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The effect of leg ischemia/reperfusion injury on the liver in an experimental breast cancer model

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Abstract

Background/Aim: Ischemia/reperfusion (I/R) injury occurs during breast cancer surgery, especially those involving a modified radical mastectomy, lumpectomy, and axillary lymph node dissection. Tissue damage and stress due to I/R alter immune system functions, especially those of the myeloid cells. The immunologic impact of this I/R injury on myeloid-derived cancerous cells remains unknown. We sought to investigate the effect of I/R injury in the extremity close the breast tumor location on myeloid cell population in the liver and liver metastasis.

Methods: 4T1 breast tumors were created in the left inguinal breast region of the experimental animals. When the tumor reached 0.5 cm in diameter, ischemia was produced on the left down-extremity for 90 min and reperfusion was induced for short (3 days), middle (7 days), and long terms (14 days). At the end of the reperfusion period, proximal limbs and livers were harvested. The limb and liver samples were histopathologically examined with H&E staining. Immune cell percentages were determined in the liver by flow cytometry.

Results: There was an increase in muscle fiber degeneration and disorganization in the I/R induced proximal legs on days 3 and 7 of I/R in both tumor free and tumor bearing animals with a further impact in tumor bearing mice. Even though I/R injury did not affect tumor metastasis to the liver, it had an impact on liver myeloid cell percentages in both tumor free and tumor bearing animals. Additionally, tumor bearing mice demonstrated higher myeloid cell percentages in both the pre-I/R and post-I/R experimental groups. There was a remarkable change in the levels of granulocytic, and monocytic myeloid cells and macrophages due to the I/R injury.

Conclusion: With the formation of short-term I/R injury in a distant site, tumor development and/or seeding to metastasis sites after surgery could be prevented. This study contributes to the understanding of the inflammatory process after I/R injury occurring during interventions.

Keywords: Breast cancer, Ischemia, I/R injury, Myeloid cells, MDSCs, Liver

Introduction

Breast cancer is the most common cancer among women and affects 2.1 million women every year, worldwide. It ranks the first cancer-related death among women, with about 15% [1]. In 2018, 22.345 women were diagnosed with breast cancer and 5.452 women lost their lives in Turkey [2]. Various methods were used in the treatment of breast cancer including surgery, radiation therapy, chemotherapy, hormone therapy, targeted drug therapy, and immunotherapy. Most women undergo breast cancer surgery, and many receive adjuvant therapies such as chemotherapy, hormone, or radiation therapy. However, during breast cancer surgery, especially those involving modified radical mastectomy, lumpectomy, axillary lymph node dissection, upper extremity and surrounding tissues are exposed to ischemia. When the surgery is completed, blood supply restarts, which leads to I/R damage.

The term "ischemia" was first expressed in early the 19th century as a restriction in blood supply to tissues by the occlusion of arterial flow. Restoration of blood flow to a previously ischemic tissue or organ is called reperfusion. The longer ischemic duration results in worse clinical complications such as functional failure of tissues and organs due to insufficient oxygen uptake [3]. Because of this, it has tremendous side effects, and increases morbidity and mortality rates. I/R injury is not restricted to the prior ischemic site, also accounts for remote organ damage, which brings out serious results like multiple organ failure [4-6]. Complications related to immune system causing tissue damage begins at reperfusion [7]. The main factors which cause distant organ damage by exiting the ischemic area and entering circulation are leukocytes, inflammatory mediators, and reactive oxygen species (ROS). The production of reactive nitrogen species is also effective in cellular and systemic response to ischemia. These reactive molecules induce cellular damage and structural changes and lead to apoptotic/necrotic cell death [8, 9].

In response to various stimuli, leukocytes migrating to the I/R region and pro-inflammatory molecules disrupt the cellular contact, thereby increasing vascular permeability [10], resulting in even more infiltration of leukocytes into the I/R region [11]. Among the leukocytes, the role of granulocytes in I/R injury is critical, and neutralizing the antibodies against CD11b and/or CD18 were utilized to prevent this damage [12]. Neutrophil granulocytes are the fastest migrating and extensively found cell group in the I/R region. They further induce tissue damage by the production of high levels of ROS, hydrolytic enzymes and pro-inflammatory cytokines and chemokines (IL-1β, IL-6, IL-12, IFN-γ, TNF-α, and MCP-1) [13, 14]. Although the contribution of monocytes and macrophages to the inflammatory process in the I/R region is limited and overshadowed by neutrophils, these cells are also known to migrate to damaged areas [15]. It is undeniable that myeloid cells play the central role in I/R damage, because they are both the foremost and fastest responding immune cell group.

