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# The Fas/Fap-1/Cav-1 complex regulates IL-1RA secretion in mesenchymal stem cells to accelerate wound healing

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# Abstract

Mesenchymal stem cells (MSCs) are capable of secreting exosomes, extracellular vesicles, and cytokines to regulate cell and tissue homeostasis. However, it is unknown whether MSCs use a specific exocytotic fusion mechanism to secrete exosomes and cytokines. We show that Fas binds with Fas-associated phosphatase–1 (Fap-1) and caveolin-1 (Cav-1) to activate a common soluble *N*-ethylmaleimide–sensitive factor (NSF) attachment protein receptor (SNARE)–mediated membrane fusion mechanism to release small extracellular vesicles (sEVs) in MSCs. Moreover, we reveal that MSCs produce and secrete interleukin-1 receptor antagonist (IL-1RA) associated with sEVs to maintain rapid wound healing in the gingiva via the Fas/Fap-1/Cav-1 cascade. Tumor necrosis factor– $\alpha$  (TNF- $\alpha$ ) serves as an activator to up-regulate Fas and Fap-1 expression via the nuclear factor  $\kappa$ B pathway to promote IL-1RA release. This study identifies a previously unknown Fas/Fap-1/Cav-1 axis that regulates SNARE-mediated sEV and IL-1RA secretion in stem cells, which contributes to accelerated wound healing.

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# INTRODUCTION

Mesenchymal stem cells (MSCs) are capable of self-renewal and differentiation into mesenchymal and non-mesenchymal lineages (1, 2). MSCs have been used therapeutically for tissue regeneration and auto-immune disease treatment (3–12). Recent emerging evidence shows that multiple mechanisms contribute to MSC-based therapies in which secretion of cytokines, growth factors, and small extracellular vesicles (sEVs) such as exosomes may serve as paracrine or autocrine mediators to regulate immune responses and tissue regeneration (13–15). MSCs have been identified to secrete at least three types of exosomes (16); these exosomes represent an important mode of intercellular communication. It is well known that a variety of cells, including neuroendocrine, endocrine, exocrine, and immune cells, use different mechanisms to secrete EVs and that soluble *N*-ethylmaleimide–sensitive factor (NSF) attachment protein receptor (SNARE)–dependent exocytotic fusion plays a major role in the exocytosis of these EVs (17, 18). Dysfunction of the secretion process may lead to various human diseases, such as schizophrenia, Alzheimer's disease, diabetes, and albinism (19–22). However, it is unknown whether and, if so, how stem cells use an exocytotic fusion mechanism to secrete sEVs.

Mammalian adult cutaneous wound healing can be regulated by numerous secreted factors including cytokines, growth factors, chemokines, and EVs (23, 24). MSC-derived exosomes play an important role in cutaneous wound healing, by reducing heat stress-induced apoptosis (25) and promoting collagen synthesis and angiogenesis (26). Compared to adult cutaneous wounds, fetal wounds heal faster, are more akin to a scarless regeneration, and display mild inflammation with fewer inflammatory cells and lower expression of proinflammatory factors (27). Like fetal wounds, oral gingival/mucosal wounds heal faster than cutaneous wounds and exhibit minimal scar formation (28), which may be due to reduced inflammatory cell infiltration and proinflammatory factor production in the gingiva (29–31). Thus, the secretion profile of MSCs may control accelerated wound healing in the gingiva. Gingiva-derived MSCs (GMSCs) have a distinct neural crest origin and show characteristics of self-renewal, multipotent differentiation, and immunomodulatory capacities both in vitro and in vivo (32, 33). However, it is unknown whether GMSCs have a unique secretory mechanism to regulate wound healing in the gingiva. Here, we show that GMSCs use the Fas/Fas-associated phosphatase-1 (Fap-1)/caveolin-1 (Cav-1) complex to activate SNARE-mediated membrane fusion to secrete higher amounts of interleukin-1 receptor antagonist (IL-1RA)-expressing sEVs to accelerate wound healing in the gingiva.

# RESULTS

#### GMSCs produce and secrete higher amounts of IL-1RA-expressing sEVs than skin MSCs

To explore whether MSCs use a unique mechanism to secrete sEVs, we first isolated exosome-like sEVs from the culture supernatant of several MSCs, including mouse and human bone marrow MSCs (BMMSCs), GMSCs, and skin MSCs (SMSCs). We found that both mouse and human GMSCs secreted higher amounts of sEV-associated proteins, and SMSCs secreted lower amounts of sEV-associated proteins in the culture supernatant compared to GMSCs (Fig. 1A and fig. S1A). We verified that these exosome-like sEVs showed standard exosome microstructure and expressed exosome-associated proteins CD63,

CD9, and CD81 (Fig. 1B and fig. S1B). Cytokine array analysis showed that GMSC-derived sEVs contained higher amounts of IL-1RA, a natural inhibitor of the proinflammatory cytokine IL-1 $\beta$  (34), compared to sEVs from SMSCs (fig. S1C). Because wound healing in the gingiva is faster than that in the skin (28) and MSC-derived exosomes play an important role in cutaneous wound healing (25), we hypothesized that secretion of higher amounts of sEVs and IL-1RA by GMSCs may contribute to a quick wound healing process in the gingiva. To further confirm that sEVs contain IL-1RA, a differential centrifugation approach, followed by sucrose cushion to isolate sEVs at a higher purity grade (35, 36), was used to isolate different sizes of EVs in the culture supernatant of GMSCs and SMSCs (Fig. 1C). We showed that cell lysate, cell debris and large EVs, and sEVs from GMSCs and SMSCs expressed IL-1RA; however, IL-1RA was not expressed in mixed EVs (Fig. 1D and fig. S1D), suggesting that IL-1RA might be released via sEVs. To rule out potential contributions from nonvesicular particles, we loaded the crude EV fraction from differential centrifugation on a sucrose gradient (36). We found that IL-1RA mainly appeared in 3 light fractions out of the 10 high-speed ultra-centrifugation fractions and matched the expression with CD63, CD9, and CD81 (fig. S1E), which was consistent with a previous report that MSC-derived sEVs usually float in gradients with 1.10 to 1.18 g/ml (37). To demonstrate that the sEV-associated secretion is the major pathway of IL-1RA secretion, we collected the supernatant from each step of differential centrifugation as described (fig. S1F). After the initial steps of centrifugation, we found that the supernatants collected from SN I, SN II, and SN III sections contained similar amounts of IL-1RA. However, the amount of IL-1RA reduced from 134.07 pg/ml in SN III to 12.22 pg/ml in SN IV after ultracentrifugation (fig. S1G). We next showed that the isolated sEVs expressed equal amounts of IL-1RA compared with the concentrated supernatant collected before ultracentrifugation (SN III), but the concentrated proteins from the ultracentrifuged supernatant (SN IV) showed a markedly decreased amount of IL-1RA (fig. S1H). We further performed immunogold electron microscopy to show that IL-1RA was detected on the surface of purified exosome-like EVs (fig. S1I). These results support our conclusion that IL-1RA is secreted by sEVs.

To further confirm the presence of IL-1RA–positive sEVs, we transfected GMSCs with IL-1RA–enhanced green fluorescent protein (EGFP) plasmids and then used superresolution stimulated emission depletion (STED) microscopy to show colocalization of IL-1RA with CD63 and CD81 (Fig. 1E). To verify EV–IL-1RA exocytosis, GMSCs were cotransfected with plasmids expressing IL-1RA–EGFP fusion protein and CD63-mCherry fluorescent protein, and colocalization was observed by total internal reflection fluorescence (TIRF) microscopy (Fig. 1F). The sequential fluorescent images displayed fusion of individual IL-1RA–EGFP/CD63-mCherry double-positive exosome-like EVs with the plasma membrane (Fig. 1F). Moreover, we found that IL-1RA–EGFP/CD63-mCherry double-positive exosome-like EVs fused with the plasma membrane in living GMSCs (movie S1).

Next, we showed that GMSCs secreted a higher amount of IL-1RA in the culture supernatant compared to SMSCs, as assessed by enzyme-linked immunosorbent assay (ELISA) (Fig. 1G). Western blotting showed that both human and mouse GMSCs expressed elevated IL-1RA relative to SMSCs (Fig. 1H and fig. S1J). IL-1RA was coexpressed with MSC markers CD105, CD44, and CD90 in GMSCs and SMSCs (Fig. 1I and fig. S1K).

