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Non-Thermal Atmospheric Pressure Argon-Sourced Plasma Flux Promotes Wound Healing of Burn Wounds and Burn Wounds with Infection in Mice through the Anti-Inflammatory Macrophages

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Citation: Dang, C.P.; Weawseetong, S.; Charoensappakit, A.; Sae-Khow, K.; Thong-Aram, D.; Leelahavanichkul, A. Non-Thermal Atmospheric Pressure Argon-Sourced Plasma Flux Promotes Wound Healing of Burn Wounds and Burn Wounds with Infection in Mice through the Anti-Inflammatory Macrophages. *Appl. Sci.* **2021**, *11*, 5343. https://doi.org/10.3390/app11125343

Academic Editor: Emilio Martines

Received: 2 May 2021
Accepted: 7 June 2021
Published: 9 June 2021

Abstract: Plasma medicine is the utilization of gas ionization that might be beneficial for the treatment of burn wounds, a healthcare problem with a significant mortality rate. Due to a lack of information on the impact of plasma flux in immune cells and a high prevalence of bacterial infection in burn wounds, non-thermal argon-based plasma flux was tested on macrophages (RAW246.7) and in mouse models of burn wounds with or without *Staphylococcus aureus* infection. Accordingly, plasma flux enhanced reactive oxygen species (ROS), using dihydroethidium assay, and decreased abundance of NF-κB-p65 (Western blot analysis) in non-stimulating macrophages. In parallel, plasma flux upregulated *IL-10* gene expression (an anti-inflammatory cytokine) in lipopolysaccharide (LPS)-induced inflammatory macrophages, while downregulating the pro-inflammatory cytokines (*IL-1β* and *IL-6*). Additionally, plasma flux improved the migratory function of fibroblasts (L929) (fibroblast scratch assay) but not fibroblast proliferation. Moreover, once daily plasma flux administration for 7 days promoted the healing process in burn wounds with or without infection (wound area and wound rank score). Additionally, plasma flux reduced tissue cytokines (TNF-α and IL-6) in burn wounds with infection and promoted collagen in burn wounds without infection. In conclusion, plasma flux induced anti-inflammatory macrophages and promoted the burn-wound healing process partly through the decrease in macrophage NF-κB. Hence, plasma flux treatment should be tested in patients with burn wounds.

Keywords: non-thermal plasma; inflammation; macrophage; burn wounds

1. Introduction

Plasma is a completely or partly ionized gas that is categorized as the fourth state of matter in addition to solid, liquid and gas [1]. A plasma is a state of matter that is similar to a gas in terms of its ability to change volume and shape, but unlike a gas, it is made up of groups of positively and negatively charged particles [1]. Plasma flux is utilized in medicine (plasma medicine) [1] and may be operated at normal atmospheric pressure with temperatures between 30 and 40 °C, which are appropriate for use on live organisms without toxicity and are referred to as "non-thermal plasma therapy" [2]. Generating plasma by ionization of gas can induce various active molecules, including reactive oxygen species

(ROS) and reactive nitrogen species (RNS), without heat production [3]. Concentrations of the active molecules are adjustable by the different properties of plasma depending on different kinds of gas sources, power supply voltages and currents that are suitable for several specific purposes, ranging from enhanced cell proliferation to the induction of programmed cell death [4–7].

Non-thermal plasma does not destroy normal cells but selectively eliminates only severely injured cells or cancer cells, at least in part, due to the prominent stress-induced ROS in these cells [8–10]. Additionally, reactive species molecules of non-thermal plasma also demonstrate bactericidal activity, regardless of the antibiotic resistant properties of the organisms [11]. Plasma flux is used in numerous medical situations (malignancy, wound care, and organismal management) due to its ability for selective elimination and enhanced proliferation of abnormal and healthy cells, respectively, with additive bactericidal activity [2]. As such, plasma flux enhanced re-epithelialization, collagen synthesis, angiogenesis and the anti-inflammatory status in a rodent wound model [12], partly through the induction of several growth factors in keratinocytes [13]. Hence, non-thermal plasma therapy is an emerging treatment strategy [1,2,12], especially for diabetic wounds [14] and burn injuries [15]. Indeed, a burn wound, a dermal injury from extreme insults (heat, erosive agents or electricity) is classified upon the depth of the wound damage (superficial, partial thickness and full thickness) and leads to several severe complications, including hypovolemia, hypermetabolism, gut permeability defect and opportunistic infections [16]. Unfortunately, the prevalence of burn wounds remains high, particularly among low- and middle-income households, with chronic wound care and treatment for sequelae having a disproportionately large economic cost [17]. In order to reduce burn injury mortality rates and country burdens, proper wound care is needed. Interestingly, secondary bacterial infection in burn wounds is one of the major complications, especially with a, currently, high prevalence of anti-microbial resistance [18]. Fortunately, the antibiotic resistant organisms are still vulnerable to microbial eradication with several physical strategies, including ultraviolet light, radiation and non-thermal plasma [19,20]. Hence, the evaluation of plasma flux on infected burn wounds is interesting. Despite extensive evaluations of plasma flux in several models of traumatic wounds [21], data of non-thermal plasma on infected burn wounds are still lacking.

In parallel, macrophages are the immune cells responsible for either organismal control or the wound healing process, which can be manipulated by non-thermal plasma therapy as demonstrated in a solid cancer model [22]. As such, macrophages are immune cells with pleomorphic functions, referred to as “macrophage polarization”, that consist of pro-inflammatory M1 and anti-inflammatory M2 polarization [23]. The induction of macrophage polarization into a proper direction for each situation might be beneficial. For example, an acceleration of M1 and M2 for the conditions with immune exhaustion (tumor micro-environments) and hyper-immune responses (infection), respectively, might improve clinical outcomes [24]. In wounds with infection, pro-inflammatory M1 polarization is necessary for organismal control but excessive pro-inflammation worsens the wound healing process [25]. With proper organismal control, M2 macrophages or anti-inflammatory macrophages enhance several processes (anti-inflammation, debris removal and matrix remodeling) and promote wound healing [25]. Because (i) non-thermal plasma can induce an anti-inflammatory wound status with bactericidal activity that might be beneficial for wound healing and (ii) due to the lack of data of the effect of plasma on macrophage function, plasma flux treatment in burn wounds with infection is interesting. Hence, non-thermal plasma was tested with in vitro experiments, using macrophages (RAW264.7 cell line) and fibroblasts (L929 cell line), and also evaluated in vivo, using burn wound mouse models with or without infection.