The relationship between cancer development and inflammation has long been investigated [16]. Epidemiological evidence indicates the connection between inflammation and cancer development. For example, longer periods of inflammation induce dysplasia [17]. Furthermore, chronic inflammation-like processes are the characteristics of tumor micro-environment [18]. Secreted various cytokines, chemokines or growth factors (M-CSF, GM-CSF, PGE2, VEGF, IL-1β, IL-4, IL-6, IL-10 and IL-13) induce hematopoiesis and the production of myeloid cells [19]. However, these cells enter the circulation before their maturation step; therefore, they disrupt inflammatory response due to insufficient immune stimulation and/or immune suppression. Myeloid cells which regulate immune responses by diverse mechanisms are called myeloid-derived suppressor cells (MDSC) and divided into subtypes as PMN-MDSC: CD14$^-$CD11b$^+$CD15$^+$ (or CD66b$^+$), E-MDSC: Lin$^-$(CD3/14/15/19/56)/HLA-DR$^-$/CD33$^+$, M-MDSC: CD11b$^+$CD14$^+$HLA-DR $^{low/-}$ CD15$^-$ in human; PMN-MDSC: Gr-1$^+$CD11b$^+$Ly6C^{lo}Ly6G$^+$ and M-MDSC: Gr-1$^+$CD11b$^+$Ly6C^{hi}Ly6G$^-$ in mice [20]. The presence and increase of MDSCs have also been shown in peripheral blood and tumor samples of cancer patients. The ratio of MDSCs in peripheral blood augments when cancer is in the progress. For example, the percentage of MDSCs is 1.96% in stage 1–2, 2.46% in stage 3 and 3.77% in stage 4 breast cancer [21].

MDSCs are gathered in the liver in addition to the spleen, bone marrow, blood, and the tumor. Liver hematopoiesis also contributes to the expansion of MDSCs in this organ [22]. It was indicated that intravenous injection of exogenous bone marrow-derived mononuclear cells into mice with colorectal cancer are able to migrate to the liver and induce tumor liver metastasis [23]. However, the contribution of hepatic I/R injury to cancer growth and metastasis in the liver has been explored in different studies [24]. Moreover, hepatic ischemia enhances the number of metastatic nodules in the liver in experimental rat and mice colon cancer models [25-27]. In other preclinical experimental studies, the impact of hepatic I/R injury on tumor growth and metastasis in different tumor models such as hepatocellular carcinoma and pancreatic cancer has been investigated [28, 29]. In hepatic ischemia, cytokines, growth factors, and adhesion molecules produced in the I/R region are the mediators of induction of tumor progression and liver metastasis [24]. However, none of these studies demonstrated the impact of I/R injury in a distant organ on tumor liver metastasis. Although immune suppression capacity, therefore, the tumor-promoting effect of MDSCs is enhanced under hypoxia, information about how these cells behave during I/R injury is limited [30]. In this study, we assumed that I/R damage to a distant organ affects the level of myeloid cells in the liver as a host repair response in the presence of a tumor and might affect tumor growth and liver metastasis. Therefore, we herein established an extremity I/R model in mice harboring breast tumors in the mammary fat pad and showed that I/R can induce the accumulation of MDSCs, especially G-MDSCs, in the liver and tumor growth without an effect on tumor metastasis to the liver.

Materials and methods

This study was conducted at Hacettepe University Experimental Animal Application and Research Center (Ankara, Turkey) after obtaining the approval of the Experimental Animals Local Ethics Committee of the university (Approval

No: 2015/46-07). All animal experiments were performed according to the World Medical Association Code of Ethics (Helsinki Declaration).

### Animal model and cell lines

Female BALB/c mice, 6–8 weeks old, (Kobay A.S., Ankara, Turkey) were housed under standard conditions. Animals were divided into 8 groups: Extremity I/R early (Day 3 (n = 5)), middle (Day 7 (n = 6)), and late (Day 14 (n = 6)), breast cancer + I/R early (Day 3 (n = 5)), middle (Day 7 (n = 6)), and late (Day 14 (n = 6)), breast cancer only (n = 6), and no I/R control groups (n = 5).

Breast cancer cell line, 4T1, (American Type Culture Collection, LGC Promochem, Rockville, MD, USA) were cultured in RPMI 1640 medium (Biowest, Nuaillé, France) supplemented with 100 U/ml penicillin, 100 mg/ml streptomycin, and 10% FBS in humidified atmosphere with 5% CO_2 at 37°C. 4T1 cells ($5x10^4$ cells/100 µl) were subcutaneously inoculated into the left-inguinal mammary fat pad of BALB/c mice.

