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CHEMOKINES AND TRAFFICKING

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STRESS RESPONSE DURING THE DEVELOPMENT OF DSS-INDUCED COLITIS DECREASES THE ADHESION MOLECULES EXPRESSION IN THE BLOOD NEUTROPHIL AND WORSENS INTESTINAL INFLAMMATION

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Introduction: It is well established a positive correlation between environmental factors, such as physical and/or psychological stressors, and the relapse of the inflammatory bowel diseases. Therefore, we aimed to investigate the role of stress on the neutrophil migration during the development of a murine model of DSS-induced intestinal inflammation. **Methods and Results:** C57BL/6 male mice (n=6/group), were divided in 4 groups: Control (C), DSS (D), Stress (S) and Stress+DSS (D+S). Colitis was induced by the addition of DSS (3.5%) to the autoclaved drinking water for 5 days. Mice of the groups S and D+S were submitted to a restraint stress protocol for 2 hours on the experimental days 1, 3 and 5 of the DSS treatment. Blood and colon were collected 24h after the experimental days 1, 3 and 5. Data were expressed by mean±SD and examined by one-way ANOVA, a *P* value <0.05 was considered significant. We observed a decrease of the % of body weight in the group D+S5 compared with the groups C, S5 and D5 (C:-0.94±0.65; S5:-2.68±0.94; D5:-5.25±1.86; S+D5:-.,46±1.50) and a decrease of the colon length (C:8.5±0.3; S5:7.6±0.2; D5:5.8±0.2; S+D5:5.1±0.1) associated to an increase in the colon length/weight ratio in the group D+S5 (C:0.025±0.001; S5:0.026±0.001; D5:0.035±0.001; D+S5:0.046±0.003). Additionally, we observed that DSS increased the CD18 expression (D3:137.4±7.5; D5:149.8±17.6) and decreases the CD62L expression (D3:87.08±5.6; D5:76.86±2.7) in blood neutrophils in D3 and D5 groups when compared with the C group (data were expressed by % of the C group), however the stress prevented the change in the CD18 expression (D+S3: 120.2±16.3; D+S5:97.61±19.78) and in the CD62L expression (D+S3: 64.89±10.8; D+S5: 83.49±6.5) when compared with the D group and worsened the neutrophil migration during the experimental days. Stress augmented edema and tissue damage in the colon in the group D+S5 when compared to group D5 ($P < 0.05$) in a histological analyses. **Conclusion:** These results show that stress during the development of DSS-induced colitis is able to increase the lesion in the colon. Therefore, we hypothesize that by decreasing the neutrophil migration the organism

attempt to cope with the damage caused by DSS is impaired, which in turn leads to the worsening of the colitis.

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PLASMIN AND UROKINASE PROMOTE CELL MIGRATION VIA MEK/ERK CASCADE AND CCR2

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Introduction: Plasminogen (plg) activation with subsequent generation of plasmin (Pla) is mediated by the proteolytic activity of urokinase-type Plg activator (uPA) or tissue Plg activator (tPA). The Plg/Pla proteolytic system is associated with a variety of biological activities beyond the classical dissolution of fibrin deposits, including cell migration, tissue repair, inflammation and metastasis. In this study, we investigated the effect of Pla and uPA on cell migration *in vitro* and *in vivo* and the role of MAPK ERK1/2 and CCR2 in this process.