There are four isotypes of IL-1RA: One isoform is secreted (sIL-1RA), whereas the three others lack a consensus leader peptide and remain intracellular (icIL-1RA1, icIL-1RA2, and icIL-1RA3) (34). GMSCs express a similar amount of sIL-1RA mRNA, but a significantly higher amount of icIL-1RA mRNA compared to SMSCs (Fig. 1, J and K), suggesting that altered expression of IL-1RA is mainly caused by icIL-1RA. Because icIL-1RA does not have a signaling peptide that marks it for transport outside of the cells, it is possible that icIL-1RA is instead packaged into sEVs and transported to the extracellular microenvironment.

#### Fas controls IL-1RA–sEV release in MSCs

Our previous study showed that Fas controls monocyte chemoattractant protein-1 (MCP-1) secretion to regulate MSC-based immune therapies (4). We therefore hypothesized that Fas may control IL-1RA-sEV release to modulate wound healing. We showed that GMSCs expressed elevated Fas in the cytoplasm compared to SMSCs (Fig. 2A). GMSCs and SMSCs from Fas-deficient MRL/*lpr* mice showed reduced capacity to secrete sEV protein to the culture supernatant (Fig. 2B and fig. S2A). EVs from MRL/lpr GMSCs and SMSCs expressed reduced CD63, CD9, CD81, and IL-1RA compared to wild-type (WT) control GMSCs (Fig. 2C and fig. S2B). We further confirmed that GMSCs from MRL/lpr mice showed reduced IL-1RA secretion into the culture supernatant, along with elevated cytoplasmic accumulation of IL-1RA when compared to WT control GMSCs (Fig. 2, D and E). In addition, immunostaining showed IL-1RA colocalized with Fas where it accumulated in Fas-deficient MRL/lpr GMSCs (Fig. 2F). Overexpression of Fas in MRL/lpr GMSCs rescued IL-1RA secretion into the culture supernatant and reduced the cytoplasmic expression of IL-1RA (Fig. 2, G and H). When Fas expression was knocked down in GMSCs by small interfering RNA (siRNA), IL-1RA secretion into the culture supernatant was reduced; however, cytoplasmic IL-1RA was increased (Fig. 2, I and J), which was also observed in MRL/Ipr GMSCs. These data suggest that Fas may control IL-1RA-sEV release in GMSCs.

#### Fas bound with Fap-1 and Cav-1 controls IL-1RA-sEV release

Fap-1 is a protein tyrosine phosphatase capable of binding to the cytosolic domain of Fas (38), and Cav-1 can also bind to Fas to regulate apoptosis (39). However, it is unknown whether Fap-1 and Cav-1 are involved in Fas-controlled sEV/cytokine release. SNARE family proteins mediate the exocytosis of cellular transport vesicles with the cell membrane; some of the key molecules in this family are synaptosome-associated protein of 25 kDa (SNAP25) and vesicle-associated membrane protein (VAMP) (18). We speculated that Fas might control IL-1RA–sEV release through interaction with Fap-1, Cav-1, and SNARE proteins. We saw that Fas coimmunoprecipitated with Fap-1, Cav-1, SNAP25, and VAMP5; this coimmunoprecipitation (co-IP) was absent in Fas-deficient MRL/*lpr* GMSCs (Fig. 3A and fig. S3A). Immunostaining confirmed that Fas colocalized with Fap-1, Cav-1, SNAP25, and fig. S3B). To assess the functional role of Fap-1/Cav-1 in IL-1RA–sEV release, we examined *Fap-1* and *Cav-1* knockout GMSCs and SMSCs. *Fap-1* and *Cav-1* knockout GMSCs and SMSCs showed reduced sEV secretion into the culture supernatant (Fig. 3C and fig. S3C). sEVs from *Fap-1* and *Cav-1* knockout GMSCs and SMSCs and SMSCs expressed reduced CD63, CD9, CD81,

and IL-1RA compared to control GMSCs and SMSCs, respectively (Fig. 3D and fig. S3D). To further dissect the functional role of Fas/Fap-1/Cav-1, we showed that there was no difference in the expression of Fap-1 and Cav-1 in Fas-deficient MRL/*lpr* GMSCs (Fig. 3E), but there was reduced secretion of exosome-associated proteins and IL-1RA into the culture supernatant and there were elevated amounts of IL-1RA in the cytoplasm (Fig. 2, B to E). Knockout of Fap-1 or Cav-1 in GMSCs reduced IL-1RA secretion into the culture supernatant and increased cytoplasmic IL-1RA, but failed to affect the expression of Fas/ Cav-1 or Fas/Fap-1 (Fig. 3, E and F). Knockdown of Fas, Fap-1, or Cav-1 by siRNAs in GMSCs failed to affect the expression of the other two members, but decreased IL-1RA secretion into the culture supernatant with increased cytoplasmic accumulation of IL-1RA (fig. S3, E and F), which is the same as that observed in MRL/lpr, Fap-1, and Cav-1 knockout GMSCs. These data suggest that Fas/Fap-1/Cav-1 control IL-1RA release. In addition, knockdown of Fas, Fap-1, or Cav-1 failed to affect the expression of SNAP25 and VAMP5 (fig. S3E). To test whether SNAP25 and VAMP5 are also involved in Fas/Fap-1/ Cav-1-controlled IL-1RA release, we showed that knockdown of SNAP25 or VAMP5 by siRNA failed to affect the expression of Fas, Fap-1, and Cav-1 (fig. S3E), but reduced IL-1RA secretion into the culture supernatant along with increased cytoplasmic accumulation of IL-1RA (fig. S3, E and F). These data suggest that Fas/Fap-1/Cav-1 machinery may control IL-1RA release via SNAP25/VAMP5.

We next determined how Fas binds to the downstream complex SNAP25/VAMP5 and how Fas/Fap-1/Cav-1 bind together to control exosome/IL-1RA release. We performed IP using Fas-deficient MRL/*lpr*, *Fap-1* knockout, and *Cav-1* knockout GMSCs to show that knocking out *Fap-1* failed to reduce the co-IP of Fas with Cav-1, SNAP25, and VAMP5, whereas knockout of *Cav-1* failed to affect the co-IP of Fas with Fap-1 but resulted in the reduction of Fas co-IP with SNAP25 and VAMP5 (Fig. 3G and fig. S3G). These data suggest that the binding of Fas with SNAP25/VAMP5 is mediated by Cav-1. To test how Fap-1 binds with the other proteins, we showed that Fas-deficient MRL/*lpr* GMSCs failed to affect the co-IP of Fap-1 with Cav-1, SNAP25, and VAMP5, whereas knocking out *Cav-1* expression failed to affect the co-IP of Fap-1 with Fas, but resulted in the reduction of Fap-1 co-IP with SNAP25 and VAMP5 (Fig. 3H and fig. S3H). We next used immunocytofluorescence staining to confirm that Fap-1 was colocalized with SNAP25 and VAMP5 (fig. S3I). These data suggest that the binding of Fap-1 with SNAP25 models with SNAP25 and VAMP5 (Fig. S3I). These data suggest that the binding of Fap-1 with SNAP25 and VAMP5 (Fig. S3I). These data suggest that the binding of Fap-1 with SNAP25 models with SNAP

To examine how Cav-1 binds with the other proteins, we showed that MRL/*lpr* or *Fap-1* knockout GMSCs failed to affect the co-IP of Cav-1 with Fap-1 or Fas and also failed to affect the co-IP of Cav-1 with SNAP25 and VAMP5 (Fig. 3I and fig. S3J). Immunocytofluorescence staining further confirmed that Cav-1 was colocalized with SNAP25 and VAMP5 (fig. S3K). Together, these data suggest that Fas/Fap-1/Cav-1 bind together, but these events occur independently of one another; that the binding between Cav-1 and SNAP25/VAMP5 is independent of Cav-1 binding with Fas/Fap-1; and that the binding of Fas/Fap-1 with SNAP25/VAMP5 is mediated by Cav-1. Fas-deficient MRL/*lpr* and *Fap-1* knockout GMSCs had marked clustering of Cav-1 expression in the cytoplasm, but *Cav-1* knockout failed to affect the colocalization or the distribution of Fas and Fap-1 (Fig. 3J). These data imply that Fas/Fap-1 may bind to Cav-1 and control its trans-location, thereby controlling SNAP25/VAMP5-related IL-1RA–sEV secretion (fig. S3L).

