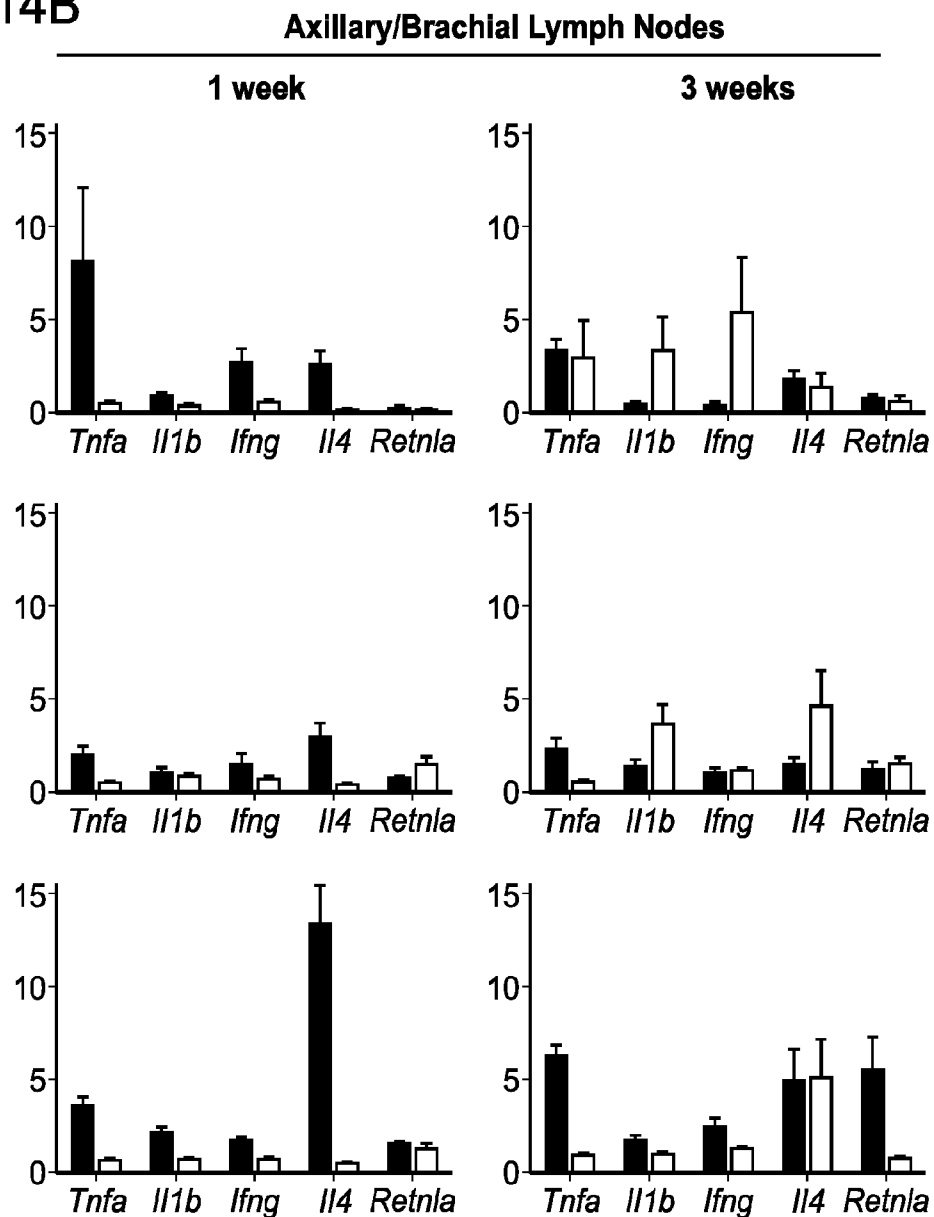
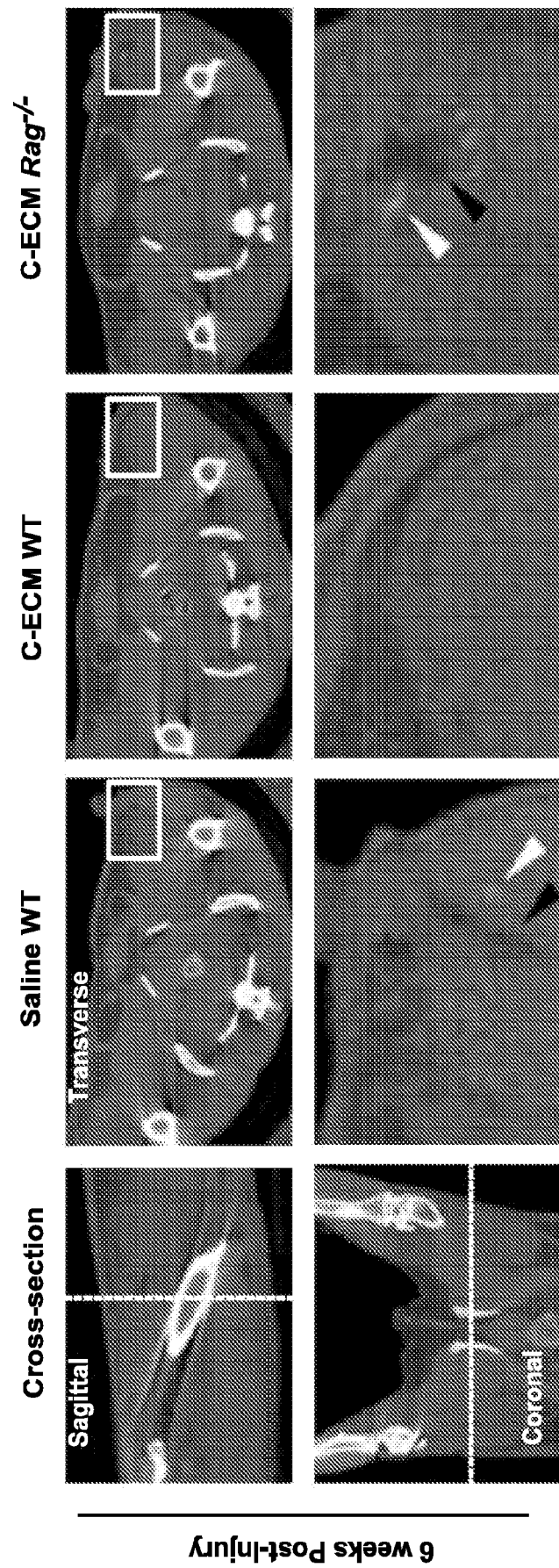


FIG. 14C

	Inguinal LN		Axillary/Brachial LN	
	1 week	3 week	1 week	3 week
Collagen	0.0061	0.4101	0.0049	0.0915
B-ECM	0.0158	0.1408	0.0007	0.0911
C-ECM	< 0.001	0.0006	< 0.001	0.0013