Methods and Results: We performed *in vitro* migration assay (wound healing assay) in culture of MEFs (Mouse Embryonic Fibroblasts). The cells were treated with Pla (2µg/mL) or uPA (1µg/mL) at different times, or pretreated with the MEK1/2 inhibitor UO126 (15µM) 30 min. before Pla/uPA and processed for microscopic counting of migrating cells or western blot analysis for phosphorylated ERK1/2. BALB/C mice were challenged by i.pl. (intrapleural) injection of Pla (2µg/cavity) and the cells present in the pleural cavity harvested at different times after treatment and processed for total and differential leukocyte counts and western blot analysis for P-ERK1/2. The migration of MEFs into the scratch after Pla or uPA was already visible at 5h post treatment (hpt.) with a highest migration observed at 10 up to 20 hpt, which was associated with an increased ERK1/2

phosphorylation. The pretreatment with UO126 inhibited cell migration(mean ± SEM of the number of migrated cells at 10h; MEFs untreated: 40.8 ± 3.8; Pla: 59.8 ± 4.8; Pla+UO: 38 ± 2.6; u-PA: 78.5 ± 3.3; uPA+UO: 35.8 ± 2.6; *n*=10; *P*<0.05).. The injection of Pla induced a time-dependent influx of leukocytes into the pleural cavity of mice(PBS: 9.0 ± 0.57; Pla 6h: 13.87 ± 0.77; Pla 24h: 22.9 ± 2.01; Pla 48h: 40.1 ± 2.9 *n*= 4; *P*<0.05). that was consistent with MCP-1 increase and was MEK dependent. Leukocyte influx induced by Pla was impaired in CCR2-/- mice.

ConclusionOur results showed that migration of MEFs is induced by plasmin and urokinase in a time-dependent manner and inhibited by UO126. Moreover, it is shown that Pla induces leukocyte traffic into the pleural cavity of mice dependent on CCR2 and ERK1/2 phosphorylation *in vivo*.

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Co-regulation between leukotriene B_4 and CCL2 generation enhances γδ T lymphocyte migration during inflammation

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Introduction: γδ T cells comprise a unique T cell subset, which play important roles in immune surveillance, especially against infection and cancer. These cells accumulate in injured tissue, a phenomenon mediated by adhesion molecules and chemoattractant mediators, such as bioactive lipids and chemokines. Previous reports from our group demonstrate that γδ T cell trafficking into sites of inflammation is mainly orchestrated by leukotriene (LT)B_4 and CCL2. Moreover, data in the literature have shown that CCL2 can regulate LTB_4 synthesis and *vice versa*. In the present study, we have further investigated the cooperation between CCL2 and LTB_4 during LPS-induced inflammation examining its impact on γδ T cell migration.

Methods and Results: The intra-pleural (i.pl) administration of LTB_4 triggered CCL2 production in mouse pleura and *vice versa* (LTB_4 0.5 μg/cav.; CCL2 500 ng/cav.). Accordingly, during LPS-induced inflammation (250ng/cav.; i.pl.), the antagonism of LTB_4 receptor BLT1 (CP105,696, 4 mg/kg; i.p.) diminished CCL2 production, as well as CCL2 neutralization (10 μg/mouse; i.p.) impaired LTB_4 generation. When directly injected in the pleura, both mediators induced γδ T cell influx; however LTB_4 i.pl. administration into CCR2 KO mice triggered a discrete γδ T cell accumulation. Inasmuch, CCL2 i.pl. injection failed to induce γδ T cell influx into 5-lipoxygenase (LO) deficient mouse pleura. The expression of CCR2 and BLT1 on γδ T cells was upregulated *in vitro* under anti-CD3 (5 μg/ml) stimulation and *in vivo* during LPS-induced pleural inflammation. LPS i.pl. stimulation also induced increased numbers of $BLT1^+$, $CCR2^+$ and $BLT1^+/CCR2^+$ γδ T cells in mouse

pleura. Interestingly, approximately 60% of $CCR2^+$ γδ T cells co-expressed BLT1. Moreover, i*n vitro* stimulation of γδ T cells with sub-optimal concentrations of LTB_4 plus CCL2 (0.1 nM - 1 nM) induced intracellular calcium influx, suggesting that these mediators synergize in promoting cell mobilization. Indeed, the combination of both mediators induced γδ T cell migration in higher extent rather than towards a single stimulus *in vitro*. Our results demonstrate that LTB_4 and CCL2 mutually regulate their production during inflammation and cooperate for optimal γδ T cell tissue accumulation.

