Dissecting the microenvironment around biosynthetic scaffolds in murine skin wound healing

Chen Hu^{a,#}, Chenyu Chu^{a,#}, Li Liu^b, Shue Jin^c, Renli Yang^a, Shengan Rung^a,
Jidong Li^c, Yili Qu^a, Yi Man^{a,*}

^a Department of Oral Implantology & State Key Laboratory of Oral Diseases & National Clinical Research Center for Oral Diseases, West China Hospital of Stomatology, Sichuan University, ,
Chengdu, Sichuan 610041, China

^b State Key Laboratory of Biotherapy, West China Hospital, Sichuan University, Collaborative Innovation Center for Biotherapy, Chengdu, Sichuan 610041, China

^c Research Center for Nano-Biomaterials, Analytical and Testing Center, Sichuan University, Chengdu 610064, China.

[#] These authors contributed equally to this work

^{*} Corresponding to Professor. Chair. **Yi Man**, E-mail: manyi780203@126.com

Corresponding author:

Yi Man, DDS, PhD
Professor and Chair
Department of Oral Implantology
State Key Laboratory of Oral Diseases, West China Hospital of Stomatology, Sichuan University, 14#, 3rd section, Renmin South Road
Chengdu 610041, China
Tel: 0086-28-85503571
Fax: 0086-28-85483678
E-mail: manyi780203@126.com

25 **Abstract:** Structural properties of biomaterials play critical roles in guiding cell
 26 behaviors and influence the immune response against them. We fabricated electrospun
 27 membranes with three types of surface topography (Random, Aligned, and Latticed).
 28 The aligned membranes showed immunomodulatory ability, and led to faster wound
 29 healing, reduced fibrotic response and enhanced regeneration of cutaneous
 30 appendages when used in skin wound repair. Based on that, we performed single-cell
 31 RNA sequencing analysis on cells from wounded mouse skin in the presence or
 32 absence of the Aligned scaffold. Keratinocytes, fibroblasts, and immune cells
 33 including neutrophils, monocytes, macrophages, dendritic cells, and T cells showed
 34 diverse cellular heterogeneity. More hair follicle progenitor cells, inner root sheath
 35 cells (anagen-related) and fibroblast subsets were found in the Aligned group, which
 36 corresponded to the improved regeneration of hair follicles and faster wound closure
 37 in the presence of scaffold. Immune responses towards the biomaterial differed from
 38 that of control group. In aligned samples, infiltrated macrophages and neutrophils
 39 were reduced, whereas more effector T cells were recruited. The time course of
 40 immune response was possibly advanced towards an adaptive immunity-dominant
 41 stage by the scaffold. The microenvironment around scaffold involved intricate
 42 interplay of immune cells and cutaneous cells, and wound healing was the
 43 comprehensive results of numerous influencing factors working together.

44 **Key words:** Surface topography, immune microenvironment, immunomodulatory
 45 ability, cellular heterogeneity

46

47 **Introduction**

48 Biomaterials and devices implanted in the body have a broad spectrum of clinical
 49 applications including tissue regeneration, cell transplantation, controlled drug release,
 50 and continuous monitoring of physiological conditions (1, 2). Among the components
 51 of a cell microenvironment, structural features (macroscale, microscale and nanoscale
 52 features) play critical roles in guiding cell behaviors (3). Electrospinning technology
 53 has been widely applied in preparing scaffolds due to its simplicity, capacity to form
 54 fibers on the micro- and nanoscale, structural control of electrospun membranes and
 55 cost-effectiveness (4). Electrospun nanofibers could be assembled into well-ordered
 56 nanofiber meshes with different morphology, e.g. parallel alignment or latticed
 57 patterns of nanofibers (5, 6), and have been preferentially applied to the regeneration
 58 of diverse tissues like skin and bone (6, 7). Upon implantation of a material, cells of
 59 both the innate and adaptive immune system have a role in the host response (8).
 60 Previous studies illustrated the type 1 (pro-inflammatory) immune polarization driven
 61 by T helper 1 (Th1) cells, and the induced pro-inflammatory M1 activation of
 62 macrophages (stimulated by IL-2 and IFN- γ from Th1 cells). By contrast, in type 2
 63 immune response, T helper 2 (Th2) cells produce cytokines like IL-4 and IL-13,
 64 which regulate the polarization of macrophages towards an anti-inflammatory M2
 65 activation (9, 10). More recently, a type 17 immune response was reported to promote
 66 chronic fibrosis in tissue around implants (11, 12). In summary, previous studies
 67 usually focused on certain types of immune cells, like macrophages, and explored
 68 their roles in the host response. However, the immune response is jointly regulated by

69 various immune cells, whose phenotype and function are dictated by external and
70 internal signals. An overview of different immune cells in the microenvironment will
71 aid in comprehensive understanding of the immune responses elicited by scaffolds.
72 Technological advances such as single-cell RNA sequencing (scRNA-seq) (13, 14)
73 have enabled cell populations, functions, and the nuances of their phenotypes in vivo
74 to be studied at a high resolution. By changing the collector, we developed
75 poly(lactic-co-glycolic acid)-fish collagen (PLGA-FC) hybrid electrospun scaffolds
76 with three types of surface topography, i.e. the group with randomly oriented fibers
77 (Random group), the group with mesh-like topography in macroscale and randomly
78 oriented fibers in microscale (Latticed group), and the group with aligned fibers
79 (Aligned group). We explored the regenerative outcomes of these scaffolds in
80 rat/mouse dorsal skin excisional wounds, and evaluated their immunomodulatory
81 properties. The scaffold with the best performance was further investigated.
82 Microenvironment around the scaffold was probed by scRNA-seq. Heterogeneity of
83 keratinocytes, fibroblasts, and immune cell populations, cellular functions, and their
84 interactions in vivo were explored.

85 **Results**

86 **Evaluation of wound healing in a rat skin wound model**

87 We fabricated scaffolds with random, aligned and latticed patterns of fibers (**Fig. 1A**),
88 and placed them below the full-thickness excisional wound (diameter=6mm) on rat
89 dorsal skin (SD rat). The Ctrl group received no scaffolds (**Fig. 1C**). Biophysical
90 properties of the scaffolds were summarized in **Fig. S1**. Workflow for evaluating

wound healing was summarized in Fig. 1B. Wound healing rate was significantly accelerated by the aligned membranes (**Fig. 1D-E**). The Latticed group showed delayed wound healing with the largest residual wound area left at day 7. On day 14, basically all groups achieved complete closure of the wound. Above the scaffolds, surrounding epithelium formed epithelial tongue as the first layer advancing towards the wound (15). On day 7, the Aligned group presented the fastest coverage of the wound, leaving the smallest gap width, whereas the latticed membrane seemed to impede the advancement of surrounding epithelium (**Fig. 1F-G, Fig. S2**). On day 14, re-epithelialization was completed in all groups except for the Latticed group. All groups showed matured stratified epithelium (**Fig. 1F**). Immunofluorescent staining for Krt5 (keratin secreted by keratinocytes in the basal layer) and Krt10 (keratin secreted by differentiated keratinocytes in the supra-basal layers) showed that on day 7, the Aligned group had the largest area of newly formed keratinized epithelium, and more keratinocytes were undifferentiated (Krt5-positive). On day 14, all groups revealed formation of stratified epithelium (**Fig. 1H-I**) (15). For the dermis part, wound space below epithelium was filled with granulation tissue on day 7. Collagen kept being deposited and remodeled (**Fig. S2**). On day 28, the Aligned group had more regenerated hair follicles and sebaceous glands than other treatment groups (**Fig. 1F**). The scaffolds were placed subcutaneously in rats to evaluate host response against them. Thickness of fibrotic capsules around scaffolds was measured at day 3, 7 and 14. The Aligned group had the smallest fibrotic capsule thickness at all time points (**Fig. 1J-K, Fig. S3**). The bulk-tissue RNA-Seq analysis for samples harvested

on day 7 (n=3 for each group) identified transcripts corresponding to 34459 genes, distributed over 6 orders of magnitude of expression level. Principal component analysis (PCA) of the data revealed that gene expression profiles of the Random and Aligned samples were more similar, compared to that of Latticed and Ctrl samples (**Fig. 1L**). In Gene ontology (GO) analysis, random and aligned scaffolds induced up-regulated expression of genes associated with immune responses when compared with the Ctrl samples, whereas the Latticed group did not (**Fig. 1M**). Kyoto Encyclopedia of Genes and Genomes (KEGG) analysis also revealed gene enrichment in an immune-related pathway “Cytokine-cytokine receptor interaction” (KEGGID rno04060) (**Fig. S4A-B**) (16). Real-time Quantitative Polymerase Chain Reaction (qPCR) confirmed these elevated genes observed in Aligned and Random groups (**Fig. S4C**). The data suggested that random and aligned membranes were able to modulate the local immune microenvironment. To explore whether the scaffolds had similar performance in other species, we applied them in mouse skin wound model.