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FIG. 15



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FIG. 16A

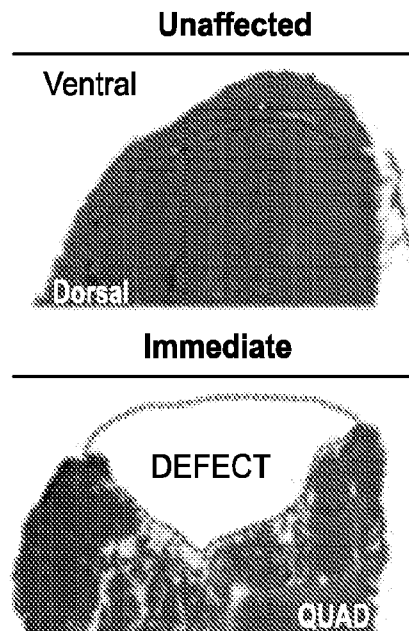
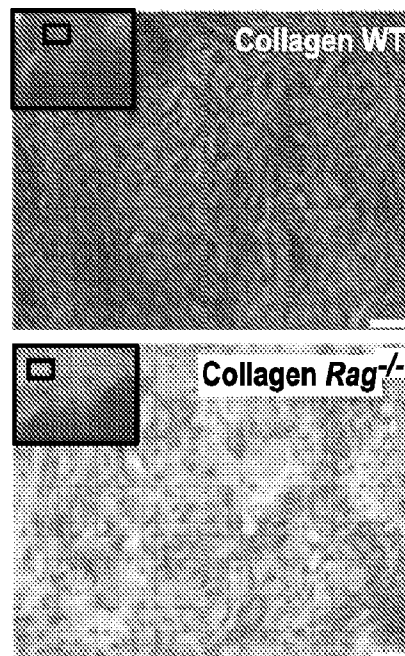
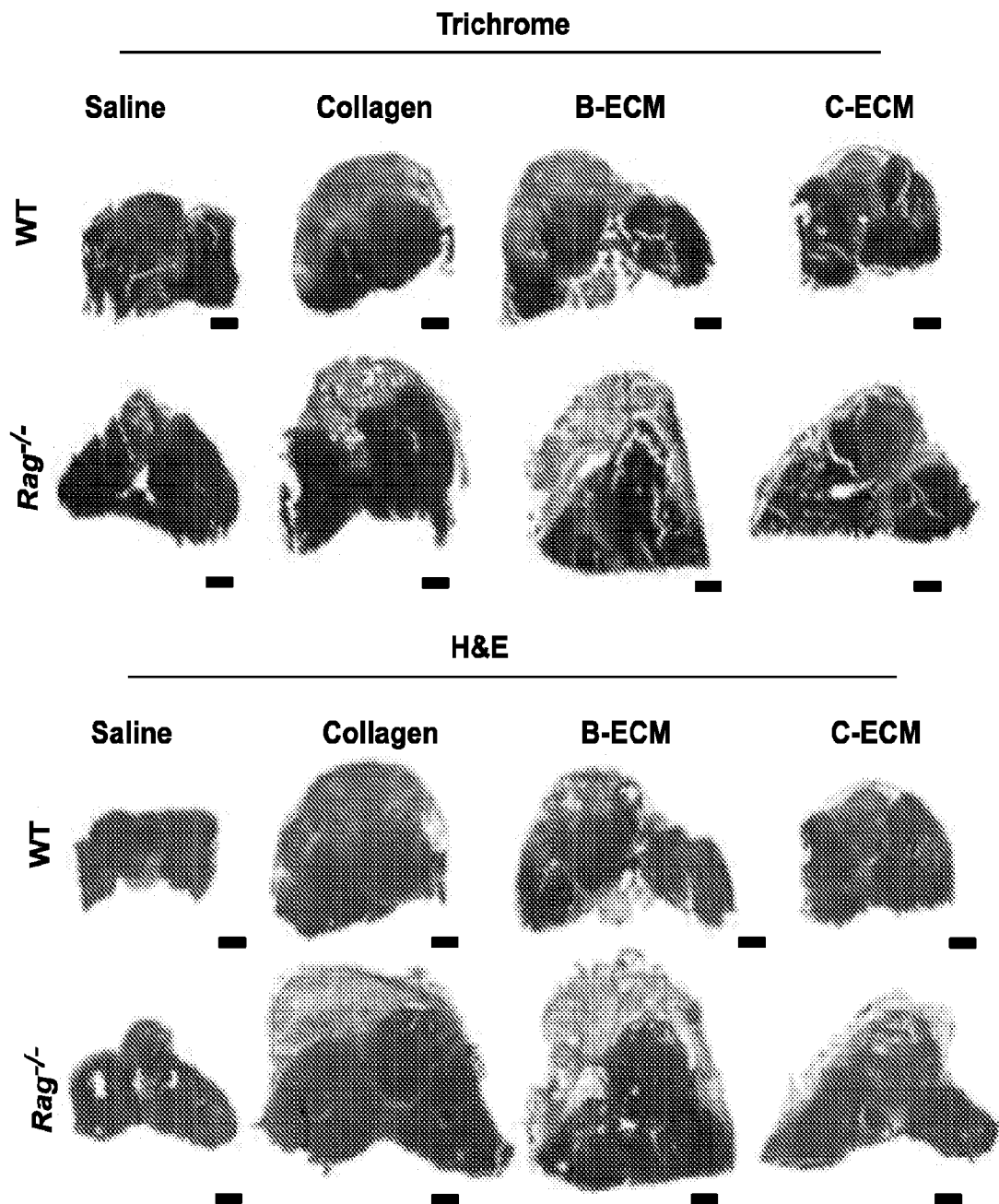


FIG. 16B



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FIG. 16C



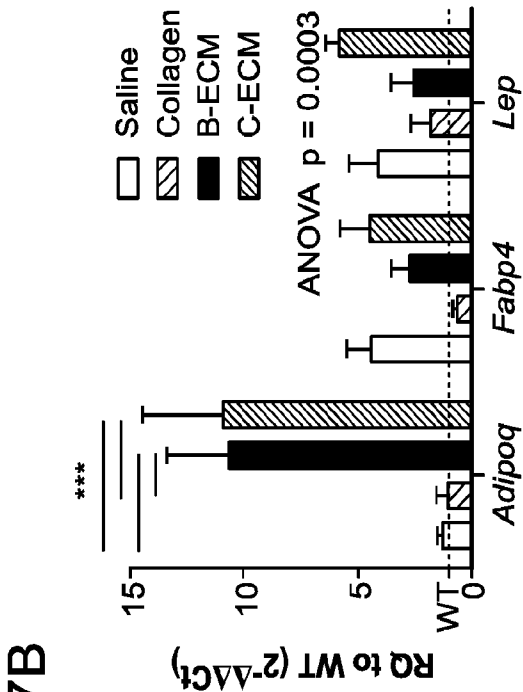
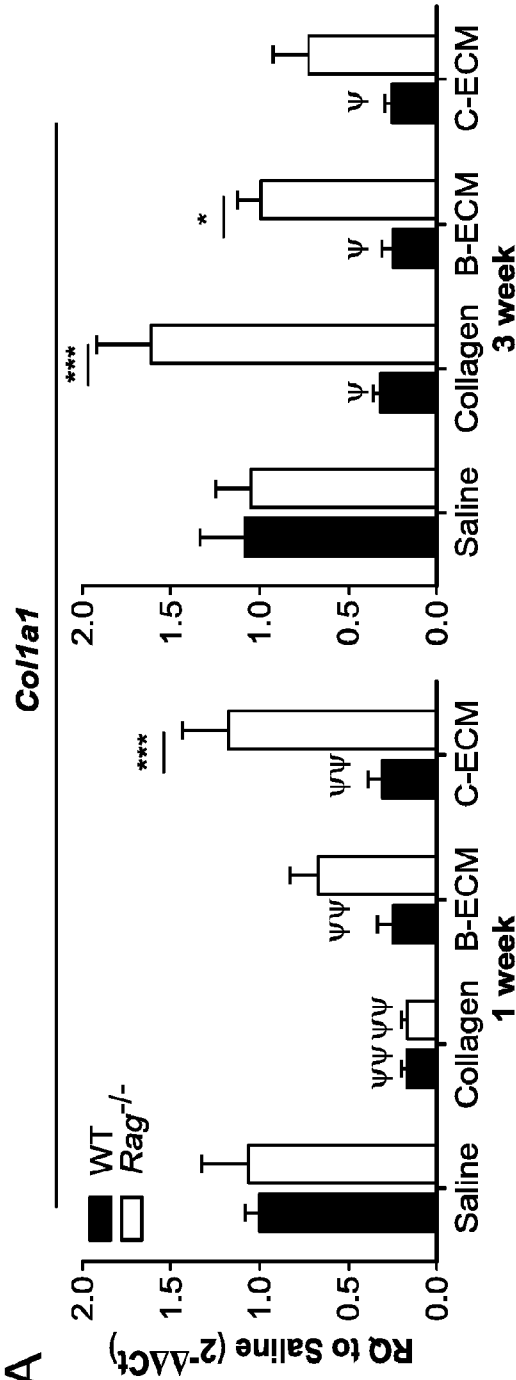