Conclusion: Herein we show that LTB_4 and CCL2 reciprocally upregulate each other's production and synergize in mediating γδ T cell activation and migration. Financial support**:** CAPES, FAPERJ, FIOCRUZ, CNPq.

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MEMBRANE VESICLES FROM BONE MARROW-DERIVED MACROPHAGES INDUCE IL-12 PRODUCTION BY MACROPHAGES IN VITRO

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Introduction: Membrane vesicles (VMs) are structures with a phospolipid bilayer membrane and 100-1000 nm in diameter. These vesicles are released from cells upon activation of surface receptors and apoptosis. The production of VMs by dendritic cells, mast cells, macrophages, B and T lymphocytes has been extensively reported in the literature (Science Signaling. 1 (6), pe8, 2008). They also express MHC class II, membrane surface molecules and carry antigens (Nature Reviews of Imunology 2,569-579, 2002). The aim of this study is to investigate the role of macrophage-derived VMs as immunomodulatory particles.

Methods and Results: Cells were collected from the bone marrow of 4-7 week-old Balb/c, differentiated into macrophages in RPMI containing 10% bovine fetal serum and 30% GM-CSF and incubated for 6 days. To isolate the VMs, the supernatant of the culture was centrifuged twice at 1500 g for 10 minutes and at 8000 g for 5 minutes to remove residual cells and cell debris. The VMs were pellet twice at 100.000g for 45 minutes followed by resuspension in HBSS. They were characterized by flow cytometry and by membrane staining of the cells from which they were derived. They were spherical and had diameters of 150 to 300 nm, as assessed by electron microscopy. Macrophage incubation with VMs was done for 48 hours. There was a statistically significant increase in the production of IL-12 by VMs-treated macrophages.

Conclusion: Many cellular types release VMs and depending of the source they can carry antigens and/or membrane surface molecules, therefore inducing diverse immune responses. The bone marrow-derived macrophages spontaneously released *in vitro* VMs that induced other macrophages to produce the proinflamatory cytokine IL-12. It's tempting to speculate that this phenomenon, if it happens with macrophages *in vivo*, could modulate the immune system in favor of a Th1 immune response.

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CD8$^{\pm}$ T CELLS EXPRESSING CCR7 ARE CRITICAL FOR SURVIVAL IN A CHRONIC MOUSE MODEL OF *TOXOPLASMA GONDII* ENCEPHALITIS

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The immunopathogenesis of toxoplasmosis has been extensively studied, yet remains poorly understood. Noor *et al* recently reported 100% mortality in the acute phase of infection in mice lacking the homeostatic chemokine receptor Ccr7 after IP inoculation with 10^4 tachyzoites of *T. gondii* (Prugniaud strain), which was associated with impaired IFN-γ production in the brain. Since *Toxoplasma* encephalitis, a major clinical manifestation of infection, occurs in the chronic phase in both humans and in the model, the role of Ccr7 could not fully be defined in this study. To address this gap, we infected C57Bl/6 mice orally with 10 cysts of *T. gondii* strain ME49, which allowed 50% of mice to survive into the chronic phase. Parasite burden was greatest in the brain, but focal neurological signs were absent. Brains from chronically infected wt and *Ccr7* ko mice had similar percentages of activated microglia, total $CD4^+$ and $CD8^+$ T cells, and activated IFN-γ^+ TNF-α^- and IFN-γ^+TNF-α^+ T cell subsets. Consistent with this, there was no difference between ko and wt in IFN-γ concentrations either in serum or brain during either acute or chronic infection. Also, *Ccr7* ko mice expressed higher brain levels than wt mice of the parasite control molecule iNOS. Yet Ccr7 ko mice still had higher brain parasitism (cyst number and parasite DNA levels). Cell transfer experiments showed that CD8$^{\pm}$ T cells expressing Ccr7 were sufficient to rescue *Ccr7* ko mice from death. Further work will be required to define how lymphocytes become activated in the absence of Ccr7 and traffic to the brain in the model, and why they fail to control the parasite after entry.