2. Materials and Methods

2.1. Animal Model and Plasma Flux Generator

C57BL/6 mice, purchased from Nomura Siam International (Pathumwan, Bangkok, Thailand), were used following an approved animal protocol from the Institutional Animal Care and Use Committee of the Faculty of Medicine, Chulalongkorn University, Bangkok, Thailand. The burn wound mouse models with and without infection were performed in accordance with the previous publications [26,27]. Briefly, in burn wound models without infection, an aluminum rod 1 cm in diameter was heated to 100 °C, using a dry block heater (Thermo fisher Scientific, Waltham, MA, USA) for 5 min before placing on a shaved area at the dorsal part of the mice under isoflurane anesthesia. After that, mice with the heated rod attached to the back were turned ventral upward to allow their own body weight to function as a controlled pressure on the heated rod for 20 s. In the infected wound model, the heated rod was placed with the same procedures of the non-infected wound model. Then, 1×10^7 CFU of *Staphylococcus aureus* (ATCC 25923, the American Type Culture Collection, Manassas, VA, USA) in 0.1 mL normal saline (NSS) was directly spread onto the wound 30 min after the injury, in accordance with a previous publication [26]. After 24 h, necrotic tissue at the burn wounds was removed in both wound models. Subsequently, treatment by non-thermal plasma flux for 30 s along with a visual evaluation of the wound was performed daily until the 7th day of experiments, when the mice were sacrificed via cardiac puncture under isoflurane anesthesia with sample collection (blood and tissue at the wound). For the in-house plasma generator, the machine was designed following the principle of atmospheric pressure plasma with alternating current (AC) electricity at 13 kHz in frequency, using argon gas flow to release the ionized, enriched gas [28] as demonstrated in the diagram (Figure 1A,B). In brief, the plasma jet was designed with two electrodes. The inner electrode was a tungsten rod with a diameter of 1 mm, while the outer electrode was a thin copper sheet in the shape of a ring, surrounding the borosilicate glass syringe as the dielectric material. To prevent arc generation during operation, the outer electrode was wrapped with a rubber band. The distance between the plasma source and substrate was 5 mm. The current of the output was approximately 0.5 mA with the square waveform. Argon flow was used during operation at a speed of 1.5 L/min.

2.2. Wound Injury Score and Gut Permeability Determination

The wound was macroscopically evaluated by direct visualization based on percentage of the injury area compared with the initial injury area at 24 h post-injury [29]. Due to the circular-shape of the wound, the wound area was calculated using wound diameters, which were measured by a Vernier caliper (Thermo fisher Scientific, Waltham, MA, USA). Additionally, the wound rank score, a score for determination of inflammatory signs, was evaluated like a previous publication [29]. Briefly, the burn wound was assessed through 4 scoring criteria, including wound length and depth (wound closure assessment), wellness and redness (inflammatory degree evaluation), using a semi-quantitative scale of 0–3 for each characteristic. Because a gut permeability defect (gut leakage) in severe burn wounds is possible [30,31], leaky gut was also tested in the models. As such, fluorescent isothiocyanate-dextran or FITC-dextran at 4.4 kDa (FD4, Sigma, St. Louis, MO, USA), a non-gastrointestinal absorbable substance, at 12.5 mg in 500 µL sterile phosphate buffer solution (PBS) was orally administered at 3 h prior to the sacrifice. Then serum FITC-dextran was analyzed by fluorescent spectrometry with a Varioskan Flash microplate reader (Thermo fisher Scientific, Waltham, MA, USA). Presentation of an intestinal, non-absorbable substance in serum after oral administration demonstrated a gut permeability defect [32].

2.3. Mouse Sample Analysis

The inflammatory cytokines in serum and in the wound tissue were evaluated, using Enzyme-linked Immunosorbent (ELISA) assay (Biolegend, San Diego, CA, USA). For cytokines in wound tissue, fresh skin samples were washed in PBS, weighted, homogenized

and thoroughly sonicated. After that, a supernatant from the samples was used for cytokine evaluation. For bacterial burdens in the wound, tissue from the wound were weighed and minced into small pieces before being dissolved in PBS (1 g tissue per 1 mL PBS). After that, the samples were directly streaked onto a tryptic soy agar (TSA) plate (Oxoid, Thermo fisher Scientific, Waltham, MA, USA) in serial dilutions and incubated for 24 h at 37 °C before colony enumeration.