FIG. 17C

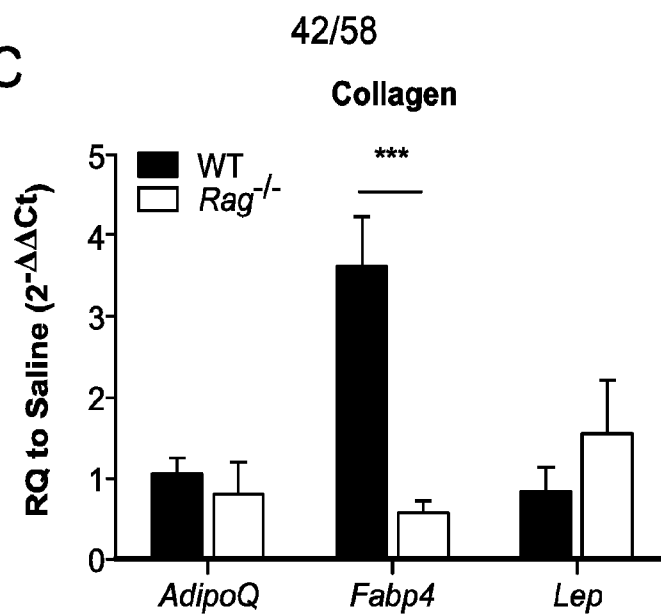


FIG. 17D

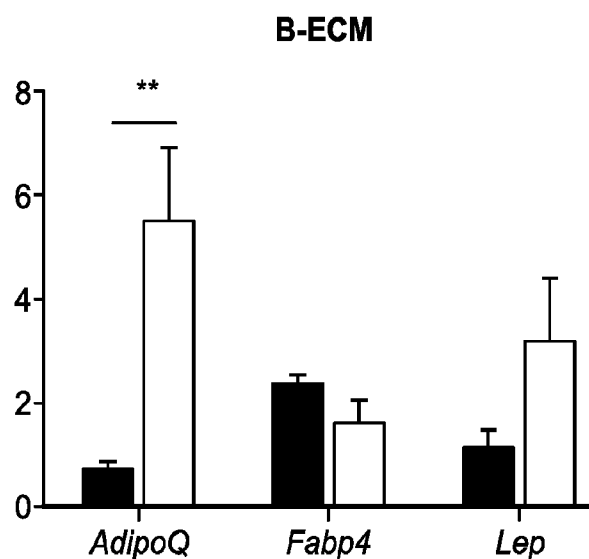
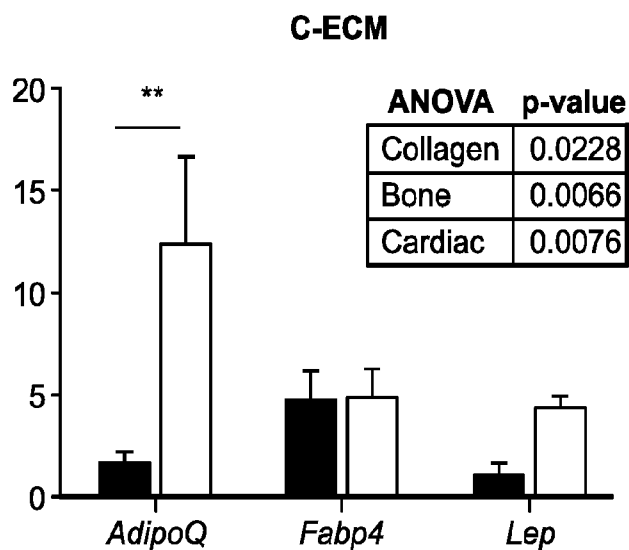


FIG. 17E



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FIG. 17F

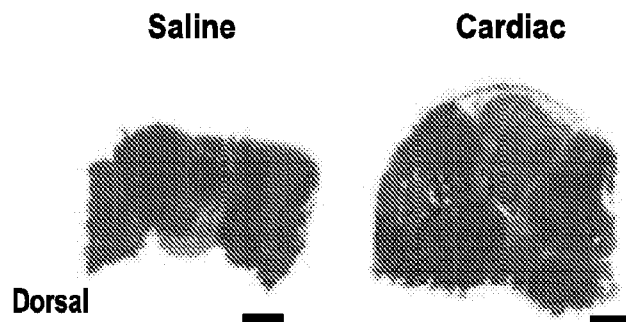


FIG. 17G

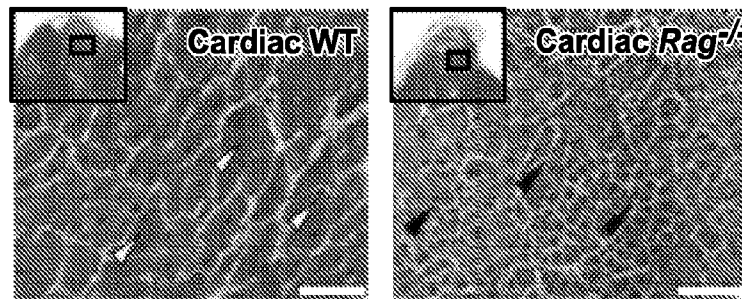


FIG. 17H

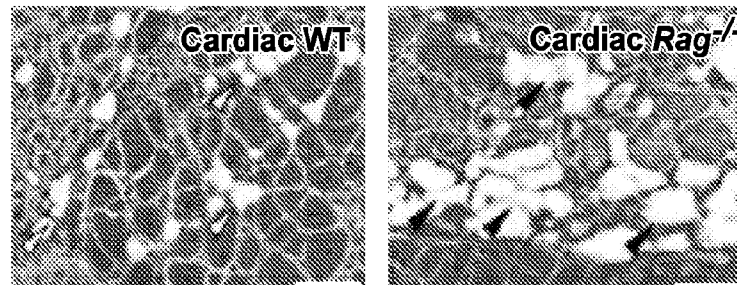
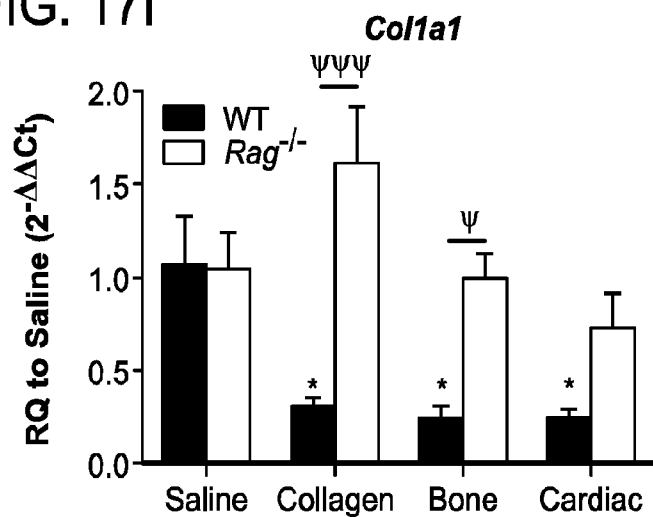
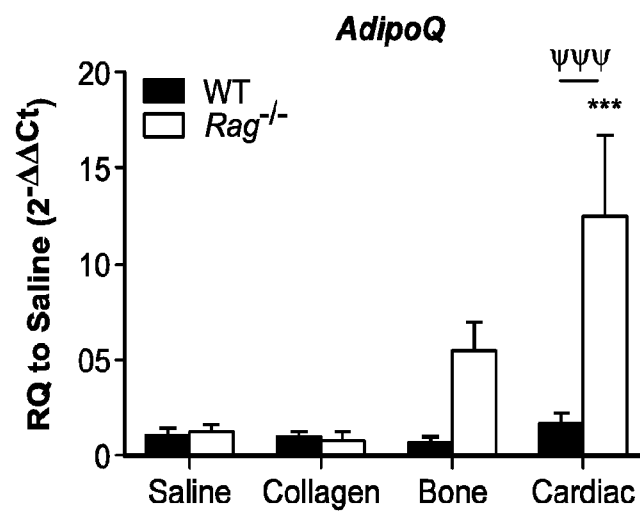


FIG. 17I



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FIG. 17J



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FIG. 18A

Gene	Saline vs		
	B-ECM	C-ECM	Collagen
Cd1d1	0.029	0.029	0.200
Cd4	0.343	0.343	0.029
Cd8a	0.029	0.029	0.029
Ifng	0.029	0.029	0.029
Tbx21	0.029	0.029	0.029
Gata3	0.886	0.343	0.029
Il4	0.029	0.029	0.029
Il5	0.686	0.886	0.686
Il17a	0.200	0.486	0.029
Il21	1.000	0.886	0.029
Il22;Il17a	0.486	0.486	0.343
Irf4	0.029	0.029	0.486
Rorc	0.029	0.029	0.486
Foxp3	0.343	0.886	0.029
Il10	0.029	0.029	0.029
Il2ra	0.486	0.886	0.029
Tgfb1	0.029	0.029	1.000
Cd28	0.029	0.029	0.029
Cd40lg	0.029	0.029	0.029
Ctla4	0.200	0.886	0.029
Fasl	0.029	0.029	0.029
Pdcd1	0.029	0.029	0.029
Tnfrsf9	1.000	0.886	0.200
Cxcl12	0.343	0.486	0.686
Cxcr4	0.057	0.029	0.057
Il15	1.000	0.686	0.114
Il18	0.886	0.686	0.886
Tnf	1.000	0.200	0.029
Il2	0.029	0.114	0.343
Socs5	0.057	0.029	0.029
Bad	0.343	0.886	0.057
Cblb	0.029	0.029	0.029
Ccnd3	0.686	0.200	0.029
Cd27	0.114	0.057	0.686
Glmn	0.029	0.029	0.029
Icos	0.029	0.029	0.486
Jag2	0.114	0.057	0.029
Sit1	0.029	0.029	0.029
Sla2	0.200	0.114	0.486
Spp1	0.057	0.029	0.029
Tnfsf14	0.029	0.029	0.114
Tslp	0.029	0.309	0.309

