Published in final edited form as:

Immunobiology. 2020 January ; 225(1): 151862. doi:10.1016/j.imbio.2019.10.011.

CD163 overexpression using a macrophage-directed gene therapy approach improves wound healing in *ex vivo* and *in vivo* human skin models.

David Wilson Ferreira^{1,2,3}, Cristina Ulecia-Morón^{1,4}, Perla Abigail Alvarado-Vázquez^{5,6}, Katharine Cunnane⁵, Carolina Moracho-Vilriales⁷, Rachel L. Grosick⁷, Thiago Mattar Cunha², E. Alfonso Romero-Sandoval^{5,*}

¹These authors contributed equally to this article

²Department of Pharmacology, University of São Paulo, Ribeirao Preto Medical School, 3900 Bandeirantes Ave., Ribeirão Preto, SP, 14049-900, Brazil

³Department of Neurobiology, University of Pittsburgh School of Medicine, 3501 Fifth Ave - BST3, 6th floor, Pittsburgh, PA, 15260, USA. Current address

⁴Center for Biomedical Research Network on Mental Health (CIBERSAM); Avenida Monforte de Lemos, 3-5. Pabellón 11. Planta 0. 28029 Madrid. Department of Pharmacology and Toxicology, School of Medicine, and Instituto Universitario de Investigación en Neuroquímica (IUIN). Complutense University of Madrid. Avenida Complutense s/n. 28040 Madrid

⁵Department of Anesthesiology, Wake Forest School of Medicine, 1 Medical Center Blvd, Winston-Salem, NC, 27157, USA

⁶Department of Medical Biochemistry and Microbiology, BMC, Uppsala University, Husargatan 3, Uppsala, 75123, Sweden

⁷Department of Pharmaceutical and Administrative Sciences, Presbyterian College School of Pharmacy, 307 N Broad St., Clinton, SC, 29325, USA

Abstract

Large tissue damage or wounds cause serious comorbidities and represent a major burden for patients, families, and health systems. Due to the pivotal role of immune cells in the proper resolution of inflammation and tissue repair, we focus our current study on the interaction of macrophages with skin cells, and specifically on the effects of CD163 gene induction in macrophages in wound healing. We hypothesize that the over-expression of the scavenger receptor gene CD163 in human macrophages would result in a more efficient wound healing process.

*Corresponding author: Edgar Alfonso Romero-Sandoval, M.D., Ph.D., earomero.sandoval@gmail.com, Address: Wake Forest School of Medicine, 1 Medical Center Blvd, Winston-Salem, NC 27157, USA. Phone: 336-716-2725.

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Conflict of interest

Declarations of interest: none.

The authors have no conflict of interest to declare.

Using 3D human wounded skin organotypic tissues, we observed that CD163 overexpression in THP-1 and human primary macrophages induced a more efficient re-epithelization when compared to control cells. Using human primary skin cells and an *in vitro* scratch assay we observed that CD163 overexpression in THP-1 macrophages promoted a more rapid and efficient wound healing process through a unique interaction with fibroblasts. The addition of CD163-blocking antibody, but not isotype control, blocked the efficient wound healing process induced by CD163 overexpression in macrophages. We found that the co-culture of skin cells and CD163 overexpressing macrophages reduced monocyte chemoattractant protein (MCP)-1 and enhanced tumor growth factor (TGF)- α , without altering interleukin (IL)-6 or TGF- β . Our findings show that CD163 induces a more efficient wound healing and seems to promote a wound milieu with a pro-resolution molecular profile. Our studies set the foundation to study this approach in *in vivo* clinically relevant settings to test its effects in wound healing processes such as acute major injuries, large surgeries, or chronic ulcers.

Keywords

Gene induction; Nanotechnology; Nanoparticle; Inflammation; Fibroblasts; Postsurgical pain; Keratinocytes

1. Introduction

Chronic ulcers are more common in patients with metabolic conditions (i.e. diabetes, obesity, etc.), representing a large financial burden for families and public health systems (Nussbaum, et al., 2018, Sen, et al., 2009). However, the number of surgeries performed annually in the world is approximately 310 million (Weiser, et al., 2016). In the United States, the number of annual surgeries is estimated to be 48 million (Hall, et al., 2017). This number of surgical procedures in the United States is larger than the number of adults suffering from diabetes (over 9% of the adult population, (Prevention, 2017)), indicating that acute wounds pose a significant and more frequent problem than chronic diabetic wounds. In the context of chronic diseases such as diabetes or chronic vein disease, there are complex metabolic aberrant cellular phenotypes that give rise to chronic wounds (Sindrilaru, et al., 2011, Loots, et al., 1998). However, after acute injuries such as surgeries the degree of invasiveness determines the duration of wound healing. A large proportion of surgeries involve large tissue damage, such as mastectomies, thoracotomies, arthroplasties, amputations, etc. These surgical wounds are considered to be acute, but due to their damage extent and therefore the large period needed for tissue repair, they are more prone to result in chronic postoperative pain than smaller surgeries (Kehlet, et al., 2006). The direct nerve injury, along with the prolonged exposure of peripheral sensory neurons to pro-inflammatory factors results in a persistent neuronal sensitization and subsequent chronic pain (Waxman and Zamponi, 2014, Woolf, 2010). Thus, acute wounds that last longer to heal not only represent a reduction in quality of life and an increased risk of other comorbidities, but also a significant cost in healthcare and lack of productivity (Sen, et al., 2009, Gaskin and Richard, 2012).

Following tissue damage (surgeries, ulcers, burns, wounds, etc.) a series of molecular and cellular events take place to restore homeostasis. A precise dynamic and well-orchestrated immune response is pivotal for the development of inflammation, host protection from external pathogens, tissue remodeling, and eventual tissue repair and wound healing. An alteration in this coordinated process could result in a continuous local inflammatory state and subsequently a hindered resolution of inflammation, which constitutes the pathophysiological basis of delayed wound healing or the development of chronic wounds. One of the key immune cells critically involved in wound healing are macrophages (Kim and Nair, 2019, Koh and DiPietro, 2011). Macrophages are activated during the early stages of tissue damage producing pro-inflammatory mediators and increasing their phagocytic activity to eliminate necrotic tissue and protect the wound from infection (Kim and Nair, 2019, Shi and Pamer, 2011). At a later stage, macrophages coordinate the resolution of the inflammatory process and promote wound healing. In this phase, the macrophage phenotype switches from a pro-inflammatory state to an anti-inflammatory or pro-resolution state, which orchestrates the resolution of inflammation, tissue repair, and tissue healing (Mantovani, et al., 2013, Mosser, 2003). An imbalance in this macrophage phenotypic transition can lead to persistent or chronic inflammation (Shi and Pamer, 2011, Classen, et al., 2009). In the context of acute injuries in which macrophages are not metabolically impaired, such as major surgeries with extensive tissue damage, a prolonged inflammatory process could result in chronic inflammation that subsequently impedes the resolution of acute pain (Waxman and Zamponi, 2014, Woolf, 2010, Fujiwara and Kobayashi, 2005, Robinson-Papp, et al., 2015, Lavand'homme, 2015). Thus, fostering a more efficient resolution of inflammation and wound healing following major acute injuries could result in a reduced rate of chronic postoperative pain. Our current study sought to harness macrophage functions to improve wound healing via CD163 gene induction.