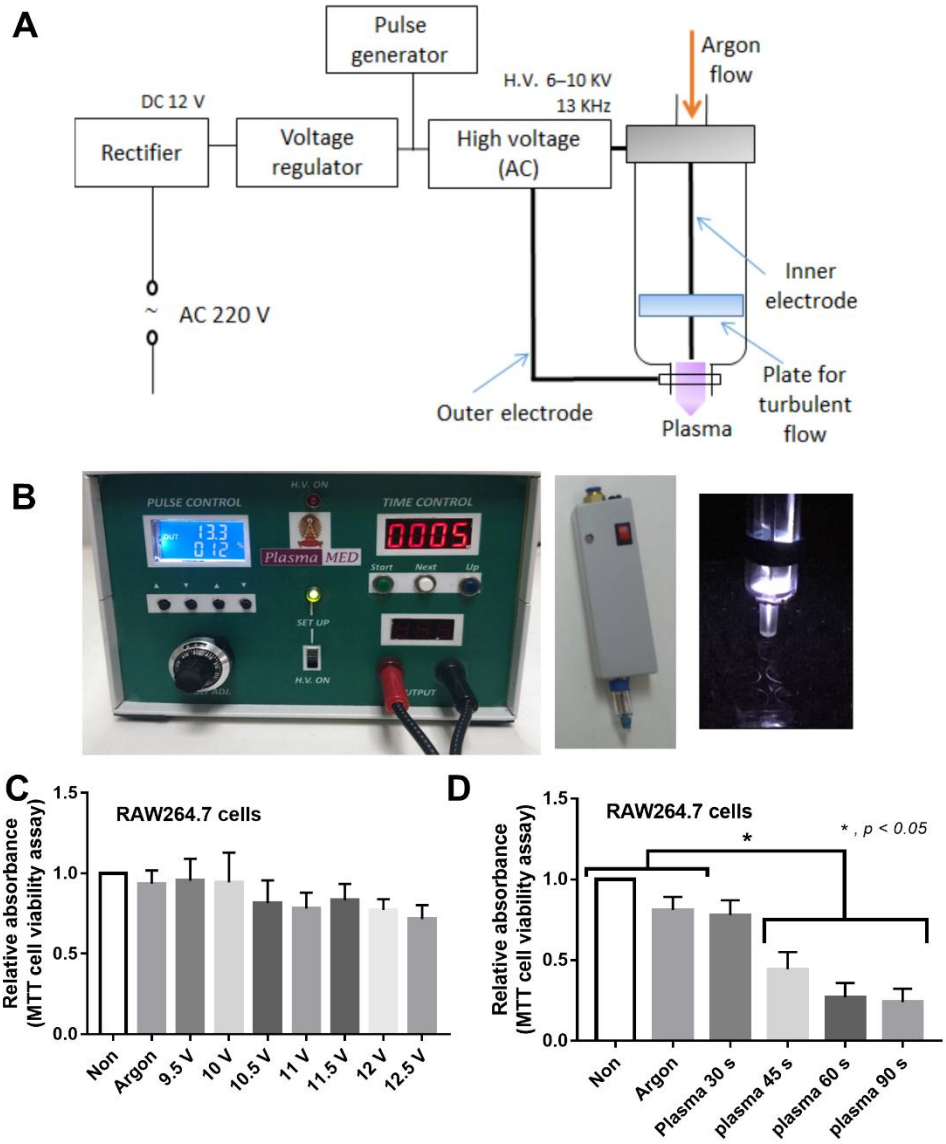

Figure 1. Schematic diagram of non-thermal atmospheric pressure plasma. Component of plasma flux generator in schematic diagram (**A**) and the representative pictures of machine (**B**, left side) with an electrode-containing syringe (**B**, middle) and the electrode during plasma flux releasing (**B**, right side) are demonstrated. Cell viability test (MTT assay) in RAW264.7 cells after plasma flux treatment at 30 s at different voltage levels (**C**) and at 10 voltage with different exposure durations (in seconds) (**D**) compared with cells without stimulation (Non) or stimulation with argon gas alone without non-thermal plasma (Argon) are demonstrated. Independent triplicated experiments were performed.

2.4. The In Vitro Experiments on Macrophages

Murine macrophages (RAW264.7) (TIB-71TM) (ATCC) were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Thermo fisher Scientific, Waltham, MA, USA) and Penicillin-Streptomycin in 5% carbon dioxide (CO^2) at 37 °C overnight. After that, the non-stimulated cells were used as a control group. Meanwhile, in the stimulated groups, macrophages at

2×10^5 cell/well were administered by plasma flux or argon gas alone (another control group) with different intensities and durations before cell collection. To determine the proper dose of plasma flux, the cell viability test after plasma flux administration was performed following a published protocol, using tetrazolium dye 3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium (MTT) (Thermo fisher Scientific, Waltham, MA, USA) [33]. In brief, the cells in the 96-well plate at the indicated time-points were incubated with a MTT solution in the dark at 37 °C. After 2 h, the MTT solution was replaced by dimethyl sulfoxide (DMSO; Thermo fisher Scientific, Waltham, MA, USA) and the dissolving purple color was measured by a Varioskan Flash microplate reader (Thermo fisher Scientific, Waltham, MA, USA) at OD 570 nm.

With the selected proper dose and duration of the plasma flux treatment, reactive oxygen species (ROS) in macrophages were determined by a dihydroethidium (DHE) fluorescent dye (Sigma-Aldrich, St. Louis, MS, USA) following a previous protocol [34]. In brief, DHE diluted in FBS free media at a concentration of 20 μM was incubated with the cells at 37 °C for 20 min before analysis by the Varioskan Flash microplate reader (Thermo fisher Scientific, Waltham, MA, USA) at OD 520 nm. In parallel, the protein abundance of the possible downstream molecules of the ROS stimulation, including AMP-activated protein kinase (αAMPK) and nuclear factor kappa B (NF-κB), was determined by Western blot analysis following a previous protocol [35]. Briefly, protein was extracted from the samples using a lysis buffer (radioimmunoprecipitation assay buffer; RIPA) in supplement with inhibitors of protease and phosphatase (Thermo fisher Scientific, Waltham, MA, USA). The protein concentration was measured via Bradford assay. Then, the samples were segregated in 10% SDS (sodium dodecyl sulfate) polyacrylamide gel and transferred into the nitrocellulose membrane. Thereafter, several primary antibodies against αAMPK, phosphorylated αAMPK, NF-κB p65, phosphorylated NF-κB p65 or the internal control β-actin (Cell signaling, Beverly, MA, USA) were incubated with the membrane prior to horseradish peroxidase (HRP)-conjugated second antibodies and visualized by chemiluminescence (Thermo fisher Scientific, Waltham, MA, USA). The qualification of the band intensity was calculated by Image Studio Lite Ver 5.2 software.

To test the effect of plasma flux on inflammatory responses, lipopolysaccharide (LPS), a potent inflammatory stimulator from Gram-negative bacteria, using LPS of *Escherichia coli* 026: B6 (Sigma-Aldrich) at 100 ng/mL, or media control was added after plasma flux administration. The cell supernatant was used for cytokines measurement using ELISA assay (Biolegend) and the cells were collected to determine expression of several genes by quantitative polymerase chain reaction (qPCR) following a previous protocol [36], using Trizol and SYBR Green reagents. The list of primers is demonstrated in Table 1.

2.5. The In Vitro Experiments on Fibroblasts

Murine fibroblasts (L929) (CCL1™) (ATCC) were cultured in modified DMEM overnight, similar to RAW246.7 cells. For fibroblast scratch wound assay, the cells were seeded in a 24-well plate with 1×10^5 cell/well and incubated overnight to gain a monolayer with the cell confluence higher than 80%, following a previous publication [37]. The scratch in the culture plates was performed by gently scraping, using a 200 μL pipette tip, on the cell layer. Then, the cell debris was washed with warm PBS and incubated with modified DMEM followed by plasma flux or argon gas without plasma. The gap between two edges of scratch was photographed over different time points to represent wound closure of the fibroblast.

In parallel, the impact of plasma on fibroblast proliferation was evaluated through the reduction in carboxy-fluorescein diacetate succinimidyl ester (CFDA-SE), a long-term fluorescent dye tracer of cells, in the daughter cells as previously published [38]. In brief, the fibroblasts were stained with 2 μM CFDA-SE (Sigma-Aldrich) and diluted in warm PBS for 15 min at 37 °C in the dark. Then, the cells were washed twice by PBS and further incubated with warm media for 6 h prior to plasma flux treatment. The daughter cells were collected at 48 h after the plasma treatment for detecting fluorescent intensity by flow cytometry BD LSRII cytometer (BD Biosciences), using FlowJo software.