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FIG. 18B

Gene Group	Saline vs		
	B-ECM	C-ECM	Collagen
NKT	0.024	0.025	0.208
Th	0.328	0.35	0.023
CTL	0.024	0.025	0.023
Th1	0.024	0.025	0.023
Th2	0.087	0.079	0.057
Th17	0.12	0.12	0.112
Treg	0.75	0.085	0.048
T cell act	0.076	0.078	0.052
Inflamm	0.251	0.152	0.144
IL2	0.059	0.053	0.045

FIG. 19A

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Gene	WT			<i>Rag</i> ^{-/-}		
	Saline vs			Saline vs		
	B-ECM	C-ECM	Collagen	B-ECM	C-ECM	Collagen
Ccr2	1.000	0.343	0.057	0.686	0.029	0.343
Csf1r	0.029	0.114	0.886	0.029	0.114	0.057
Csf2rb	0.200	0.029	0.029	0.114	0.686	0.029
Emr1	0.057	0.029	0.886	0.343	1.000	0.886
Arg1	0.029	0.029	0.029	0.029	0.114	0.029
Cebpb	0.114	0.029	0.029	0.029	0.057	0.029
Igf1	0.029	0.029	1.000	0.343	0.029	0.343
Il10	0.029	0.029	0.029	0.029	0.200	0.029
Il10ra	0.029	0.029	0.686	0.114	0.686	0.057
Il12a	0.029	0.029	0.029	1.000	1.000	0.343
Il12b	0.114	0.486	0.343	0.029	0.114	1.000
Il1r1	0.057	0.029	0.029	0.343	0.343	0.029
Il4ra	0.200	0.886	0.029	0.886	0.486	0.029
Mmp9	0.029	0.029	0.686	0.686	0.114	0.886
Retnla	0.029	0.029	0.114	0.029	0.029	0.057
Stat3	0.029	0.029	0.200	0.029	0.114	0.029
Stat6	0.343	0.114	0.057	0.029	0.343	0.029
Tgfb1	1.000	0.057	1.000	0.886	0.486	0.114
Timp1	0.029	0.029	0.029	0.029	0.486	0.343
Vegfa	0.486	0.343	0.057	0.029	0.029	0.029
Ccl5	0.029	0.029	0.029	0.114	0.114	0.200
Cybb	0.029	0.029	0.029	0.029	0.200	0.029
Ebi3	0.686	0.029	0.029	0.029	0.486	0.029
H2-Ab1	1.000	0.200	0.200	1.000	0.486	0.886
Hif1a	0.886	0.057	0.114	0.029	1.000	0.486
Ido1	1.000	0.686	0.029	0.029	0.343	0.029
Ifngr1	0.486	0.686	1.000	0.886	0.114	0.029
Il1b	0.029	0.057	0.029	0.686	0.029	0.114
Il23a	0.029	0.029	0.029	0.029	0.029	0.057
Il6	0.486	0.114	0.686	0.029	0.029	0.029
Myd88	0.686	0.029	0.029	0.686	0.057	0.029
Nos2	0.029	0.029	0.029	0.029	0.343	0.200
Ptgs2	0.057	0.114	0.029	0.029	0.057	0.029
Stat1	0.029	0.114	0.886	0.029	0.057	0.029
Tnf	0.886	0.057	0.486	0.686	0.029	0.029
Casp1	0.029	0.029	0.029	0.029	0.029	0.029
Cd274	0.029	0.029	0.029	0.343	0.057	0.029
Ctsk	0.029	0.029	0.029	0.057	0.057	0.486
Il17ra	0.486	0.029	0.029	0.029	0.029	0.200
Il23r	0.686	0.343	0.343	0.029	0.029	0.029
Mmp16	0.029	0.029	0.029	0.029	0.057	0.029
S100a8	0.114	0.029	0.029	0.029	0.200	0.029

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FIG. 19B

WT vs <i>Rag</i> ^{-/-}			
Gene	B-ECM	C-ECM	Collagen
Ccr2	0.71	0.843	0.002
Csf1r	0.991	0.111	<0.001
Csf2rb	0.023	0.004	<0.001
Arg1	<0.001	<0.001	<0.001
Cebpb	0.008	<0.001	<0.001
Igf1	0.007	<0.001	0.724
Il10	0.004	<0.001	<0.001
Il12a	0.021	0.12	0.568
Il12b	0.413	0.234	0.032
Il1r1	0.237	0.393	<0.001
Il4ra	0.131	0.022	<0.001
Mmp9	0.002	0.029	0.435
Retnla	0.001	<0.001	0.002
Stat6	0.289	0.051	0.008
Tgfb1	0.261	0.979	0.003
Timp1	<0.001	<0.001	<0.001
Vegfa	0.011	0.006	<0.001
Ccl5	<0.001	<0.001	<0.001
Cybb	0.021	0.004	0.005
Ebi3	0.142	0.008	<0.001
H2-Ab1	0.115	0.003	<0.001
Hif1a	0.071	0.211	0.003
Ifngr1	0.413	0.028	<0.001
Il1b	0.051	0.276	<0.001
Il23a	0.06	0.023	0.119
Myd88	0.021	<0.001	<0.001
Nos2	0.039	<0.001	0.08
Stat1	0.002	0.787	<0.001
Tnf	0.336	0.033	0.022
Casp1	<0.001	0.007	<0.001
Cd274	<0.001	<0.001	0.007
Ctsk	<0.001	<0.001	<0.001
Il17ra	0.775	0.022	<0.001
Mmp16	0.063	0.748	<0.001
S100a8	0.177	0.042	0.358