CD163 has been proposed as a specific marker for monocytes/macrophages with an anti-inflammatory phenotype. CD163 is a cell-surface glycoprotein receptor that was identified as the "hemoglobin (Hb) scavenger receptor" HbSR (Kristiansen, et al., 2001) for the uptake of Hb released into the plasma and complexed to haptoglobin (Hp) during intravascular hemolysis. When hemoglobin-haptoglobin (Hb-Hp) complexes bind to CD163 in macrophages, they release anti-inflammatory mediators (Kowal, et al., 2011). CD163 expression is highly dependent on the stage of differentiation or activation status of macrophages (Lau, et al., 2004). CD163-expressing macrophages are found during the healing process of acute inflammation, in chronic inflammation, and in wound healing tissues (Verschure, et al., 1989). We have shown that the blockade of CD163 receptor using monoclonal antibodies that binds specifically to the scavenger receptor results in the production of inflammatory mediators, indicating that the CD163 receptor is implicated in the induction of anti-inflammatory responses (Alvarado-Vazquez, et al., 2017). The association of CD163 with the resolution of inflammation has also been temporally associated with the tissue repair phase in a blister injury model in humans (Philippidis, et al., 2004), indicating that CD163 could be a marker of a pro-resolution phenotype in macrophages. We have demonstrated that CD163 induction in human primary macrophages under inflammatory conditions produces changes in cytokine secretion in favor of an anti-inflammatory phenotype (Alvarado-Vazquez, et al., 2017, Alvarado-Vazquez, et al., 2019).

However, the cellular phenotype transitions in macrophages seems to be tissue or disease-dependent (Mantovani, et al., 2013), and whether CD163-induced phenotype is causatively related to wound healing remains elusive. This study directly addresses this possibility.

Thus far, the potential direct interaction between macrophages and skin cells in the context of wound healing via CD163 has not yet been explored. Therefore, we hypothesize that CD163 gene induction in macrophages will result in a pro-resolution phenotype that would promote a more efficient wound re-epithelization. We used *ex vivo* human organotypic 3D skin tissues and *in vitro* models of wound healing with human primary macrophages, keratinocytes, and fibroblasts. We conducted gene induction in macrophages using nanotechnology as a cell-directed gene therapy approach to target preferentially macrophages, as we have successfully done (Bernal, et al., 2017). Specifically, we used polyethylenimine (PEI) grafted with a mannose receptor ligand (Man-PEI) to induce CD163 gene expression as previously done in our laboratory (Alvarado-Vazquez, et al., 2017, Alvarado-Vazquez, et al., 2019). This modified nanoparticle, Man-PEI, preferentially target cells that express mannose receptors [MR, (Bernal, et al., 2017, Diebold, et al., 1999)]. Interestingly, mannose receptors are expressed primarily in macrophages, but not in undifferentiated monocytes (Ernst, 1998). This technology has been successfully used in HIV positive patients, which provides an enhanced clinical relevance to our approach (Liszewicz, et al., 2012, Liszewicz, et al., 2005). We tested our hypothesis following these specific aims: 1) Evaluate the role of CD163-overexpressing macrophages in the skin re-epithelialization process using 3D (full-thickness) wounded organotypic human tissue; 2) Investigate the functional cell interactions among CD163-overexpressing human macrophages, fibroblasts and/or keratinocytes using the scratch assay - an *in vitro* wound healing model; 3) Determine whether the induction of a more efficient skin cell wound healing by macrophages is specifically due to the overexpression of CD163 utilizing a CD163-blocking antibody; and 4) Determine whether CD163 gene and protein induction in human macrophages produces changes in the release of inflammatory mediators when co-cultured with human primary keratinocytes and fibroblasts.

2. Material and methods

2.1. 3D organotypic human tissue and (H&E) staining.

Small rodents and humans possess very different skin anatomical features, and different skin physiological and pathophysiological mechanisms during wound healing (Zomer and Trentin, 2018). Thus, we sought to utilize a clinically relevant and translational model for our studies instead of rodent models. Hence, we used a 3D organotypic human wounded skin tissue model (de Andrade Lima Chaves, et al., 2014, Nayak, et al., 2013). This organotypic tissues have an organizational and architectural structure that exhibit *in vivo*-like morphological and growth characteristics, mimicking key aspects of cell interactions found in *in vivo* settings in humans (Hu, et al., 2010, Safferling, et al., 2013). The 3D organotypic tissue is derived from human neonatal foreskin tissue in which the epidermis and dermis contain, respectively, functional human keratinocytes and fibroblasts. The tissues consisted of three layers, including the stratum corneum, the epidermis, and the dermis (Safferling, et al., 2013). The presence of these cells allows the skin layers to be mitotically

and metabolically active (Hu, et al., 2010). The epidermal and dermal layers, therefore, exhibit *in vivo*-like morphological and growth characteristics (Safferling, et al., 2013). Despite all these advantageous characteristics, these 3D organotypic tissues do not completely recapitulate an *in vivo* system (e.g. organizational structure, different anatomical skin locations, adult skin properties, etc.), and therefore the interpretation of our results should take this into consideration. Three dimensional full-thickness organotypic human tissues (EpiDermFT) were obtained from MatTek (Ashland, MA). Tissues arrived with a circular wound (3 mm diameter) in the center and were allocated to specific groups in a randomized manner. After an 18-hour incubation/equilibration, organotypic tissues were fixed into 10% formalin (pH 7.4) overnight and stored in PBS (pH 7.4) the following day, and these experiments were used as the baseline time point. After 18 hours of incubation/equilibration period, the remaining tissues were treated with the addition of macrophages (200 μ L, final concentration of 100,000 cells/mL) transfected with either a plasmid encoding CD163 (M-pCD163) or an empty vector (M-pEmpty) and incubated at 37°C in a 5% CO₂ atmosphere. Tissues treated with pEmpty or pCD163 were removed on days 1, 3 and 6 (THP-1 macrophages) and on days 1 and 3 (human primary macrophages), fixed with formalin overnight, and placed into PBS. Tissues were placed in fresh PBS until hematoxylin and eosin (H&E) staining were performed. These procedures were outsourced to the company that generated the tissues (MatTek, Ashland, MA). The company returned slides with mounted tissue slices to our laboratory. Digital pictures were taken of each tissue section (3 section per tissue) at 5X and 20X augmentations to be analyzed using the SigmaScan Pro program (Systat Software Inc., San Jose, CA) for length of re-epithelialization, width of original wound, width of remaining gap, keratinocyte cellularity, and thickness of the newly formed epithelium. The percent (%) wound closure was measured by the following equation: [(length of the original wound – length of the remaining gap)/length of the original wound]*100. The keratinocyte cellularity (epidermis) was quantified by counting cells in pictures (between 1 and 4 pictures) of the re-epithelialized tissue near the unwounded area and the tissue closest to the gap. One picture was taken close to the unwounded area on either side. If the wound was closed or close to being closed, an additional picture was taken in the center of the initially wounded area. The number of keratinocytes in each tissue was calculated as the number of cells in 250 μ m of re-epithelialized tissue. Three sections were analyzed per tissue, and their values were averaged. The same images were used to analyze thickness of the new epithelium. First, we divided the tissues in three sections: the right edge of the growing epithelium, the left edge of the growing epithelium, and the center of the wound. Second, we measured thickness at three different points in each of the segments distributed equally across the analyzed tissue segment. If the wound was not fully closed and there was no epithelium in the center region, thickness was reported as zero for its respective three measurements. Thus, in total we analyzed nine points across the tissue to have a good representation of the thickness throughout the entire new epithelium. The area under the curve (AUC, μ m²) of these nine points was calculated and compared among groups.