Wound healing in mouse

Workflow for evaluating wound healing in C57BL/6 mouse was summarized in Fig. 2A. We placed three types of scaffolds below the full-thickness excisional wound (diameter=6mm) (**Fig. 2B**). The Ctrl group received no scaffolds. Wound coverage was faster in the Aligned (residual wound area=29.29±4.81%) and Ctrl group (20.80±4.66%) on day 7. On day 14, basically all groups achieved complete closure of the wound (**Fig. 2C-D**). The Aligned group had the smallest gap width on day 7, followed by the Ctrl group (**Fig. 2E-F**). On day 14, re-epithelialization was completed

135 in all groups except for some samples of the Latticed group. All groups showed
136 matured stratified epithelium. Regeneration of hair follicles was seen in Aligned
137 samples after 14 days of healing (**Fig. 2E**). Scaffolds were also placed subcutaneously
138 to evaluate the host response against them. The aligned membranes had the smallest
139 capsule thickness over the observation period (**Fig. 2G, Fig. S5**). Bulk-tissue
140 RNA-Seq for Aligned and Ctrl samples harvested on day 7 (n=3 for each group) was
141 performed. Compared with Ctrl, the Aligned group showed increased genes enriched
142 in inflammatory response, leukocyte chemotaxis and migration (**Fig. 2I**). KEGG
143 analysis revealed elevated gene expression in immuno-related signaling pathways for
144 the Aligned group (KEGGID mmu04657, mmu04668, mmu04060 and mmu04064)
145 (**Fig. 2J**). To summarize, in both rat and mouse models, the aligned membranes led to
146 faster wound healing, reduced fibrotic response and enhanced regeneration of
147 cutaneous appendages compared to other membranes. Meanwhile, they showed
148 immunomodulatory properties. We therefore sought to explore how aligned
149 membranes regulated peri-implant microenvironment. Here, we used scRNA-seq to
150 sequence cells from the wounded murine full-thickness skin 7 days post-wounding.

151 **Single-cell transcriptome analysis of full-thickness skin after wounding and** 152 **scaffold placement**

153 We isolated cells from the Aligned and Ctrl samples (n=4 biological replicates in each
154 group), and applied them to the 10X scRNA-seq platform (**Fig. 3A**). A total of 8,982
155 cells in the Ctrl group and 9,593 cells in the Aligned group were captured. After cell
156 filtering, 17,181 single cell transcriptomes were included in the final dataset (8,869

for the Aligned group and 8,312 for the Ctrl group) (**Fig. S6**). We first computationally pooled cells from Ctrl and Aligned groups to create a virtual aggregate. Unsupervised clustering using Seurat categorized the cells into 26 clusters based on global gene expression patterns (Fig. S7), which were assigned to thirteen main classes of cells (**Fig. 3B**): keratinocytes (KER), fibroblasts (FIB), sebocytes (SEB), smooth muscle cells (SMC), endothelial cells (EC), Schwann cells (SC), melanocytes (MEL), innate lymphoid cells (ILC), monocyte-macrophages (MAC), T cells (TC), neutrophils (NEU), dendritic cells (DC) and B cells (BC). Marker genes for each cell cluster were shown in the heatmap (**Fig. 3C**). The composition of each cell cluster was listed so that the proportion of cells from two groups could be identified across all cell clusters (**Fig. 3B**). As smooth muscle cells (57% from the Aligned group and 43% from the Ctrl group) could hardly regenerate at 7 days post-wounding, we regarded a proportion within $50 \pm 7\%$ as equilibrium between two groups. Genes related to macrophages (Itgam, Cd68, Arg1, Mrc1) were significantly higher expressed by the Ctrl samples (**Fig. 3D**).

Sub-clustering of keratinocytes reveals a higher proportion of hair follicle progenitor cells from the Aligned group

We selected cells that were in the first-level clustering defined as keratinocyte, and subjected them to a second round of unsupervised clustering (**Fig. 4A**). Terminal cornification of epidermis is achieved by keratinocytes passing through basal layers, differentiated layers, and cornified layers (15). In our study, the inter-follicular epithelium was composed of the basal layer cells (IFEB, Krt5^{hi}Krt14^{hi}), supra-basal

layer cells (Krt10^{hi}Krt1^{hi} IFED1, Krt10^{hi}Mt4^{hi} IFED2), and cornified layer (Lor⁺ IFED3) cells (**Fig. 4A-B**). The location of keratinocyte subsets was marked in **Fig. 4C**. Hair follicles in this study were in second telogen since the mice were 8 to 10 weeks old when they were sacrificed (17). However, anagen hair follicle gene signatures were also found, possibly resulting from hair follicle regeneration after wounding. Upper hair follicle cells were separated into three subsets named uHF1 (Krt79^{hi}Krt17^{hi}), uHF2 (Klk10⁺Krt79^{hi}), and uHFB (the upper hair follicle basal layer cells, Sostdc1^{hi}Apoe^{hi}). The hair follicle progenitor cells (Krt28^{hi}Lhx2^{hi}Mki67^{hi} HFP) were highly proliferative. Germinative layer cells (Mt2^{hi}Dcn^{hi}) belonging to anagen hair follicles also expressed high cell proliferation related genes like Top2a and Birc5. Meanwhile, they had a basal cell gene signature (Krt5^{hi}Krt14^{hi}). The inner root sheath (IRS) and cortex cells were characterized by Krt28, Krt27, Krt73, and Krt25, markers for the Henle and Huxley layers of anagen hair follicles (18). Cells of the inner bulge layer (IB) and outer bulge layer (OB) expressed their typical gene signatures (**Fig. 4A-C**). When analyzing the inter-group differences, the Aligned group contributed to a larger proportion of hair follicle progenitor cells (HFP) and highly proliferative inner root sheath cells (IRS) (**Fig. 4A**). The overall difference analysis also revealed up-regulation of genes (Krt28, Sox18) related to hair follicle stem cells in the Aligned group (**Fig. 3D**). This might explain the enhanced hair follicle regeneration observed in Aligned groups either on rat or mouse models.

Inter-group differences in fibroblasts suggest more active ECM formation in the

Aligned group

201 The dermis consists of several layers: the papillary dermis lies closest to the epidermis,
 202 the underlying reticular dermis contains the bulk of the fibrillary extracellular matrix,
 203 and beneath the reticular dermis lies the hypodermis (19). Fibroblasts from different
 204 layers presented distinct gene signatures (**Fig. 4D**). There were four populations of
 205 papillary fibroblasts ($\text{Crabp1}^+\text{Col23a1}^+$) (20, 21). Dermal fibroblasts expressed
 206 increased Ccl11 and Dcn . The $\text{Gpx3}^+\text{Plac8}^{\text{hi}}$ subset was identified as hypodermis
 207 fibroblast located to the adventitia (HDF1), and the $\text{Gpx3}^+\text{Plac8}^{\text{lo}}$ subset (HDF2) was
 208 around the panniculus carnosus muscle (18). Contractile myo-fibroblasts (Acta2^+ MF)
 209 expressed elevated genes associated with cell proliferation (Birc5^{hi}). Composition of
 210 each subset revealed that more papillary fibroblasts (PF2), dermal fibroblasts (DF),
 211 hypodermis fibroblasts (HDF2) and myo-fibroblasts (MF) were from the Aligned
 212 group, suggesting more robust ECM formation in the presence of scaffolds.

213 **Neutrophils and dendritic cells are more abundant in the Ctrl samples**

214 After wounding, neutrophils close to the focus of injury migrate toward the nidus,
 215 followed by those recruited from more than 200 μm from the site of tissue injury (22).
 216 Three neutrophil subsets were identified in our study (**Fig. 4E**). Peripheral blood
 217 neutrophils (PBN) expressed typical gene signatures including Csf3r , Pglyrp1 , Il1b ,
 218 and Retnlg . Neutrophil 2 (Neu2) expressed elevated Cd14 and Ccl3 , and was
 219 identified as antimicrobial phagocytic neutrophils. Neutrophil 1 (Neu1) expressed
 220 elevated Ccr1 , a chemokine receptor that mediates neutrophil migration.
 221 Correspondingly, PBN showed gene enrichment in leukocyte chemotaxis and
 222 migration; Neu1 expressed genes enriched in leukocyte/granulocyte chemotaxis and

223 migration, and Neu2 was enriched in anti-bacterial biological processes (**Fig. 4F**).
 224 According to the correlation analysis, Neu 1 and Neu 2 were derived from PBN in
 225 circulation (**Fig. 4G**). In Neu1 and Neu2, more cells belonged to the Ctrl group (**Fig.**
 226 **4E**), suggesting more significant Neu1 and Neu2 infiltration in the Ctrl samples at a
 227 proliferative stage (7 days post-wounding). Dendritic cells were classified into two
 228 subpopulations (**Fig. 4H**). The subset derived from monocytes (DC) was
 229 characterized by increased Cd207 and Cd86 (10, 23). The Langerhans cell subset bore
 230 a keratinocyte gene signature, probably transferred from the resident
 231 microenvironment (**Fig. 4I**) (24). Both DC and LC presented elevated major
 232 histocompatibility complex (MHC) molecules, suggesting an antigen presenting
 233 function of them (**Fig. 4J**). LCs contained more Ctrl-derived cells, indicating
 234 differences in Langerhans cell infiltration between groups.