FIG. 19C

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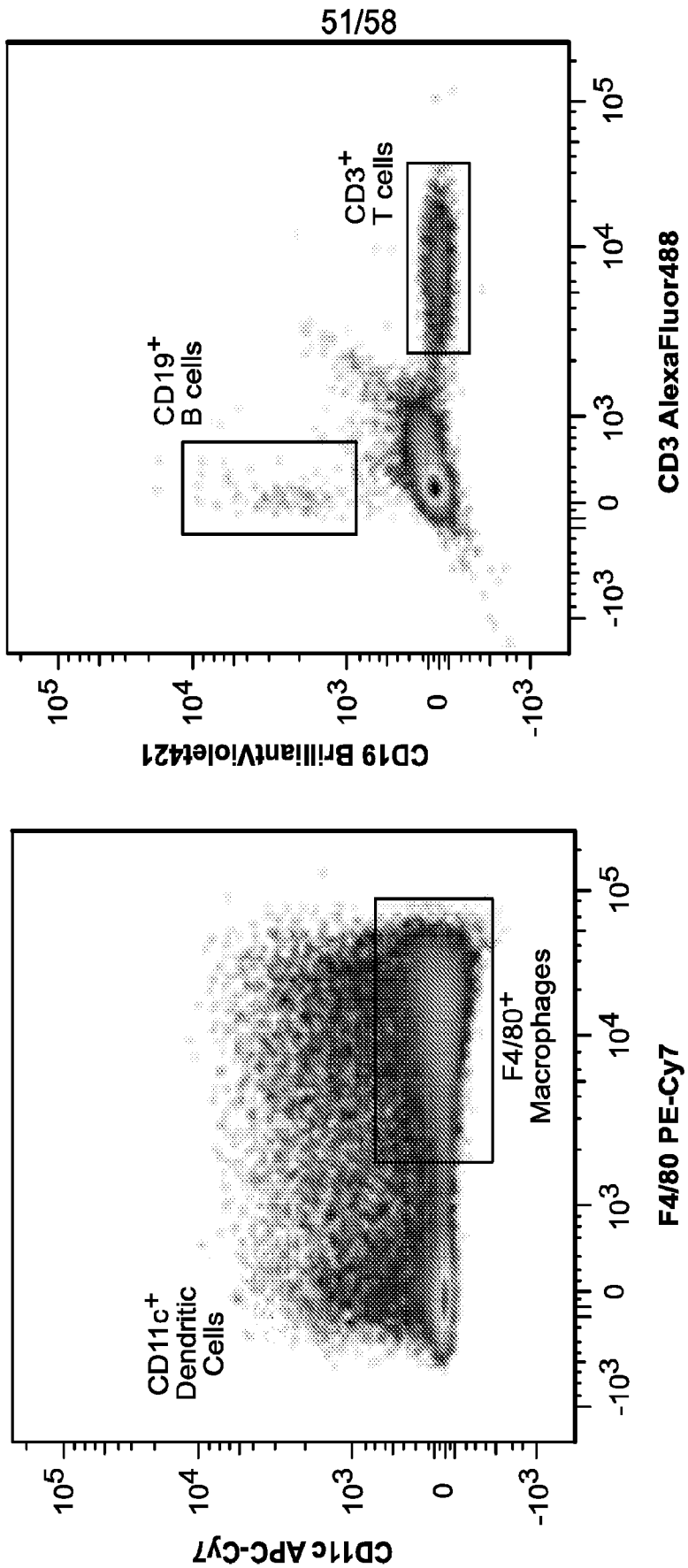
Gene	WT			<i>Rag</i> ^{-/-}	
	Saline vs			Saline vs	
	Bone	Cardiac	Collagen	Bone	Cardiac
Ccr2	1.000	0.343	0.057	0.686	0.029
Csf1r	0.029	0.114	0.886	0.029	0.029
Csf2rb	0.200	0.029	0.029	0.343	0.057
Emr1	0.057	0.029	0.886	0.029	0.029
Arg1	0.029	0.029	0.029	0.114	0.114
Cebpb	0.114	0.029	0.029	0.057	0.029
Igf1	0.029	0.029	1.000	0.114	0.029
Il10	0.029	0.029	0.029	0.686	0.343
Il10ra	0.029	0.029	0.686	0.029	0.029
Il12a	0.029	0.029	0.029	0.343	0.886
Il12b	0.114	0.486	0.343	1.000	0.486
Il1r1	0.057	0.029	0.029	0.886	0.114
Il4ra	0.200	0.886	0.029	0.029	0.029
Mmp9	0.029	0.029	0.686	0.114	0.486
Retnla	0.029	0.029	0.114	0.029	0.343
Stat3	0.029	0.029	0.200	0.029	0.029
Stat6	0.343	0.114	0.057	0.057	0.029
Tgfb1	1.000	0.057	1.000	0.029	0.029
Timp1	0.029	0.029	0.029	0.343	0.114
Vegfa	0.486	0.343	0.057	0.029	0.114
Ccl5	0.029	0.029	0.029	0.343	0.200
Cybb	0.029	0.029	0.029	0.029	0.029
Ebi3	0.686	0.029	0.029	0.200	0.200
H2-Ab1	1.000	0.200	0.200	0.029	0.029
Hif1a	0.886	0.057	0.114	0.114	0.029
Ido1	1.000	0.686	0.029	0.686	0.486
Ifngr1	0.486	0.686	1.000	0.057	0.029
Il1b	0.029	0.057	0.029	1.000	1.000
Il23a	0.029	0.029	0.029	1.000	0.486
Il6	0.486	0.114	0.686	0.343	0.886
Myd88	0.686	0.029	0.029	0.029	0.029
Nos2	0.029	0.029	0.029	0.114	1.000
Ptgs2	0.057	0.114	0.029	1.000	0.486
Stat1	0.029	0.114	0.886	0.343	0.029
Tnf	0.886	0.057	0.486	0.343	0.343
Casp1	0.029	0.029	0.029	0.029	0.029
Cd274	0.029	0.029	0.029	0.886	0.886
Ctsk	0.029	0.029	0.029	0.486	0.114
Il17ra	0.486	0.029	0.029	0.029	0.029
Il23r	0.686	0.343	0.343	0.686	0.686
Mmp16	0.029	0.029	0.029	0.114	0.029
S100a8	0.114	0.029	0.029	0.886	0.114

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FIG. 19D

WT vs <i>Rag</i> ^{-/-}		
Gene	Bone	Cardiac
Csf2rb	0.037	0.009
Arg1	<0.001	<0.001
Cebpb	0.02	<0.001
Igf1	<0.001	<0.001
Il10	0.002	<0.001
Il12a	0.033	0.149
Il4ra	0.128	0.022
Mmp9	0.002	0.03
Retnla	<0.001	<0.001
Stat6	0.181	0.017
Timp1	<0.001	<0.001
Vegfa	0.015	0.009
Ccl5	0.002	<0.001
Cybb	0.019	0.004
Ebi3	0.176	0.016
H2-Ab1	0.145	0.007
Hif1a	0.025	0.113
Ifngr1	0.284	0.006
Il23a	0.059	0.023
Myd88	0.018	<0.001
Nos2	0.039	<0.001
Stat1	0.004	0.796
Casp1	<0.001	0.007
Cd274	0.002	<0.001
Ctsk	<0.001	<0.001
Il17ra	0.744	0.012
S100a8	0.181	0.045