# Tumor necrosis factor– $\alpha$ activated IL-1RA–sEV release via up-regulation of Fas/Fap-1 expression in MSCs

Because tumor necrosis factor-a (TNF-a) can increase the production of MSC-secreted growth factors (40), we used TNF- $\alpha$  and interferon- $\gamma$  (IFN- $\gamma$ ) to treat GMSCs and found that only TNF-a significantly enhanced the secretion of IL-1RA in the culture supernatant in a dose-dependent manner (P < 0.01) (Fig. 4A). Moreover, we found that TNF- $\alpha$ -treated GMSCs and SMSCs showed increased capacity to secrete sEV-associated protein to the culture supernatant (Fig. 4B and fig. S4A). sEV-associated protein from TNF-a-treated GMSCs and SMSCs expressed elevated amounts of CD63, CD9, CD81, and IL-1RA compared to control GMSCs (Fig. 4C and fig. S4B). To determine whether TNF-a affects Fas/Fap-1–controlled sEV/IL-1RA release, we showed that TNF-a treatment up-regulated the expression of Fas, Fap-1, and IL-1RA, but not Cav-1 in control and MRL/lpr GMSCs (Fig. 4D). TNF-a treatment-induced secretion of IL-1RA and sEV-associated protein was significantly reduced in MRL/lpr GMSCs when compared to control GMSCs (P < 0.001) (Fig. 4E and fig. S4C). These data suggest that TNF-a may serve as an activator to enhance IL-1RA-EV release through up-regulation of Fas and Fap-1. To confirm that TNF-a. regulates Fas, we used siRNA to knock down Fas expression in GMSCs and showed that TNF-a treatment resulted in reduced secretion of IL-1RA into the culture supernatant with elevated intracellular IL-1RA compared to control GMSCs (fig. S4, D and E). Fap-1 and Cav-1 knockout GMSCs showed that TNF-a treatment resulted in elevated intracellular Fas and IL-1RA in Fap-1 knockout GMSCs and elevated intracellular Fas, Fap-1, and IL-1RA in Cav-1 knockout GMSCs (Fig. 4, F and G). TNF-a treatment-induced secretion of IL-1RA and exosome protein in the culture supernatant was significantly reduced in Fap-1 and Cav-1 knockout GMSCs when compared to control GMSCs (P < 0.001) (Fig. 4H and fig. S4F). Fas and Cav-1 translocated to the cell membrane region at 0.5 to 1 hour after TNF-a treatment (Fig. 4I). Fap-1 was also distributed near the membrane region along with Cav-1 at 0.5 to 1 hour after TNF-a treatment (fig. S4G). Western blotting showed that TNF-a treatment increased the expression of Fas and Cav-1 in the cell membrane as well as Fas and Fap-1 in the cytoplasm in GMSCs, but decreased the expression of Cav-1 in the cytoplasm (Fig. 4J). These data indicate that TNF-a treatment induces the membrane translocation of Fas and Cav-1 to control Fas/Fap-1/Cav-1-mediated IL-1RA release. Because TNF-a is able to activate the nuclear factor  $\kappa B$  (NF- $\kappa B$ ) pathway (41) and NF- $\kappa B$  is capable of regulating the transcription of Fas and Fap-1 (42), we further showed that treatment with an NF- $\kappa$ B inhibitor, pyrrolidinedithiocarbamate (PDTC), was able to block TNF-a-induced IL-1RA release in GMSCs (fig. S4H).

We next determined whether TNF-a could activate IL-1RA–EV release. When GMSCs were transfected with plasmids expressing IL-1RA–EGFP and the vesicular organelles were stained with LysoTracker TIRF microscopy showed that IL-1RA–EGFP overlapped with LysoTracker-positive EVs (fig. S4I). After 0.5 hours of TNF-a treatment, IL-1RA–EGFP exosome-like EVs fused with the plasma membrane in living GMSCs (movie S2). Sequential fluorescent images displayed fusion of individual IL-1RA–EGFP–positive exosome-like EVs to the plasma membrane in response to TNF-a treatment (fig. S4I). To confirm TNF-a–activated IL-1RA–positive exosome-like EV release, GMSCs were cotransfected with plasmids expressing IL-1RA–EGFP fusion protein and CD63-mCherry

fluorescent protein (Fig. 4K). After 0.5 hours of TNF-α treatment, colocalization of IL-1RA–EGFP and CD63-mCherry exosome-like EVs was observed by TIRF microscopy (Fig. 4K). Moreover, we found intensive IL-1RA–EGFP/CD63-mCherry double-positive exosome-like EVs fused with the plasma membrane in living GMSCs (movie S3), and sequential fluorescent images displayed fusion of individual IL-1RA–EGFP/CD63-mCherry double-positive exosome-like EVs to the plasma membrane after TNF-α treatment (Fig. 4K). These data indicate that TNF-α up-regulates Fas/Fap-1 expression via the NF-κB pathway to induce the membrane translocation of Cav-1, thereby enhancing IL-1RA release in GMSCs (fig. S4J).

#### GMSCs produce high amounts of IL-1RA to facilitate wound healing in the gingiva

Wound healing in the gingiva is faster than that in the skin (28), and we showed that GMSCs and gingival wound tissue have higher expression of IL-1RA than SMSCs and skin wound tissue, respectively (Fig. 1, G and H, and fig. S5A). We examined whether the IL-1RA produced by GMSCs contributed to accelerated wound healing in the gingiva using immunohistofluorescence staining and found that GMSCs expressed IL-1RA at the margins of wound areas (fig. S5B). Staining showed that IL-1RA was coexpressed with neural crestderived MSCs in gingival wound tissue in Wnt1-Cre-tdTomato mice, further confirming that GMSCs produce IL-1RA (fig. S5C). A previous study reported that IL-1RA knockout mice showed impaired cutaneous wound healing (43). We found that gingival healing was significantly delayed in *IL-1RA* knockout mice at 3, 5, and 7 days post-wounding compared to WT control mice (3 days, P<0.01; 5 days, P<0.001; 7 days, P<0.001) (Fig. 5, A and B, and fig. S5D). Moreover, injection of IL-1RA neutralizing antibody significantly inhibited gingival wound healing at 3, 5, and 7 days after wound creation compared to the control mice (3 days, P<0.05; 5 days, P<0.001; 7 days, P<0.001) (Fig. 5C and fig. S5E). Because IL-1RA was also detected in SMSCs near the margin of cutaneous wound areas (fig. S5F), we showed that *IL-1RA* knockout mice have delayed wound healing in a fullthickness square cutaneous wound model at 5, 7, 10, and 14 days after wound creation when compared to WT control mice (fig. S5, G to I). This effect was confirmed in a circular cutaneous wound model (fig. S5J). These data suggest that MSC-secreted IL-1RA plays a crucial role in gingival and cutaneous wound healing.

To further examine the role of IL-1RA in wound healing, anakinra, a Food and Drug Administration (FDA)–approved form of recombinant IL-1RA, was locally injected into wounds. We found that gingival wound healing was significantly accelerated by administration of anakinra at 3, 5, and 7 days after wound creation compared to the control group (3 days, P < 0.001; 5 days, P < 0.001; 7 days, P < 0.01) (Fig. 5D and fig. S5, K and L). Moreover, administration of anakinra significantly promoted cutaneous wound healing at 5, 7, 10, and 14 days after wound creation compared to the control group (P < 0.001) (Fig. 5, E and F, and fig. S5M). This effect was also confirmed in a circular cutaneous wound model (fig. S5N).

To confirm the effect of GMSC-secreted IL-1RA–sEV on wound healing, we isolated sEVs from WT and *IL-1RA* knockout GMSCs and locally injected them into the gingival wounds in *IL-1RA* knockout mice. We found that administration of WT control GMSC-derived

sEVs significantly promoted gingival wound healing (P < 0.001), whereas injection of IL-1RA knockout GMSC-derived sEVs failed to accelerate the wound healing (Fig. 5, G and H, and fig. S5O). To confirm the effect of TNF-a on GMSC sEV secretion, we isolated sEVs from TNF-a-treated GMSCs and used these sEVs to treat gingival wounds. We found that local injection of TNF-a-treated GMSC-derived sEVs promoted the closure of wound healing at 7 days after wound creation in *IL-1RA* knockout mice as compared to injection of WT control sEVs (Fig. 5, G and H). Similar to gingival wound healing, we isolated sEVs from WT and IL-IRA knockout SMSCs and locally injected these sEVs into the cutaneous wounds in IL-1RA knockout mice. We found that administration of WT SMSC-derived sEVs significantly promoted cutaneous wound healing at 3, 5, 7, 10, and 14 days after wounding in *IL-1RA* knockout mice (P < 0.05), whereas injection of *IL-1RA* knockout SMSC-derived sEVs failed to accelerate the wound healing (fig. S5, P to R). To confirm the effect of TNF-a on SMSC sEVs, we isolated sEVs from TNF-a-treated SMSCs and locally injected these sEVs into cutaneous wounds. We found that administration of TNF-a-treated SMSC-derived sEVs further potentiated wound healing at 7, 10, and 14 days after wound creation in *IL-1RA* knockout mice as compared to injection of sEVs from WT SMSCs (fig. S5, P to R). These results suggest that MSC-derived IL-1RA-EVs can serve as a therapeutic agent for wound healing.