235 **Macrophage heterogeneity and their down-regulation by scaffolds**

236 To explore the heterogeneity of macrophages in vivo and their inter-group differences,
 237 we subjected them to further unsupervised sub-clustering. Four subsets were
 238 determined (**Fig. 5A**). The subset that showed increased anti-inflammatory genes
 239 (Ccl8, Fcrl2, C1qa and Mrc1) was named anti-inflammatory macrophages (AIM) (25,
 240 26). Another subset characterized by elevated expression of pro-inflammatory genes
 241 (Ptgs2, Ccl3, Inhba, Nos2) was named pro-inflammatory macrophages (PIM) (27, 28).
 242 The monocyte subset (Mono) showed higher Ly6c2, Plac8, Cd14, and Clec4e,
 243 indicating inflammatory responses against lesions and microorganism (29, 30). The
 244 Cytip^{hi}H2-Eb1^{hi} subset of cells was defined as monocyte-derived dendritic cells

(M-DC) (**Fig. 5B**) (31). We further found that canonical M1 and M2 markers were not entirely consistent with computationally determined AIM and PIM. Arg1, a canonical M2 marker, was expressed by basically all monocyte-macrophage subsets (AIM, PIM, Mono), and was regarded as a pan-macrophage marker in this study (**Fig. 5B**). Expression of another type 2 gene, Socs3, did not parallel Mrc1 expression either. Similar disparity was found in the expression of canonical type 1 genes. M-DC, rather than PIM, expressed more Cd86. Neither was the expression of Cd86 correlated with Nfkbiz. Expression of other genes associated with fibrotic or regenerative macrophage subsets in a scaffold immune microenvironment did not correspond with these clusters either (**Fig. 5B**) (12). It was determined that Ly6c2, Arg1, Mrc1 and Nos2 were sufficient to distinguish the computationally determined AIM, PIM and Mono subsets. We performed flow cytometry on cells isolated from the Aligned and Ctrl samples using Cd68 (a monocyte-macrophage marker, also expressed by some neutrophils and dendritic cells) and the proposed markers (Ly6c2, Arg1, Mrc1 and Nos2). The Cd68⁺ cells were selected to create a t-distributed stochastic neighbor embedding (tSNE) plot. We then identified Arg1⁺ macrophages expressing the surface markers Mrc1 and Nos2 in the gated dataset to represent AIM and PIM, respectively. The Arg1⁺Ly6c2⁺ monocytes were also identified (Mono). The three terminal clusters (AIM, PIM, and Mono) could be separated, indicating that the subsets can be identified experimentally using flow cytometry (**Fig. 5C**). Pseudo-temporal trajectory (Monocle 2) of the four subsets revealed that monocytes developed into M-DCs and polarized macrophages (AIM and PIM). Although AIM and PIM expressed distinct

gene signatures, they were highly correlated (**Fig. 5D-E**). The Ctrl samples contributed to a larger proportion of cells in all subsets (**Fig. 5A**). Therefore, the scaffold might play a role in the reduction of macrophage infiltration at the proliferative stage.

Sub-clustering of T cells revealed a novel T cell population and more effector T cells in the Aligned group

T cells were clustered into four subsets (**Fig. 5F**). T cells characterized by increased Cd7, Cd3g, and Areg were named as Early T cells (ET) (32). The subset adjacent to ET expressed elevated Xcl1 and Sult2b1 (associated with T cell activation), and was named Activated T cell 1 (AT1) (33). AT1 also expressed increased Areg, Ctla2a, and Ctla2b, genes related to immune homeostasis and immuno-suppression (34, 35). Another activated T cell subset (AT2) expressed up-regulated genes associated with cytotoxic T cells (Cd8b1), Th1 cells (Ifng, Ptpn18), and Th17 cells (Il17a, Il17f) (36, 37). Therefore, AT2 might include multiple effector T cells. A novel T cell subset connecting AT2 and ET was featured by high expression of Birc5, Mki67 and Stmn1, markers for cell proliferation, and was named Proliferating T cell (PT) (**Fig. 5F-G**). In GO analysis, both ET and AT1 were enriched in T cell activation. AT1 was also found elevation of T cell receptor signaling pathway, suggesting that these T cells played a role in antigen recognition (**Fig. 5G**). We found three terminally differentiated clusters stemming from two precursors (ET and AT1) in pseudo-time analysis. PT was differentiated from AT1 and ET after the first branch point, and might be a transitional status between early T cells and effector T cells. At the second branch, cells

differentiated into two terminal clusters. One belonged to AT1 and ET, and another one was the AT2 population (**Fig. 5H**). Correlation analysis showed that ET and AT1 were highly correlated (**Fig. 5I**). In the ET and AT1 populations, more cells were from Ctrl samples, whereas a larger number of cells in AT2 and PT were from aligned samples.

Receptor-ligand analysis reveals intricate interactions among immune cells, keratinocytes and fibroblasts

To explore potential interactions among immune cells, keratinocytes and fibroblasts, we ran CellChat analysis on these datasets (38). For anti-inflammatory macrophages (AIM), broadly speaking, “pro-inflammatory” signals like Tnf, Visfatin, Rankl, EGFR and C3a signaling, and “anti-inflammatory” signals including Spp1, Lgals9, Sema3, Chemerin, Il2, Il13 and Il10 signaling were involved either in a paracrine or autocrine way. Signals like Mif, Ccl, Csf, Edn, and Cxcl12 signaling exerted protective or deleterious effects depending on their specific roles (**Fig. 6E**) (25, 39-50). Among T cell populations (**Fig. 6F**), ET communicated with other T cells mainly through Tgfb signaling, mediating ET cell tolerance and immune homeostasis (51). The novel PT population secreted Wnt11 signals that bound to Fzd5 receptor on ET. Inner bulge layer keratinocytes secreted Cxcl16 that targeted Cxcr6 on ET, chemoattracting ET to the wound area (52). For fibroblast populations, HDF1 had the most frequent interaction with other cells (**Fig. 6G**). In addition to the mentioned signals, OSM signaling was also activated and induced the expression of the pro-inflammatory cytokine IL-6 (53). T cells and macrophages secreted Tgfb1 and Tgfb2 to target

311 HDF1 and PF1, leading to proliferation and differentiation of fibroblasts into
 312 myo-fibroblasts (54). Among keratinocytes, IFEB and IB1 had the most frequent
 313 contact with other cells (**Fig. 6H, Fig. S8I-M**). IFEB received Tgfb1 and Tgfb2
 314 (signals playing key roles in regulating epithelial-to-mesenchymal transition (EMT)
 315 for keratinocytes) from T cells, macrophages and germinative layer (GL)
 316 keratinocytes (55). Interactive plots for other types of cells were summarized in Fig.
 317 S8. The communication between immune cells and cutaneous cells was complicated,
 318 and wound healing was the comprehensive results of numerous influencing factors
 319 working together.

320 **Discussion**

321 For this study, we fabricated electrospun membranes with three types of surface
 322 topography (Random, Aligned, and Latticed). The aligned membranes led to faster
 323 wound healing, reduced fibrotic response and enhanced regeneration of cutaneous
 324 appendages compared to other scaffolds. Meanwhile, the aligned membranes
 325 exhibited immunomodulatory properties (**Fig. 6A**). Based on that, we generated
 326 single-cell transcriptomes from wounded mouse skin to investigate the
 327 microenvironment around scaffolds. The skin wound repair process was classically
 328 divided into four phases: hemostasis (hours), inflammation (days), proliferation (1-2
 329 weeks), and remodeling (>2 weeks). 7 days post-wounding was a transitional time
 330 point when the innate immune response subsided and activity of adaptive immune
 331 cells increased (10). In the Ctrl samples, the infiltration of T cells achieved a similar
 332 extent with macrophages (**Fig. 6B Overall distribution**). However, in Aligned group,

333 infiltrated macrophages were much fewer than T cells, and more terminally
334 differentiated effector T cells were present (**Fig. 6B**). According to the timeframe of
335 innate and adaptive immune responses (10), the process of innate immunity seemed to
336 be alleviated earlier, and adaptive immune response was advanced in the presence of
337 aligned scaffolds (**Fig. 6C**). In the immune microenvironment around aligned
338 scaffolds (**Fig. 6D**), damage-associated molecular patterns (DAMPs),
339 pathogen-associated molecular patterns (PAMPs) and antigens from cell debris,
340 pathogens, and foreign agents (scaffold) triggered innate and adaptive immune
341 responses. Neutrophils from circulation (PBN) quickly migrated to the wound area.
342 The infiltrated neutrophils (Neu1 and Neu2) phagocytosed dying cells and
343 microorganisms, and secreted chemo-attractants like Ccr1 and Ccl4 to recruit more
344 leukocytes and lymphoid cells (52). Circulating monocytes (Mono) were also
345 recruited to the wound area, and differentiated into pro-inflammatory macrophages
346 (PIM), clearing dying neutrophils and debris. Meanwhile, anti-inflammatory
347 macrophages (AIM) chemo-attracted more cells via chemokines like Ccl8 and Ccl6.
348 Part of monocytes differentiated into dendritic cells (M-DC). Tissue resident
349 Langerhans cells (LC), together with M-DC, functioned as antigen-presenting cells
350 (APCs). In the antigen specific signal, LC and DC processed antigens into peptides,
351 and presented them by MHC class II molecules (H2-Aa, H2-Eb1) on the cell surface.
352 T cells (ET and AT1) bound to the MHC molecules through surface receptors (TCR),
353 and differentiated into effector T cells (Cd4⁺ AT2 cells). The co-stimulatory signal was
354 characterized by the engagement of Cd28 receptor on T cells (AT2, Cd8b1⁺Cd28^{hi})