FIG. 20A



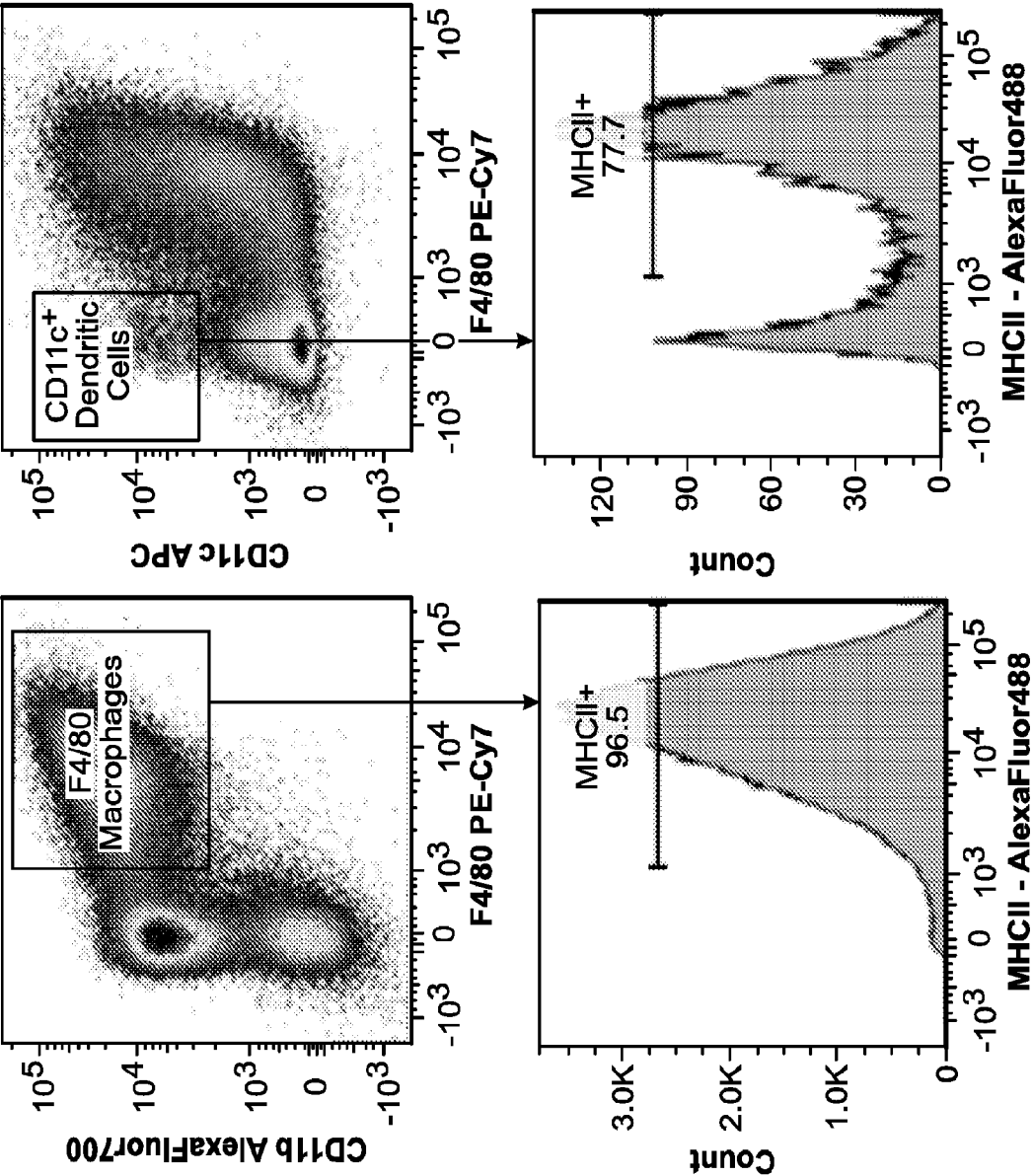
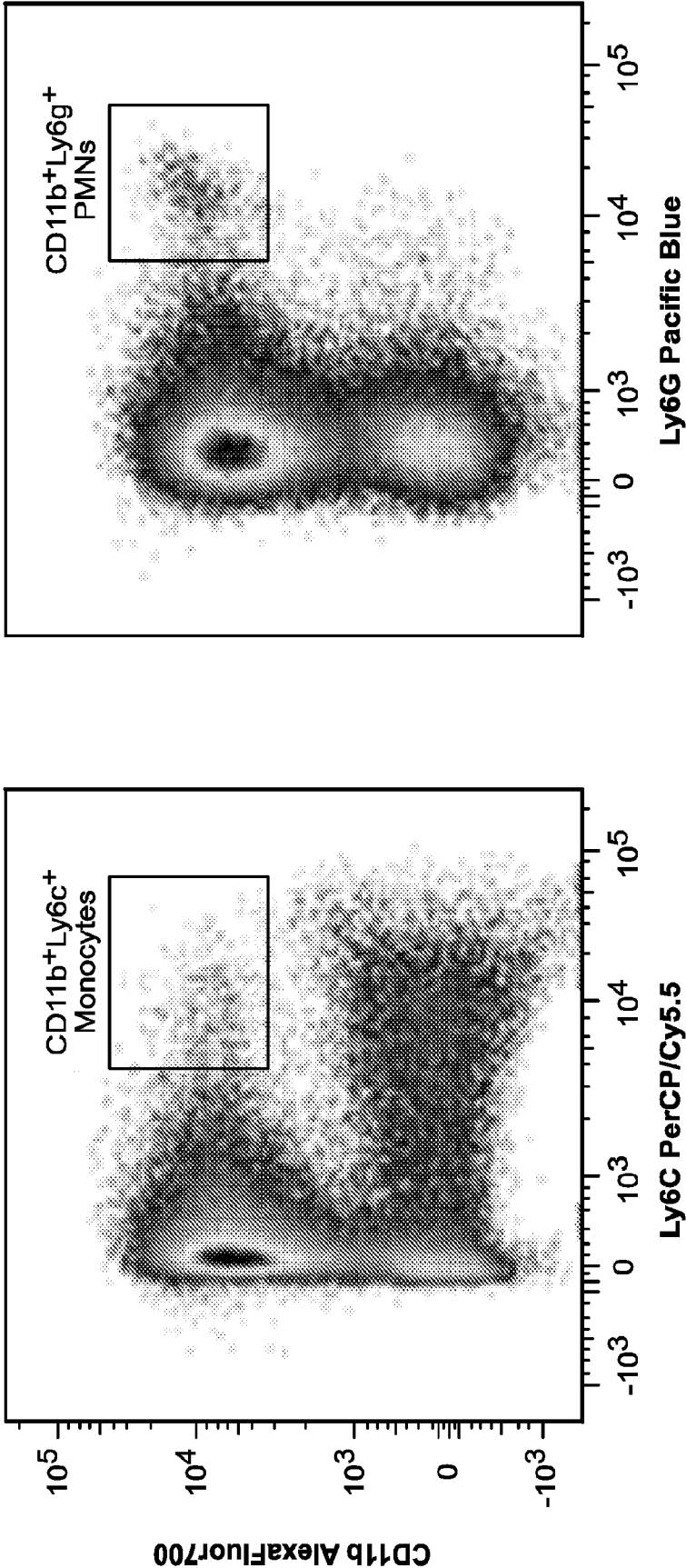


FIG. 20B

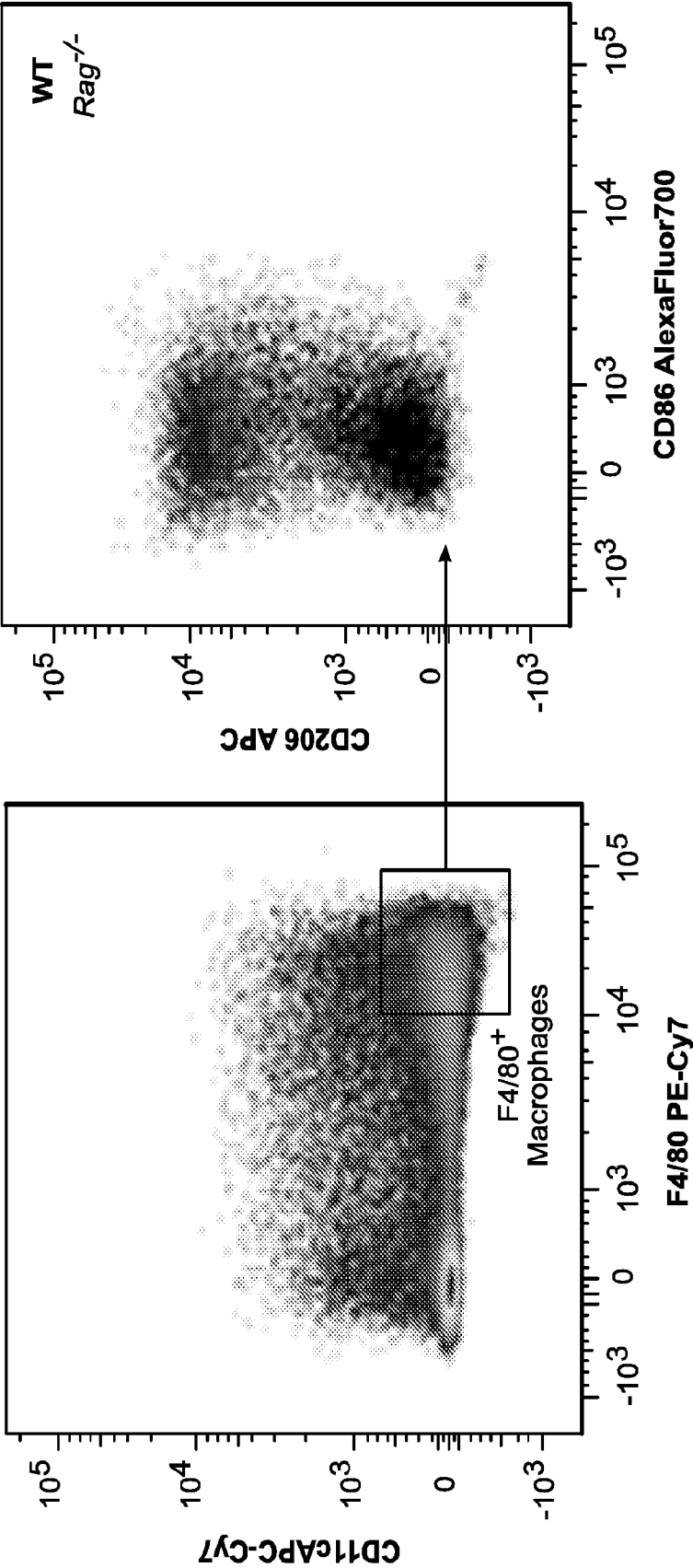
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FIG. 20C



