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Effects of dermal wounding on distal primary tumor immunobiology in mice

Leah M. Pyter^{*,1,2,3,4}, Daniel B. McKim¹, Yasmin Husain⁴, Humberto Calero⁴, Jonathan P. Godbout^{1,3}, John F. Sheridan^{1,5}, Phillip T. Marucha^{4,#}, and Christopher G. Engeland^{4,+}

¹Institute for Behavioral Medicine Research, Ohio State University Wexner Medical Center, Columbus, OH, USA

²Departments of Psychiatry and Behavioral Health, Ohio State University, Columbus, OH, USA

³Department of Neuroscience, Ohio State University, Columbus, OH, USA

⁴Center for Wound Healing and Tissue Regeneration, College of Dentistry, University of Illinois at Chicago, Chicago, IL, USA

⁵Department of Biosciences, College of Dentistry, Ohio State University, Columbus, OH, USA

Abstract

Background—Before primary oral tumors are treated, various prophylactic procedures that require tissue repair are often necessary (e.g., biopsies, tooth extractions, radiation, tracheotomies). Wound healing and tumor growth harness similar immune/inflammatory mechanisms. Our previous work indicates that tumors impair wound healing, although the extent to which tissue repair conversely influences tumor growth is poorly understood. Here, we test the hypothesis that dermal wound healing exacerbates primary tumor growth and influences tumor immunobiology.

Materials and Methods—Female, immunocompetent mice were inoculated subcutaneously with murine oral cancer cells (AT-84) to induce flank tumors. Half of the mice received dermal excisional wounds (four × 3.5 mm diameter) on their dorsum 16 days later, while skin of controls remained intact. Tumor and blood tissues were harvested 1 and 5 days post-wounding and tumor myeloid cell populations and inflammatory gene expression were measured. Circulating myeloid cells, cytokines, and corticosterone were also quantified.

*Corresponding author: Leah M. Pyter, leah.pyter@osumc.edu, 219 IBMR, 460 Medical Center Dr., Columbus OH 43210, t. 614.293.3496, f. 614.366.2097.

#Current address: College of Dentistry, Oregon Health and Sciences University, Portland, OR, USA

+Current address: Department of Biobehavioral Health and College of Nursing, Pennsylvania State University, University Park, PA, USA

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Results—Wounding increased tumor mass, early tumor infiltration of macrophages, and tumor inflammatory gene expression. While wounding attenuated tumor growth-induced increases in circulating myeloid cells, no effects of wounding on circulating cytokine/endocrine measures were observed.

Conclusions—These results indicate that modest skin immune/inflammatory processes can enhance distal tumor growth and alter innate tumor immunity. The implication for this work is that, in the presence of a tumor, the benefits of tissue-damaging procedures that occur clinically must be weighed against the potential consequences for tumor biology.

Keywords

oral cancer; tissue repair; myeloid cells; trafficking; cytokines

INTRODUCTION

Oral cancer is diagnosed most often in >40 year-olds who use tobacco/alcohol, although a new and rapidly growing population of young adults with human papilloma virus-positive oral cancer has also emerged. Despite treatment improvements, the 5-year survival rate has stagnated around 50% for the last 40 years (1). Diagnosis of oral cancer is often relatively late and it is a diffuse cancer; hence, tissue-damaging treatments (e.g., surgery, radiation) are common, extensive, and numerous. Prophylactic procedures that require healing (e.g., tooth extractions and other dental surgery, portacaths, feeding tubes, tracheotomies, biopsies) are often part of the early stages of treatment in order to stabilize oral health before attempting to treat the tumor (29). Even for operable oral tumors, primary tumor recurrence is common (21), indicating that cancer cells remain *in situ* after surgery. Taken together, wound healing is a frequent clinical occurrence in the presence of primary tumor cells. Partly because these tissue-damaging procedures are status quo and considered unavoidable, there is little understanding of how these two concurrent processes involving the innate immune system (i.e., tissue repair and tumor growth) may interact with each other. In our recent paper, we reported a detrimental effect of tumors on the rate of skin repair (27). Conversely, here we focus here on the effects of modest dermal wounding on primary tumor size, as well as, tumor innate immunobiology and signaling.