Table 1. List of the primers for macrophage polarization and glycolysis pathway are demonstrated.

Primers		
β-actin	Forward	5′-CGGTTCCGATGCCCTGAGGCTCTT-3′
	Reward	5′-CGTCACACTTCATGATGGAATTGA-3′
Inducible nitric oxide synthase (*iNOS*)	Forward	5′-CCCTTCCGAAGTTTCTGGCAGCAGC-3′
	Reward	5′-GGCTGTCAGAGCCTCGTGGCTTTG-3′
Arginase 1 (*Arg-1*)	Forward	5′-CAGAAGAATG GAAGAGTCAG-3′
	Reward	5′-CAGATATGCA GGGA GTCACC-3′
Found in inflammatory zone (*Fizz*)	Forward	5′-GCCAGGTCCTGGAACCTTTC-3′
	Reward	5′-GGAGCAGGGAGATGCAGATGAG-3′
Interleukin-1β (*IL-1β*)	Forward	5′-GAAATGCCACCTTTTGACAGTG-3′
	Reward	5′-TGGATGCTCTCATCAGGACAG-3′
Interleukin-10 (*IL-10*)	Forward	5′-GCTCTTACTGACTGGCATGAG-3′
	Reward	5′-CGCAGCTCTAGGAGCATGTG-3′
Transforming growth factor-β (*TGF-β*)	Forward	5′-CAGAGCTGCGCTTGCAGAG-3′
	Reward	5′-GTCAGCAGCCGGTTACCAAG-3′

2.6. Statistical Analysis

GraphPad Prism 5.0 (GraphPad Software, Inc., San Diego, CA, USA) was used to generate graphs and statistical analysis of the experiments. The in vitro experiments were performed in three independent times and demonstrated by mean ± standard error (SEM). Data analysis between two or more groups was assessed by Student's t-test or one-way analysis of variance (ANOVA) with Tukey's analysis, respectively. The significance was determined by a p value of less than 0.05.

3. Results

The administration of non-thermal plasma flux promoted the healing process of burn wounds through several wound-healing promotion factors (anti-inflammatory macrophages and improved fibroblast migration).

3.1. Non-Thermal Plasma Flux Induced Anti-Inflammatory Macrophages and Fibroblast Migration, but Not Fibroblast Proliferation

An in-house non-thermal plasma flux generator, using argon gas in atmospheric pressure with adjustable plasma properties (electrical frequency and voltage), was developed (Figure 1A,B). Due to the importance of macrophages on the wound healing process, different plasma properties, titrated by voltages and durations, were applied on macrophages (RAW246.7 cell line) before the cell viability test (MTT assay, see method) to determine the proper dose of plasma flux. With the duration of the plasma treatment at 30 s, all of the selected input voltages, in a range between 9.5 and 12.5 volts (V), demonstrated a non-statistical difference on cell viability when compared with the control non-treatment (Non) or argon gas alone without non-thermal plasma (Argon) (Figure 1C). However, the power supply voltage below 10 V showed a tendency of more survived macrophages (Figure 1C). Input with 10 V (corresponding with the output of 10 kV) was applied in the different durations, from 30 s to 90 s, and a duration of less than 30 s demonstrated a decreased number of macrophage cell deaths (Figure 1D). Notably, argon gas alone did not affect the macrophages and only argon with 90 s is shown in Figure 1D. Subsequently, non-thermal plasma with 10 V input (10 kV output) for 30 s was selected for further experiments. Because of the well-known enhanced ROS production by plasma flux [3], ROS in macrophages, using DHE fluorescent dye (see method), was measured. As expected, DHE in the plasma-treated macrophages was higher than the control conditions (Non and Argon) (Figure 2A).

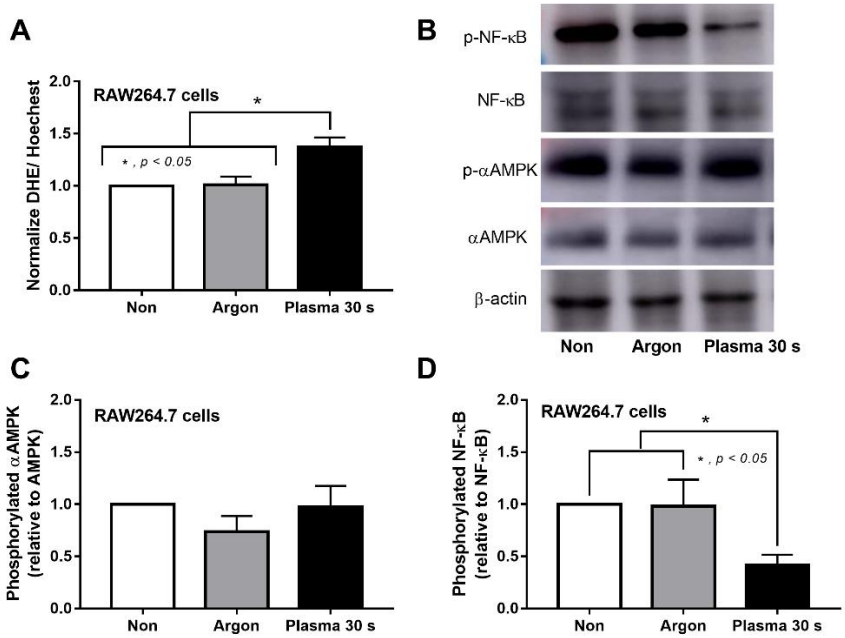

Figure 2. Characteristics of macrophages (RAW264.7 cells) without stimulation (Non) or after stimulation with argon gas alone (Argon) or plasma flux for 30 s (Plasma 30 s) as evaluated by intracellular reactive oxygen species (ROS) using dihydroethidium (DHE) fluorescent dye (neutralized by Hoechest nucleus staining dye) (**A**) and abundance of phosphorylated NF-κB-p65 (p-NF-κB) and phosphorylated-αAMPK (p-αAMPK) with the representative pictures of Western blot analysis (**B**–**D**) are demonstrated. Independently triplicated experiments were performed.