Previous studies reported that diabetic mice showed a delayed wound healing process (44, 45). It would therefore be interesting to know whether diabetic GMSCs have similar characteristics to normal GMSCs. We showed that GMSCs from diabetic mice had reduced capacity to secrete sEV proteins to the culture supernatant (fig. S6A). Moreover, GMSCs from diabetic mice showed reduced IL-1RA secretion into the culture supernatant, along with elevated cytoplasmic accumulation of IL-1RA and decreased Fas expression when compared to WT GMSCs (fig. S6, B and C). Streptozotocin-induced type 1 diabetic mice exhibited significantly delayed gingival and skin wound healing compared to WT mice (gingival, P < 0.001; skin, P < 0.05) (fig. S6, D to I). Injection of WT GMSC-derived sEVs partially rescued the delayed wound healing; however, injection of *IL-1RA* knockout GMSC-derived sEVs failed to rescue the delayed wound healing in diabetic mice (fig. S6, D to I). These results suggest that sEVs containing IL-1RA can partially ameliorate the delayed wound healing process in diabetic mice.

#### Fas-controlled IL-1RA secretion regulates wound healing in the gingiva and skin

Next, we examined whether Fas-controlled IL-1RA release governs the wound healing process. Immunohistofluorescence staining showed that Fas was coexpressed with IL-1RA in both gingival and cutaneous wound sites in WT mice (fig. S7, A and B). Fas-deficient MRL/*Ipr* mice exhibited significantly delayed gingival wound healing at 5 and 7 days after wounding compared to WT control mice (P < 0.001), and injection of anakinra partially rescued the delayed wound healing (Fig. 6, A and B, and fig. S7C). In addition, MRL/*Ipr* mice exhibited significantly delayed cutaneous wound healing at 5, 7, 10, and 14 days after wounding compared to the WT control mice (5 days, P < 0.01; 7 days, P < 0.01; 14 days, P < 0.001), and injection of anakinra partially rescued the delayed wound healing in MRL/*Ipr* mice (Fig. 6, C and D, and fig. S7D). These results suggest that Fas may control the IL-1RA release in MSCs and, thus, govern the wound healing process. In summary, these results

suggest that MSCs use the Fas/Fap-1/Cav-1 axis to regulate SNARE-mediated IL-1RA–sEV release to regulate wound healing in the gingiva. TNF-a serves as an activator to up-regulate Fas and Fap-1 to enhance IL-1RA–sEV release (fig. S8).

# DISCUSSION

The trafficking of secretory vesicles to the plasma membrane of eukaryotic cells is essential for intercellular communication through the release of a variety of extracellularly acting molecules. Secretory vesicles and some extracellular molecules (plasma proteins, antibodies, and extracellular matrix components) are also secreted by a constitutive exocytotic pathway (17, 46). Regulation of exocytosis has been studied in a wide range of cell types specialized to secrete large amounts of secretory products, including neuroendocrine, endocrine, and exocrine cells. Most exocytotic secretory processes are mediated through mechanisms with SNARE protein–dependent exocytotic fusion (17, 18). Cells that use this mechanism include astrocytes, which are cells of the central nervous system that secrete chemical mediators (47), and  $\beta$  cells, which are cells of the endocrine system that secrete insulin (48). However, it is unknown whether and how stem cells use exocytotic fusion mechanisms to secrete EVs.

The secretion of cytokines by immune cells in response to inflammatory or infectious stimuli plays an important role in the regulation of immune response. The trafficking machinery and secretory (exocytotic) pathways are complex and highly regulated in immune cells. The majority of secretory cytokines contain N-terminal or internal signal peptides that direct their sorting into the endoplasmic reticulum (ER) for synthesis as either soluble or transmembrane precursors and are then transported to the extracellular space or the plasma membrane through the ER-Golgi secretory pathway, which is called the conventional secretory pathway (49). Secretion of TNF by macrophages is an example of the conventional secretory pathway that proceeds via granule-mediated and constitutive routes. MSCs secrete a variety of growth factors, immunomodulatory cytokines, and exosomes (13–15, 24), serving as paracrine regulators. However, whether MSCs serve as secretory cells, using a unique mechanism to release cytokines and EVs, is largely unknown.

Here, we used MSCs as a model to dissect a distinctive cytokine secretion mechanism in stem cells in which Fas/Fap1/Cav1 machinery controls SNAP25/VAMP5–associated IL-1RA–sEV exocytosis. Cav-1 is the main component of lipid rafts that dynamically participate in a number of cellular processes, including signal transduction, lipid regulation, and membrane trafficking (50). Cav-1 is known to bind to Fas and is perhaps involved in the apoptotic process (39). As expected, *Cav-1* knockout mice showed impaired cutaneous wound healing (51). Cav-1 also binds with SNAP25 and is associated with neurosecretion (52). It is known that Fap-1, a protein tyrosine phosphatase, binds with Fas (38). We found that Fap-1 also binds with Cav-1. Recent studies reveal that interactions between synaptotagmin-1 and the SNARE complex can promote neuronal exocytotic fusion (53). Rab27a and Rab27b control multivesicular endosomes docking at the plasma membrane to regulate exosome secretion in HeLa cells (54). Our findings identify a role for the Fas/Fap-1/Cav-1 complex in MSC-mediated IL-1RA–sEV secretion.

The surrounding microenvironment may stimulate paracrine factor production to promote MSC-mediated tissue homeostasis. Once MSCs have encountered the microenvironment of injured tissues, many factors, including inflammatory cytokines (such as TNF- $\alpha$ , IFN- $\gamma$ , and IL-1), toxins of infectious agents, and hypoxia, can stimulate the release of various regulatory molecules (14). Elevation of internal  $Ca^{2+}$  concentrations can activate astrocytes to initiate inter-cellular communication by SNARE-dependent glutamate vesicle release (55), and glucose stimulates SNARE-dependent insulin vesicle exocytosis in  $\beta$  cells (48). Components of the microenvironment, including the wound milieu, may stimulate paracrine factor production to promote MSC-mediated tissue repair and immune regulation. We found that TNF-a is able to activate sEV/IL-1RA release via up-regulating Fas/Fap-1 expression. It is known that the interaction of TNF- $\alpha$  with TNF- $\alpha$ R may activate the NF- $\kappa$ B pathway and induce the expression of prosurvival genes including B cell lymphoma 2 (Bcl-2), X-linked inhibitor of apoptosis protein (XIAP), and the FLICE-inhibitory protein (FLIP); furthermore, FLIP can directly inhibit caspase-mediated cell apoptosis (41, 56). Here, we found that TNF-a activates IL-1RA release in MSCs via the NF-kB pathway to promote wound healing. Supporting evidence for this conclusion came from a previous study that also showed that TNF-a could promote human MSCs to secrete growth factors such as vascular endothelial growth factor (40).

Oral gingival/mucosal wounds heal faster than cutaneous ones, with minimal scar formation (28) and reduced inflammatory cell infiltration (29–31). A previous study reported the beneficial effects of MSCs on wound healing, by promoting M2 macrophage polarization (57). Recent studies showed that MSC-derived exosomes are capable of transporting active proteins and microRNAs to communicate with the extracellular environment (13, 16, 58, 59). We also showed that sEVs from GMSCs and SMSCs contain many cytokines, among which GMSC-derived sEVs contain a significantly higher amount of IL-1RA. Given that IL-1RA is a natural inhibitor of the proinflammatory cytokine IL-1 $\beta$ , it modulates a variety of IL-1-related immune and inflammatory responses (60-62), thus contributing to the rapid wound healing of the gingiva. MSC-produced IL-1RA mediates the anti-inflammatory and antifibrotic effect during lung injury in mice (63). Also, MSC-derived IL-1RA promotes macrophage polarization and inhibits B cell differentiation (64). IL-1RA-deficient mice show delayed cutaneous wound healing, spontaneous and lethal arteritis, and destructive arthritis (43, 65, 66). Delivery of anakinra, a commercially available form of IL-1RA approved by the FDA for treating rheumatoid arthritis and neonatal-onset multisystem inflammatory disease (67, 68), into the wound area can reduce fibrotic invasion (69).