Dermal tissue repair is a coordinated and complex innate immune process involving rapid recruitment of neutrophils and then macrophages to the wound site (26, 37). Mounting evidence indicates that these same myeloid cells modulate tumor initiation, growth, and metastasis (7, 10, 35). Indeed, solid tumors have been dubbed the “wounds that never heal” (8). Therefore, both wounds and tumors solicit overlapping innate immune resources, which potentially create a competitive immune energetic trade-off scenario and/or an overall heightened state of inflammation. These immune processes are largely studied independently of one another, even though clinically they co-occur in cancer patients. One relevant line of basic and clinical research indicates that surgery incites metastases via the shedding and enriching of tumor cells, the neuroendocrine responses to surgery, and by causing suppression of cell-mediated immune function (e.g., natural killer cells [NK], T-cells) (3, 11, 12, 15). Given the frequency of tissue damage that occurs prior to and during primary oral cancer removal, this study aimed to test the hypothesis that active wounds

promote primary tumor growth by activating pro-neoplastic myeloid cell resources in tumors. This investigation is important because standard tissue-damaging clinical treatments may have a larger impact on primary tumor growth than is currently assumed. Understanding these mechanistic interactions will inform both pre-surgery and surgery treatment strategies, and will provide insight into targets/plans for limiting or preventing unnecessary complications.

MATERIALS AND METHODS

Animals

Nulliparous female C3H mice (Charles River, Wilmington, MA) were housed 3–5/cage and acclimated to the temperature-controlled ($21 \pm 1^\circ\text{C}$) vivarium for 1–2 weeks under a 14:10 light:dark cycle (lights on at 06:00 h). Females were used to prevent non-experimental wounding due to fighting. Rodent chow (Harlan 7912) and water were available *ad libitum* throughout the study and shredded paper was provided for nest building. All animal experiments were approved by the University of Illinois at Chicago or Ohio State University Institutional Animal Care and Use Committees and carried out in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (25). All efforts were made to minimize animal suffering and to reduce the number of mice used. Experiment 1 assessed tissue masses, tumor gene expression, and circulating cytokines and corticosterone (n=40). Experiment 2 assessed immune cell populations in the blood and tumors using flow cytometry (n=21). Figure 1 is an illustration of the experimental timeline.

Cells

The murine oral cancer cell line AT-84 originating from C3H mice (14) was generously provided by Dr. Shulin Li at MD Anderson, Houston, TX and grown in RPMI-1640 with 10% FBS, 2 mM L-glutamine, 0.1 mM non-essential amino acids, 10 mM N-2-HEPES buffer, 100 units penicillin/ml, and 100 μg streptomycin/ml at 37°C with 5% CO_2 . As this cell line is not commercially available, its authenticity has been tested using microscopic morphological assessment, as well as, growth curve analyses and mycoplasma testing (IDEXX BioResearch, Columbia, MO 65201).

Tumor Induction

Between 8–10 weeks of age, mice were acclimated to handling three times over two weeks. Next, all mice were anesthetized (100 mg/kg ketamine mixed with 10 mg/kg xylazine, i.p.) and injected in the flank (s.c.) with a 150 μl suspension of 1.5×10^6 AT-84 cells. Flank tumors were used to avoid both the potentially confounding complications of oral tumors (i.e., eating, grooming, breathing) (21) and of metastatic disease that results from oral tumor placement (23). Ear notches were made at this time for identification purposes. This procedure results in oral squamous cell carcinomas (14, 23, 36), the most common type of oral cancer in humans (9), that do not metastasize (23). This is a validated syngeneic model, allowing for the immune system to remain intact and eliminating the confounding consumption problems of a tumor located in the oral cavity. Body mass was measured twice/week.

Dermal Wounding Procedure and Analysis

Wounding treatment began 16 d post-tumor induction, when average tumor dimensions were $\sim 0.5 \text{ cm}^2$. All mice were anesthetized (same ketamine/xylazine cocktail), the dorsal skin was shaved and cleaned with alcohol. In half of the mice, four full-thickness excisional wounds were placed through the dorsal skin using a 3.5 mm sterile biopsy punch tool (Miltex Instrument Company, Plainsboro, NJ, USA) just below the shoulder blades. The other half of the mice received the same surgical preparation and anesthesia experience, minus the excisions (unwounded controls).