355 with Cd86 ligands on APCs (Cd86⁺ DC and LC) (10, 56). With regard to the
 356 influences that immune microenvironment had on tissue generation, immune cells
 357 sent out a variety of signals (like Grn, Tgfb, Areg/Hbegf) that modulated behaviors of
 358 keratinocytes and fibroblasts (**Fig. 6**). On the other hand, keratinocytes and fibroblasts
 359 secreted chemotactic, pro- or anti-inflammatory signals that regulated immune cell
 360 polarization and function. Immune cells themselves also interact with each other via
 361 numerous signals. The communication network of immune cells and cutaneous cells
 362 was complicated, and wound healing was the comprehensive results of these factors.
 363

364 **Acknowledgement**

365 This work was supported by Research and Develop Program, West China Hospital of
366 Stomatology Sichuan University (No.LCYJ2019-19); The Fundamental Research
367 Funds for The Central Universities (No. 2082604401239); National Key Research and
368 Development Program of China (No. 2016YFA0201703/2016YFA0201700)

369 **Statement of conflict of interest**

370 There are no conflicts of interest related to this manuscript.

371

Figure legends:

Fig. 1

Fig. 1 Evaluation of rat skin wound healing implanted with three types of

electrospun scaffolds at 7 days post-wounding. (A) Surface topography of three

376 types of electrospun membranes. (B) Workflow for evaluating rat skin wound healing.
377 (C) Surgical processes for the rat skin excisional wound model. (D, E) Residual
378 wound area at each time point. (F) Histological view of Ctrl group and groups
379 implanted with three types of scaffolds. (G) Semi-quantitative evaluation of gap width.
380 (H) Immunofluorescent staining using Krt5 (red) and Krt10 (green). (I)
381 Semi-quantitative evaluation of the fluorescent area. (J) Evaluation of foreign body
382 reaction around biomaterials. FBGCs lined up on Random and Latticed membranes,
383 whereas fewer of them appeared on Aligned ones. (K) Thickness of fibrotic capsules.
384 (L-N) PCA and GO analysis revealed immunomodulatory effects for aligned and
385 random scaffolds. ****P < 0.0001, **P < 0.01, and *P < 0.05 by ANOVA for data in
386 (E), (G) and (K).
387

Fig. 2

Fig. 2 Evaluation of mouse skin wound healing implanted with three types of

electrospun scaffolds at 7 days post-wounding. (A) Workflow for evaluating mouse

skin wound healing. (B) Surgical processes for mouse skin excisional wound model.

392 (C, D) Residual wound area at each time point. (E) Histological analysis of Ctrl group
 393 and groups implanted with three types of scaffolds. (F) Semi-quantitative evaluation
 394 of gap width. (G) Evaluation of foreign body reaction around biomaterials. (H)
 395 Thickness of fibrotic capsules. (I, J) GO and KEGG analysis revealed
 396 immunomodulatory effects for aligned scaffolds. ****P < 0.0001, ***P < 0.001, and
 397 *P < 0.05 by ANOVA for data in (D), (F) and (H).
 398

Fig. 3

Fig. 3 Overview of the single-cell transcriptome analysis. (A) Workflow for single-cell experiment. (B) Cells were categorized into thirteen main classes. Cell populations in each class, number of cells (%), and composition of aligned and Ctrl cells were listed. (C) Marker genes for different cell classes. (D) Gene expression differences between Ctrl and Aligned groups.

Fig. 4 Further analysis of keratinocytes, fibroblasts, neutrophils and dendritic cells. (A) Sub-clustering of keratinocytes revealed twelve subsets. Marker genes and composition for each subset was listed. (B) Marker genes for each keratinocyte subset.

410 (C) Location of different keratinocyte subsets. Color of each cell corresponds with its
 411 cell type in (A). (D) Sub-clustering of fibroblasts revealed eight subsets. The marker
 412 genes and composition for each subset was listed. (E) Sub-clustering of neutrophils
 413 revealed three subsets. (F) Marker genes for neutrophil subsets and their enriched
 414 gene sets in GO analysis. (G) Correlation analysis of neutrophil subsets. (H)
 415 Sub-clustering of dendritic cells revealed two subsets. (I) Marker genes for dendritic
 416 cell subsets. (J) Expression of genes associated with antigen presenting in dendritic
 417 cells.
 418

419

420 **Fig. 5 Further analysis of macrophages and T cells.** (A) Sub-clustering of

421 macrophages revealed four subsets. Marker genes and composition for each subset

422 was listed. (B) Marker genes for each macrophage/monocyte subset and their enriched

423 gene sets in GO analysis. Expression of canonical M1 and M2 markers and other
424 proposed markers were shown. (C) In vivo flow cytometry strategy using Cd68 to
425 identify monocytes and macrophages. Arg1⁺ macrophages expressing the surface
426 markers Mrc1 and Nos2 in the gated dataset represented AIM and PIM, respectively.
427 The Arg1+Ly6c2⁺ monocytes were also identified (Mono). (D) Pseudo-temporal
428 ordering of macrophage/monocytes, and the distribution of four subsets along the
429 trajectory. (E) Correlation analysis of macrophage/monocyte subsets. (F)
430 Sub-clustering of T cells revealed four subsets. (G) Marker genes for T cell subsets
431 and their enriched gene sets in GO analysis. AT2 subset expressed gene signatures of
432 cytotoxic T cells, Th1 cells, and Th17 cells. (H) Pseudo-temporal ordering of T cells,
433 and the distribution of four subsets along the trajectory. (I) Correlation analysis of T
434 cell subsets.
435

Fig. 6 Summary of the differences in wound healing and immune responses

between the Aligned group and Ctrl group. (A) The aligned membranes had

immunomodulatory properties, and led to faster wound healing, reduced fibrotic

440 response and enhanced regeneration of skin appendages. (B) Pie plot for overall
 441 immune cells in two groups. For each cell type, differences in cell amount between
 442 aligned and Ctrl groups. (C) Timeframe of innate and adaptive immune responses
 443 occurred in Aligned and Ctrl groups. (D) The immune microenvironment around
 444 aligned scaffolds. (E) Cell-cell interaction plots according to receptor-ligand analysis
 445 via CellChat.
 446

447 **References**

- 448 1. X. Li, B. Cho, R. Martin, M. Seu, C. Zhang, Z. Zhou, J. S. Choi, X. Jiang, L.
449 Chen, G. Walia, J. Yan, M. Callanan, H. Liu, K. Colbert, J.
450 Morrisette-McAlmon, W. Grayson, S. Reddy, J. M. Sacks, H. Q. Mao,
451 Nanofiber-hydrogel composite-mediated angiogenesis for soft tissue
452 reconstruction. *Sci Transl Med* **11**, eaau6210 (2019).
- 453 2. A. Mandal, A. V. Boopathy, L. K. W. Lam, K. D. Moynihan, M. E. Welch, N.
454 R. Bennett, M. E. Turvey, N. Thai, J. H. Van, J. C. Love, P. T. Hammond, D. J.
455 Irvine, Cell and fluid sampling microneedle patches for monitoring
456 skin-resident immunity. *Sci Transl Med* **10**, eaar2227 (2018).
- 457 3. G. Huang, F. Li, X. Zhao, Y. Ma, Y. Li, M. Lin, G. Jin, T. J. Lu, G. M. Genin, F.
458 Xu, Functional and Biomimetic Materials for Engineering of the
459 Three-Dimensional Cell Microenvironment. *Chem Rev* **117**, 12764-12850
460 (2017).
- 461 4. H. Li, Y. Xu, H. Xu, J. Chang, Electrospun membranes: control of the
462 structure and structure related applications in tissue regeneration and drug
463 delivery. *J Mater Chem B* **2**, 5492-5510 (2014).
- 464 5. Y. Wang, H. Shi, J. Qiao, Y. Tian, M. Wu, W. Zhang, Y. Lin, Z. Niu, Y. Huang,
465 Electrospun tubular scaffold with circumferentially aligned nanofibers for
466 regulating smooth muscle cell growth. *ACS Appl Mater Interfaces* **6**,
467 2958-2962 (2014).
- 468 6. A. Martins, M. L. Alves da Silva, S. Faria, A. P. Marques, R. L. Reis, N. M.