It is well known that, unlike human skin wounds, mouse cutaneous wounds heal largely via contraction, due to the nature of their loose skin with dense hair follicles. This might explain why we only observed significant differences of wound closure at the later stages (5 days after surgery). We found that *IL-1RA* knockout mice exhibited delayed wound healing in both the gingiva and skin. Blockage of IL-1RA by submucosal injection of IL-1RA neutralizing antibody at wound sites resulted in impaired gingival wound healing. Conversely, injection of anakinra accelerated gingival and cutaneous wound healing, thus highlighting the role of IL-1RA in promoting wound healing. MSC-derived exosomes may also affect the wound healing process by other mechanisms, such as by delivery of Wnt4 to promote proliferation of skin cells and by promoting collagen synthesis and angiogenesis in

the wounded area (25, 26). We found that WT MSC-derived, but not IL-1RA knockout MSC-derived, EVs promoted delayed wound healing in IL-1RA knockout or diabetic mice. These results suggest that MSC-derived IL-1RA-sEV plays an important role in gingiva and cutaneous wound healing. Exosome membrane proteins can interact with receptors in a target cell or can be cleaved by proteases in the extracellular space to act as soluble ligands that bind to the target cell surface (70). A previous study showed that exosomes carry active Wnt proteins on their surface to induce Wnt signaling (71). Here, we found that IL-1RA was detected on exosome-like EV membranes. This might explain why IL-1RA-sEV effectively communicated with the extracellular environment in wound areas. In addition, sEVs may contain the consensus leader peptide-containing isoform of IL-1RA, which may bind to a receptor on sEV surface after targeting the luminal side of ER-Golgi secretory vesicles. Further research is required to dissect the detailed mechanism(s) by which intracellular isoforms of IL-1RA are shuttled to the membrane of exosome-like EVs. Other oral cavity factors such as saliva and the microbiome may also affect gingival wound healing, and the effect of epidermal stem cells is particularly important in the process of epithelialization during cutaneous wound healing. Nevertheless, these studies support the notion that IL-1RA-sEV plays a crucial role in regulating wound healing in both the gingiva and the skin and suggest that anakinra and IL-1RA-positive sEVs can be used to improve cutaneous wound healing.

Here, we found that MSCs release IL-1RA using a mechanism by which Fas/Fap-1/Cav-1 regulate dynamic exocytosis of IL-1RA–EV. TNF-a serves as an activator to promote IL-1RA–sEV exocytosis from MSCs. Our previous study showed that MSC-derived sEVs are able to rescue Fas-deficient MSCs through a Fas reuse mechanism to improve miR-29b release in recipient MSCs (58). These pieces of evidence suggest that sEVs play a crucial role in biological cross-talk between MSCs and surrounding cells or recipient cells. This study identifies an sEV/cytokine secretion mechanism used by MSCs that may play a crucial role in MSC-based wound healing therapies.

# MATERIALS AND METHODS

#### Study design

Here, we used MSCs as a model to examine whether stem cells have a unique exocytic fusion mechanism to release EVs and whether gingival MSCs secrete a high amount of EVs with IL-1RA to contribute to accelerated wound healing. Three experimental studies were designed: (i) We identified GMSC production and secretion of IL-1RA–expressing sEVs using ultracentrifugation and sucrose gradient, immunoelectron microscopy, colocalization of IL-1RA with EV markers observed by STED microscopy, living cell exocytosis of IL-1RA–EGFP/CD63-mCherry double-positive exosome-like EVs observed by TIRF microscopy, ELISA, and Western blotting; (ii) we used Western blotting, ELISA, immunocytofluorescence staining, and co-IP in WT, gene knockout, and siRNA-treated MSCs to reveal that MSCs use the Fas/Fap-1/Cav-1 axis to regulate SNARE-mediated EVs and IL-1RA/sEVs in accelerating gingival and cutaneous wound healing in WT, *IL-1RA* knockout, MRL/*Ipr*, and diabetic mouse models. The sample sizes for the in vivo studies

were based on the resource equation because the effect size was unknown. Exact numbers for each experiment are included in the figure legends. The investigators were not blinded when conducting or evaluating the experiments. Mice were randomly assigned to the treatment and control groups. Individual subject-level data for experiments where n < 20 are included in table S1.

## Animals

Female C57BL/6J, C3H/HeJ, B6.129S-*II1rn<sup>tm1Dih</sup>*/J (*IL-1RA* knockout), C3MRL-*Fas<sup>lpr</sup>*/J (MRL/*lpr*), and *Cav1<sup>tm1Mls</sup>*/J (*Cav-1* knockout) mice were purchased from the Jackson Laboratory. Age-matched 8- to 10-week female mice from the same background were used in all experiments. All animal experiments were performed under institutionally approved protocols for the use of animal research (University of Pennsylvania Institutional Animal Care and Use Committee, #805478).

#### Antibodies and reagents

Anti–IL-1RA and VAMP5 antibodies were purchased from Abcam. Anti-Fas antibody was purchased from Millipore. Anti–Fap-1, Cav-1, SNAP25, CD105, CD90, CD44, CD63, CD9, and CD81 antibodies were purchased from Santa Cruz Biotechnology. Anti–p-NF- $\kappa$ B p65 and NF- $\kappa$ B p65 antibodies were purchased from Cell Signaling. Anti– $\beta$ -actin antibody was purchased from Sigma-Aldrich. Alexa Fluor 488 and Alexa Fluor 568 secondary antibodies were purchased from Invitrogen. Protein A/G PLUS-Agarose was purchased from Santa Cruz Biotechnology. NF- $\kappa$ B inhibitor ammonium PDTC was purchased from Sigma-Aldrich.

#### Isolation of mouse and human bone marrow, gingival, and skin MSCs

Stem cells from mouse and human tissue were isolated and cultured as reported by other groups (72, 73) and our previous studies (4, 9, 32, 33, 74-80). Briefly, gingival and skin tissues from mice were gently separated, minced, and digested with solution containing collagenase type I (2 mg/ml) (Worthington Biochemical) and dispase II (4 mg/ml) (Roche Diagnostics) in phosphate-buffered saline (PBS) for 1 hour at 37°C. Bone marrow cells were flushed out from the bone cavities of femurs and tibias with 2% heat-inactivated fetal bovine serum (FBS; Equitech-Bio) in PBS. Single-cell suspensions from the gingiva, skin, or bone marrow were obtained by passing the cells through a 70- $\mu$ m strainer (BD Biosciences). All nucleated cells were seeded on 100-mm culture dishes (Corning) in complete media containing a-minimum essential medium (a-MEM) (Invitrogen) supplemented with 20% FBS, 2 mM l-glutamine (Invitrogen), 55 µM 2-mercaptoethanol (Invitrogen), penicillin (100 U ml<sup>-1</sup>), and streptomycin (100  $\mu$ g ml<sup>-1</sup>) (Invitrogen), followed by an initial incubation for 48 hours at 37°C and 5% CO2. The cultures were washed with PBS twice to eliminate the nonadherent cells. Attached cells were cultured for another 12 days under the same conditions in the complete medium mentioned above. Skin MSCs were isolated and cultured using the same procedure described for gingival MSCs. These single colonies were passaged with frequent medium changes to eliminate potential hematopoietic cell contamination (72, 73). We further identified these MSCs and demonstrated that BMMSCs, GMSCs, and SMSCs showed the capacity of self-renewal, assessed by fibroblast colony-forming units and high rate of proliferation (fig. S9). Flow cytometric analysis confirmed that these MSCs

were positive for the MSC surface markers CD44, CD90, CD105, and Sca-1, but were negative for the hematological markers CD34 and CD45 (fig. S9). These MSCs also showed the capacity for multipotent differentiation, including osteogenic, adipogenic, chondrogenic, and neurogenic differentiation (fig. S9).