Tissue collection

Tumors from half of the mice in both wounded and unwounded treatment groups were harvested on Day 1 and the other half on Day 5 post-wound (Exp. 1: $n=10/\text{group/time}$; Exp. 2: $n=5-6/\text{group/time}$) following terminal anesthetization (ketamine/xylazine) and exsanguination. In Experiment 1, trunk blood was collected via heparinized tubes, kept on ice until centrifugation (4°C , 20 min at 3,000 rpm), and then frozen at -80°C for circulating cytokine and corticosterone concentration measurement. Tumors were weighed and flash frozen for quantitative RT-PCR analyses. In Experiment 2, mice were asphyxiated using CO_2 and blood was collected in heparin-lined syringes via cardiac puncture and processed for flow cytometry as previously described (39). Tumors were removed and a mid-line section was minced and collected in an enzymatic digestion solution (3.3 mg/ml each of collagenase I, collagenase XI, hyaluronidase) until further processing for flow cytometry (20).

Plasma cytokine concentrations

To assess potential systemic inflammatory alterations due to wounding, IL-1 β , IL-6, TNF α , IL-10, GM-CSF, and CCL2 (MCP-1) were measured in plasma samples using multiplex fluorescent bead array (Mouse Inflammatory 5-Plex Panel; Invitrogen, Frederick, MD, USA) according the manufacturer's protocol.

Plasma corticosterone concentrations

To determine the potential role of anti-inflammatory glucocorticoids in wounding-induced changes in tumor immunobiology, plasma corticosterone was measured in duplicate via EIA according to the manufacturer's instructions (Enzo Life Sciences, Plymouth Meeting, PA, USA) after 1:30 dilution. Intrassay and interassay variations were each $<10\%$.

Quantitative RT-PCR

Total tumor RNA was extracted by the Trizol method (Invitrogen, Carlsbad, CA). RNA concentrations were measured and 260/280 ratios were verified as between 1.8–2.0 (NanoDrop, DE, USA). Total RNA was reverse transcribed using SuperScript First-Strand kits (Invitrogen, NY, USA) according to the manufacturer's protocol. RNase H ($1 \mu\text{l}$) was added to each sample and incubated at 37°C for 20 min and then $1 \mu\text{l}$ cDNA was diluted 1:5 with RNase-free water and stored in -20°C for qRT-PCR. Five genes of interest were chosen based on their role in tumor inflammation (*IL-1 β* , *Tnfa*, *IL-6*, *IL-10*), and specifically, myeloid-derived suppressor cell activity (*Arginase-1*) (6). Mouse TaqMan Gene

Expression Assays were purchased from Applied Biosystems (Carlsbad, CA, USA) with probes labeled with 6-FAM and MGB (non-fluorescent quencher) at the 5' and 3' ends, respectively: *Il-1 β* (Mm00434228_m1), *Il-6* (Mm00446190_m1), *Il-10* (Mm00439614_m1), *Tnfa* (Mm00443260_g1), *Arg-1* (Mm00475988_m1), *Gapdh* (Mm99999915_g1; labeled with VIC). The universal two-step RT-PCR cycling conditions used on the 7000 Sequence Detection System (Applied Biosystems) were: 50 °C (2 min), 95 °C (10 min), 40 cycles of 95 °C (15 s) and 60 °C (1 min). Relative gene expression of individual samples run in duplicate was calculated by the comparative C_T method (2^{-CT}).

Flow cytometry

Tumor sections were rotated at 37 °C for 1 h. The digest was filtered through a 70- μ m nylon cell strainer, rinsed, centrifuged, resuspended, and then the total number of cells was determined with a BD Coulter Particle Count and Size Analyzer (Beckman Coulter). Staining of cell surface antigens for blood and tumor samples was performed as previously described (38, 39). In brief, Fc receptors were blocked with anti-CD16/CD32 antibody (eBioscience, San Diego, CA, USA) and then cells were incubated with the appropriate conjugated antibodies (CD11b [M1/70], Ly6C [AL-21], Ly6G [1A8]; BD Biosciences, San Jose, CA, USA) for 1 h at 4°C. Cells were washed and then resuspended in FACS buffer for analysis. Nonspecific binding was assessed using isotype-matched antibodies. Antigen expression was determined using a Becton-Dickinson FACSCalibur four-color cytometer (BD Biosciences). Data were analyzed using FlowJo software (Tree Star, Ashland, OR, USA) and positive labeling for each antibody was determined based on isotype-stained controls.

Statistical analysis

Statistical comparisons of differences in tissue mass, gene expression, and flow cytometry data were analyzed using 2-way ANOVAs and Student's *t*-tests with Statview version 5.0.1 software (Scientific Computing, Cary, NC, USA) when variance was normal. Nonparametric Mann-Whitney *U* tests were used when variance was not normally distributed. Repeated measures ANOVAs were used to compare changes in body mass over time. Pearson's correlations were used to relate spleen mass to tumor mass. Data were determined to be statistically significant when $p < 0.05$ and are presented as mean $\pm$ standard error of the mean (SEM).