Anesthesia and surgical procedure

For all groups, 5 mg/kg Xylazine (Alfazyne- %2) and 150 mg/kg ketamine hydrochloride (Ketalar- %5) were injected intraperitoneally for general anesthesia. Thirty minutes after the induction of anesthesia, the McGivney Ligasure was used to apply the ORB on the left limb [31] (Figure 1A). Supplemental anesthesia was administered to keep the mice under anesthesia throughout the duration of ischemia. Ischemia was induced on the hindlimbs for 90 min in the first 6 groups, and ORB was cut to induce reperfusion for 3, 7, and 14 days. Mice in 4th, 5th and 6th groups were first administered 4T1 cells into left mammary pad s.c. (Figure 1B). Tumors were monitored twice a week and when the tumor reached 0.5 cm in diameter, 90 min of ischemia was created. Mice in the 7th group were only inoculated with tumor cells while mice in 8th group served as controls. At the end of reperfusion, animals were euthanized (Figure 1B). Both limbs, livers and tumors were harvested. Body weights were followed biweekly. Limbs were collected just under the region where ORB was applied and the skin was removed.

Figure 1: Establishment the model of hind limb I/R injury. A) Representative position of ORB and MHL on mouse hind limb and operative view of hind limb ischemia injury. Orthodontic rubber band (ORB) is applied by McGiveny hemorrhoidal ligator (MHL). Red arrow indicates the position of ORB. B) Animals were inoculated with 4T1 cells s.c. ($5 x 10^4$ cells/100 µl) into left mammary fat pad and 90 min of ischemia was induced on the left hind limb when 4T1 tumors were 0.5 cm in diameter in (n =23 mice). Mice were sacrificed after 3-, 7- and 14-days following reperfusion and livers were harvested for histological and immunological assessment. n=6 mice were only injected with tumor cells. I/R injury was induced in n=18 mice w/o tumor challenge and n=5 mice left as controls.
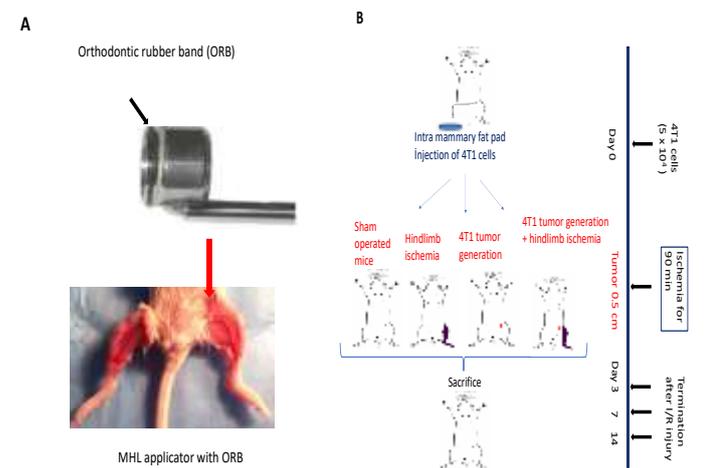
Immunological Analyses

Cell suspensions from the livers and tumors were obtained with mechanical agitation in phosphate buffered saline (PBS) and passed through 40 µm pore-sized filters. The leukocytes in the cell suspension were further separated by Ficoll-1119 (Sigma, Steinheim, Germany) density gradient centrifugation. The cells were labeled with monoclonal antibodies against CD45 (clone 30/F11), CD11b (clone M1/70), Gr-1 (clone RB6-8C5), Ly6C (clone HK1.4), Ly6G (clone 1A8), and F4/80 (clone BM8) and isotype controls were used (Biolegend, San Diego, CA, USA). The percentage of positive cells was calculated by comparison with the appropriate isotype-matched antibody controls. Studies and analyses were conducted on a FACS Aria II flow cytometer (Becton Dickinson, San Jose, CA, USA) and FACS Diva software, respectively.

Histopathology and immunohistochemistry

Proximal limb tissues were harvested, fixed in 3.7% formaldehyde, embedded in paraffin, sliced, and stained with hematoxylin and eosin for histopathological evaluation. Histopathological scores for muscle fiber degeneration and disorganization were found in 5 tumor-free mice in the no I/R group, 8 in 3 days post-I/R, 5 in 7 days post-I/R, 6 in 14 days post-I/R and among tumor bearing mice, 6 in the no I/R group, 5 in 3 days post-I/R, 4 in 7 days post-I/R, and 6 in 14 days post-I/R groups. They were scored as severe = 3, mild = 2, and occasional = 1. In the liver, metastatic loci and myeloid cell groups were examined. For granulocytic myeloid cells, Ly6G marker was analyzed by immunohistochemical staining. Metastatic foci were determined within 4 µm sections cut across the liver. The geometric mean of the largest vertical and horizontal dimensions of these foci was calculated as a measure of the size. A metastasis score was calculated for each animal by considering the frequency and size of foci (metastasis score per liver = number of the foci x average size of the foci).