- 469 Neves, The influence of patterned nanofiber meshes on human mesenchymal
470 stem cell osteogenesis. *Macromol Biosci* **11**, 978-987 (2011).
- 471 7. K. T. Kurpinski, J. T. Stephenson, R. R. Janairo, H. Lee, S. Li, The effect of
472 fiber alignment and heparin coating on cell infiltration into nanofibrous PLLA
473 scaffolds. *Biomaterials* **31**, 3536-3542 (2010).
- 474 8. C. Chu, L. Liu, S. Rung, Y. Wang, Y. Ma, C. Hu, X. Zhao, Y. Man, Y. Qu,
475 Modulation of foreign body reaction and macrophage phenotypes concerning
476 microenvironment. *J Biomed Mater Res A* **108**, 127-135 (2020).
- 477 9. K. Sadtler, A. Singh, M. T. Wolf, X. Wang, D. M. Pardoll, J. H. Elisseeff,
478 Design, clinical translation and immunological response of biomaterials in
479 regenerative medicine. *Nat Rev Mater* **1**, 16040 (2016).
- 480 10. M. O. Dellacherie, B. R. Seo, D. J. Mooney, Macroscale biomaterials
481 strategies for local immunomodulation. *Nat Rev Mater* **4**, 379-397 (2019).
- 482 11. L. Chung, D. R. Maestas, Jr., A. Lebid, A. Mageau, G. D. Rosson, X. Wu, M.
483 T. Wolf, A. J. Tam, I. Vanderzee, X. Wang, J. I. Andorko, H. Zhang, R. Narain,
484 K. Sadtler, H. Fan, D. Cihakova, C. J. Le Saux, F. Housseau, D. M. Pardoll, J.
485 H. Elisseeff, Interleukin 17 and senescent cells regulate the foreign body
486 response to synthetic material implants in mice and humans. *Sci Transl Med*
487 **12**, eaax3799 (2020).
- 488 12. S. D. Sommerfeld, C. Cherry, R. M. Schwab, L. Chung, D. R. Maestas, P.
489 Laffont, J. E. Stein, A. Tam, S. Ganguly, F. Housseau, J. M. Taube, D. M.
490 Pardoll, P. Cahan, J. H. Elisseeff, Interleukin-36 gamma-producing

- macrophages drive IL-17-mediated fibrosis. *Sci Immunol* **4**, eaax4783 (2019).
13. T. Stuart, A. Butler, P. Hoffman, C. Hafemeister, E. Papalexi, W. M. Mauck, 3rd, Y. Hao, M. Stoeckius, P. Smibert, R. Satija, Comprehensive Integration of Single-Cell Data. *Cell* **177**, 1888-1902 e1821 (2019).
14. H. Wu, S. Han, B. Wu, X. Du, Z. Sheng, J. Lin, X. Chen, K. Zhao, V. Bunpetch, Y. Chen, M. Zeng, E. V. Alakpa, Y. Ma, X. Lei, J. Huang, X. Zou, H. Ouyang, Single-cell mass cytometry reveals in vivo immunological response to surgical biomaterials. *Appl Mater Today* **16**, 169-178 (2019).
15. I. Pastar, O. Stojadinovic, N. C. Yin, H. Ramirez, A. G. Nusbaum, A. Sawaya, S. B. Patel, L. Khalid, R. R. Isseroff, M. Tomic-Canic, Epithelialization in Wound Healing: A Comprehensive Review. *Adv Wound Care (New Rochelle)* **3**, 445-464 (2014).
16. R. Tokunaga, W. Zhang, M. Naseem, A. Puccini, M. D. Berger, S. Soni, M. McSkane, H. Baba, H. J. Lenz, CXCL9, CXCL10, CXCL11/CXCR3 axis for immune activation - A target for novel cancer therapy. *Cancer Treat Rev* **63**, 40-47 (2018).
17. P. Myung, M. Ito, Dissecting the bulge in hair regeneration. *J Clin Invest* **122**, 448-454 (2012).
18. S. Joost, K. Annusver, T. Jacob, X. Sun, T. Dalessandri, U. Sivan, I. Sequeira, R. Sandberg, M. Kasper, The Molecular Anatomy of Mouse Skin during Hair Growth and Rest. *Cell Stem Cell* **26**, 441-457.e447 (2020).
19. C. Philippeos, S. B. Telerman, B. Oules, A. O. Pisco, T. J. Shaw, R. Elgueta, G.

- 513 Lombardi, R. R. Driskell, M. Soldin, M. D. Lynch, F. M. Watt, Spatial and
514 Single-Cell Transcriptional Profiling Identifies Functionally Distinct Human
515 Dermal Fibroblast Subpopulations. *J Invest Dermatol* **138**, 811-825 (2018).
- 516 20. T. Tabib, C. Morse, T. Wang, W. Chen, R. Lafyatis, SFRP2/DPP4 and
517 FMO1/LSP1 Define Major Fibroblast Populations in Human Skin. *J Invest*
518 *Dermatol* **138**, 802-810 (2018).
- 519 21. C. F. Guerrero-Juarez, P. H. Dedhia, S. Jin, R. Ruiz-Vega, D. Ma, Y. Liu, K.
520 Yamaga, O. Shestova, D. L. Gay, Z. Yang, K. Kessenbrock, Q. Nie, W. S. Pear,
521 G. Cotsarelis, M. V. Plikus, Single-cell analysis reveals fibroblast
522 heterogeneity and myeloid-derived adipocyte progenitors in murine skin
523 wounds. *Nat Commun* **10**, 650 (2019).
- 524 22. W. M. Nauseef, N. Borregaard, Neutrophils at work. *Nat Immunol* **15**, 602-611
525 (2014).
- 526 23. K. Takahara, Y. Omatsu, Y. Yashima, Y. Maeda, S. Tanaka, T. Iyoda, B. E.
527 Clausen, K. Matsubara, J. Letterio, R. M. Steinman, Y. Matsuda, K. Inaba,
528 Identification and expression of mouse Langerin (CD207) in dendritic cells.
529 *Int Immunol* **14**, 433-444 (2002).
- 530 24. Q. Su, B. Z. Igyarto, Keratinocytes Share Gene Expression Fingerprint with
531 Epidermal Langerhans Cells via mRNA Transfer. *J Invest Dermatol* **139**,
532 2313-2323 e2318 (2019).
- 533 25. S. S. Bohlson, S. D. O'Conner, H. J. Hulsebus, M. M. Ho, D. A. Fraser,
534 Complement, clq, and clq-related molecules regulate macrophage

- 535 polarization. *Front Immunol* **5**, 402 (2014).
- 536 26. S. A. Islam, D. S. Chang, R. A. Colvin, M. H. Byrne, M. L. McCully, B.
537 Moser, S. A. Lira, I. F. Charo, A. D. Luster, Mouse CCL8, a CCR8 agonist,
538 promotes atopic dermatitis by recruiting IL-5+ T(H)2 cells. *Nat Immunol* **12**,
539 167-177 (2011).
- 540 27. M. Carrion, S. Perez-Garcia, C. Martinez, Y. Juarranz, L. Estrada-Capetillo, A.
541 Puig-Kroger, R. P. Gomariz, I. Gutierrez-Canas, VIP impairs acquisition of the
542 macrophage proinflammatory polarization profile. *J Leukoc Biol* **100**,
543 1385-1393 (2016).
- 544 28. J. E. Kanter, F. Kramer, S. Barnhart, M. M. Averill, A. Vivekanandan-Giri, T.
545 Vickery, L. O. Li, L. Becker, W. Yuan, A. Chait, K. R. Braun, S. Potter-Perigo,
546 S. Sanda, T. N. Wight, S. Pennathur, C. N. Serhan, J. W. Heinecke, R. A.
547 Coleman, K. E. Bornfeldt, Diabetes promotes an inflammatory macrophage
548 phenotype and atherosclerosis through acyl-CoA synthetase 1. *Proc Natl Acad*
549 *Sci U S A* **109**, E715-724 (2012).
- 550 29. M. Clement, G. Basatemur, L. Masters, L. Baker, P. Bruneval, T. Iwawaki, M.
551 Kneilling, S. Yamasaki, J. Goodall, Z. Mallat, Necrotic Cell Sensor Clec4e
552 Promotes a Proatherogenic Macrophage Phenotype Through Activation of the
553 Unfolded Protein Response. *Circulation* **134**, 1039-1051 (2016).
- 554 30. A. M. Soler-Rodriguez, H. Zhang, H. S. Lichenstein, N. Qureshi, D. W. Niesel,
555 S. E. Crowe, J. W. Peterson, G. R. Klimpel, Neutrophil activation by bacterial
556 lipoprotein versus lipopolysaccharide: differential requirements for serum and