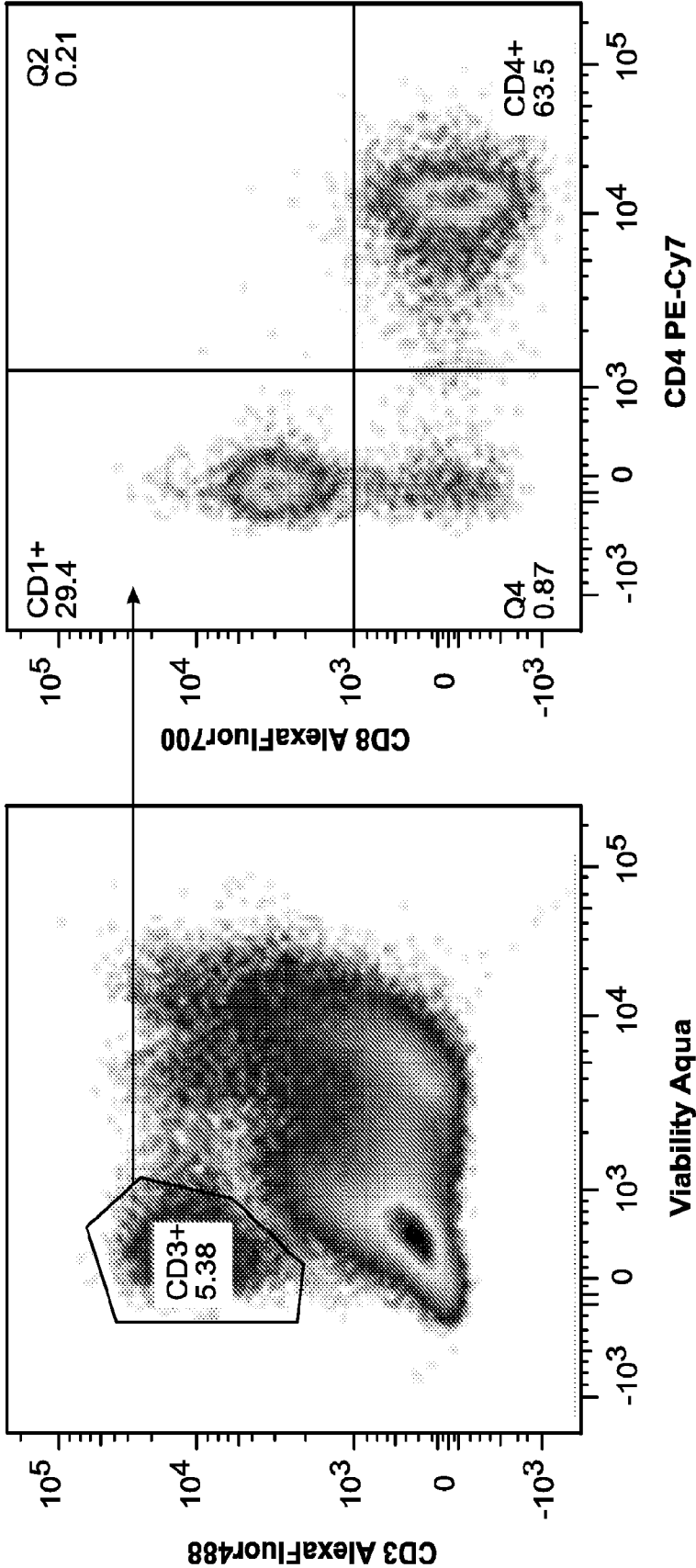
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FIG. 20D