Relative-quantitative RT-PCR

Total RNA (5 µg) was isolated (Animal Tissue RNA Purification Kit, Norgen Biotek, Thorold, ON, Canada) and converted into cDNA (RevertAid First Strand cDNA Synthesis Kit, Thermo, Waltham, MA, USA). Real-time PCR (SsoAdvanced Universal SYBR Green supermix, Bio-Rad, Hercules, CA, USA) was performed with the forward and reverse primer oligonucleotides, respectively, specifically designed for IL-1β, 5'-TGCCACCTTTTGACAGTGATG-3' and 5'-AAGGTCCACGGGAAAGACAC-3'; calciumbinding protein A9 (S100A9), 5'-AGATGGCCAACAAAGCACCT-3' and 5'-TCTCTTTCTTCATAAAGGTTGCCA-3'; matrix metalloproteinase 9 (MMP-9), 5'-GCGGTCCTCACCATGAGTCC-3' and 5'-TAGCGGTACAAGTATGCCTCTGC-3'; vascular endothelial growth factor A (VEGFA), 5'-CAGATCATGCGGATCAAACCTC-3' and 5'-TTGTTCTGTCTTTCTTTGGTCTGC-3'; CCL2 5'-AGCTGTAGTTTTTGTCACCAAGC-3' and 5'-GTGCTTGAGGTGGTTGTGGA-3'; βactin, 5'-GGCACCACACCTTCTACAATG-3' and 5'-GGGGTGTTGAAGGTCTCAAAC-3'. For each gene of interest, threshold cycle (Ct) was determined and normalized according to the housekeeping β-actin Ct.

Statistical analysis

Statistical analyses were performed using Student's t-test and one-way ANOVA where appropriate (SPSS software, IBM, Turkey). For each test, *P*-values ≤0.05, ≤0.01, ≤0.001 and ≤0.0001 were considered statistically significant, more significant, significant, most significant, respectively ((*), (**), (***), and (****), respectively). Unless otherwise noted, the data are shown as mean ± SEM.

Results

The effect of I/R injury on muscle damage in the proximal limb tissues

Histopathological sections from the extremities were examined for muscle fiber degeneration and disorganization. Muscle fiber degeneration was 50% with score 3, 37.5% with score 2, and 12.5% with score 1 on day 3 post-I/R in the proximal limb of tumor free animals, while it was 80% with score 3 and 20% with score 2 in tumor-bearing mice (Figure 2A). Muscle fiber disorganization in the proximal leg of tumor-bearing animals was 40% with score 3, 40% with score 2 and 20% with score 1. In tumor-free mice, it was 50% with score 3, 12.5% with score 2 and 37.5% with score 1 on day 3 post-I/R (Figure 2B). Muscle fiber degeneration was 100% with score 3 in the proximal leg of tumor-bearing animals and 40% with score 3, 60% with score 2 on day 7 post-I/R in tumor-free mice (Figure 2A). In the proximal leg of tumor free animals, muscle fiber disorganization was 40% with score 3, and 60% with score 1, whereas in tumor-bearing mice, it was 100% with score 3 on day 7 post-I/R (Figure 2B). Furthermore, muscle fiber degeneration was 83.3% with score 2, and 16.6% with score 1 in tumor free mice, whereas in tumor-bearing mice, it was 100% with score 2 on day 14 post-I/R (Figure 2A). Muscle fiber disorganization in the proximal leg of tumor-bearing animals was 66.6% with a score 2 and 33.3% with score 1. In tumor-free mice, it was 33.3% with score 2 and 66.6% with score 1 on day 14 post-I/R (Figure 2B). The histopathological analysis of the proximal limb tissues demonstrated that the I/R procedure induces muscle fiber degeneration and disorganization in both tumor bearing and tumor-free animals with a higher impact on tumor-bearing mice and on day 7 post-I/R (Figure 2).

The effect of I/R injury on the liver

The breast tumor cells may spread locally or metastasize via the lymphatics and blood vessels. Frequently, breast cancer metastasizes to the liver and the lung through blood vessels [32]. Therefore, we first examined the effect of I/R injury on liver metastasis. Liver metastasis was not found in cancer-bearing mice. Moreover, the liver weights were not altered significantly among the tumor-free and tumor-bearing groups except on day 14 post-I/R (Figure 3A).

It is known that myeloid-derived cells have a tumor-promoting function in hematopoiesis-related organs, such as the liver, in cancer patients. Hence, we examined the liver tissue histologically. There was an average of 3 myeloid cell groups in the control group, 14 in the breast cancer group, 3 on day 3 post-I/R, 4 on day 7 post-I/R, and 2 on day 14 post-I/R groups. However, there was 11 on day 3 post-I/R, 30 on day 7 post-I/R and 85 on day 14 post-I/R in tumor-bearing animals.