RESULTS

Tissue masses

Body mass increased over time ($F_{2,76}=78.7$, $p<0.0001$; Figure 2A) and was not modified by wounding ($p>0.05$). Tumor mass relative to body mass (absolute mean $\pm$ SEM, Day 1: no wound 0.19 ± 0.03 , wound 0.18 ± 0.03 ; Day 5: no wound 0.3 ± 0.03 , wound 0.4 ± 0.03 g) increased with time in mice that were wounded ($t_{22}=3.8$, $p<0.01$; Figure 2B), but did not in unwounded mice ($p>0.05$). Relative (and absolute, mean $\pm$ SEM, Day 1: no wound 0.13 ± 0.005 , wound 0.12 ± 0.008 ; Day 5: no wound 0.15 ± 0.004 , wound 0.17 ± 0.02 g) spleen mass increased over time ($F_{1,26}=8.8$, $p < 0.01$; Figure 2C), however wounding did not statistically alter spleen mass ($p>0.05$). Relative (and absolute) masses of spleens and tumors

were significantly correlated with each other among both wounded and unwounded mice ($r=0.49$, $n=29$, $p<0.01$; data not shown).

Tumor flow cytometry

Tumor neutrophil ($F_{1,22}=26.7$, $p<0.001$) and macrophage/monocyte ($M\Phi/Mo$; $F_{1,20}=9.7$, $p<0.01$) infiltrate increased over tumor growth (Day 1 to Day 5) in all mice (Figure 3). The $M\Phi/Mo$ increase over time was largely driven by the unwounded mice ($U=4$, $p<0.05$) and attenuated in the wounded mice ($p>0.05$). Wounding tended to interact with time for $M\Phi/Mo$ infiltration ($F_{1,20}=2.7$, $p=0.1$), driven primarily by the significant increase in tumor $M\Phi/Mo$ infiltration that occurred one day after wounding compared to mice that were not wounded ($U=5$, $p<0.05$). Neutrophil infiltration was not affected by wounding ($p>0.05$).

Blood flow cytometry

Circulating monocytes ($F_{1,17}=15.8$, $p<0.01$; Table 1) and neutrophils increased over tumor growth (Day 1 to Day 5), although this did not reach statistical significance for neutrophils ($p=0.08$). The monocyte increase over time was driven more by the unwounded mice ($t_7=3.9$, $p<0.01$) than by the wounded mice ($p>0.05$). Based on the *a priori* hypothesis, neutrophils also significantly increased in the bloodstream within unwounded ($t_7=2.6$, $p<0.05$) more than wounded mice ($p>0.05$).

Blood cytokines and hormones

Wounding did not alter any of the circulating cytokine or corticosterone concentrations measured over time ($p>0.05$ in all cases; Table 1).

Tumor qPCR

Wounding altered whole tumor gene expression of several inflammatory genes over time including *Il-1 β* ($p>0.05$), *Il-6* ($F_{1,35}=13.5$, $p<0.001$), *Il-10* ($F_{1,33}=4.5$, $p<0.05$), *Arginase* ($F_{1,34}=12.3$, $p<0.01$), and *Tnfa* ($F_{1,35}=9.2$, $p<0.01$; Figure 4). Several of these interactions consisted of initial increases in tumor gene expression on Day 1 post-wounding (*Il-6*, *Il-10*, *Arginase*; $p<0.05$ in each case) compared to unwounded mice. For *Il-6*, this was followed by a significant decline in gene expression on Day 5 post-wounding ($p<0.05$). Conversely, wounding consistently reduced tumor expression of *Tnfa* ($p<0.01$).

DISCUSSION

The potential for standard-of-care tissue repair events to influence primary tumor growth by altering local immune cell populations is an understudied, but clinically-relevant concern. Indeed, our previous study indicated that the inverse relationship was relevant, that tumors altered the immunology and rate of dermal wound healing relative to tumor-free controls (27). This present study demonstrated that modest excisional skin wounds significantly increased distal primary tumor mass in mice relative to unwounded controls. Increases in spleen mass (often correlated with tumor mass) supported this finding. Previous studies in mice indicate that more invasive and damaging surgical wounds (e.g., intramuscular, longer surgery time) also increase the metastatic spread of primary tumors (33) and is potentially modulated by sex (males > females). The present finding that small, superficial skin wounds

enlarge the size of distal tumors suggests that simple tissue-damaging procedures, which frequently occur before tumor resection, have an impact on primary tumor growth and biology. Such prophylactic procedures are particularly common prior to oral tumor treatment given the anatomical complexity and vital functions of this body region (e.g., respiration, speech, consumption, social aesthetics).