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DENGUE VIRUS REQUIRES THE CC-CHEMOKINE RECEPTOR CCR5 FOR THE DEVELOPMENT OF INFECTION AND DISEASE IN AN EXPERIMENTAL MODEL OF DENGUE IN MICE

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Introduction: Dengue virus (DENV) infection is the most important human arboviroses. Infection is characterized by a systemic inflammatory response and hematological alterations that may evolve with shock and death in severe cases. Recent clinical and experimental data have shown an association between components of the chemokine network and severity of Dengue. Here we investigated the role of the chemokine receptor CCR5 in a murine model of dengue virus infection and disease. **Methods and results:** Untreated wild-type (WT) mice, CCR5 antagonist- treated WT mice and CCR5 KO mice were inoculated with 100 PFU of mouse-adapted DENV. Survival rates, platelet counts, hematocrit indexes and liver histopathology were evaluated. Viral load was measured in organs, blood and murine primary macrophages culture samples, treated or not with CCR5 antagonist Met-RANTES or Pertussis toxin (PTX). This set of experiments was approved by CETEA-UFMG (133/2009). CCR5 deficiency or blockade with antagonists prevented dengue associated disease and mortality. CCR5 KO mice and WT mice treated before infection with a small molecule CCR5 receptor antagonist presented increased survival rates. All hematological parameters and tissue damage in the liver were reduced in CCR5 KO mice and WT mice pre-treated with Met-RANTES compared to WT ($p<0.01$). Importantly, viral load was reduced in tissues of CCR5 KO mice at different time points after infection, contrary to the viral load observed in WT mice, which continually increased during infection ($p<0,001$). Treatment with either CCR5 antagonists 3 days after infection was ineffective. *In vitro* assays with primary murine macrophages, target-cells for DENV, revealed reduced viral load in CCR5 KO cells compared to WT. Likewise, pre-treatment with Met-RANTES or PTX reduced viral load in WT cells. **Conclusion:** The lack of CCR5 cannot prevent DENV infection *in vitro* but prevent progression of infection and development of disease in vivo. The reduced viral load in CCR5 KO tissues since early phases of infection and the sole success of treatment with CCR5 antagonists before infection led us to the hypothesis that CCR5 is involved in initial phases of the DENV lifecycle. Further studies will determine the role of CCR5 in DENV infection.

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TITLE: CD-49d HAS MAJOR ROLE UPON T CELL TRAFFICK IN DUCHENNE MUSCULAR DYSTROPHY PATIENTS

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Introduction Recent years have seen significant increase in the participation of chronic degenerative diseases as a cause of mortality. The most prevalent in children and adolescents is Duchenne Muscular Dystrophy (DMD), which affects 1 in 3,500 live male births. It is caused by the absence of functional dystrophin, due to mutations in the dystrophin gene. Although the cause of DMD is genetic, there is accumulating data suggesting that an immune response may play a role in pathophysiology of this disease. In a recent work, our group described an increased percentage of circulating CD4+CD49dhigh and T CD8+CD49dhigh T lymphocytes, and such an increase correlates with more rapid progression of the disease. CD49d can drive T cells to the site of inflammation favoring migration and adhesion to the muscle tissue and muscle damage by interacting more strongly with the fiber and extracellular matrix proteins. **Methods and Results** DMD patients were selected in IPPMG-UFRJ. All individuals were male at 4 to 20 years old and with no health problem. This study was approved by the Ethical Committee of UFRJ and all subjects signed an informed consent to be enrolled in the study. Peripheral blood mononuclear cells were separated by Ficoll gradient and labeled for protein profile by flow cytometry. We found the higher migration/adhesion capacity of T cells from DMD patients *in vitro* driven by VCAM-1, fibronectin, muscle fibers and transendothelial migration, compared with healthy subjects. The blockade of CD49d with monoclonal antibody could impair their migratory and adhesive responses in all experiments performed. Also, the expression of others integrins, such as CD49a, CD49e, CD49f and CD11a were not different between patients and healthy controls. **Conclusion** Our data indicates that CD49d expression in high levels, but not in the others integrins evaluated, contribute to transendothelial and fibronectin migration and intramuscular positioning of T lymphocytes, ultimately being involved in the inflammatory process seen in DMD patients. In this context, the use of CD49d inhibitors can be envisioned as an immunotherapeutic option to ameliorate the quality of life of DMD patients.