Figure 2: Analysis of muscle damage in I/R injury induced proximal limb tissues. A) Muscle fiber degeneration in the proximal hind limb of tumor free animals without I/R injury (n = 5), on days 3 (n = 8), 7 (n = 5), and 14 (n = 6) post-I/R and 4T1 tumor bearing animals without I/R injury (n = 6), on days 3 (n = 5), 7 (n = 4), and 14 (n = 6). B) Muscle fiber disorganization in the proximal hind limb of tumor-free animals without I/R injury (n = 5), on days 3 (n = 8), 7 (n = 5), and 14 (n = 6) post-I/R and 4T1 tumor bearing animals without I/R injury (n = 6), on days 3 (n = 5), 7 (n = 4), and 14 (n = 6). Muscle fiber degeneration and disorganization scores were determined by hematoxylin and eosin staining. C) Representative H and E-stained histological images, 1 out of all samples in each group are shown.
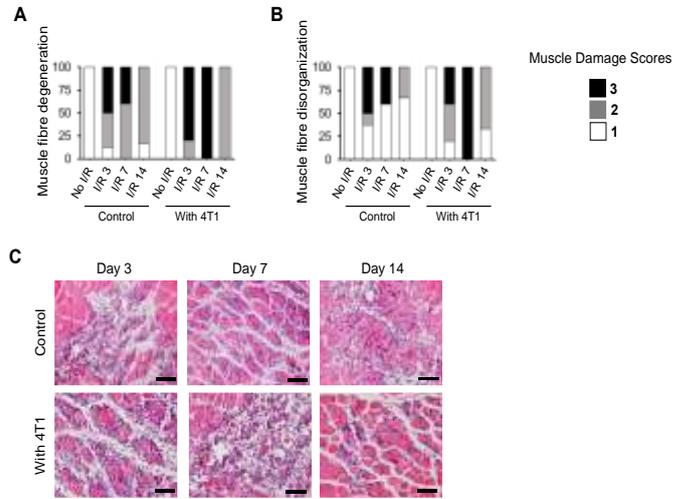

Figure 3: Hind limb I/R injury influence on the liver myeloid cell population percentages in tumor free and tumor-bearing mice. A) Liver weights were measured and average weight of livers in each group were graphed. The data are shown as mean ± SEM. B) Low-density cells (<1.077 g/mL) were isolated from fresh liver samples and CD45+ CD11b+ Gr-1+ total MDSCs, CD45+ CD11b+ Gr-1+ Ly6G+ Ly6C+ MDSCs, CD45+ CD11b+ Gr-1+ Ly6G-Ly6C+ MDSCs and CD45+ CD11b+ F4/80+ macrophages were assessed by flow cytometry. The data are shown as mean ± SEM. Significance was determined by one-way ANOVA (*p<0.05; **p<0.01; ***p<0.001; ****p<0.0001) and only significant values were indicated.
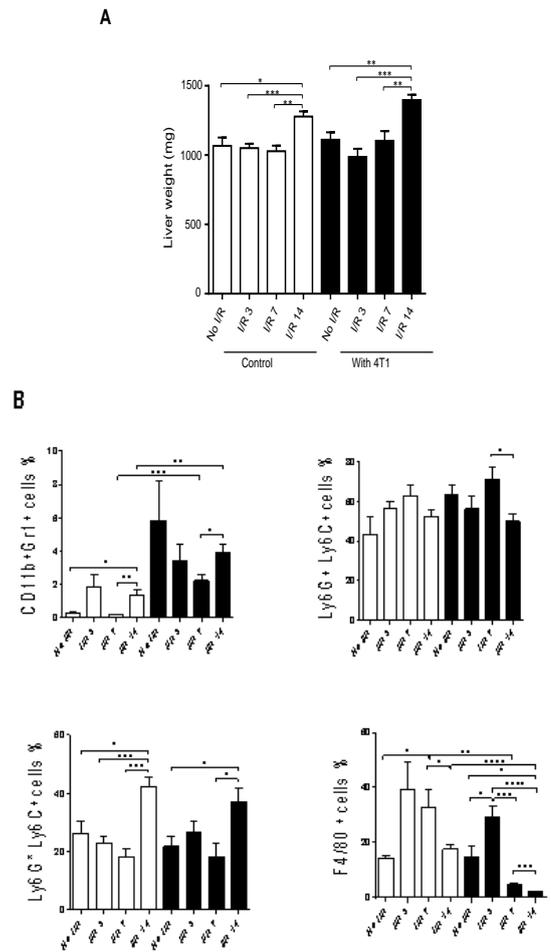
Due to the difference in the number of myeloid cell groups in liver sections, the percentage of myeloid cells was analyzed by flow cytometry. There was 0.22% (0.06%) of CD11b $^+$ Gr1 $^+$ total myeloid cells on average before the induction of I/R injury in the livers of tumor free animals (Figure

3B). This level was increased to the average of 1.83 (0.65) by day 3 after the I/R, reduced to 0.16 (0.02) by day 7 post - I/R, and enhanced to 1.29 (0.35) on day 14 post - I/R in tumor free mice. Total myeloid cell percentage on day 14 after the I/R was significantly more than the control I/R and day 7-post - I/R groups ($P<0.05$ and 0.02, respectively). However, there was 5.85% (2.2%) CD11b$^+$ Gr1$^+$ total myeloid cells in tumor-bearing animals before the I/R and this percentage reduced to 3.4 (0.95) on day 3 after the I/R, 2.2 (0.34) on day 7 post-I/R (Figure 3B). However, CD11b$^+$ Gr1$^+$ cell percentage was increased significantly to 3.92 (0.48) on day 14 compared to day 7 after the I/R ($P<0.05$). Furthermore, there was significantly more liver myeloid cell level on day 7 and 14 in tumor-bearing animals compared with tumor-bearing mice ($P<0.001$ and 0.02, respectively) (Figure 3B).