2.2. THP-1 cell culture, cell differentiation and stimulation.

Immortalized human acute monocytic leukemia cells (THP-1) were kept at 37°C, 5% CO₂ and in Roswell Park Memorial Institute 1640 media (RPMI 1640, Gibco, Grand Island, NY)

with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin. Cells were incubated with 60 ng/mL phorbol-12-myristate-13-acetate (PMA, Sigma, St. Louis, MO) for 36 hours to differentiate them into macrophages (Landry, et al., 2012). 250,000 cells/mL were plated for 1 hour and then challenged them with LPS (5 µg/mL, *Escherichia coli* O111:B4, Sigma, St. Louis, MO) as we have previously described (Alvarado-Vazquez, et al., 2017, Bernal, et al., 2017). Some cells were also treated simultaneously with plasmids as described below.

2.3. Human primary monocytes cell culture, cell differentiation and stimulation.

We used human peripheral blood CD14⁺ monocytes obtained from a commercial source (LONZA, Lonza Walkersville, MD). Cells from two screened and healthy male donors were used (41-year-old Asian and 42-year-old Caucasian). Cells of each donor were used separately in different assays and experiments. In all cases, cells were thawed, fed every 2-3 days, and maintained in culture for a total of 5-6 days before stimulation or transfection. Cells were processed as described previously by our group (Alvarado-Vazquez, et al., 2017) using RPMI with 10% FBS, 1% penicillin/streptomycin, and 1% sodium pyruvate. Cells were differentiated into macrophages using M-CSF (100 ng/mL, eBioscience, San Diego, CA) for 5-6 days (37°C, 5% CO₂). Macrophages were collected using 500 µL of trypsin per well. We plated 250,000 cells/mL for 1 hour and then we challenge them with LPS. Some cells were also treated simultaneously with plasmids as described below.

2.4. THP-1 and human primary macrophage cell transfection using Man-PEI nanoparticles.

A nanoparticle (polyethylenimine, PEI) grafted with a mannose receptor ligand (Man-PEI; Polyplus Transfection, New York, NY) was used for cell transfection and gene induction as described previously by our laboratory (Alvarado-Vazquez, et al., 2017, Bernal, et al., 2017). Man-PEI was mixed with a cDNA plasmid (pCMV6-XL4 vector, pCD163 or pEmpty, 0.5 µg of plasmid in NaCl, Origene, Rockville, MD) as per manufacturer's instructions. We (Alvarado-Vazquez, et al., 2017, Bernal, et al., 2017) and others (Lisziewicz, et al., 2001) have shown that a nitrogen/DNA phosphate (N/P) ratio of 5 produces gene induction with minimal toxic or immunogenic effects, and this was the N/P ratio used for our current studies. Man-PEI and plasmid complexes (100 µL) were added together with LPS to macrophages (250,000 cells/well). Then, after an incubation period of 48 hours we performed the CD163 mRNA or protein assessments. Macrophages were added to wound healing preparations at this time point, 48 hours after transfection, once CD163 consistently was overexpressed (see below) with our intervention.

2.5. Human primary fibroblasts and keratinocytes cell culture.

Human primary keratinocytes and fibroblasts derived from foreskin of three male neonates discarded after circumcision were a gift from Dr. Kim Creek, University of South Carolina, Columbia (IRB approval number: 2008-10). In all cases, the media was changed every 4-5 days and the cells were cultured through three passages. We used the cells of the second and third passages in all the experiments. Cells were cultured as previously described by our group (Bort, et al., 2017). Briefly, we use DMEM/F12 medium with 2.5 mM of L-glutamine, 10% FBS, and 15 mM of HEPES (Gibco-Life Technologies™, Grand Island, NY) for fibroblast cultures, and for keratinocytes we used a Dermal Cell Basal medium

supplemented with growing factors contained in a keratinocyte growing kit (Catalog number: PCS-200-049, American Type Culture Collection, ATCC, Manassas, VA). Cells were grown separately (37°C, 5% CO₂) until they were completely confluent. Then, cells were trypsinized (Trypsin/EDTA, ethylenediaminetetraacetic acid) and plated at 100,000 cells/mL/well for mono-cellular cultures, or at 50,000 cells/mL/well for fibroblasts and keratinocytes co-culture experiments, i.e. keratinocytes and fibroblasts together.

2.6. Cell viability.

THP-1, human primary macrophages, fibroblasts and keratinocytes were evaluated using the Trypan blue assay (Life Technologies, Grand Island, NY) as described previously (Alvarado-Vazquez, et al., 2017, Bort, et al., 2017). Cells were mixed in 50% Trypan blue and 50% cell culture media and total cells were counted and compared with stained cell counts, which were considered dead cells to obtain the percent of dead cells in our preparations. All our experiments were performed in preparations with more than 90% viable cells.

2.7. Macrophage Co-Culture and Wound Healing Assay.

The effects of THP-1 or human primary macrophages in the scratch assay were tested by co-cultures using skin cells. We cultured human primary keratinocytes and/or fibroblasts and determined the rate in which they can fill a scratch gap made in the culture that mimics wound healing (Walter, et al., 2010, Li, et al., 2015, Liang, et al., 2007). All cell cultures were conducted at 37°C and 5% CO₂. Fibroblast and/or keratinocyte were incubated for 24 hours, and then macrophages were added to the corresponding groups (except for the control groups - fibroblasts and/or keratinocytes only). Previously, macrophages were challenged with LPS and transfected with pEmpty or pCD163 for 48 hours, time in which they were added to the skin cell preparations. For the control group, macrophages received LPS for 48 hours without plasmid transfections. We added 100,000 cells/mL/well of macrophages to the respective groups of skin cell preparations using RPMI with 10% FBS, 1% penicillin/streptomycin (THP-1 and primary macrophages), and 1% sodium pyruvate (primary macrophages only). Then, cells were left for 1 hour and a scratch was made with the tip of a 1 µL pipette. The final volume of medium after the scratch was of 1 mL. We used Dermal Cell Basal medium supplemented with growing factors contained in a keratinocyte growing kit (Catalog number: PCS-200-049, American Type Culture Collection, ATCC, Manassas, VA) for the keratinocyte (K) group and keratinocyte+fibroblast (K + F) group, and DMEM/F12 medium with 2.5 mM of L-glutamine, 10% FBS, and 15 mM of HEPES (Gibco-Life Technologies™, Grand Island, NY) for the fibroblast (F) group. Due to the limited access to primary monocytes, we did not include the control macrophage group (macrophage) for primary cells, cells stimulated with LPS for 48 hours without transfection. The scratch was made consistently in each condition and pictures were taken consistently in the same scratch location.