Human bone marrow aspirates from healthy human adult volunteers (20 to 35 years of age) were purchased from AllCells LLC. Human GMSCs and SMSCs were isolated from the gingival and skin tissues obtained as remnants of discarded human tissues under an approved Institutional Review Board protocol at the University of Pennsylvania. Human gingival and skin tissues were treated aseptically and incubated overnight at 4°C with dispase (2 mg/ml) to separate the epithelial and lower spinous layers. The tissues were minced into 1- to 3- mm<sup>2</sup> fragments and digested at 37°C for 2 hours in sterile PBS containing collagenase IV (4 mg/ml) (Worthington Biochemical). The dissociated cell suspension was filtered through a 70- $\mu$ m cell strainer; plated in 100-mm culture dishes with  $\alpha$ -MEM containing 10% FBS, penicillin (100 U ml<sup>-1</sup>), streptomycin (100  $\mu$ g ml<sup>-1</sup>), 2 mM l-glutamine, and 10 mM l-ascorbic acid phosphate; and cultured at 37°C in a humidified tissue culture incubator with 5% CO<sub>2</sub>. After 48 hours, the nonadherent cells were removed. The plastic-adherent confluent cells were passaged with 0.05% trypsin containing 1 mM EDTA and continuously subcultured and maintained in the complete growth medium.

#### Isolation and characterization of EVs

Cells were cultured in exosome-depleted medium (complete medium depleted of FBSderived exosomes by overnight centrifugation at 100,000g) for 48 hours. EVs from culture supernatants of  $12 \times 10^6$  MSCs were isolated by differential centrifugation, as described in the literature (35), at 300g for 10 min, 3000g for 10 min, 20,000g for 30 min, and 120,000g for 70 min. After differential centrifugation, we used a sucrose cushion to purify sEVs as described in previous literature (35, 36, 81, 82). Briefly, a crude sEV pellet from  $12 \times 10^6$ cells was resuspended in PBS, underlain by a single cushion composed of 30% sucrose prepared in tris/D<sub>2</sub>O, and centrifuged at 110,000g for 3 hours at 4°C. The ability of D<sub>2</sub>O to readily diffuse with H<sub>2</sub>O across the discontinuous interphase allowed for the formation of a continuous minigradient between 1.10 and 1.18 g/cm<sup>3</sup> (82), which matched the ideal gradients for MSC exosome isolation (37). The sEVs captured within the sucrose layer were collected, washed with PBS twice, and centrifuged at 110.000g for 90 min at 4°C. The purified sEVs were further verified by transmission electron microscopy and Western blotting analysis. For analysis of exosome protein secretion, we used a Bradford protein assay (Bio-Rad Laboratories) to measure the amount of total protein in purified exosomes from  $12 \times 10^6$  MSCs. Total exosome protein was normalized to  $1 \times 10^6$  cells to show the amount as micrograms per  $1 \times 10^6$  cells. To further confirm exosomes containing IL-1RA, sucrose gradient-purified exosomes were collected as previously described (37, 83). Briefly, a crude exosome pellet from  $24 \times 10^6$  cells was mixed with 2.5 M sucrose, loaded at the bottom of an ultracentrifuge tube, and overlaid with a gradient of decreasing concentrations of sucrose (2.0 to 0.4 M). Tubes were centrifuged for 16 hours at 4°C at 200,000g. After centrifugation, 10 fractions were collected from the top of the tube. Total proteins from the concentrated fractions were loaded for Western blotting analysis to detect IL-1RA.

#### Wound healing in mice

Full-thickness circular gingival wounds with a diameter of 1.5 mm were made in the palates of the mice with a biopsy punch. Full-thickness square excision wounds  $(1 \text{ cm} \times 1 \text{ cm})$  were created by marking the area of the wound on the mid-backs of the mice with a fine marker and a ruler, lifting the skin with a pair of a forceps, and excising the full-thickness skin along the lines with a pair of surgical scissors. To create circular cutaneous wounds, 4-mmdiameter full-thickness wounds were made in the mid-backs of the mice with a biopsy punch. After the surgery on day 1, the wounds were topically submucosally injected with placebo (0.9% saline), IL-1RA neutralizing antibody (10  $\mu$ g per mouse), or IL-1RA drug (anakinra, 100  $\mu$ g per mouse for gingival wounds and 500  $\mu$ g per mouse for cutaneous wounds). For in vivo sEV treatments of gingival wounds, the wounds were submucosally injected with either placebo or sEVs (40  $\mu$ g) suspended in PBS (40  $\mu$ l). For cutaneous wounds, the wounds were subcutaneously injected with either placebo or sEVs  $(200 \mu g)$ suspended in PBS (200  $\mu$ l) on the basis of previous reports (25, 26). The gingival wound images were taken via a stereoscope after sacrifice of the animal at the indicated time point. A series of digital photographs of the cutaneous wounds was taken, including a ruler for scale. At the indicated time points, the wound areas (for gingival wounds) or percentage of wound closure (for cutaneous wounds) was quantified on photographs using Adobe Photoshop 7.0.1 software (Adobe Systems). Changes in the area of the cutaneous wounds were expressed as a percentage of the initial wound area.

#### Western blotting

Cells and purified exosomes were lysed in M-PER mammalian protein extraction reagent (Thermo Fisher Scientific) with protease and phosphatase inhibitors (Roche), and proteins were quantified using a protein concentration assay (Bio-Rad Laboratories). For Western blotting of cell lysis, 20  $\mu$ g of proteins was separated by SDS-polyacrylamide gel electrophoresis and transferred to 0.2-µm nitrocellulose membranes (Millipore). For Western blotting analysis of exosome, exosome protein was isolated from the culture supernatant of 4  $\times$  10<sup>6</sup> cells, and 10 µg of total exosome proteins was loaded. The membranes were blocked with 5% nonfat dry milk and 0.1% Tween 20 for 1 hour, followed by incubation overnight with the primary antibodies diluted in blocking solution according to the manufacturer's instructions. Antibodies to mouse Fas (05-351) were purchased from Millipore. Antibodies to mouse Fap-1 (sc-15356), Cav-1 (sc-894), and SNAP25 (sc-7538) were purchased from Santa Cruz Biotechnology. Antibodies to mouse IL-1RA (ab124962) and VAMP5 (ab85581) were purchased from Abcam. Antibody to mouse β-actin (A5441) was purchased from Sigma-Aldrich. The membranes were then incubated under room temperature for 1 hour in species-related horseradish peroxidase-conjugated secondary antibody (Santa Cruz Biotechnology) diluted at 1:10,000 in blocking solution. Immunoreactive proteins were detected using SuperSignal West Pico Chemiluminescent Substrate (Thermo Fisher Scientific) and Biomax film (Kodak); the sensitivity of this substrate can be used to detect low-picogram amounts of protein in polyvinylidene difluoride membrane. The relative density was measured using ImageJ 1.49v software (Wayne Rasband). The quantification of Western blotting for total EV protein was normalized against the control group or GMSC group, and the quantification of the other Western blotting experiments was normalized

against loading control  $\beta$ -actin. Western blotting films corresponding to Figs. 1 to 4 are shown in database S1.

#### Immunoprecipitation

Cells were lysed in M-PER mammalian protein extraction reagent (Thermo Fisher Scientific) with protease and phosphatase inhibitors (Roche), and proteins were quantified using a protein concentration assay (Bio-Rad Laboratories). One microgram of the appropriate control immunoglobulin G, together with 20  $\mu$ l of resuspended protein A/G PLUS-Agarose, was added to precleared lysate and incubated at 4°C for 30 min. Pellet beads were centrifuged at 2500 rpm for 5 min at 4°C, and the supernatant with total cellular protein (300  $\mu$ g) was transferred to a fresh centrifuge tube on ice. Primary antibody was added and incubated overnight at 4°C. Twenty microliters of resuspended protein A/G PLUS-Agarose was added at 4°C. After 2 hours, the tubes were centrifuged at 2500 rpm for 5 min at 4°C and the immunoprecipitates were collected. The beads were pelleted and washed with radioimmunoprecipitation assay buffer. Beads were then pelleted, washed, and resuspended in 40  $\mu$ l of electrophoresis sample buffer. The samples were boiled at 90°C for 5 min, and 20- $\mu$ l aliquots were subjected to Western blotting analysis.