We further examined the sub-populations of myeloid cells and determined that the amount of Ly6G$^+$C$^+$ cell percentages enhanced to an average of 56.8 (2.75) on day 3, 62.9 (5.54) on day 7 compared to the control I/R group 43.2 (7.92) and reduced to 52.2 (3.4) on day 14 after the I/R (Figure 3B). In contrast, there was 63.4 (4.87)% of Ly6G$^+$C$^+$ cells before I/R in tumor-bearing animals and this percentage decreased to 56.3 (5.68)% on day 3, increased to 70.9 (6.01)% on day 7 and diminished to 49.8 (4.57)% on day 14 post - I/R. The difference in the percentage of Ly6G$^+$C$^+$ cells between day 7 and day 14-post - I/R was significant ($P<0.05$) (Figure 3B). There were augmented percentages of Ly6G$^+$C$^+$ cells in tumor bearing mice compared to tumor free animals before the I/R and on day 7 after the I/R while lower percentages on day 3 and 14 after the I/R. The amount of Ly6G$^-$C$^+$ cells were 26.1 (3.65)% on average before the induction of I/R injury in tumor free mice, which reduced to an average of 22.8 (1.83)% by day 3 and 18.1 (2.85)% by day 7 after the I/R. On the contrary, these levels were increased to 42.1 (3.3)% by day 14 post - I/R. Ly6G$^-$C$^+$ myeloid cell percentage on day 14 after the I/R was significantly more than the control no I/R, day 3 and 7 post-I/R groups ($P<0.05$, 0.001 and 0.001, respectively) (Figure 3B). The percentage of Ly6G$^-$C$^+$ cells were 21.6 (3.32)% on average before the I/R injury in tumor-inoculated mice, and increased to 26.7 (3.5) by day 3, decreased to 18.1 (4.41) by days 7 and increased to 34.3 (5.15) by day 14 after the I/R. Ly6G$^-$C$^+$ cell percentage on day 14 after the I/R was significantly more than the control no I/R, and day 7 post - I/R groups ($P<0.05$) (Figure 3B). There were reduced percentages of Ly6G$^-$C$^+$ cells in tumor-bearing mice compared to tumor free animals before the I/R and on day 14 after the I/R, while a higher percentage was observed on day 3 and the same percentage was seen on day 7 post- I/R.

Moreover, the percentage of CD11b$^+$ F480$^+$ macrophages was 14.3 (0.76)% in tumor free animals before the I/R and increased to 39.3 (8.43)% on day 3 post – I/R. Then, their levels decreased to 32.8 (6.24)% on day 7 and 17.6 (1.7)% on day 14 (Figure 3B). However, day 7 post-I/R level was still significantly higher than before I/R control and on day 14 after the I/R ($P<0.05$) (Figure 3B). In tumor-bearing mice, the macrophage level was 14.8 (3.58)% before the ischemia. This percentage significantly increased on day 3 post - I/R (29.1 (3.78) compared to before I/R control, day 7 and day 14 post-I/R groups ($P<0.05$, 0.001 and 0.0001, respectively) since the levels

were decreased to 4.8 (0.47)% on day 7 and 1.99 (0.26)% on day 14. Furthermore, macrophage level on day 14 after I/R was significantly lower than before I/R control and day 7 groups ($P<0.05$ and 0.001, respectively) (Figure 3B). Macrophage percentages in tumor-inoculated mice were lower than tumor-free mice in all groups except for the before I/R control group with a significant difference on days 7 and 14 after the ischemia ($P=0.02$ and $P<0.001$, respectively) (Figure 3B).