2.8. Functional blockade of CD163 using antibody.

We used a monoclonal anti-CD163 antibody (Clone RM3/1) to block functional properties of CD163, as it has been shown elsewhere in vitro in monocytes (Philippidis, et al., 2004, Moura, et al., 2012), and we have successfully used to block the transition of macrophages

to an anti-inflammatory-like phenotype (Alvarado-Vazquez, et al., 2017). In our current study we incubated skin cells co-cultured with THP-1 cells with either anti-CD163 antibody clone RM3/1 or its isotype antibody mouse IgG1 (20 µg/mL, Santa Cruz Biotechnology, Santa Cruz, CA, sc-33715, sc-3877 respectively). We applied antibody treatments two times, at 0 and 9 hours after the scratch procedure.

2.9. Imaging and image analysis after scratch assay.

For our current study we followed the methodology previously described by our group (Bort, et al., 2017). Briefly, two marks were made at the bottom of each well and they were used to make the scratch consistently between them. These marks served as a guide to take microscopic images (bright-light) across time accurately on the same scratch location to identify the scratch edges over time. Pictures (4X) of the scratch were taken under the microscope using a Leica Microscope Imaging Software (Leica Microsystems, Buffalo Grove, IL) at different time points after the scratch procedure: 0, 2, 4, 17, and 19 hours for the experiment using THP-1 cells; 0, 12, 15, 17 and 19 hours for the experiment using human primary macrophages; and 0, 12, 15 and 17 hours for the experiment using THP-1 cells plus antibody treatment. Pictures of scratches were processed with SigmaScan Pro (Systat Software Inc., San Jose, CA). Intensity thresholds were set for each image to specifically mark the open area of the scratch, which excludes the cells, ensuring the measurement of the scratch gap. A consistent size area of analysis (square shaped) was set for all images at time 0, and this analysis area was used at all time points leaving the visible scratch gap in the center of this area all the times to ensure accuracy to obtain the gap area. The gap area with no cells was quantified as the number of pixels, which was show a smaller size as the scratched area was closing.

2.10. Immunocytochemistry and microscopic imaging.

CD163 protein induction was confirmed using the procedures described elsewhere by our group (Alvarado-Vazquez, et al., 2017). Briefly, macrophages were seeded in a well containing a rounded coverslip previously treated with fibronectin, they cells were stimulated with LPS and transfected with plasmids for 48 hours as described before. In a separate set of experiments previously stimulated (LPS) and transfected macrophages (for 48 hours) were co-cultured with skin cells on fibronectin-treated coverslips and processed for the scratch assay for an additional period of 17 hours using the following conditions: 50,000 cells/mL/well of fibroblasts plus 50,000 cells/mL/well of keratinocytes for the K + F control group, and 100,000 cells/mL/well of pEmpty-transfected THP-1 macrophages (K+F +M-pEmpty group) or pCD163-transfected THP-1 macrophages (K+F+M-pCD163 group) in conjunction with fibroblasts plus keratinocytes. Subsequently, cells were fixed (4% formaldehyde) for 30 minutes, permeabilized (0.25% Triton-X100) for 5 minutes, and blocked (0.5% FBS) for 1 hour. A mouse antibody against human CD163 (Serotec, Raleigh, NC, 1:150) was added and left in the preparation overnight at 4°C. Then, a goat anti-mouse secondary antibody conjugated to Alexa 555 (Life technologies, Grand Island, NY, 1:1,000) was added for 1 hour. The preparation was mounted in an anti-fade medium (Vectashield; Vector Laboratories, Burlingame CA, USA) with 4',6-diamidino-2-phenylindole dihydrochloridehydrate (DAPI, Sigma, St. Louis, MO) for cell nuclei staining. Images were taken in a Leica DMIL microscope (Model: 11521258) and a Leica DFC345 FX Digital

Camera (Leica Microsystems Inc., Buffalo Grove, IL). Individual cells were used to quantify their fluorescence intensities for CD163 (background was subtracted) using Sigma Scan Pro software (Systat Software Inc., San Jose, CA). The images were prepared with the Adobe Photoshop software.

2.11. RNA isolation, cDNA synthesis and quantitative real time-PCR (qRT-PCR).

THP-1 or human primary macrophages alone were collected at 48 hours after transfection with pEmpty or pCD163. For skin cells and macrophages co-cultures, cells were collected at 17 hours after scratch assay for the different groups (K + F, K+F+macrophages, K+F+M-pEmpty and K+F+M-pCD163). We used the cell lysis buffer included in the Reliaprep™ RNA Cell Miniprep System kit (Promega, Madison, WI) to harvest cell preparations and stored the samples at -80°C until further use. The mRNA isolation was performed following the instructions provided in the Reliaprep™ RNA Cell Miniprep System kit (Promega, Madison, WI). We measured levels of mRNA following the methods described by our group (Alvarado-Vazquez, et al., 2017). We used Script™ Reverse Transcription Supermix (BioRad, Hercules, CA) to synthesize cDNA as follows: 5 min at 25°C , 30 min at 42°C and 5 min at 85°C . We used SsoAdvanced™ Universal SYBR Green Supermix (BioRad) to measure CD163 (57°C), CD11b (60°C) and β -actin (57°C) as follows: 1 cycle of 98°C for 30 sec, 45 cycles of 98°C for 15 sec followed by 30 sec of the primer-specific annealing temperature. Table 1 shows the primers used in our studies. Duplicate samples were processed using the CFX96 Real-Time PCR system (Bio-Rad, Hercules, CA). The levels of mRNA were normalized to β -actin levels. We followed the ddCt method (Livak and Schmittgen, 2001) to determine gene changes (fold change).

2.12. ELISA.

Supernatants from co-cultured cells were collected 17 hours after scratch assay and stored at -80°C until enzyme-linked immunosorbent assays (ELISA) were performed according to instructions provided by the manufacturers. Transforming growth factor-beta (TGF- β), interleukin (IL)-10 tumor necrosis factor-alpha (TNF- α), IL-6 and monocyte chemoattractant protein (MCP)-1 concentrations were measured with ELISA kits (Human TGF β /IL-10/TNF- α /IL-6/CCL2 (MCP-1) ELISA Ready-SET-Go!, eBioscience, San Diego, CA). Their levels of sensitivity are 8 pg/mL for human TGF- β , 2 pg/mL for human tumor necrosis factor-alpha, IL-6 and 7 pg/mL for human CCL2 (MCP-1). The soluble form of CD163 (sCD163), TGF- α and IL-1 β were quantified using specific ELISA kits (Human CD163/TGF-alpha ELISA kit, R&D systems, Minneapolis, MN) and (Human IL-1beta ELISA Kit, Invitrogen - Life Technologies, Carlsbad, CA). Their levels of sensitivity are 0.613 ng/mL for human sCD163, 7.1 pg/mL for human TGF-alpha and 2 pg/mL for human IL-1beta. Levels of TNF- α , sCD163, and IL-10 are not shown because they were under the detection limit of the standard curve (non-detected). All values were reported in pg/mL.