- 557 CD14. *J Immunol* **164**, 2674-2683 (2000).
- 558 31. L. Grosche, C. Drassner, P. Muhl-Zurbes, L. Kamm, V. T. K. Le-Trilling, M.
559 Trilling, A. Steinkasserer, C. S. Heilingloh, Human Cytomegalovirus-Induced
560 Degradation of CYTIP Modulates Dendritic Cell Adhesion and Migration.
561 *Front Immunol* **8**, 461 (2017).
- 562 32. B. Gerby, E. Clappier, F. Armstrong, C. Deswarte, J. Calvo, S. Poglio, J.
563 Soulier, N. Boissel, T. Leblanc, A. Baruchel, J. Landman-Parker, P. H. Roméo,
564 P. Ballerini, F. Pflumio, Expression of CD34 and CD7 on human T-cell acute
565 lymphoblastic leukemia discriminates functionally heterogeneous cell
566 populations. *Leukemia* **25**, 1249-1258 (2011).
- 567 33. S. J. Bensinger, M. N. Bradley, S. B. Joseph, N. Zelcer, E. M. Janssen, M. A.
568 Hausner, R. Shih, J. S. Parks, P. A. Edwards, B. D. Jamieson, P. Tontonoz,
569 LXR signaling couples sterol metabolism to proliferation in the acquired
570 immune response. *Cell* **134**, 97-111 (2008).
- 571 34. D. M. W. Zaiss, W. C. Gause, L. C. Osborne, D. Artis, Emerging functions of
572 amphiregulin in orchestrating immunity, inflammation, and tissue repair.
573 *Immunity* **42**, 216-226 (2015).
- 574 35. B. Mahata, X. Zhang, A. A. Kolodziejczyk, V. Proserpio, L. Haim-Vilmovsky,
575 A. E. Taylor, D. Hebenstreit, F. A. Dingler, V. Moignard, B. Gottgens, W. Arlt,
576 A. N. McKenzie, S. A. Teichmann, Single-cell RNA sequencing reveals T
577 helper cells synthesizing steroids de novo to contribute to immune
578 homeostasis. *Cell Rep* **7**, 1130-1142 (2014).

- 579 36. P. Castro-Sanchez, R. Ramirez-Munoz, P. Roda-Navarro, Gene Expression
580 Profiles of Human Phosphotyrosine Phosphatases Consequent to Th1
581 Polarisation and Effector Function. *J Immunol Res* **2017**, 8701042 (2017).
- 582 37. I. C. Boothby, J. N. Cohen, M. D. Rosenblum, Regulatory T cells in skin
583 injury: At the crossroads of tolerance and tissue repair. *Sci Immunol* **5**,
584 eaaz9631 (2020).
- 585 38. S. Jin, C. F. Guerrero-Juarez, L. Zhang, I. Chang, P. Myung, M. V. Plikus, Q.
586 Nie, Inference and analysis of cell-cell communication using CellChat.
587 *bioRxiv*, (2020).
- 588 39. M. Croft, The role of TNF superfamily members in T-cell function and
589 diseases. *Nat Rev Immunol* **9**, 271-285 (2009).
- 590 40. J. D. Klement, A. V. Paschall, P. S. Redd, M. L. Ibrahim, C. Lu, D. Yang, E.
591 Celis, S. I. Abrams, K. Ozato, K. Liu, An osteopontin/CD44 immune
592 checkpoint controls CD8⁺ T cell activation and tumor immune evasion. *J Clin*
593 *Invest* **128**, 5549-5560 (2018).
- 594 41. C. Wu, T. Thalhamer, R. F. Franca, S. Xiao, C. Wang, C. Hotta, C. Zhu, M.
595 Hirashima, A. C. Anderson, V. K. Kuchroo, Galectin-9-CD44 interaction
596 enhances stability and function of adaptive regulatory T cells. *Immunity* **41**,
597 270-282 (2014).
- 598 42. M. Hayashi, T. Nakashima, M. Taniguchi, T. Kodama, A. Kumanogoh, H.
599 Takayanagi, Osteoprotection by semaphorin 3A. *Nature* **485**, 69-74 (2012).
- 600 43. D. S. Shouval, A. Biswas, J. A. Goettel, K. McCann, E. Conaway, N. S. Redhu,

601 I. D. Mascanfroni, Z. Al Adham, S. Lavoie, M. Ibourk, D. D. Nguyen, J. N.
602 Samsom, J. C. Escher, R. Somech, B. Weiss, R. Beier, L. S. Conklin, C. L.
603 Ebens, F. G. Santos, A. R. Ferreira, M. Sherlock, A. K. Bhan, W. Muller, J. R.
604 Mora, F. J. Quintana, C. Klein, A. M. Muise, B. H. Horwitz, S. B. Snapper,
605 Interleukin-10 receptor signaling in innate immune cells regulates mucosal
606 immune tolerance and anti-inflammatory macrophage function. *Immunity* **40**,
607 706-719 (2014).

608 44. S. S. Jankauskas, D. W. L. Wong, R. Bucala, S. Djudjaj, P. Boor, Evolving
609 complexity of MIF signaling. *Cell Signal* **57**, 76-88 (2019).

610 45. W. Lin, D. Xu, C. D. Austin, P. Caplazi, K. Senger, Y. Sun, S. Jeet, J. Young, D.
611 Delarosa, E. Suto, Z. Huang, J. Zhang, D. Yan, C. Corzo, K. Barck, S. Rajan,
612 C. Looney, V. Gandham, J. Lesch, W. C. Liang, E. Mai, H. Ngu, N. Ratti, Y.
613 Chen, D. Misner, T. Lin, D. Danilenko, P. Katavolos, E. Doudemont, H. Uppal,
614 J. Eastham, J. Mak, P. E. de Almeida, K. Bao, A. Hadadianpour, M. Keir, R. A.
615 D. Carano, L. Diehl, M. Xu, Y. Wu, R. M. Weimer, J. DeVoss, W. P. Lee, M.
616 Balazs, K. Walsh, K. R. Alatsis, F. Martin, A. A. Zarrin, Function of CSF1 and
617 IL34 in Macrophage Homeostasis, Inflammation, and Cancer. *Front Immunol*
618 **10**, 2019 (2019).

619 46. S. F. Nie, L. F. Zha, Q. Fan, Y. H. Liao, H. S. Zhang, Q. W. Chen, F. Wang, T.
620 T. Tang, N. Xia, C. Q. Xu, J. Y. Zhang, Y. Z. Lu, Z. P. Zeng, J. Jiao, Y. Y. Li, T.
621 Xie, W. J. Zhang, D. Wang, C. C. Wang, J. J. Fa, H. B. Xiong, J. Ye, Q. Yang,
622 P. Y. Wang, S. H. Tian, Q. L. Lv, Q. X. Li, J. Qian, B. Li, G. Wu, Y. X. Wu, Y.

- 623 Yang, X. P. Yang, Y. Hu, Q. K. Wang, X. Cheng, X. Tu, Genetic Regulation of
624 the Thymic Stromal Lymphopoietin (TSLP)/TSLP Receptor (TSLPR) Gene
625 Expression and Influence of Epistatic Interactions Between IL-33 and the
626 TSLP/TSLPR Axis on Risk of Coronary Artery Disease. *Front Immunol* **9**,
627 1775 (2018).
- 628 47. S. Avniel, Z. Arik, A. Maly, A. Sagie, H. B. Basst, M. D. Yahana, I. D. Weiss,
629 B. Pal, O. Wald, D. Ad-El, N. Fujii, F. Arenzana-Seisdedos, S. Jung, E. Galun,
630 E. Gur, A. Peled, Involvement of the CXCL12/CXCR4 pathway in the
631 recovery of skin following burns. *J Invest Dermatol* **126**, 468-476 (2006).
- 632 48. A. Stofkova, Resistin and visfatin: regulators of insulin sensitivity,
633 inflammation and immunity. *Endocr Regul* **44**, 25-36 (2010).
- 634 49. D. M. Hardbower, K. Singh, M. Asim, T. G. Verriere, D. Olivares-Villagomez,
635 D. P. Barry, M. M. Allaman, M. K. Washington, R. M. Peek, Jr., M. B.
636 Piazzuelo, K. T. Wilson, EGFR regulates macrophage activation and function in
637 bacterial infection. *J Clin Invest* **126**, 3296-3312 (2016).
- 638 50. M. C. Walsh, Y. Choi, Biology of the RANKL-RANK-OPG System in
639 Immunity, Bone, and Beyond. *Front Immunol* **5**, 511 (2014).
- 640 51. J. C. Marie, D. Liggitt, A. Y. Rudensky, Cellular mechanisms of fatal
641 early-onset autoimmunity in mice with the T cell-specific targeting of
642 transforming growth factor-beta receptor. *Immunity* **25**, 441-454 (2006).
- 643 52. S. K. Bromley, T. R. Mempel, A. D. Luster, Orchestrating the orchestrators:
644 chemokines in control of T cell traffic. *Nat Immunol* **9**, 970-980 (2008).

- 645 53. B. Le Goff, S. Singbrant, B. A. Tonkin, T. J. Martin, E. Romas, N. A. Sims, N.
646 C. Walsh, Oncostatin M acting via OSMR, augments the actions of IL-1 and
647 TNF in synovial fibroblasts. *Cytokine* **68**, 101-109 (2014).
- 648 54. V. Miguel, O. Busnadiego, M. Fierro-Fernandez, S. Lamas, Protective role for
649 miR-9-5p in the fibrogenic transformation of human dermal fibroblasts.
650 *Fibrogenesis Tissue Repair* **9**, 7 (2016).
- 651 55. S. Liarte, A. Bernabe-Garcia, F. J. Nicolas, Human Skin Keratinocytes on
652 Sustained TGF-beta Stimulation Reveal Partial EMT Features and Weaken
653 Growth Arrest Responses. *Cells* **9**, 255 (2020).
- 654 56. M. L. Kapsenberg, Dendritic-cell control of pathogen-driven T-cell
655 polarization. *Nat Rev Immunol* **3**, 984-993 (2003).