In parallel, the possible downstream signals of ROS, including AMPK (a protein sensing cell energy shortage and anti-inflammatory mediator) [39] and NF-κB (a common proinflammatory transcriptional factor) [40] was determined. Accordingly, the abundance of activated NF-κB (ratio of phosphorylated NF-κB-p65/NF-κB-p65), but not AMPK (ratio of phosphorylated αAMPK/total αAMPK), decreased after plasma flux administration (Figure 2B–D), implying a modulation of macrophage responses without cell energy alteration. Because of (i) an important transcriptional factor for cytokine production of NF-κB [40] and (ii) a potent pro-inflammatory activating property of lipopolysaccharide (LPS), an organismal molecule [41], the effect of plasma flux on macrophages with or without LPS stimulation was tested. Accordingly, LPS induced pro-inflammatory cytokines (TNF-α and IL-6) with the peak levels at 24 h post-stimulation (Figure 3A,B). Meanwhile, LPS induced pro-inflammatory macrophage polarization as evaluated by expression of *IL-1β* (peak level at 3–6 h post-LPS) and *iNOS* (peak level at 24 h Post-LPS) (Figure 3C,D). In parallel, LPS enhanced anti-inflammatory markers, including *IL-10* (similarly, between 3 and 24 h post-LPS) and *Arg-1* (peak level at 24 h post-LPS), but not *Fizz* and *TGF-β* (Figure 3E–H). Upregulation of anti-inflammatory *IL-10* was highest in LPS-stimulated macrophages with plasma flux (Figure 3E). Without LPS (PBS control), plasma flux mildly induced anti-inflammatory macrophages as determined by the upregulated *Fizz*, but not other genes (*IL-10*, *Arg-1* and *TGF-β*) (Figure 3E–H).

Due to the importance of fibroblasts in the wound healing process [9], the impact of plasma on fibroblasts (L929 cell line) was further tested. As such, wound closure as determined by fibroblast migration (the scratch wound assay), using the distance between each edge of fibroblast monolayer in cell culture plates, was lesser in the plasma treated group at 24 h post-treatment (Figure 4A,B). However, plasma flux did not enhance fibroblast cell proliferation as evaluated by CFDA-SE fluorescent staining (Figure 4C,D).

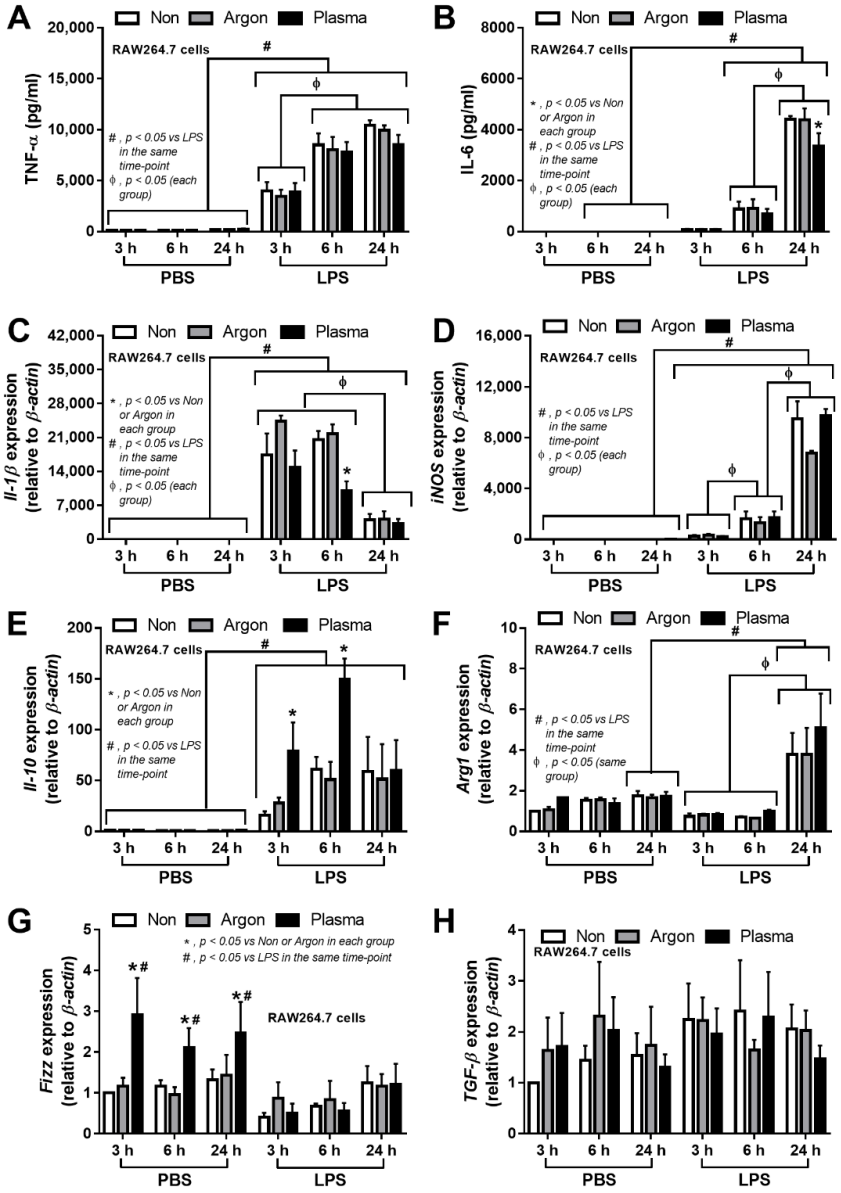

Figure 3. Responses of plasma-pretreated macrophages (RAW264.7 cells) followed by stimulation with LPS or phosphate buffer saline (PBS) (negative control) at different time-points as evaluated by supernatant cytokines (TNF-α, IL-6) (**A**,**B**), gene expression of M1 macrophage polarization markers (*IL-1β* and *iNOS*) (**C**,**D**) and M2 macrophage polarization markers (*IL-10*, *Arg1*, *Fizz* and *TGF-β*) (**E**–**H**) are demonstrated. Independently triplicated experiments were performed.