Previous work on the interaction between surgery and metastases focuses primarily on NK and T-cell-mediated mechanisms (12, 28). However, tissue repair and tumor growth involve other overlapping immune processes, including inflammation (31). Therefore, the shared activation of immune/inflammatory mechanisms may drive the observed increases in primary tumor growth within wounded mice. Indeed, the early inflammatory phase of wounding (captured on Day 1 post-wounding) corresponded to elevations in tumor macrophage infiltration, as well as increases in tumor cytokine gene expression. This mirroring of the wound inflammatory phase by the tumor could reflect an overall shift toward systemic inflammation or more localized immune changes in the tumors.

With respect to the former possibility (systemic inflammation), wounding did not exacerbate, but rather attenuated, the observed elevations in circulating myeloid cells as tumors grew over time. In contrast, burn wound models characterized by larger damaged skin surface area and bacterial infection induce marked myelopoiesis from the bone marrow into the blood (13, 30). In humans, circulating monocytes decrease after surgery, which may be explained by the glucocorticoids released from the perceived stress of undergoing surgery (2).

Furthermore, effects of the present wounding procedures on circulating cytokine and glucocorticoid concentrations were lacking. In contrast, routine surgeries, which promote immune responses relevant to metastases in humans (NK activity suppression), increase circulating inflammatory cytokines and glucocorticoids (2, 12). This difference is likely due to the increased invasiveness of surgical wounds and the anticipated stress relative to the current rodent model. Norepinephrine has also been shown to play a role in these metastatic responses (4, 11) and in burn wound models (34). Taken together, while systemic signaling between the wounds and tumor remains a putative mechanism by which wounding increases tumor size, evidence supporting this possibility was not observed using this experimental paradigm.

In contrast to the hypothesis that wounding may have increased systemic inflammation, evidence of wounds affecting local tumor immunobiology was observed. Tumor MΦ/Mo quantity increased one day after wounding, relative to tumors from unwounded mice. This suggests that tumor immune cell infiltration and/or retention was elevated by distal tissue repair, as opposed to the possibility that macrophages already present in the tumor simply underwent a wounding-induced phenotypic switch.

Increased infiltration or retention of tumor MΦ/Mo could be driven by the observed coincident increases in tumor cytokine signals (*Il-6*, *Il-10*, *Arg*). Indeed, cytokines cause the local endothelial permeability, reactivity, and adhesion molecule expression that are required for immune cell infiltration from the vasculature (22). Tumor inflammatory gene expression

is also associated with tumor growth in general by promoting cell survival, invasion, and angiogenesis (5). For example, the observed elevations in tumor *Il-10* and *Arginase* mRNA are consistent with those secreted by tumor-associated macrophages that promote tumor progression (32). In contrast to the other inflammatory markers examined, *Tnfa* mRNA was decidedly reduced in tumors of wounded mice. Considering that this is inconsistent with the other immune/inflammatory observations, tumor *Tnfa* likely functions as originally described (i.e., “tumor necrosis factor”), by disrupting tumor growth through impaired angiogenesis (17). It is important to note that the present gene expression data stemmed from whole tumor samples (e.g., tumor cells, fibroblasts, endothelial cells, immune cells) and were not necessarily specific to tumor-infiltrating immune cells. Overall, the wounding-induced increases in tumor macrophages and associated pro-tumor/immune infiltration signaling may have contributed to the concomitant increased tumor growth.

Interpreting the data over time (Day 1 to 5) is complicated by the simultaneous dynamics of a healing wound and a growing tumor. By Day 5 post-wounding, tumor size had increased, which may be underpinned the increases in circulating and tumor-infiltrating myeloid cells in all mice over time (16). Of note, the initial (Day 1) tumor MΦ/Mo cell increase that was specific to wounded mice was somewhat attenuated on Day 5 relative. A similar pattern emerged among the gene expression data (e.g., *Il-6*, *Arginase*). In the future, greater sample sizes and a study design that controls for either the dynamics of wound healing or tumor growth would determine the significance of these Day 5 attenuations in wounded mice. An important consideration for the clinical relevance of these findings is the role of the tumor microenvironment given that the present tumors were ectopic due to the general health complications of experimental tumors in the oral cavity. Furthermore, conclusions based on these findings may be restricted to female mice as sex hormones are an important modulator of both immune cell function (18) and tumorigenesis (24). Indeed, relevant to the initial increases in tumor inflammatory genes with wounding, adult female mice are characterized by elevated inflammatory responses relative to males (19).