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FIG. 20E



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FIG. 21

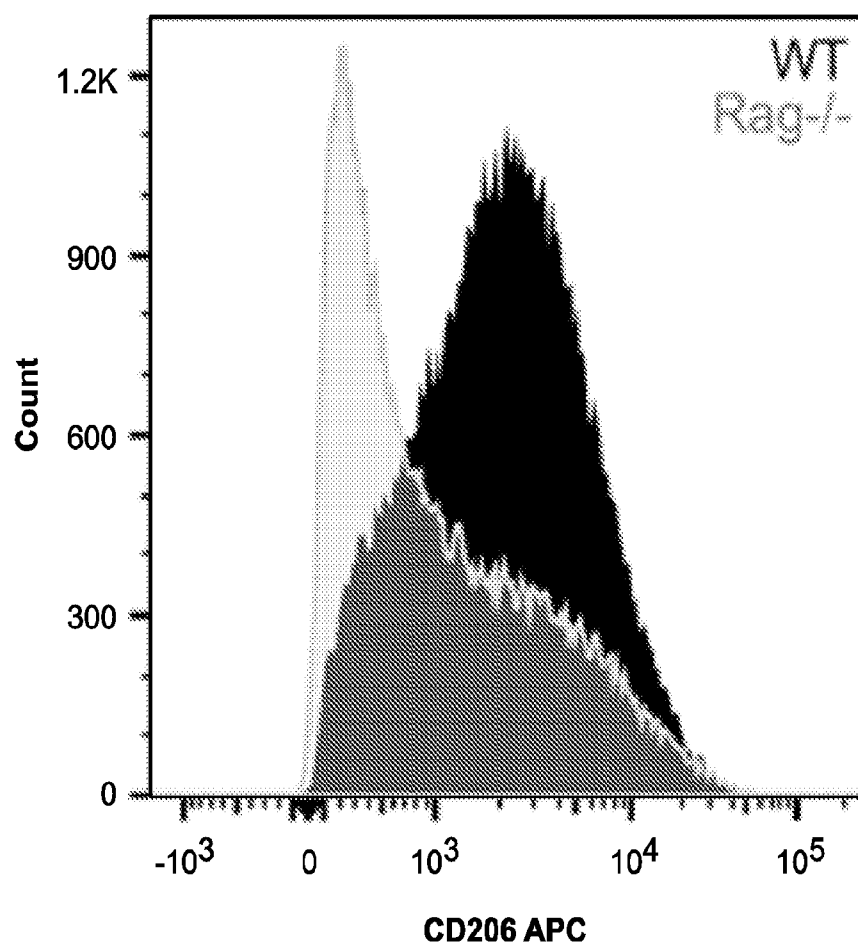


FIG. 22A

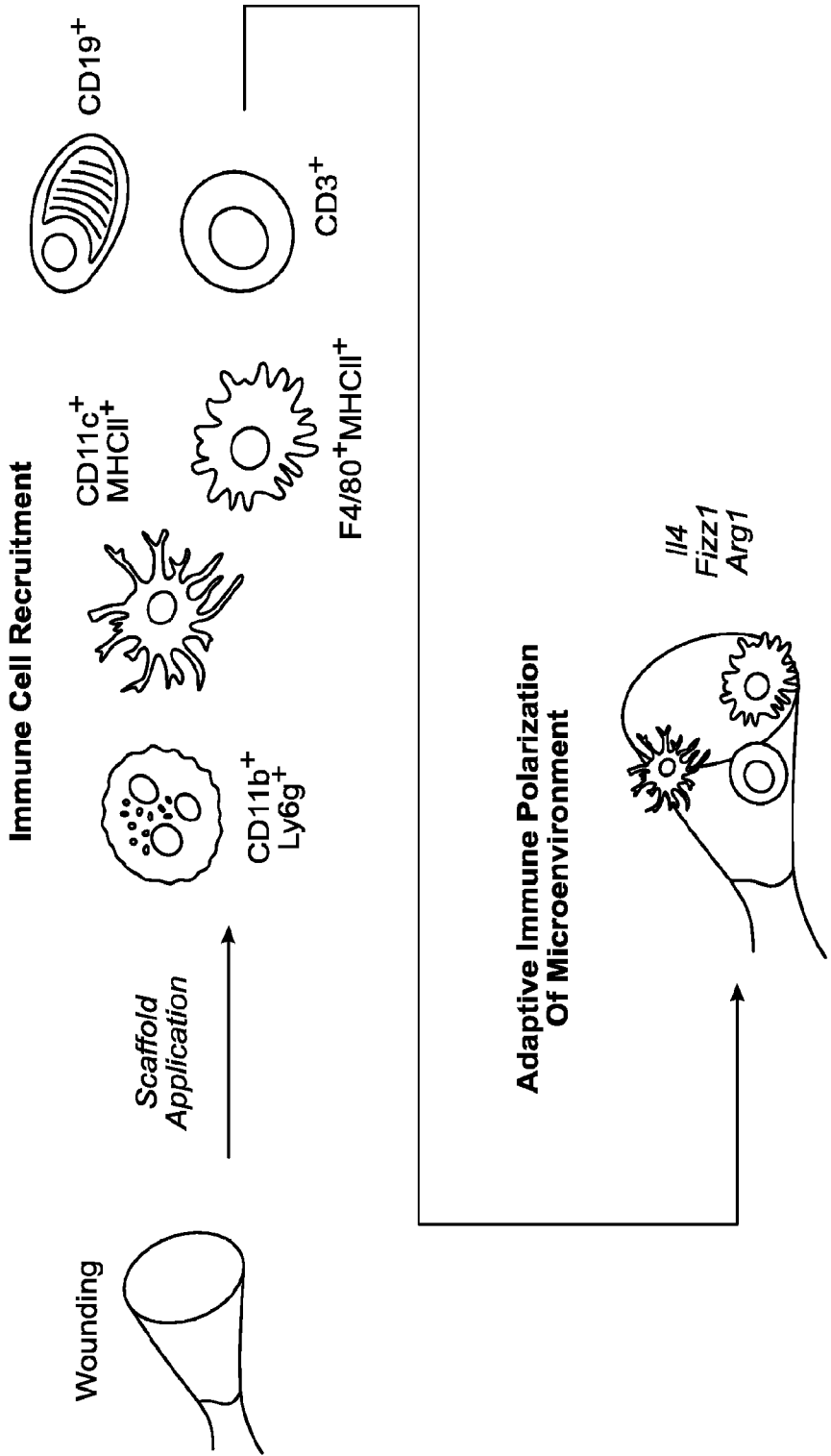
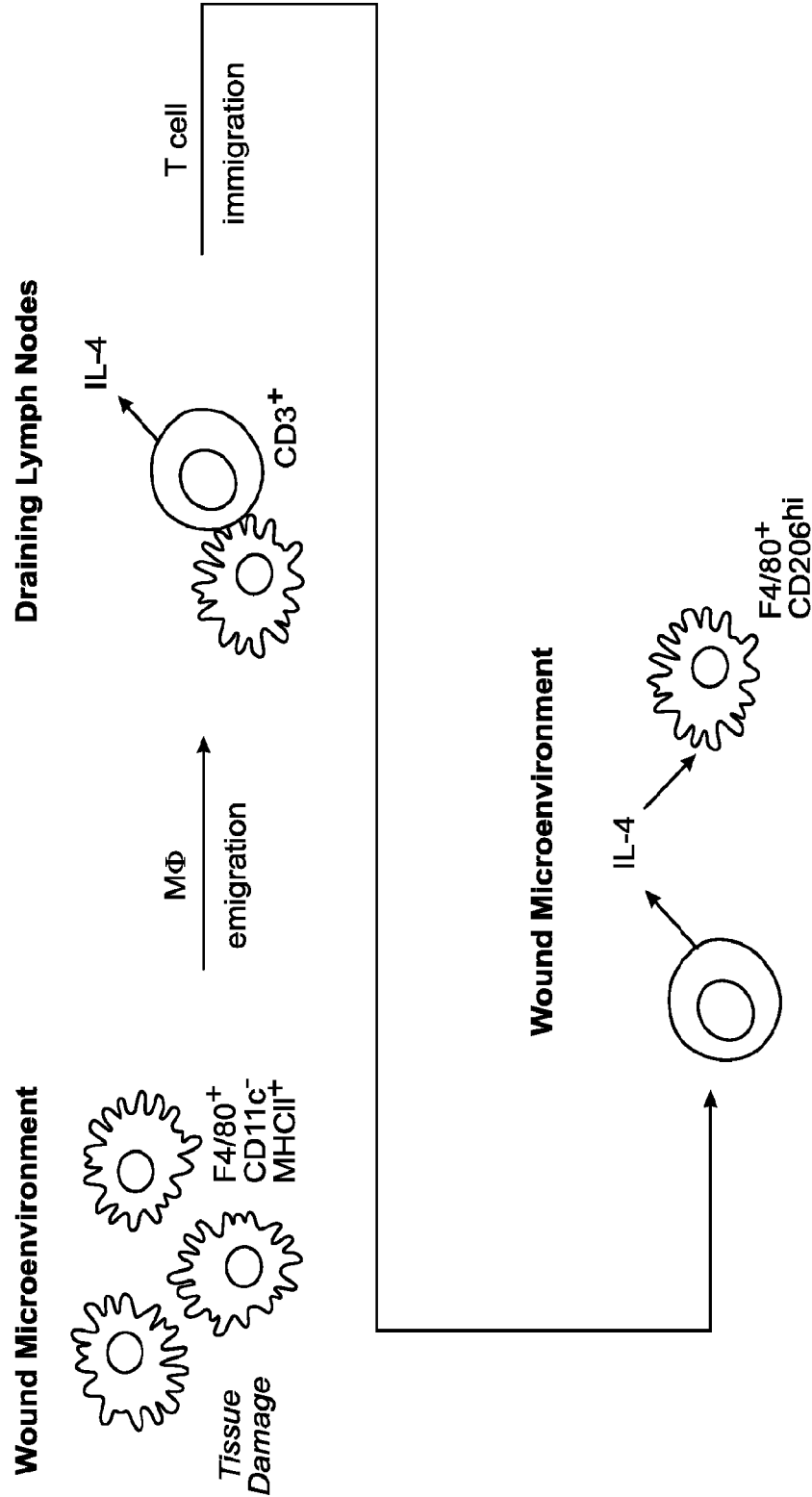


FIG. 22B



INTERNATIONAL SEARCH REPORT

International application No.

PCT/US2016/045720

Box No. I Nucleotide and/or amino acid sequence(s) (Continuation of item 1.c of the first sheet)

1. With regard to any nucleotide and/or amino acid sequence disclosed in the international application, the international search was carried out on the basis of a sequence listing:

a. ☐ forming part of the international application as filed:

☐ in the form of an Annex C/ST.25 text file.

☐ on paper or in the form of an image file.

b. ☐ furnished together with the international application under PCT Rule 13ter.1(a) for the purposes of international search only in the form of an Annex C/ST.25 text file.

c. ☒ furnished subsequent to the international filing date for the purposes of international search only:

☒ in the form of an Annex C/ST.25 text file (Rule 13ter.1(a)).

☐ on paper or in the form of an image file (Rule 13ter.1(b) and Administrative Instructions, Section 713).

2. ☒ In addition, in the case that more than one version or copy of a sequence listing has been filed or furnished, the required statements that the information in the subsequent or additional copies is identical to that forming part of the application as filed or does not go beyond the application as filed, as appropriate, were furnished.

3. Additional comments:

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US2016/045720

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
3. ☒ Claims Nos.: 48-53, 66
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying additional fees, this Authority did not invite payment of additional fees.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.
- ☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.
- ☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US2016/045720

A. CLASSIFICATION OF SUBJECT MATTER IPC(8) - A61L 15/22; A61L 15/32; A61L 27/24 (2016.01) CPC - A61L 15/22; A61L 15/32; A61L 15/325; A61L 15/40; A61L 27/3633; A61L 27/54 (2016.11) According to International Patent Classification (IPC) or to both national classification and IPC		
B. FIELDS SEARCHED Minimum documentation searched (classification system followed by classification symbols) IPC - A61L 15/22; A61L 15/32; A61L 27/24 CPC - A61L 15/22; A61L 15/32; A61L 15/325; A61L 15/40; A61L 27/3633; A61L 27/54 Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched USPC - 424/423; 424/447 (keyword delimited) Electronic data base consulted during the international search (name of data base and, where practicable, search terms used) Orbit, Google Patents, Google Scholar, PubMed Search terms used: Wound healing; Immunomodulation; Scaffold		
C. DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 2010/0303886 A1 (JANIS) 02 December 2010 (02.12.2010) entire document	1-3, 5-14, 16-26, 28-35, 37-47, 54-59, 61-65
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Y		4, 15, 27, 36, 60
Y	BOEHLER et al. "Tissue engineering tools for modulation of the immune response," Biotechniques, 31 October 2011 (31.10.2011), Vol. 51, No. 4, Pgs. 1-20 (originally Pgs. 239-254). entire document.	4, 15, 27, 36, 60
A	US 2015/0139960 A1 (DERMAGENESIS LLC) 21 May 2015 (21.05.2015) entire document	1-47, 54-65
A	US 2009/0202617 A1 (WARD et al) 13 August 2009 (13.08.2009) entire document	1-47, 54-65
A	US 2013/0323201 A1 (WISE et al) 05 December 2013 (05.12.2013) entire document	1-47, 54-65
☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.		
* Special categories of cited documents: "A" document defining the general state of the art which is not considered to be of particular relevance "E" earlier application or patent but published on or after the international filing date "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified) "O" document referring to an oral disclosure, use, exhibition or other means "P" document published prior to the international filing date but later than the priority date claimed "T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention "X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone "Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art "&" document member of the same patent family		
Date of the actual completion of the international search 23 November 2016		Date of mailing of the international search report 13 DEC 2016
Name and mailing address of the ISA/US Mail Stop PCT, Attn: ISA/US, Commissioner for Patents P.O. Box 1450, Alexandria, VA 22313-1450 Facsimile No. 571-273-8300		Authorized officer Blaine R. Copenheaver PCT Helpdesk: 571-272-4300 PCT OSP: 571-272-7774