Tumorigenesis after ischemia/reperfusion injury

Since MDSC are implicated in tumor progression [33], next, we evaluated the impact of the limb I/R injury on the tumor size and composition of the tumor microenvironment. A positive influence of the limb I/R on the tumor growth (a change in tumor size, 82 (2.7)% was observed on day 7, compared to a group of tumor-bearing mice without the I/R injury (change in tumor size, 19 (1.2) % (Figure 4A). Accordingly, the expression of angiogenesis- related MMP-9 and VEGFA genes was enhanced in the tumor tissue from the animals with I/R injury, whereas the expression of inflammatory factors S100A9 and IL-1β was decreased. The mRNA level of CCL2 was not changed (Figure 4B). In the tumor tissue, no difference was observed in the percentage of myeloid cells. Albeit insignificant, the amount of tumor-infiltrating Ly6G+Ly6C+ granulocytic cells on day 7 tended to decrease (tumor-bearing group without I/R, 24.3 (3.82)%; tumor-bearing group with I/R, 18.55 (0.46)% (Figure 4C and D). It might be speculated that the accumulation of G-MDSC into the I/R limb tissue reduced the influx of these cells into the tumor microenvironment. Hence, the expression pattern of the genes studied may indicate a relationship between tumor growth, angiogenesis and inflammatory regulation in the tumor tissue upon the I/R response.

Figure 4: Influence of the hind limb I/R on 4T1 breast tumors. A) The change observed in the tumor growth on days 3 and 7 is plotted. A group of tumor-bearing animals without I/R injury served as a control group (n=5). B) In the tumor tissues on the day 7 post-I/R, expression of the inflammation-associated genes was studied by semiquantitative real time PCR. The data were normalized, and percent change was calculated in comparison to the data obtained from the tumors from the control mice that did not undergo the I/R procedure (n=5). C) The percentage of Gr-1+, Ly6G+Ly6C+, Ly6GLy6C+ myeloid cells amongst tumor-infiltrating CD45+CD11b+ cells and D) representative counter plots from a mouse out of 6 used are shown. The data are shown as mean ± SEM. Significance was determined by one-way ANOVA (ns, not significant; *$P<0.05$).
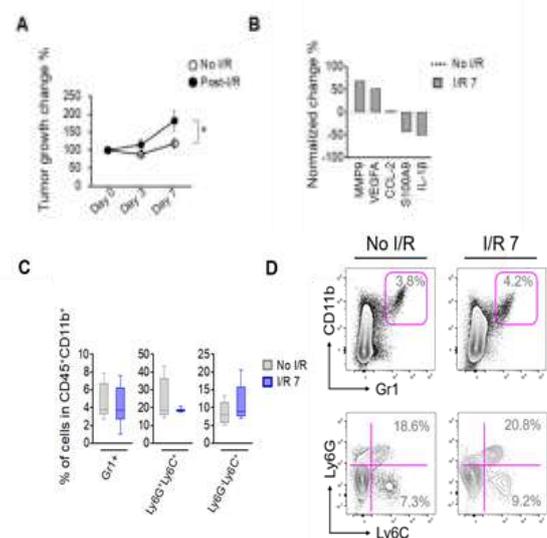
Discussion

Although significant advances have been made in the diagnosis and treatment of breast cancer, a rational number of patients still develop resistances. I/R injury might develop regionally and/or in the extremities during great operations such as breast cancer surgery. It is known that the tissue damage and stress caused by this injury affect the functional character of the immune system, especially myeloid cells, and these cells display tumor-promoting function in the tumor microenvironment and in the organs related to hematopoiesis.

First of all, in this study, breast tumors were developed in the left inguinal breast region of experimental animals. Afterwards, short, middle and long term I/R injury was formed on left down-extremity. The short, middle, and long-term effect of the injury in tumor-bearing and tumor-free mice was evaluated histopathologically with sections taken from the extremities. It was observed that muscle fiber degeneration and disorganization increased gradually on the 3rd and 7th days while decreased on day 14 after ischemia. Our result is consistent with the literature findings since I/R injury is responsible for about 40% of the muscle damage [39]. Moreover, in the presence of breast tumor, the muscles were more damaged in the proximal limbs. This damage is a result of increased oxidative stress due to ROS production and facilitated probably by neutrophils since neutrophils accumulate more at the ischemic site in tumor bearing mice than in the controls [39]. This might be due to hematopoiesis induced by local inflammation within the tumor microenvironment. However, it is necessary to determine the cell types and functional characteristics of the cells in the extremities.

MDSCs play an important role in tumor development. Liver hematopoiesis contributes to the expansion of MDSCs in this organ [22] and these cells have tumor-promoting function in hematopoiesis-related organs of cancer patients. Therefore, liver weights were measured, and no significant difference was found between tumor free and tumor-bearing groups. However, liver weights on day 14 post-I/R were significantly higher than the control no I/R groups, short and mid-term groups. This might be a result of tumor or immune cell migration. Therefore, myeloid cell groups were analyzed histologically within the livers. It has been determined that locus numbers of myeloid cells were more in breast cancer bearing mice than in control animals, and more in ischemia-performed mice than the controls. After all, we sought to investigate the percentage of total and subpopulations of MDSCs within the liver by flow cytometry. Total myeloid cell percentages were enhanced on day 3 post-I/R, diminished to the level previous of the ischemia on day 7 post-I/R and again came up to higher percent on day 14 post-I/R in tumor free mice. The decrease in the myeloid cell populations within the liver on day 7 post-I/R suggests that cells might be migrating to the I/R region as a repair mechanism (40) as the level increased on day 14 post-I/R. On the other hand, there was already high levels of myeloid cells before the I/R and this percent decreased gradually on days 3 and 7 post-I/R and increased back on day 14 post-I/R in tumor harboring animals. Additionally, tumor-bearing mice demonstrated higher myeloid cell percentages at each experimental timepoint. The higher level of total myeloid cells prior to the I/R injury is most probably due to the presence of tumor. Later on, they progressively might have migrated to the