656 **Materials and methods**

657 **Electrospinning of polymer scaffolds**

658 We used poly(lactic-co-glycolic acid) (PLGA) (LA/GA = 75:25, Mw = 105 kDa,
659 dispersity is 1.897) produced by Jinan Daigang Biomaterial Co., Ltd. (Shandong,
660 China) and FC (from fish scale and skin) obtained from Sangon Biotech Co., Ltd.
661 (Shanghai, China) to fabricate scaffolds by electrospinning. The PLGA (20% w/v) and
662 FC (2% w/v) solution (dissolved in 1,1,1,3,3,3-Hexafluoro-2-propanol (HFIP) solvent
663 (Aladdin Co., Ltd. (Shanghai, China)) were loaded into a plastic syringe fitted with a
664 flat-tipped 21G needle (inner diameter=0.5mm). A high voltage of 7kV and a distance
665 of 16 cm were applied between the needle and the collector. For randomly oriented
666 fibers (Random group), the electrostatically charged fiber was ejected toward the

667 grounded flat collector in the high electric field, forming a membrane deposited on the
668 aluminium foil. For the Latticed group, an electroconductive chess-like wire net was
669 used as the collector. For the Aligned group, the rotational speed of the collecting
670 drum is set at 2800rpm. Finalized scaffolds were approximately 30–90 μm in
671 thickness. To crosslink FC, the membranes were immersed in 50 mM of EDS/NHS
672 and 10 mM of MES ethanol solution for 24 h at 4 °C. Then membranes were washed
673 three times with 75% ethanol and dried in vacuum oven for 24 h. Subsequently, the
674 prepared membranes were sterilized by γ -irradiation for in vitro and in vivo
675 experiments.

676 **Characterization of scaffolds**

677 Scanning electron microscopy (SEM; JEOL, JSM-6510LV, Japan) was employed to
678 observe the surface morphology of the electrospun membranes. Image-Pro Plus was
679 applied to quantitatively measure the fiber diameter and distribution from the SEM
680 images obtained. The surface wetting behavior of the membranes were characterized
681 by measuring the water contact angles (Chengde Dingsheng, JY-82B, China). Five
682 samples were tested for each type of membrane to obtain an average value. The
683 tensile properties of the membranes were tested under a constant upper clamp at speed
684 of 15 mm/min. All tensile tests follow the criteria of “Plastics-Determination of
685 tensile properties of films” (GB/T 1040.3–2006, corresponding with ISO 1184–1983).

686 **Cell culture and cell viability test**

687 L929 mouse fibroblast cells and Human oral keratinocytes (HOK) were used for
688 viability tests. Cells were cultured in medium containing RPMI 1640 medium

689 (HyClone) supplemented with 10% fetal bovine serum (Gibco), and were kept at 37 °C
690 in humidified 5% CO₂/95% air. The cell viability was determined by Cell Counting
691 Kit-8 (CCK-8, Dojindo Laboratories, Kumamoto, Japan). Electrospun membranes
692 were cut into squares (edge length=5mm) and placed in the bottom of 96-well plates
693 (n=3 for each group). L929 cells and HOK cells were seeded onto membranes at
694 4×10^4 cells/ml. Cells were co-cultured with membranes for 1, 3, and 5 days. Blank
695 wells seeded with equal amount of cells were used as Ctrl. 10μl CCK-8 solution was
696 added to each well, and the plates were incubated at 37 °C for 1h. After incubation,
697 the Abs at 450nm was measured to determine the cell viability using a micro-plate
698 reader (Multiskan, Thermo, USA).

699 **Experimental model**

700 **Excisional wound model**

701 The protocol of the present experiment was approved by Institution Review Board of
702 West China Hospital of Stomatology (No.WCHSIRB-D-2017-033-R1) Animals
703 included Sprague Dawley male rat at ages from 7 to 8 weeks and C57BL/6 male mice
704 at ages from 7 to 9 weeks (Chengdu Dossy Experimental Animals Co., LTD.). Hair at
705 the surgical area was removed. Full-thickness circular excisional wound
706 (diameter=6mm) was created at the dorsal skin of rats/mice. Random, aligned and
707 latticed electrospun scaffolds were trimmed into circular shape (diameter=8mm), and
708 placed below the wound. The Control group did not receive any implants. A sterile
709 Tegaderm film (3M) was placed above the wound to protect the wound area. Then
710 annular silicone splints (inner diameter=8mm, outer diameter=12mm) were sutured

711 with the Tegaderm film and underlying skin in order to minimize the contraction of
712 the dorsal muscle. After healing for 1, 2 and 4 weeks, animals were euthanized for
713 sample harvest. Using the residual wound as center, a round skin sample (diameter
714 =10mm) containing all the layers of skin was harvested.

715 **Model for subcutaneous implant placement**

716 The surgical area on dorsal skin was shaved and aseptically prepared. Three
717 horizontal incisions of approximately 10 mm were made and subcutaneous pockets
718 were created for membrane implantation. Then random, align and lattice scaffolds
719 were placed into the pockets. After implantation, the incisions were sutured with
720 interrupted sutures. After recovering for 3, 7, and 14 days, samples including scaffolds
721 and the whole layer of skin at surgical sites were together harvested.

722 **Specimen harvest for scRNA-seq**

723 We obtained skin samples by cutting off skin at the wound area (circular,
724 diameter=10mm). Subcutaneous tissues were removed. A total of four samples were
725 harvested in each group. The tissues were washed in a 100 mm petri dish containing
726 20 ml of phosphate-buffered saline (PBS). Then they were transferred to a 50 mm
727 petri dish containing 100μL of Enzyme G (Epidermis Dissociation Kit mouse,
728 Miltenyi) and 3.9 ml of PBS buffer with the dermal side facing downwards. Tissues
729 were digested for 16 hours at 4°C. Then they were transferred into a 50 mm petri dish
730 containing 4mL of 1×Buffer S (Miltenyi). Epidermis was peeled off from the skin
731 using tweezers, and was cut into pieces. Enzyme mix containing 3.9 ml of 1×Buffer S,
732 100 μl of Enzyme P, and 20 μl of Enzyme A (Miltenyi) stored in a gentleMACS™ C

733 Tube was used to digest the epidermis pieces for 20 minutes at 37°C. Then 4 ml of
734 PBS that contained 0.5% bovine serum albumin (BSA) was added. A gentleMACS
735 Dissociator (Miltenyi) was applied to automatically dissociate the epidermis (Program
736 B). The sample was passed through a 70µm cell strainer (Corning), centrifuged at
737 300×g for 10 minutes at room temperature, and resuspended with PBS that contained
738 0.5% BSA. Cells were gently washed twice and stored in an ice box. For the dermis
739 part, they were first cut into pieces (diameter < 1mm). The tissue was mixed with
740 10ml enzyme mix containing Type I Collagenase (3125u/mL) (Gibco) and 2.5ml
741 trypsin (Gibco), and poured into a gentleMACS™ C Tube. After dissociating the
742 tissue on gentleMACS Dissociator for 37s (Skin mode), another 10ml enzyme mix
743 was added. The sample was digested for 2.5 hours at 37°C in a rotary machine
744 (Peglab). Then the dermis sample was passed through a 70µm cell strainer (Corning),
745 centrifuged at 300×g for 5 minutes at room temperature, and resuspended with 3ml
746 red blood cell lysis buffer (Solarbio). After 3 minutes, the cell suspension was
747 centrifuged and gently resuspended with RPMI 1640 medium (Hyclone). Cells were
748 gently washed twice with PBS containing 0.5% BSA and stored in an ice box. The
749 epidermis and dermis cell solutions were mixed together as a whole. The sample was
750 centrifuged, and resuspended with 100 µl Dead Cell Removal MicroBeads (Miltenyi).
751 After incubation for 15min at room temperature, the cell suspension was diluted in
752 3ml 1×Binding buffer (Miltenyi). LS columns (Miltenyi) and a magnetic stand
753 (Miltenyi) were used for removal of dead cells and debris. The negatively selected
754 live cells passed through the column, and were resuspended with PBS containing 0.05%

755 BSA. Finally, we proceeded with the 10x Genomics® Single Cell Protocol.

756 **Single-cell encapsulation and library generation**

757 Single cells were encapsulated in water-in-oil emulsion along with gel beads coated
758 with unique molecular barcodes using the 10× Genomics Chromium Single-Cell
759 Platform. For single-cell RNA library generation, the manufacturers' protocol was
760 performed. (10×Single Cell 3' v3) Sequencing was performed using Illumina 1.9
761 mode with 94574 reads per cell.