#### siRNA knockdown, CRISPR/Cas9 knockout, and cytokine treatments

For siRNA knockdown, GMSCs  $(0.2 \times 10^6)$  were seeded on a six-well culture plate. Fas, FAP-1, Cav-1, SNAP25, and VAMP5 siRNAs (Santa Cruz Biotechnology) were used to treat the GMSCs according to the manufacturer's instructions. Nontargeting control siRNAs (Santa Cruz Biotechnology) were used as negative controls. Fap-1 CRISPR (clustered regularly interspaced short palindromic repeats)/Cas9 (CRISPR-associated protein 9) knockout plasmid (sc-422505, Santa Cruz Biotechnology) was used to knock out Fap-1 expression in GMSCs according to the manufacturer's instructions. Briefly, GMSCs  $(0.2 \times$  $10^{6}$ ) were seeded on a six-well culture plate. The cells were allowed to grow to 40 to 80% confluence and then transfected with Fap-1 CRISPR/Cas9 knockout plasmids using Lipofectamine LTX with Plus reagent (Life Technologies) according to the manufacturer's instructions. Scrambled guide RNA CRISPR/Cas9 plasmids were used as a negative control. The efficiency of siRNA knockdown and CRISPR/Cas9 knockout was confirmed by Western blot analysis. For cytokine treatments, GMSCs were treated with different concentrations of IFN-y and TNF-a (Peprotech; 0, 20, 50, 100, and 200 ng/ml) or TNF-a (20 ng/ml) for 24 hours. After transfection or cytokine treatment, cells were used for protein extraction for Western immunoblotting, and the culture supernatants were used for ELISA.

#### Plasmid transfection, TIRF, and STED microscopy

For protein tracing, IL-1RA–EGFP fusion protein expression plasmids (System Biosciences) and CD63-mCherry fusion protein expression plasmids (a gift from C.G., University of Pennsylvania) were used. Empty plasmids with the same backbone were used as a control. The cells were transfected with plasmids using Lipofectamine LTX with Plus reagent (Life Technologies) according to the manufacturer's instructions. For IL-1RA–positive vesicle colocalization studies, WT GMSCs were transfected with plasmids expressing IL-1RA–EGFP fusion protein and fixed with 4% paraformaldehyde. Next, the cells were incubated with anti-CD63 or anti-CD81 antibodies and imaged using a super-resolution STED

microscope (Leica Microsystems). To visualize IL-1RA–positive vesicle dynamics, WT GMSCs were transfected with plasmids expressing IL-1RA–EGFP fusion protein, and the vesicular organelles were stained with LysoTracker. To visualize IL-1RA–positive exosome dynamics, WT GMSCs were cotransfected with plasmids expressing IL-1RA–EGFP fusion protein and CD63-mCherry fluorescent protein. Forty-eight hours after transfection, the GMSCs were treated with TNF- $\alpha$  (20 ng/ml), and the dynamics of IL-1RA–positive microvesicle exocytosis were monitored in living GMSCs using TIRF microscopy. Briefly, about  $3.0 \times 10^4$  transfected cells were plated on an eight-well chambered cover glass (Lab-Tek II, Nunc) in the imaging medium Dulbecco's modified Eagle's medium–Hepes without phenol red (Thermo Fisher Scientific). Cells were imaged at a rate of 1 frame/s using a Leica TIRF microscope equipped with adaptive focus control and an environmental control system set to 5% CO<sub>2</sub> and 37°C. Fluorophores were excited with solid-state 488- and 561-nm lasers, TIRF angle was set to 90 nm depth, and images were acquired using a Photometrics Evolve EMCCD camera. Images were analyzed and processed using FIJI software.

#### Statistics

Comparisons between two groups were analyzed using independent unpaired two-tailed Student's *t* tests, and comparisons between more than two groups were analyzed using one-way analysis of variance (ANOVA) with Bonferroni correction. *P* values less than 0.05 were considered statistically significant.

# **Supplementary Material**

Refer to Web version on PubMed Central for supplementary material.

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#### Fig. 1. Murine gingival MSCs and skin MSCs produce and secrete IL-1RA–EV.

(A) Total protein contained within small extracellular vesicles (sEVs) isolated from the culture supernatant of  $1 \times 10^6$  murine human bone marrow mesenchymal stem cells (BMMSCs), gingiva-derived MSCs (GMSCs), and skin MSCs (SMSCs) (n = 3). (B) Western blotting and semi-quantification analysis of CD63, CD9, and CD81 expression from sEVs isolated from GMSCs and SMSCs. (C) Differential centrifugation and sucrose cushion procedure for the isolation of EVs from MSC culture supernatants (SN). (D) Interleukin-1 receptor antagonist (IL-1RA), CD63, CD9, and CD81 expression in lysates from fractions corresponding to (C). (E) Super-resolution stimulated emission depletion staining and quantification for IL-1RA-enhanced green fluorescent protein (EGFP) (green), CD63 (red), and CD81 (red) in GMSCs transfected with plasmids containing IL-1RA-EGFP fusion protein. The lower right box is a higher magnification of the boxed region in the merged image; colocalization of IL-1RA with CD63 or CD81 is shown in yellow (n = 5). Scale bar,  $10 \,\mu\text{m}$ . (F) Total internal reflection fluorescence (TIRF) microscopy images from GMSCs cotransfected with plasmids expressing IL-1RA-EGFP (green) and CD63-mCherry (red). The top right panel is a higher magnification of the boxed region in the left image; colocalization of IL-1RA-EGFP and CD63-mCherry is shown in yellow. The bottom panels (1 to 4) show sequential images from live-cell imaging. Arrows indicate two individual IL-1RA-positive vesicle fusion events. Scale bar, 10 µm. (G) Enzyme-linked immunosorbent assay (ELISA) of IL-1RA from the culture supernatant of GMSCs and SMSCs (n = 3). (H) Western blotting and semi-quantification analysis of IL-1RA expressed by GMSCs and SMSCs. (I) Immunocytofluorescence staining of IL-1RA (green) and the

MSC marker CD105 (red) in GMSCs and SMSCs. Scale bar, 20  $\mu$ m. (**J** and **K**) Real-time polymerase chain reaction analysis of soluble IL-1RA (sIL-1RA) mRNA (J) and intracellular IL-1RA (icIL-1RA) mRNA (K) in GMSCs and SMSCs. All results are representative of data generated in at least three independent experiments (J and K) (n = 6). \*\*P < 0.01, \*\*\*P < 0.001. Error bars are means  $\pm$  SD. Data were analyzed using one-way analysis of variance (ANOVA) with Bonferroni correction (A), or independent un-paired two-tailed Student's *t* tests (B, G, H, J, and K).

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#### Fig. 2. Fas controls IL-1RA–sEV secretion in murine MSCs.

(A) Western blotting and semi-quantification of Fas expression in GMSCs and SMSCs (n =3). (B) Secreted sEV-associated protein quantification from Fas-deficient MRL/*lpr* and wildtype (WT) control GMSCs (n = 5). (C) Western blotting and semi-quantification analysis of CD63, CD9, CD81, and IL-1RA from sEV from Fas-deficient MRL/lpr and WT control GMSCs. sEV-associated proteins from culture supernatants of equal numbers of cells in control and MRL/lpr GMSC groups were loaded (n = 3). (D) ELISA analysis of secreted IL-1RA from the culture supernatant from WT control and Fas-deficient MRL/lpr GMSCs (n = 3). (E) Western blotting and semi-quantification analysis of cytoplasmic IL-1RA from WT control and Fas-deficient MRL/lprGMSCs (n = 3). (F) Immunocytofluorescent double staining of IL-1RA (green) and Fas (red) in WT control and Fas-deficient MRL/Ipr GMSCs. Dashed lines indicate the cell edge. Scale bar, 20 µm. (G) ELISA analysis of IL-1RA secretion in the culture supernatant of MRL/lpr and Fas-overexpressing MRL/lpr GMSCs (n = 5). (**H**) Western blotting and semi-quantification analysis of cytoplasmic IL-1RA from MRL/lpr and Fas-overexpressing MRL/lpr GMSCs (n = 3). (I) ELISA analysis of IL-1RA secretion in the culture supernatant from WT control GMSCs treated with and without Fas small interfering (siRNA) (n = 5). (J) Western blotting and semi-quantification of cytoplasmic IL-1RA and Fas from WT control GMSCs treated with or without Fas siRNA (n = 3). All results are representative of data generated from at least three independent experiments. \*\*P < 0.01, \*\*\*P < 0.001. Error bars are means  $\pm$  SD. All data were analyzed using independent unpaired two-tailed Student's t tests.