3.2. Plasma Flux Promoted Wound Healing in Burn Wounds of Mice, Regardless of Infection

For the in vivo experiments, two mouse models of burn wounds with and without infection by *Staphylococcus aureus*, the most common secondary infection in burn wounds [42], were used. Notably, the susceptibility against plasma flux of *S. aureus* and methicillin resistant *S. aureus* (MRSA) were non-different, as an approximately 30% reduction in colony count (by culture at 24 h) in both strains after a 30 s plasma flux exposure was found (data not shown). As such, the macroscopic wound monitoring at 7 days post-injury demonstrated a higher area of the wound with prominent inflammation (wound rank score, see method) in burn wounds with bacterial infection, compared with the non-infected wound (Figure 5A–C). With the plasma flux treatment, the wound area was smaller with fewer prominent inflammatory signs in the burn wound models either with or without bacterial infection (Figure 5A–C). However, our models were not severe enough to demonstrate the systemic effect of burn wounds or infected burn wounds as indicated by the non-

difference in serum cytokines and the gut permeability defect when compared with the control group (Figure 5D–F), which is different from other publications [30,43]. At 7 days, plasma flux attenuated tissue pro-inflammatory cytokines (TNF-α and IL-6), but not anti-inflammatory IL-10, in the bacterial infected burn wounds but not in the non-infected burn wounds (Figure 6A–C). These data support an anti-inflammatory effect of plasma during the wound healing process. Despite the bactericidal activity of plasma flux in vitro [44], plasma flux did not reduce bacterial burdens in burn wounds with or without infection (Figure 6D). Moreover, the accumulation of collagen, an indicator of improved wound healing [45], as determined by Masson's trichrome staining, was higher in plasma-treated non-infected burn wounds compared with the control group (Figure 6E). However, the collagen deposition was not different between the plasma treatment and control group in the infected burn wound model (Figure 6E). These data imply that plasma flux promotes wound healing in infected burn wounds partly through anti-inflammation (skin cytokines) (Figure 6A,B), while improving the wound condition in non-infected burn wounds, at least in part, through accelerated collagen deposition (Figure 6E).

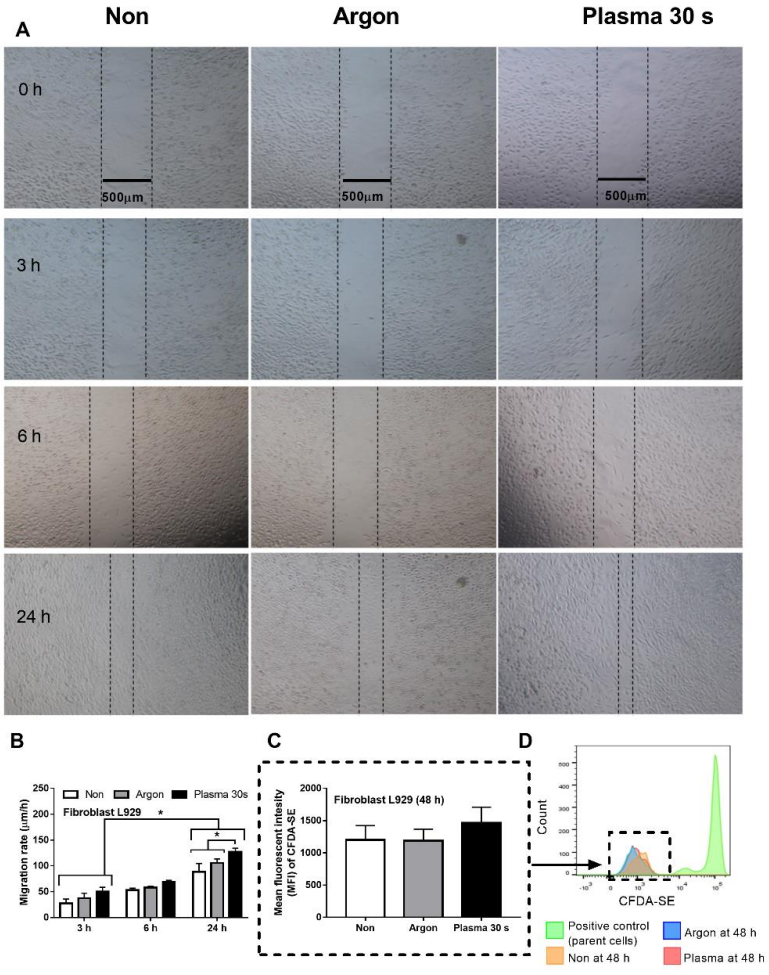

Figure 4. Responses of fibroblasts (L929 cell line) to plasma treatment (30 s), when compared with cells without stimulation (Non) or stimulated with argon gas alone (Argon) as evaluated by fibroblast migration (fibroblast scratch assay) with the representative microscopic photographs at different time points and scoring comparison (**A**,**B**) with fibroblast proliferation, using carboxyfluorescein diacetate succinimidyl ester (CFDA-SE) fluorescent staining and the representative flow cytometry analysis for CFDA-SE (**C**,**D**) are demonstrated. Independently triplicated experiments were performed.