2.13. Statistical Analysis.

All *in vitro* and *ex vitro* experiments were performed in a blinded fashion. Data are presented as mean $\pm$ standard deviation (SD). For immunocytochemistry one experiment was considered a single observation (N) and represent the average of fluorescent intensity of multiple analyzed cells. For *in vitro* molecular studies, the number of observations (N) is the

average of technical duplicates. *In vitro* molecular experiments were repeated at least 3 times and all observations were used for statistical analysis. Due to the extensive inter-assay variability observed when primary cells are used for *in vitro* molecular assessments we powered our experiments to have 3-6 observations (N, technical duplicates) per experiment as described in detail in our previous publication using similar assays and experimental and analysis approaches (Alvarado-Vazquez, et al., 2017). Due to the delicate nature of co-culturing multiple human primary cells (keratinocytes, fibroblasts, and macrophages), which require different media and different timelines for growth and maintenance, each scratch assay or organotypic skin assay was considered a single observation (N), each experiment had 1-3 observations (depending on the groups and conditions), and the total N per group is the result of at least two experiments. Statistical analyses were performed using GraphPad Prism 6.01 (GraphPad Software, Inc., San Diego, CA). Unpaired t-test, One-way or Two-way analysis of variance (ANOVA) followed by Bonferroni's post hoc test was used as appropriate. A value of $p < 0.05$ was considered significant.

3. Results

3.1 CD163 overexpression in THP-1 and human primary macrophages.

We followed the same methods to induce CD163 overexpression in macrophages using Man-PEI (Alvarado-Vazquez, et al., 2017, Alvarado-Vazquez, et al., 2019, Bernal, et al., 2017). We based our time point for CD163 gene induction in macrophages on these previous studies from our group, in which CD163 expression is significantly increased at 48 hours and remained elevated until 96 hours of incubation. Consistently, we observed an efficient CD163 induction in THP-1 and human primary macrophages using Man-PEI 48 hours after transfection treatments at the mRNA and protein level (Fig. 1), time in which they were added to wounded organotypic tissues in our subsequent studies.

3.2 CD163 overexpressing macrophages enhances re-epithelialization in a 3D organotypic human wounded skin tissue.

The 3D organotypic human skin model allowed us to evaluate the wound healing process in a very clinical translational fashion. We used it to investigate whether CD163 overexpression in THP-1 macrophages (pCD163) improves some aspects of the wound healing process, namely re-epithelialization, epithelium cellularity, and epithelium thickness. We observed that wounded tissues incubated with either macrophage transfected with pCD163 or pEmpty displayed a robust epithelization from day 1 (>75%) and reached nearly 100% wound closure by day 3 and 6 (Fig. 2). However, the percent epithelization of the pCD163 group was significantly larger when compared to the pEmpty group on day 1 (Fig. 2). We also assessed the keratinocyte cellularity and thickness of the newly formed epithelium in tissues incubated with THP-1 macrophages transfected with pCD163 or pEmpty on days 1 and 3. No changes in keratinocyte cellularity and epithelium thickness were found in pCD163 vs. pEmpty groups on day 1 (data not shown). However, on day 3 we found a small increase of keratinocyte cellularity in pCD163 group (22.03 ± 1.11 cells/250 μm , $n=3$) when compared to the pEmpty group (15.89 ± 3.27 cells/250 μm , $n=3$), but no changes in epithelium thickness (data not shown). This increase in cellularity could be explained by the leukemic

(hyperactive) nature of THP-1 cells and their enhanced interaction with keratinocytes, features that seem potentiated by the induction of CD163-overexpression.

Subsequently, we used human primary macrophages in a separate set of wounded human organotypic tissues. We observed that wounded tissues incubated with primary macrophages overexpressing CD163 (pCD163) displayed a significantly larger epithelium on day 3 (~100%) when compared to the pEmpty group (Fig. 3). The wound epithelization was not different between groups on day 1. In this case we determined the quality of the re-epithelialized tissue by quantifying the number of epidermal keratinocytes by looking at sections of the newly formed epithelium. We found no difference in keratinocyte cellularity between pCD163 and pEmpty group on days 1 and 3 (data not shown), suggesting that CD163 overexpressing macrophages induces the growth of healthy new epithelium.

Additionally, we measured the thickness of the new epithelium in our setting, and we found that the epithelium of tissues incubated with primary macrophages overexpressing CD163 (pCD163) displayed a more uniform thickness in the new tissue when compared to the pEmpty group on day 3 (Fig. 4A–C), consistent with a faster epithelization process. We observed that the area under the curve (AUC) of tissues in the pCD163 group was larger than the AUC of tissues in the pEmpty group (Fig. 4D). To further explore the interactions of CD163-macrophages with specific skin cells we used the *in vitro* scratch assay.

3.3 Interactions of CD163 overexpressing macrophages with keratinocytes and fibroblasts in wound healing8.

To further understand the cell interactions and mechanisms underlying the wound healing process, we decided to use an *in vitro* scratch assay using human primary skin cells, namely keratinocytes and fibroblasts. Accordingly, we co-cultured keratinocytes and fibroblasts (K + F) in the absence (control) or presence of human primary macrophages transfected with either pEmpty (M-pEmpty) or pCD163 (M-pCD163) and assessed wound (scratch) closure over time at 0, 12, 15, 17, and 19 hours. Consistent with our previous findings using organotypic wounded skin tissues, M-pEmpty and M-pCD163 groups improved wound healing at 12, 15, 17 and 19 hours after scratch assay compared to the K + F alone (control) group (Fig. 5A). Furthermore, M-pCD163 group displayed a faster and more efficient wound healing process compared to the M-pEmpty group at 17 and 19 hours after scratch assay (Fig. 5A). For clarity, we show the % of wound healing process in K + F in the absence (control) or presence of M-pEmpty or pCD163 (Fig. 5B) at the time points that statistic differences were found, 17 and 19 hours after the scratch.

To further characterize the cellular interactions between macrophages and skin cells, we utilized THP-1 cells and human primary keratinocytes and fibroblast in three major conditions as follows: keratinocytes alone, fibroblasts alone, or keratinocytes and fibroblasts in co-culture (K + F). These skin cell conditions showed a time dependent gap closure, and were called “control” when compared to the following sub-groups (performed in each control condition): skin cells plus macrophages LPS-stimulated and without transfection (macrophage group), skin cells plus macrophages transfected with pEmpty (M-pEmpty group), and skin cells plus macrophages transfected with pCD163 (M-pCD163 group). This approach allowed us to assess whether macrophages or CD163 overexpression in

macrophages influence the capabilities of keratinocytes and/or fibroblasts to promote an efficient wound healing process (Fig. 6A–C).