762 **Quality control**

763 We use fastp to analyze basic statistics on the quality of the raw reads.

764 If clean reads is indispensable, then raw read sequences produced by the Illumina
765 pipeline in FASTQ format were pre-processed through Trimmomatic software which
766 can be summarized as below:

767 (1)Remove low-quality reads: scan the reads with a 4-base wide sliding window, cut
768 when the average quality per base drops below 10 (SLIDINGWINDOW: 4:10)

769 (2)Remove trailing low quality or N bases (below quality 3) (TRAILING: 3)

770 (3)Remove adapters: there are two modes to remove the adapter sequence: a.
771 alignment with the adapter sequence, the number of matching bases was greater than
772 7 and mismatch=2; b. when read1 and read2 overlapping base scoring was greater
773 than 30, we removed non-overlapping portions (ILLUMINACLIP:adapter.fa:2:30:7)

774 (4)Drop reads below the 26 bases long

775 (5)Discard those reads that did not form pairs.

776 The remaining reads that passed all the filtering steps was counted as clean reads and

777 all subsequent analyses were based on this.

778 Generation and Analysis of Single-Cell Transcriptomes

779 Raw reads were demultiplexed and mapped to the reference genome by 10X

780 Genomics Cell Ranger pipeline

781 (<https://support.10xgenomics.com/single-cell-gene-expression/software/pipelines/latest/what-is-cell-ranger>) using default parameters. All downstream single-cell analyses

782 were performed using Seurat. In brief, for each gene and each cell barcode (filtered by

783 CellRanger3.0), unique molecule identifiers were counted to construct digital

784 expression matrices. Secondary filtration by Seurat: A gene with expression in more

785 than 3 cells was considered as expressed, and each cell was required to have at least

786 200 expressed genes. We also filtered out some of the foreign cells.

787 The Seurat package was used in data normalization, dimension reduction, clustering,

788 and differential expression. We used the Seurat alignment method canonical

789 correlation analysis (CCA) [Nat. Biotechnol. 36, 411–420 (2018).] for integrated

790 analysis of datasets. For clustering, highly variable genes were selected and the

791 principal components based on those genes used to build a graph, which was

792 segmented with a resolution of 0.6.

793

794 **RNA-seq analysis**

795 The bulk tissue RNA-seq analysis was carried out by Novogene Corporation (Beijing,

796 China). RNA was extracted from tissues or cells using standard methods to make sure

797 samples were strictly controlled for quality. In terms of library construction and

798 quality control, mRNA can be obtained in two main ways: firstly, most eukaryotes'

mRNA has poly A-tailed structural, and poly A-tailed mRNA can be enriched by Oligo (dT) magnetic beads. The other is the removal of ribosomal RNA from the total RNA to obtain mRNA. Subsequently, the obtained mRNA was randomly interrupted by divalent cations in NEB Fragmentation Buffer, and the database was constructed according to the NEB general database construction method or chain specific database construction method. Upon completion of library construction, a Qubit2.0 Fluorometer was used for initial quantification, and the library was diluted to 1.5ng/ul. Then the insert size of the library was detected using Agilent 2100 bioanalyzer. After the insert size met the expectation, the effective concentration of the library was accurately quantified by qRT-PCR (the effective concentration of the library higher than 2nM) to ensure library quality. Finally, the libraries were qualified for sequencing, and Illumina sequencing was performed after pooling the different libraries according to the requirements of effective concentration and target data volume, of which the basic principle is Sequencing by Synthesis. Through z-transformation of Fragments Per Kilobase of transcript per Million mapped reads (fpkm) of the selected gene, gene expression was analyzed. Sample size for conventional, bulk RNA-Seq libraries was fixed at 3 biological replicates.

Quantitative Real-Time Polymerase Chain Reaction (qPCR).

The harvested samples were cut into pieces, and homogenated in TRIzol™ Reagent (Cat. #15596026, Invitrogen, Thermo Scientific). Concentration and ratio of total RNA were detected by NanoPhotometer NP80 (Implen, Westlake Village, CA) at wavelength of 260 nm and 280 nm. The cDNAs were synthesized using

821 PrimeScript™ RT reagent Kit with gDNA Eraser (Perfect Real Time) (Cat.
822 #RR047A), then amplified by qPCR with the specific primers (Table 1). PCR was
823 performed on QuantStudio 3 Real-Time PCR Systems (ThermoFisher Scientific,
824 Waltham, MA). Each 20 µL of PCR mixture contained 10µl of TB Green Premix Ex
825 Taq (Tli RNaseH Plus, Takara) (2X), 0.4µL of PCR Forward Primer (10µM), 0.4µl of
826 PCR Reverse Primer (10µM), 0.4µl of ROX Reference Dye (50X), 2µl Template and
827 6.8µl of Sterile purified water. Samples were incubated at 1 cycle of 95° for 30 s
828 followed by 40 cycles of 95° for 5 s and 60° for 34 s, and ended up with a cycle
829 composing of 95° for 15 s, 60° for 1 min and 95° for 15 s. Results were analyzed
830 using the comparative CT ($\Delta\Delta CT$) method to calculate gene expression fold changes
831 normalized to the levels of Gapdh gene transcripts. The experiments were repeated for
832 three times independently (n=3).

Primer	Forward sequence	Primer	Reverse sequence
Gapdh forward	AGTGCCAGCCTCGTCTCATA	Gapdh reverse	GATGGTGATGGGTTTCCCGT
Cd68 forward	TGGGGCCTCTCTGTATTG	Cd68 reverse	TCTGATGTCGGTCCTGTTT
Tgm2 forward	CGGAAGGACACGGTTAGG	Tgm2 reverse	GTATGGGACCAGGAAACCC
Cd4 forward	AACCAAAAGGTGTCTGTGC	Cd4 reverse	ACCAGCAAAGTGAAGGGA
Cd3e forward	TCTCCATCTCAGGAACCAG	Cd3e reverse	CAGGCAGGACTTTGTCATT

833 **Table1 TB Green rat mRNA primers**

834 **Fluorescence activated Cell Sorting (FACS) analysis**

835 The surface markers of macrophages and their phenotypes were examined by flow
836 cytometry to evaluate proportion and polarization of macrophages.

837 The in vivo specimens were first cut into pieces (diameter < 1mm). The tissue was

838 mixed with 10ml enzyme mix containing Type I Collagenase (3125u/mL) (Gibco) and
839 2.5ml trypsin (Gibco), and poured into a gentleMACS™ C Tube. After dissociating
840 the tissue on gentleMACS Dissociator for 37s (Skin mode), another 10ml enzyme mix
841 was added. The sample was digested for 2.5-3 hours at 37°C in a rotary machine
842 (Pepqlab). Then sample was passed through a 70µm cell strainer (Corning), centrifuged
843 at 300×g for 5 minutes at room temperature. Cells were gently washed twice with
844 PBS containing 0.05% BSA and stored in an ice box. Then the cell solutions were
845 co-incubated with antibodies against iNOS (PE, NBP2-22119PE, Novus), CD68
846 (FITC, ab134351, Abcam), Arg1 (PE/Cyanine7, Cat# 369707, Biolegend), Ly6c
847 (Alexa Fluor 700, Cat# 128023, Biolegend) and Mrc1 (Alexa Fluor 647, ab195192,
848 Abcam) at 1:400 dilution in the dark for 1 h at 4°C (100µl per antibody for each
849 sample). All samples were centrifuged at 450RCF for 5 min at 4°C. Supernatants were
850 removed by aspiration, 1ml 1XPBS solution containing 0.05% BSA was used to wash
851 the cells twice. FACS analysis was performed on NovoCyte Flow Cytometers (ACEA
852 Biosciences®, San Diego, California) and FlowJo10.5.0. The experiments were
853 repeated for three times independently (n=3).

854 **Histological and immunofluorescent staining**

855 The sections were pretreated with 1% BSA in PBS containing 0.1% Triton X 100 for
856 1 h, incubated in 1% Tween 20 for 20 min and washed again in PBS. The sections
857 were subsequently analyzed for Krt10 and Krt5, according to the manufacturers'
858 instructions. Briefly, sections were incubated for 30 min in dark. The excessive dye
859 was rinsed off with PBS. Sections were incubated with antibody isotype to exclude

860 false positive staining. Double immunofluorescence staining with primary antibodies
861 against cytokeratin 10 (ab76318, Abcam, 1:150), cytokeratin 5 (ab52635, Abcam,
862 1:200) and secondary antibodies (GB25303, GB21303, Servicebio, 1:400) was
863 performed. The immunostained specimens were further subjected to Hoechst33258
864 staining (G1011, Servicebio). At least three parallel sections were observed with
865 fluorescence microscope (ZEISS SteREO Discovery.V20, Olympus). The
866 fluorescence area measurement was conducted on five random sights of regenerated
867 epithelium with CaseViewer 2.1 and Image Pro Plus 7.0 (n = 5).

868 **Statistical Analysis**

869 Statistical significance for in vivo and in vitro data of histological analysis, qPCR and
870 FACS were analyzed by analysis of variance (ANOVA) at the 95% confidence level,
871 which were performed in GraphPad Prism 8.0 (GraphPad Software, San Diego, CA,
872 USA) and $P < 0.05$ was considered statistically significant, while $P > 0.05$ was
873 considered having no statistical differences, which was marked with NS.

874