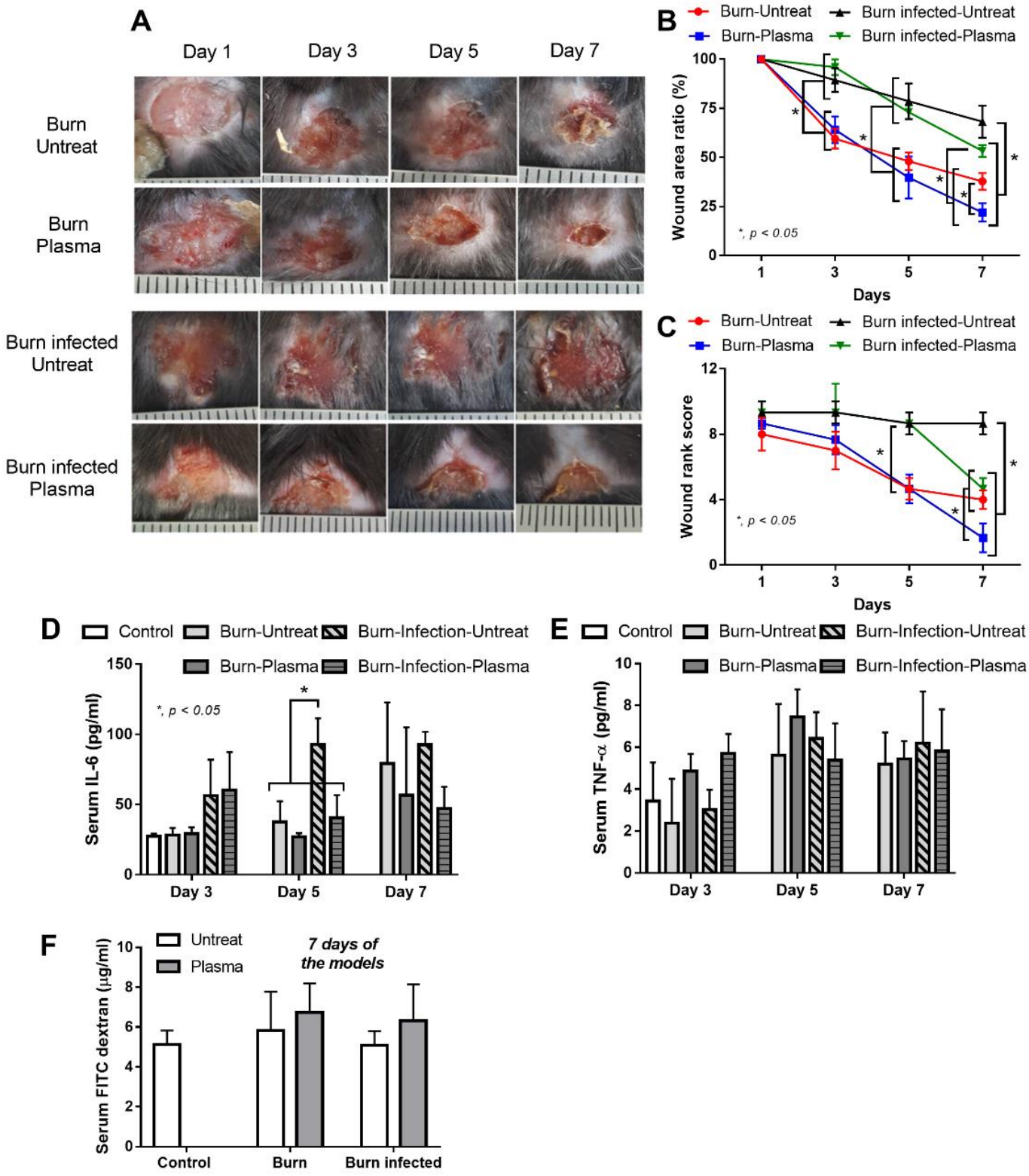

Figure 5. Characteristics of wounds in mouse models with burn injury with or without bacterial infection after treatment by plasma flux (Plasma) or Argon gas alone (Untreat) as determined by wound area and wound rank score (see method) with the representative pictures (**A**–**C**), serum cytokines (IL-6 and TNF-α) (**D**,**E**) and gut permeability measurement (FITC-dextran assay) (**F**) (n = 5–7/time-point or group) are demonstrated.

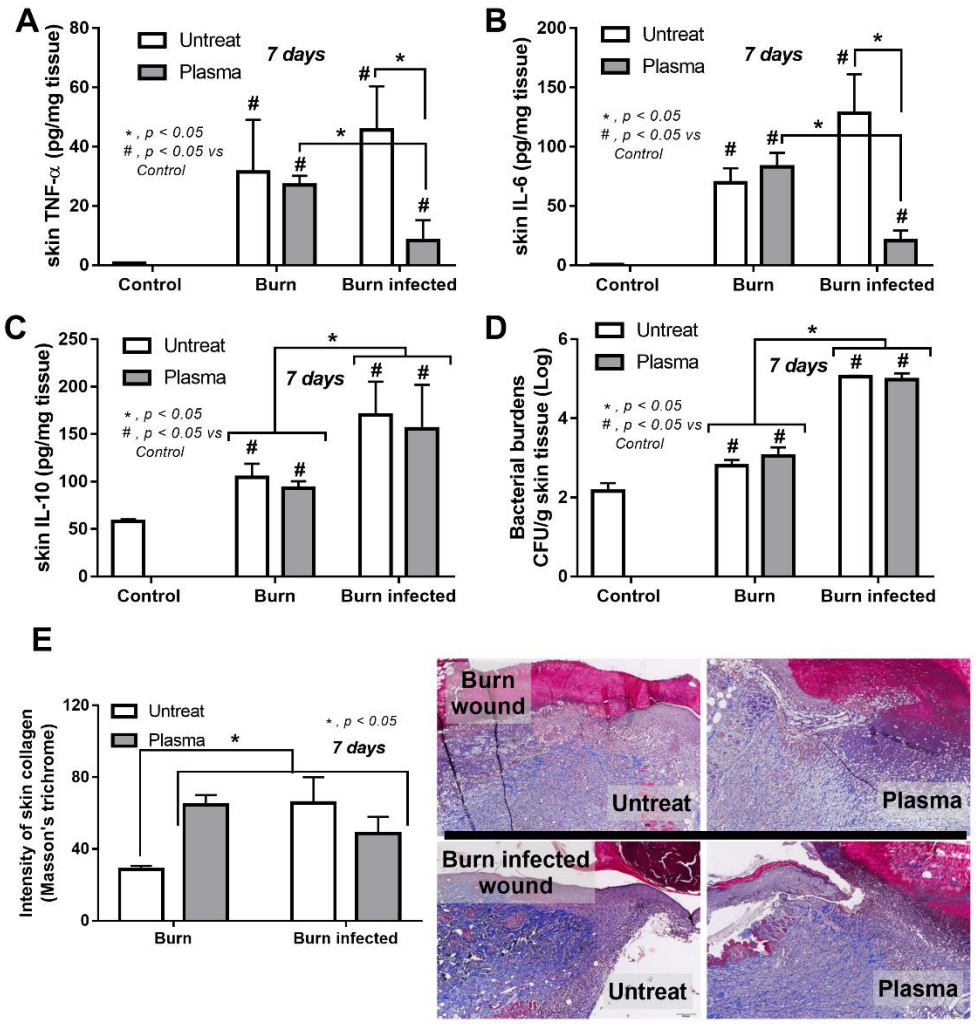

Figure 6. Characteristics of wounds in mouse models with burn injury with or without bacterial infection after treatment by plasma flux (Plasma) or argon gas alone (Untreat) as determined by cytokines from skin tissue (TNF-α, IL-6 and IL-10) (**A**–**C**), bacterial burdens (**D**) and collagen in the lesions with the representative Masson's trichrome stained histological pictures (**E**) (n = 5–7/time-point or group) are demonstrated.

4. Discussion

Argon-sourced non-thermal plasma induced anti-inflammatory macrophages that promoted wound healing in burn wound models with and without infection.