The presence of non-transfected and LPS-stimulated macrophages, pCD163-transfected macrophages, or pEmpty-transfected macrophages promoted a faster wound healing migration (reduced gap width) of keratinocyte only preparations at the early stage of the process, 4 hours after the scratch, with no further changes at later time points (Fig. 6A), suggesting that macrophages irrespective of their phenotype directly influence keratinocyte migration. The same experiment performed in fibroblast only cultures showed that the macrophage group and the M-pEmpty group did not change the gap closure over time in this type of skin cells. Interestingly, the addition of M-pCD163 produced a more efficient fibroblast wound healing migration process when compared to the M-pEmpty group at later stages, namely 19 hours after the scratch (Fig. 6B). These data suggest that macrophages directly interact with fibroblasts only when they acquire a phenotype driven by CD163 overexpression. In K + F cultures macrophages improves gap closure at 17 and 19 hours, and similarly M-pEmpty improved gap closure at 19 hours, consistent with a contribution of macrophages in skin cell wound healing. Furthermore, in K + F in the presence of M-pCD163 produced a more efficient gap closure when compared to the M-pEmpty group at 17 hours with no further changes at 19 hours (Fig. 6C), confirming the contribution of CD163 overexpressing macrophages in a more efficient wound healing process, likely due to direct interactions with fibroblasts. For clarity, we show the % of wound healing process in keratinocytes (Fig. 6D), fibroblasts (Fig. 6E), or K + F (Fig. 6F) in the absence (control) or presence of macrophages (non-transfected and LPS-stimulated), M-pEmpty or M-pCD163 at 17 and 19 hours after the scratch.

3.4 CD163 overexpression in macrophages promotes changes in cytokine production when co-cultured with keratinocytes and fibroblasts.

To further investigate the mediators responsible to a more efficient keratinocyte and fibroblast wound healing process, we measured the protein levels of pro- and anti-inflammatory factors released by keratinocytes + fibroblast co-cultures and compared them with keratinocytes + fibroblasts + macrophages (untreated) co-cultures at 17 hours after scratch assay using ELISA kits. We found that the addition of macrophages to skin cell co-cultures produced a significant increase in the levels of IL-6, MCP-1, TGF- α , and TGF- β , with no changes in IL-1 β (Table 2).

Then, we followed factors that were altered by macrophages. We compared levels of these molecules in co-cultures of keratinocytes + fibroblasts + macrophages transfected with either pEmpty (M-pEmpty group) or pCD163 (M-pCD163 group) at 17 hours after scratch assay (Figure 7). We found that the addition of M-pCD163 in skin cell co-cultures has lower levels of MCP-1 (Figure 7B) and higher levels of TGF- α (Figure 7C) when compared to skin cell co-cultured with M-pEmpty. We did not find any difference in IL-6 or TGF- β between groups (Figures 7A and D).

3.5 CD163 gene and protein induction in macrophages co-cultured with K + F.

To determine whether the co-culture of skin cells with M-pCD163 alters the overexpression of CD163, we confirmed its overexpression under these conditions 17 hours after the scratch. We observed that K + F with M-pCD163 displayed a 2,300-fold increase of CD163 mRNA when compared to the K + F with M-pEmpty control group, and K + F with macrophage LPS-stimulated and no transfection (Fig. 8A). To confirm that this CD163 overexpression was not due to an increase in the number of macrophages, we measure the mRNA levels of CD11b in all these groups. We observed that K + F without macrophages did not express CD11b, and we observed similar levels of CD11b mRNA in K + F co-cultured with either macrophages, M-pEmpty, or M-pCD163 (Fig. 8B). Additionally, we observed an increase of CD163 at the protein level in the M-pCD163 co-cultured with K + F group at 17 hours after the scratch using immunohistochemistry (Fig. 8C,D).

3.6 Blockade of CD163 prevents the efficient wound healing induced by CD163 overexpressing macrophages.

To determine whether the observed effects on the efficient wound healing process are achieved specifically by the overexpression of CD163 in macrophages when co-cultured with K + F, we used a CD163-neutralizing antibody as a functional blocker. As observed previously, the scratch gap closure occurred faster (at 15 and 17 hours) in the M-pCD163 Alone group (untreated) compared to the M-pEmpty Alone group (untreated). This gap closure effect was not observed in the M-pCD163 treated with a CD163-blocking antibody (Ab) group (Fig. 9A). In contrast, the gap closure pattern in the M-pCD163 Alone group was similar to the M-pCD163 treated with isotype antibody (Iso) control group. For clarity, we show representative images of the scratch analysis (Fig. 9B) and the percent of wound healing at the 17 hour time point separately (Fig. 9C) for groups with CD163 antibody or isotype control antibody treatments, and untreated (Alone) groups.

4. Discussion

The main findings of our studies are: first, CD163 overexpressing human primary or THP-1 macrophages promote a faster and more regular growth of epithelium with signs of normal cellularity in *ex vivo* 3D organotypic human skin tissues; second, macrophages directly interact with keratinocytes at early stages of wound healing, but only interact directly with fibroblasts at later stages when macrophages overexpress CD163; third, the efficient skin wound healing induced by CD163 is associated with changes in cytokines involved in tissue repair.

Our laboratory has recently demonstrated that LPS-stimulated THP-1 macrophages and human primary macrophages transfected with pCD163 using Man-PEI nanoparticles results in a cytokine release profile that is consistent with an anti-inflammatory phenotype (Alvarado-Vazquez, et al., 2017, Alvarado-Vazquez, et al., 2019). However, whether CD163 overexpression promotes pro-resolution functional outcomes was not known. In our current study, we build on our previous findings by demonstrating that the induction of CD163 in macrophages induces functional changes that result in a more efficient wound healing process by interactions with skin cells. Our findings are in line with the role of macrophages

when adopting an anti-inflammatory state and also a pro-resolution phenotype that promotes tissue repair (Mantovani, et al., 2013, Italiani and Boraschi, 2014, Novak and Koh, 2013, Novak and Koh, 2013, Krzyszczyk, et al., 2018). Similarly, others have shown that the presence of macrophages expressing CD163 is associated with wound healing in an experimental model in humans (Philippidis, et al., 2004, Evans, et al., 2013). We now confirm the direct causality of this association by specifically inducing and selectively blocking CD163 using 3D human skin tissues, and human primary and THP-1 macrophages with human primary skin cells.

The role of macrophages in the induction of wound healing has been extensively studied as a function of multiple subsets of macrophage phenotypes. However, what characteristics or particular phenotype is needed to promote an effective wound healing is not completely known (Krzyszczyk, et al., 2018). We show that CD163 overexpression in macrophages is sufficient to promote a more efficient wound healing process in our models. We also show that macrophages affect keratinocytes and fibroblasts differentially. Notably, we demonstrate that macrophages regardless of their CD163 expression level directly affect keratinocytes during the first hours of the wound induction promoting a more rapid migration to close the scratch gap, whilst macrophages without CD163 overexpression do not affect fibroblast at early and late stages. Interestingly, the induction of CD163 in macrophages resulted in a direct influence in fibroblasts at the later stages of the wound healing, promoting a more rapid gap closure. Accordingly, keratinocytes display a faster migration response (Seeger and Paller, 2015) and are the first cells to respond to an injury in the skin (Woodley, et al., 2015), supporting our observations. Similarly, fibroblasts respond at later stages in the wound healing process promoting a physical structure that allows the regeneration of new healthy tissue (Darby, et al., 2014). Our data strongly suggest that these skin cell coordinated functions could be orchestrated by macrophages and make them more efficient by CD163 overexpressing macrophages. Our data are in agreement with previous observations that demonstrate the active role of fibroblasts in later stages of the wound healing process, namely at time points in which macrophages adopt an anti-inflammatory phenotype (Lucas, et al., 2010). Indeed, we have demonstrated that human primary macrophages overexpressing CD163 produce less pro-inflammatory cytokines such as TNF- α , IL-6, IL-1 β , and MCP-1 when cultured alone, namely in the absence of skin cells (Alvarado-Vazquez, et al., 2017, Alvarado-Vazquez, et al., 2019, Bernal, et al., 2017), we speculate that this phenotype is retained in our current setting, and therefore this could affect the wound healing process. However, we cannot rule out the possibility that the interaction with skin cells modifies the phenotype of macrophages in a different manner, and consequently the molecular mechanisms downstream CD163 overexpression remains elusive. Nonetheless, our data provide some insight on the molecular mechanisms induced by CD163 overexpressing macrophages when co-cultured with keratinocytes and fibroblasts since we observed a milieu with pro-tissue repair features: a reduction in the pro-inflammatory chemokine MCP-1, and an increase in the pro-resolution factor TGF- α . Interestingly, we did not see a reduction or changes in the pro-inflammatory molecule IL-6 and anti-inflammatory factor TGF- β , both with pro-wound healing properties (Gallucci, et al., 2001, Lin, et al., 2003, Penn, et al., 2012). These findings show that CD163 does not obliterate the molecular capabilities of macrophages or skin cells nor alter molecular mechanisms that are necessary