Fig. 3. Fas binds with Fap-1 and Cav-1 to regulate IL-1RA-sEV release in murine GMSCs. (A) Fas co-IP of WT control and MRL/Ipr GMSC lysate. (B) Immunocytofluorescence double staining for Fas, Fap-1, and Cav-1 in WT GMSCs. (C) Secreted sEV-associated protein quantification from WT control, Fap-1, and Cav-1 knockout GMSCs (n = 3). (**D**) Western blotting and semi-quantification of CD63, CD9, CD81, and IL-1RA in sEVs from WT control, Fap-1, and Cav-1 knockout GMSCs. Culture supernatants from equal numbers of cells from control and knockout GMSCs were loaded for Western blotting analysis (n = 3). (E) Western blotting and semi-quantification of Fas, Fap-1, Cav-1, and IL-1RA expression in WT control, MRL/Ipr, Fas-deficient, Fap-1 knockout, and Cav-1 knockout GMSCs (n = 3). (F) ELISA analysis of secretion of IL-1RA in the culture supernatant from WT control, Fap-1-deficient, and Cav-1-deficient GMSCs (n = 5). (G) Immunoprecipitation (IP) and semi-quantification analysis of Fas from WT control, Fap-1-deficient, and Cav-1 knockout GMSC lysates (n = 3). (H) IP and semi-quantification analysis of Fap-1 from WT control, MRL/*lpr*, *Cav-1* knockout, and *Fap-1* knockout GMSC lysates (n = 3). (I) IP and semi-quantification analysis of Cav-1 from WT control, MRL/lpr, Fap-1 knockout, and *Cav-1* knockout GMSCs (n = 3). (J) Immunocytofluorescence staining of Cav-1 in WT, Fasdeficient MRL/lpr, and Fap-1 knockout GMSCs, and double-staining of Fap-1 and Fas in WT and Cav-1 knockout GMSCs. For A-P, Fas-deficient GMSCs from MRL/lpr mice, GMSCs with Fap-1 knocked out using a CRISPR (clustered regularly interspaced short palindromic repeats)/Cas9 (CRISPR-associated protein 9) plasmid, and Cav-1 knockout GMSCs from Cav-1<sup>-/-</sup> mice were used, and WT GMSCs served as a control. For IP, whole-

cell lysates from indicated GMSCs were immunoprecipitated with corresponding antibodies, and the immunocomplexes were subjected to Western blotting with antibodies against Fas, Fap-1, and Cav-1. All results are representative of data generated from three independent experiments. \*P < 0.05, \*\*P < 0.01, \*\*\*P < 0.001. Error bars are means  $\pm$  SD. Scale bars, 20  $\mu$ m. All data were analyzed using independent unpaired two-tailed Student's *t* tests. DAPI, 4',6-diamidino-2-phenylindole; IgG, immunoglobulin G.



Fig. 4. TNF-a up-regulates Fas/Fap-1 expression to promote IL-1RA-sEV release in murine MSCs.

(A) ELISA analysis of IL-1RA secretion into the culture supernatant from GMSCs treated with tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) or interferon- $\gamma$  (IFN- $\gamma$ ) (n = 3). (**B**) Secreted sEVassociated proteins from control or TNF-a (20 ng/ml)-treated GMSCs (n = 6). (C) Western blotting and semi-quantification of CD63, CD9, CD81, and IL-1RA expression in WT control GMSCs with or without TNF-a (20 ng/ml) treatment. sEV-associated proteins from culture supernatants of equal numbers of cells were loaded for Western blotting analysis (n =3). (D) Western blotting and semi-quantification analysis of Fas, Fap-1, Cav-1, and IL-1RA expression in WT GMSCs (left) and MRL/lpr GMSCs (right) treated with or without TNF-a (n = 3). (E) ELISA analysis of secretion of IL-1RA in the culture supernatant in control or MRL/*lpr* GMSCs treated with and without TNF- $\alpha$  (20 ng/ml) (n = 3). (F) Western blotting and semi-quantification of Fas, Cav-1, and IL-1RA in Fap-1 knockout GMSCs with and without TNF- $\alpha$  (20 ng/ml) treatment (n = 3). (G) Western blotting and semi-quantification of Fas, Fap-1, and IL-1RA in Cav-1 knockout GMSCs with and without TNF-a (20 ng/ml) treatment (n = 3). (H) ELISA analysis of IL-1RA in the culture supernatant of WT control, Fap-1, and Cav-1 knockout GMSCs treated with and without TNF-a. (20 ng/ml) (n = 3). (I) Immunocytofluorescence staining of GMSCs at various time points after TNF-a. (20 ng/ml) treatment. Scale bar, 20  $\mu$ m. (J) Western blotting and semi-quantification analysis of Fas, Fap-1, and Cav-1 in membrane and cytoplasmic fractions of GMSCs treated with and without TNF-a (20 ng/ml) (n = 3). (K) TIRF microscopy images of IL-1RA–EGFP (green)

and CD63-mCherry (red) cotransfected into WT GMSCs treated with TNF- $\alpha$  (20 ng/ml) for 0.5 hours. The top left panel is a higher magnification of the boxed region in the left image to show colocalization (yellow); the bottom panels show sequential images (1 to 4). Arrows indicate two individual IL-1RA–positive vesicle fusion events. Scale bar, 10  $\mu$ m. \**P*<0.05, \*\**P*<0.01, \*\*\**P*<0.001. Error bars are means ± SD. Data were analyzed using independent unpaired two-tailed Student's *t* tests (A to D, F, G, and J), or one-way ANOVA with Bonferroni correction (E and H).

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# Fig. 5. GMSCs produce IL-1RA, which contributes to gingival wound healing in mice.

(A) Scheme illustrating the gingival wound procedure and treatment with IL-1RA neutralizing antibody. Full-thickness circular wounds were made in the palates of WT control mice and IL-1RA<sup>-/-</sup> mice and submucosally injected one time with placebo (0.9%) saline) or IL-1RA neutralizing antibody (IL-1RA Ab, 10 µg per mouse) 1 day after wound creation. (B) Representative macroscopic images and quantification of gingival wound area in WT control and IL-1RA<sup>-/-</sup> mice. All the gingival wound is outlined in a dashed line (n =5). (C) Representative macroscopic images and quantification of gingival wound area in WT mice after treatment with or without IL-1RA Ab (n = 5). (**D**) Representative macroscopic images and quantification of gingival wound area in WT mice after treatment with or without IL-1RA drug (n = 5). (E) Scheme illustrating cutaneous wound procedure and treatment with IL-1RA drug anakinra. Full-thickness excision cutaneous wounds (1 cm × 1 cm) were created in the mid-backs of WT mice. One day after wound creation, the mice were subcutaneously injected with either placebo (0.9% saline) or the IL-1RA drug anakinra  $(500 \ \mu g \ per \ mouse)$ . (F) Representative macroscopic images and quantification of closure of full-thickness cutaneous wounds in WT mice after treatment with or without anakinra (n =5). (G) Scheme illustrating the gingival wound procedure and administration of sEVs. IL-1RA knockout mice were submucosally injected with placebo (0.9% saline) or WT GMSC-derived, IL-1RA knockout GMSC-derived, or TNF-a-activated GMSC-derived sEVs 1 day after wound creation. (H) Representative macroscopic images and quantification of gingival wound area in IL-IRA knockout mice after treatment with or without sEVs (n =

3 for day 7; n = 5 for day 5). \*P < 0.05, \*\*P < 0.01, \*\*\*P < 0.001. Error bars are means ± SD. Data were analyzed using independent unpaired two-tailed Student's *t* tests (B to F) or one-way ANOVA with Bonferroni correction (H).

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#### Fig. 6. Fas-controlled IL-1RA secretion regulates wound healing in mice.

(A) Scheme illustrating the gingival wound procedure in MRL/*lpr* mice and treatment with the IL-1RA drug anakinra. Full-thickness circular wounds were made in the palates of WT and MRL/*lpr* mice with a biopsy punch, and MRL/*lpr* mice were submucosally injected with either placebo (0.9% saline) or the IL-1RA drug anakinra (100  $\mu$ g per mouse) 1 day after wound creation. (**B**) Representative macroscopic images and quantification of gingival wound area in WT and MRL/*lpr* mice after treatment with and without anakinra (n = 5). (**C**) Representative macroscopic images and quantification of dermal wound area in WT and MRL/*lpr* mice treated with placebo (0.9% saline) or anakinra ( $500 \mu$ g per mice) injected 1 day after wound creation as in (A) (n = 3). \*\*P < 0.01, \*\*\*P < 0.01. Error bars are means ± SD. Data were analyzed using one-way ANOVA with Bonferroni correction (B) or independent unpaired two-tailed Student's *t* tests (C and D).