4.1. Non-Thermal Plasma Flux Induced Anti-Inflammatory Macrophages and Fibroblast Migration

Non-thermal atmospheric pressure plasma might be beneficial in several health topics, including dentistry [46], hematology (coagulation) [47], microbiology (microbial eradication) [48], surgery (wound healing) [49] and oncology [50]. Interestingly, the effects of plasma flux on wound healing (in burns) and angiogenesis (in cancer) are dependent on the conditions and features of non-thermal plasma [51], which may cause varying intensities of bioactive molecules [52]. Indeed, plasma flux increased macrophage ROS, a mediator of several cell activities (apoptosis, proliferation and inflammation), inhibiting NF-κB [53,54] in a variety of cells, including epithelial cells (H9C2) [55] and T cells (Jurkat cell) [56]. Here, plasma flux enhanced ROS and reduced NF-κB abundance in macrophages that might, at least in part, attenuate macrophage inflammatory responses. However, the reduced pro-inflammatory status of plasma-treated macrophages was not associated with the cell energy status, as the abundance of AMPK, a sensor of cell energy [39], was not different from control group.

Because (i) M2 polarized macrophages promote several processes of wound healing [25], (ii) excessive pro-inflammatory macrophages decelerate wound healing [57] and (iii) plasma flux could alter macrophage inflammatory responses [58], several genes are interesting to explore. Without LPS (an inflammatory activation), the macrophages in a neutral state did not produce inflammatory cytokines and plasma flux showed only a slight impact on macrophage as indicated by only the upregulation of *Fizz*, a biomarker of M2 macrophage polarization, but not other genes of M2 macrophages polarization. With LPS stimulation, plasma flux demonstrated a greater anti-inflammatory effect as indicated by reduced IL-6 production (at 24 h post-LPS), *IL-1β* downregulation (6 h post-LPS) and *IL-10* upregulation (3 and 6 h post-LPS). However, the expression of M2 associated genes was not different between the plasma flux treatment and the control group, implying that the plasma flux induced anti-inflammatory macrophages but did not profoundly activate M2 macrophage polarization. Nevertheless, the anti-inflammatory state could promote the wound healing process [25] and the wound healing promotion in our mouse models might partly have been due to the anti-inflammatory effect of plasma flux.

Due to the influence of fibroblasts in wound healing processes (fibrin clot lysis and production of extra cellular matrix and collagen [45]), the impact of plasma flux on fibroblasts was tested. Indeed, plasma flux induced fibroblast migration without enhanced fibroblast proliferation. Despite the promotion of both proliferation and migration in human fibroblast-like cells by helium plasma flux [38], our argon-based plasma flux could not induce fibroblast proliferation, possibly due to the difference in plasma sources. It was mentioned that argon-based plasma demonstrates a lower electron temperature than helium-based plasma [59]. Nevertheless, the enhanced fibroblast migration is one of the factors that promotes wound healing [60]. Notably, the non-different proliferation between the plasma-treated group and the control imply a safety of plasma flux on fibroblasts.

4.2. Non-Thermal Plasma Flux Promoted Healing Process of Burn Wounds, Regardless of Infection

Amongst the proposed plasma flux applications, wound healing enhancement is one of the most attractive treatment indications among the potential plasma flux applications [61], owing to the anti-inflammation action on epithelial cells [38,62,63]. However, other cell types, including immune cells and fibroblasts, are also important in the wound healing process [64]. Unfortunately, data on the effects of plasma flux on non-epithelial cells are still limited. Because burn wounds are associated with a high mortality rate [65], partly through immune dysregulation-induced opportunistic infection [16,66], burn wound models with and without *S. aureus* infection were used [67]. Although MRSA-infected burn wounds are the most important problem in clinical practice, a standard ATCC strain of *S. aureus*, but not clinical isolated MRSA, was used due to the concern of model reproducibility at this level of proof-of-concept experimentation.

Indeed, the plasma flux treatment attenuated burn wounds with and without infection as evaluated by the wound area and wound inflammatory score. Plasma flux might promote wound healing differently between burn wounds and infected burn wounds. There were no differences in cutaneous inflammatory cytokines between the plasma-treated and control groups in non-infected burn wounds. Plasma, on the other hand, improved wound healing in non-infected burn wounds, probably by increasing fibroblast migration and collagen formation. As such, the ability of fibroblasts to make collagen was enhanced by efficient cell migration [60]. On the other hand, plasma flux lowered pro-inflammatory cytokines in dermal tissue without affecting collagen formation in the infected burn wounds. These data imply promotion of the wound healing process through an anti-inflammatory effect in the infected burn wounds. Due to the activation by pathogen molecules, pro-inflammatory macrophages may be over-activated in infected burn wounds [68], and plasma flux adjusts the balance of the response. However, bacterial burdens in the total dermal tissue of the wound were not different between the plasma-treated and control groups. Perhaps the bactericidal impact may be restricted primarily to the surface of the lesions due to the limited depth of penetration by plasma flux. A more proper method

to determine bacterial burdens on the surface of lesions is needed. Nevertheless, our experiments support the utilization of plasma flux in burn wounds, which could be easily used in real clinical practice. More studies on patients would be interesting.

In conclusion, non-thermal atmospheric pressure argon-based plasma induced anti-inflammatory macrophages, possibly through (i) reduced NF-κB by ROS and (ii) enhanced fibroblast migration, which were responsible for wound healing promotion in murine burn wound models.

Author Contributions: C.P.D. and S.W. contributed equally to this work. S.W. designed plasma device. Conceptualization, C.P.D., S.W. and A.L.; methodology, C.P.D., S.W., A.C., K.S.-K.; investigation, C.P.D., D.T.-A., A.L.; data analysis and writing—original draft preparation, C.P.D. and S.W.; writing—review and editing, A.L.; funding acquisition, C.P.D., S.W., A.L.; supervision, A.L. All authors have read and agreed to the published version of the manuscript.

Funding: This work is supported by the Asahi Glass Foundation Grant (2020–2021) for Chulalongkorn University, the Program Management Unit for Human Resources & Institutional Development Research and Innovation-CU (Global Partnership B16F630071 and Flagship B05F630073) and National Research Council of Thailand.

Institutional Review Board Statement: The study was conducted according to the approved animal protocol (029/2563) from the Institutional Animal Care and Use Committee of the Faculty of Medicine, Chulalongkorn University, Bangkok, Thailand.

Informed Consent Statement: Not applicable.

Data Availability Statement: All data analyzed during this study are included in this published article.

Acknowledgments: The authors would like to thank Pornpimol Phuengmaung, Pratsanee Hiengrach for research assistance in bacterial field and Peerapat Visitchanakul for help in research document.

Conflicts of Interest: The authors declare no conflict of interest.

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