for the production of a healthy epithelium. Accordingly, the reduction of MCP-1 in our preparation is in line with its abundance and role in recruiting monocytes at early stages of inflammation or injury (Ding and Tredget, 2015). Indeed, MCP-1's levels are reduced as wound healing is completed (Ding and Tredget, 2015), as shown in our current study. Similarly, TGF- α is an epidermal growth factor (EGF) that promotes a faster wound closure *in vitro* (Beyeler, et al., 2014) and stimulates wound re-epithelialization (Gurtner, et al., 2008). However, our experiments cannot determine what is the source of these molecules (skin cells or macrophages). The changes we observed in these factors are modest, and perhaps this is more beneficial than a more robust effect that could result in an excessive epithelialization or keratinocyte hypercellularity, or on the opposite site of the spectrum, a delay in wound healing as observed with treatments that produce immunosuppression, i.e. glucocorticoids (Wicke, et al., 2000).

Our nanoparticle approach to induce CD163 in macrophages possesses a preferential cell-targeting capability that other gene therapy vectors do not offer (Bernal, et al., 2017, Diebold, et al., 1999). The cell target precision of our approach could also serve to avoid limitations exhibited by pharmaceutical strategies. For example, topical or systemic glucocorticoids indistinctively affect fibroblasts, keratinocyte, adipocytes, macrophages, etc. In fact, glucocorticoids negatively affect keratinocytes and fibroblasts (toxicity, accelerated cell death, reduction in mitotic capabilities, etc.) producing a reduction in skin thickness and delay in wound healing (Wicke, et al., 2000, Schoepe, et al., 2006). To avoid these negative effects, others have developed a macrophage glucocorticoid delivery system using liposomes, which promotes a strong anti-inflammatory cellular phenotype including an increase in CD163 expression, which arguably would promote a more efficient wound healing and avoid toxic effects on skin cells (Gauthier, et al., 2018). While the effects in wound healing and the potential of clinical translation of that liposome-based approach remain unknown (Sercombe, et al., 2015), the nanotechnology used in our current study has been successfully and safely used as a cell-directed gene therapy in HIV positive human patients (Liszewicz, et al., 2012, Liszewicz, et al., 2005). We have confirmed the lack of immunogenicity and toxicity of this plasmid carrier in human macrophages (Bernal, et al., 2017), and now we confirm its viability to promote a more efficient wound healing.

How CD163 overexpressing macrophages induce this local pro-wound healing and protective milieu when interacting with skin cells is an intriguing hypothesis that is the subject of the current efforts in our laboratory. There are other unanswered questions that our studies did not resolve, like the role of other molecular signaling factors, such as epidermal growth factor (EGF), macrophage colony-stimulating factor (GM-CSF), fibroblast growth factor 2 (FGF-2) and hepatocyte growth factor (HGF) in our model (Seeger and Paller, 2015).

5. Conclusions

Our current study provides a functional evidence that CD163 overexpressing macrophages promote a faster wound healing process when interacting with skin cells *in vitro* and *ex vivo* using 3D organotypic tissues. Our findings establish groundwork to further develop this cell directed gene therapy based in nanotechnology and to test our approach in clinically relevant

in vivo settings. This approach may represent the initial steps towards an alternative approach to prevent chronic postoperative pain after invasive surgeries by resolving inflammation and promoting a faster tissue repair (Woolf, 2010, Lavand'homme, 2015, Khan, et al., 2011). Future studies to determine whether CD163 overexpressing macrophages might also regulate other processes such as burns, diabetic ulcers, and chronic vein wounds (Sindrilaru, et al., 2011, Loots, et al., 1998, Stone Ii, et al., 2018) will be of paramount clinical interest.

Acknowledgments

The authors would like to acknowledge Rita Allen Foundation and American Pain Society-Pain Scholar Award (EAR-S), NIH-NIGMS R15GM109333 (EAR-S), Presbyterian College School of Pharmacy Research Summer Internship (DWF) for funding, and CAPES Foundation - Ministry of Education of Brazil, PDSE: BEX 10794/14-0 for funding DWF's scholarship.

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Figure 1. CD163 gene induction in THP-1 and human primary macrophages. mRNA levels of CD163 in THP-1 macrophages and human primary macrophages transfected with a plasmid that encodes the CD163 gene (pCD163) or an Empty vector (pEmpty) following 48 hours of transfection. Representative microscopic images of nuclear staining using DAPI (blue) and CD163 protein (red) in THP-1 macrophages and human primary macrophages transfected with pEmpty or pCD163 for 48 hours (B). Quantification of the average of fluorescence of CD163 in THP-1 macrophages and human primary macrophages transfected with pEmpty or pCD163 for 48 hours (C). Scale bar = 100 μ m. N= 3-6 per group. * $p < 0.05$ pEmpty vs. pCD163 groups, by student's t test.

Figure 2. Wound closure in organotypic tissues treated with CD163-overexpressing THP-1 macrophages.

A) Quantification of wound closure (%) in tissues without treatment (baseline, control on day 0) and in tissues in the presence of LPS-stimulated macrophages transfected with a plasmid that encodes the CD163 gene (pCD163) or an Empty vector (pEmpty) on days 1, 3 and 6 after macrophage addition. B) Representative microscopic images (5X) depicting epithelialization on day 1 after the addition of macrophages transfected with pEmpty or pCD163. N= 3-4 per group. * $P < 0.05$ pEmpty vs. M-pCD163 group, by student's t-test. 1.5-column fitting image

Figure 3. Wound closure in organotypic tissues treated with CD163-overexpressing human primary macrophages.

A) Quantification of the wound closure (%) in tissues in the presence of LPS-stimulated macrophages transfected with a plasmid that encodes the CD163 gene (pCD163) or an Empty vector (pEmpty) on days 1 and 3 after macrophage addition. B) Representative microscopic images (5X) depicting epithelization on day 3 after the addition of macrophages transfected with pEmpty or pCD163. N= 3-4 per group. *P<0.05 M-pEmpty vs. M-pCD163 group, by student's t-test. 1.5-column fitting image

Figure 4. Epithelium thickness in organotypic tissues treated with CD163-overexpressing human primary macrophages.