I/R region and by day 14 post-I/R, the percentages came up to higher levels due to the tumor effect. Furthermore, the percentage of Ly6G+C+ cells increased gradually on day 3 and 7 and reduced on day 14 post-I/R in tumor-bearing mice. However, the level of these cells decreased on day 3, increased on day 7 and reduced below to the percentage before ischemia in tumor bearing animals. Ly6G-C+ cells diminished gradually on day 3 and 7 and enhanced on day 14 post-I/R in tumor free mice. Their level increased on day 3, decreased on day 7 and augmented significantly on day 14 after ischemia in tumor inoculated mice. The gradual increase in Ly6G+C+ and decrease in Ly6G-C+ on days 3 and 7 NA may be due to the I/R affect in tumor free mice. However, in the presence of I/R injury and a tumor, Ly6G+C+ and Ly6G-C+ levels significantly changed on day 14 post-I/R. However, CD11b+ F480+ macrophage percentages were diminished progressively on day 3, 7 and 14 post-I/R in tumor free mice. In contrast, macrophage levels increased on day 3 and gradually reduced on day 7 and 14 in tumor-bearing mice. Before I/R was created, macrophage levels were similar between tumor-free and tumor-bearing groups. However, the levels dramatically augmented on day 3 post-I/R while reduced gradually on day 7 and 14 post-I/R in both groups. The level of PMN, G-MDSC and macrophages in the peripheral blood and other hematopoietic organs should be determined to understand their trafficking. Furthermore, not only myeloid cell levels but also their functionality should be studied to conclude their role in the model.

Different groups have reported that tumor development and metastasis are induced due to the induction of cytokines, growth factors and adhesion molecules in I/R injury models [24, 41, 42]. However, reperfusion injury was performed in the organ where metastasis is studied in the previously published studies. In our model, we evaluated the effect of limb I/R injury on the metastasis of breast cancer cells to the liver and liver metastasis was not observed in any group. Generation of I/R injury in extremities prevents ischemic damage in distant organs [42, 43]. Providing protection of tissues against severe I/R injury that will be induced later by the generation of short-term periods of I/R injury is called ischemic preconditioning (IPC). Therefore, I/R injury in distant organs prevents the accumulation of myeloid cells in the liver and migration of tumor cells to the liver through myeloid cells. On the other hand, it has been demonstrated that intravenous injection of exogenous bone marrow derived mononuclear cells into mice with colorectal cancer were able to migrate to the liver and induced tumor liver metastasis [23]. In our experimental approach we could not detect this effect. This might be a timing issue in which the duration of the study was insufficient to study metastasis since there were extremely high numbers of myeloid cell groups in the liver of tumor bearing mice on day 14 in the post-I/R group.

Temporary clamping of blood vessels is used to prevent bleeding and may cause I/R injury during oncological surgery [34]. The pro-tumorigenic effects of I/R and its impact on surgical wounds were previously reported to be associated with the upregulation of angiogenic factors, generation of an inflammatory environment, activation of sympathetic nervous system, and induction of hypoxia [35, 36]. In contrast to a study that reported the increment of tumor-infiltrating MDSC upon

skin incision surgery [37], here, the I/R injury did not alter the myeloid cell compartment in the tumors. MDSCs also contribute to the formation of pre-metastatic niche [19]. Even though we did not monitor the impact of limb I/R on metastatic burden in the tumor-bearing animals, a previous study indicated the surgery induced wound-healing mechanisms as a factor that restricts T cell-mediated anti-tumor responses through elevation of circulating and tumor-infiltrating myeloid cells [38]. The influence of I/R-induced G-MDSC on metastasis and anti-tumor immunity remains to be better defined.

Conclusion

This study provides a basis for actualization of more comprehensive studies and formation of new hypotheses in the future. Furthermore, these findings contribute the understanding of inflammatory processes depending on I/R injury that developed during the clinical applications. Understanding the interactions of ischemia formed tissue and tumor microenvironment as two separate sterile inflammation sites, explaining the role of I/R injury in tumor growth and metastasis is a critical necessity for development of new treatment and surgical methods. With the formation of short-term I/R injury in a distant site, tumor development and/or seeding to metastasis sites after surgery could be prevented.

Acknowledgements

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