Overall, this study provides evidence that small, distal skin wounding has an impact on primary tumor growth and immunobiology and is suggestive of potential clinical implications for standard of care practices for (oral) cancer patients. Clinical procedures that require wound healing prior to tumor resection should be carefully weighed against the potential pro-tumor effects that even distal tissue repair may confer. Further mechanistic investigation is warranted.

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Experimental Model

Figure 1. Experimental timeline

Temporal sequence of tumor and wounding treatments, tissue collection, and related photograph.

Figure 2. Effects of dermal wounding on tissue sizes during oral tumor growth in the flank
Mean $\pm$ SEM **A)** body mass, **B)** tumor mass relative to body mass, and **C)** spleen mass relative to body mass. $n = 10-12/\text{group}$; # $p < 0.05$ within wounded between days; * $p < 0.05$ between days

Figure 3. Effects of dermal wounding on absolute number of myeloid cells in oral tumors
Mean $\pm$ SEM (A) $CD11b/Ly6G^+$ macrophages/monocytes and $CD11b/Ly6G^+$ neutrophils in tumors 1 or 5 days after wounding treatment. (B) Representative flow bivariate dot plots of Ly6G and Ly6C labeling, gated by CD11b, of tumor tissue. $n = 4-6$ /group; * $p < 0.05$ between wounding treatments at one day post-wounding; # $p < 0.05$ within wounding treatment between days

Figure 4. Effects of wounding on mRNA expression of inflammatory markers in oral tumors
Mean ± SEM quantitative gene expression of **A) *IL-1β***, **B) *IL-6***, **C) *IL-10***, **D) *Arginase***, and **E) *Tnfα*** in whole tumor tissue. n = 6/group; *p<0.05 between wounding treatments

Table 1

Circulating immune and endocrine measures in wounded and unwounded tumor-bearing mice.

Blood Analyte (Mean $\pm$ SEM)	DAY 1 POST-WOUNDING		DAY 5 POST-WOUNDING	
	NO WOUND	WOUND	NO WOUND	WOUND
CD11b/Ly6C ⁺ Monocytes (cells/ml) *	$4.8 \times 10^5 \pm 5 \times 10^4$	$5.4 \times 10^5 \pm 7 \times 10^4$	$8.0 \times 10^5 \pm 6 \times 10^4$ [#]	$7.8 \times 10^5 \pm 1 \times 10^5$
CD11b/Ly6G ⁺ Neutrophils (cells/ml) *	$1.3 \times 10^6 \pm 2 \times 10^5$	$1.7 \times 10^6 \pm 2 \times 10^5$	$2.1 \times 10^6 \pm 3 \times 10^5$ [#]	$2.6 \times 10^6 \pm 7 \times 10^5$
IL-1 β (pg/ml) ^{&}	10.9 ± 1.7	10.5 ± 1.0	10.2 ± 1.0	14.5 ± 4.2
IL-6 (pg/ml) ^{&}	41.9 ± 3.0	44.9 ± 6.0	41.3 ± 1.3	40.8 ± 2.1
TNF α (pg/ml) ^{&}	8.0 ± 0.4	8.7 ± 0.3	8.8 ± 0.4	8.4 ± 0.6
IL-10 (pg/ml) ^{&}	26.2 ± 1.5	27.9 ± 2.3	27.2 ± 1.5	29.6 ± 1.0
GM-CSF (pg/ml) ^{&}	4.2 ± 0.3	4.2 ± 0.2	4.4 ± 0.3	4.5 ± 0.4
CCL2 (pg/ml) ^{&}	4.2 ± 0.7	7.8 ± 2.9	8.0 ± 1.8	8.8 ± 2.8
CORTICOSTERONE (ng/ml) ⁺	76.1 ± 8.0	72.6 ± 6.4	41.3 ± 12.1	59.6 ± 11.2

[#] p<0.05 relative to Day 1 values;

* n=4–6/group;

[&] n=5–6/group;⁺ n=5/group