Representative microscopic images (A, B) and quantification of epithelium thickness across the tissue wound (C) in the presence of LPS-stimulated human primary macrophages transfected with a plasmid that encodes the CD163 gene (pCD163) or an Empty vector (pEmpty) on day 3 after macrophage addition. The area under the curve (AUC) was calculated in every tissue and compared in both conditions (D). Scale bars = 50 μm (A, B) and 25 μm (Aa, Ab, Ba and Bb insets). The empty arrowhead indicates the absence of epithelium (Aa inset) and the solid black arrowheads and brackets (Ab, Ba and Bb insets) indicate the epithelium thickness in both conditions. N=6 per group. *P<0.05 M-pEmpty vs. M-pCD163 groups, by student's t-test. 1.5-column fitting image

Figure 5. Wound Healing assay in the presence of CD163-overexpressing human primary macrophages co-cultured with K + F.

Time course of the migration healing progression of K + F alone (control) or in the presence of LPS-stimulated human primary macrophages transfected with a plasmid that encodes the CD163 gene (M-pCD163) or an Empty vector (M-pEmpty) cultures at 0, 12, 15, 17 and 19 hours after the scratch (A). Comparison of wound healing (%) between K + F alone (control) or in the presence of M-pEmpty or M-pCD163 at 17 and 19 hours after the scratch (B). N = 10-16 per group. $\#p < 0.05$ Control vs. M-pEmpty or M-pCD163; $*p < 0.05$ M-

pEmpty vs. M-pCD163, by Two-way ANOVA and Bonferroni post-test. single-column fitting image

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Figure 6. Wound healing assay and migration temporal course of keratinocytes and/or fibroblasts cultures in the presence of CD163-overexpressing THP-1 macrophages. Time course (time-response curves) of the migration healing progression of keratinocytes (A), fibroblasts (B), keratinocytes + fibroblasts – K + F (C) alone (Control) or in the presence of LPS-stimulated non-transfected macrophages (macrophage), pEmpty-transfected macrophages (M-pEmpty) or pCD163-transfected macrophages (M-pCD163) cultures at 0, 2, 4, 17 and 19 hours after the scratch. Comparison of wound healing (%) between keratinocytes (D), fibroblasts (E), or K + F (F) alone (control) or in the presence of LPS-stimulated non-transfected macrophages (macrophage), M-pEmpty or M-pCD163 at 17 and 19 hours after the scratch. Macrophages were previously stimulated with LPS. The closure of the scratch gap (scratch width) was measured in pixels. N= 3 per group (A-F). +\$# p<0.05 all groups vs. control group; *p<0.05 M-pEmpty vs. M-pCD163 group, by Two-way ANOVA and Bonferroni post-test. 2-column fitting image

Figure 7. Cytokine/chemokine concentration in skin cells and THP-1 macrophage co-cultures using the scratch assay.

Quantification for IL-6 (A), MCP-1 (B), TGF-α (C), and TGF-β (D) protein concentration in keratinocytes + fibroblasts in the presence of LPS-stimulated THP-1 macrophages transfected with a plasmid that encodes the CD163 gene (M-pCD163) or an Empty vector (M-pEmpty). N= 5-9/group. *P<0.05 M-pEmpty vs. M-pCD163 groups, by student's t-test.

Figure 8. CD163 gene and protein inductions in THP-1 macrophages co-cultured with K + F. THP-1 macrophages were stimulated with LPS and non-transfected (macrophage) or transfected with a plasmid that encodes the CD163 gene (M-pCD163) or an Empty vector (M-pEmpty). Following 66 hours of transfection (and 17 hours after the scratch), the levels of CD163 mRNA were determined in K + F alone (control) or co-cultured with THP-1 macrophages (A) by RT-PCR. The CD11b mRNA expression was not detected in K + F alone (control) (B). The CD163 and CD11b mRNA expressions were normalized to the respective levels of β -actin expression in each group. Then all values were normalized to 1 against K+F+M-pEmpty group at 17 hours after the scratch. Representative microscopic images of nuclear staining using DAPI (blue) and CD163 protein (red) in K + F alone (control) or co-cultured with M-pCD163 or M-pEmpty (C). Quantification of the average of fluorescence of CD163 at 17 hours after the scratch (D). The quantification of average of intensity was normalized to 1 against K + F alone (control) group. N= 13-16 (A); 5-6 (B); 8-15 (D) per group. * $p < 0.05$ K+F+M-pEmpty vs. K+F+M-pCD163 group, by One-way ANOVA and Bonferroni post-test. 1.5-column fitting image

Figure 9. CD163-blocking antibody impairs the efficient wound healing process induced by CD163-overexpressing THP-1 macrophages co-cultured with K + F.

Time course of the migration healing progression of K + F in the presence of LPS-stimulated macrophages transfected with a plasmid that encodes the CD163 gene (M-pCD163) or an Empty vector (M-pEmpty) untreated (alone) or treated with CD163 antibody (Ab) or its isotype control-mouse IgG1 (Iso) at 0, 12, 15 and 17 hours after the scratch (A). Representative microscopic images (pixels in red) of K+F+M-pCD163 group untreated (alone) or treated (Ab or Iso) at 17 hours after the scratch (B). Comparison of wound healing (%) in untreated (alone) or treated (Ab or Iso) groups at 17 hours after the scratch (C). The closure of the scratch gap (scratch width) was measured in pixels. N= 12 per group. *p<0.05 M-pCD163 alone vs. M-pEmpty alone or M-pCD163 Ab; #p<0.05 M-pCD163 Iso vs. M-pCD163 Ab, by Two-way ANOVA and Bonferroni post-test. 1.5-column fitting image

Table 1.

Primers used for qRT-PCR analyses.

PCR Primers	Forward (5'-3')	Reverse (5'-3')
CD11b	CAGAGAGGAAGTAGCAGCTCCT	CTGGTCATGTTGATGAAGGTGCT
CD163	GCTACATGGCGGTGGAGACAA	ATGATGAGAGGCAGCAAGATGG
β -actin	AGAGCTACGAGCTGCCTGAC	AGCACTGTGTTGGCGTACAG

Table 2.

Molecule concentrations in co-culture supernatants.

Molecules	Keratinocytes + Fibroblasts	Keratinocytes + Fibroblasts + Macrophages
IL-1 β	30.3 $\pm$ 14.9 pg/mL	39.4 $\pm$ 15.9 pg/mL
IL-6	12.4 $\pm$ 3.4 pg/mL	142.5 $\pm$ 23.8 pg/mL *
MCP-1	879.2 $\pm$ 200.2 pg/mL	1775.0 $\pm$ 35.9 pg/mL *
TGF- α	108.8 $\pm$ 30.9 pg/mL	153.2 $\pm$ 20.3 pg/mL *
TGF- β	126.7 $\pm$ 9.2 pg/mL	140.3 $\pm$ 9.9 pg/mL *

*
P<0.05 vs. Keratinocytes + Fibroblasts group by t-test.