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GENERATION OF A CXCL1 FLOXED REPORTER MOUSE STRAIN: STUDY OF CXCL1/2-CXCR2 AXIS IN PULMONARY INFECTIONS

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Introduction: Chemokines are a family of conserved secreted proteins that mediate diverse cellular effects as chemotaxis and cell activation by binding their GPCRs. ELR+ CXC chemokines (CXCL8 in humans and CXCL1, CXCL2 in mice) bind to CXCR1 and CXCR2 receptors and promote neutrophil traffic. During lung infections – Influenza and *Streptococcus pneumoniae* – great neutrophilic response is correlated with tissue damage and mortality. Using the conditional inactivation of genes through Cre-LoxP system it is possible to study the role of a gene in specific cell and time. Aiming to understand the dynamics and role of specific cells in CXCL1 release during infection, this study performed a construction of CXCL1 floxed reporter mice. **Methods and Results:** CXCL1 targeting vector contained two loxP sites flanking exons 2 and 3 of *cxcl1* and an IRES-Tomato sequence that codifies the fluorescent protein Tomato. The fragments of *cxcl1* were amplified by PCR and cloned into two cloning vectors – first pGEMT IRES-Tomato and then pRapidFlirt – using In-Fusion cloning system. The recombination vector – containing Tomato and *cxcl1* floxed with loxP – was used in homologous recombination in V6.5 embryonic stem (ES) cells. The procedure was followed by ES cells positive clone selection through the screening with radioactive probes using Southern Blot technique. The selected positive clones were expanded to a second Southern Blot screening and were used for blastocyst injection in female pseudopregnant mouse. After mating first chimeric offspring, germline transmission of *cxl1* floxed with Tomato was achieved. In parallel, Influenza (WSN/33) and *S. pneumoniae* (ATCC 6303) infection in WT mice showed that an early and intense CXCL1 production is correlated with disease severity. Further, CXCR2 blockade using a CXCR1/2 antagonist (DF2162) decreased inflammation caused by Influenza infection and lethality (16% vs 100%) caused by *S. pneumoniae*. **Conclusion:** The importance of CXCL1-CXCR2 axis in lung infections should be further investigated. The CXCL1 floxed reporter mice is a promising tool to understand the dynamics of CXCL1 by different cell types during pulmonary infection and also in different models of inflammatory diseases. Crossing the generated mouse with cell specific Cre strains will provide more information about which cell type is more important for the production of the chemokine in distinct moments and under diverse stimuli.

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CCR4-DEFICIENT MICE EXHIBITED A HIGHER LUNG CD4+ CELLS AND WERE MORE SUSCEPTIBLE TO *Mycobacterium tuberculosis* INFECTION

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Introduction: Lymphocyte recruitment from blood to inflammatory sites is a multistep process that involves rolling on the endothelium, chemokine-induced activation, firm adhesion, and transendothelial migration into the tissue. Numerous chemokines are produced during inflammation which supports the migration of cells to tissue. Chemokine receptor 4 (CCR4) is expressed by a variety of T-cell subsets and leukocytes. As *Mycobacterium tuberculosis* uses diverse mechanisms to avoid elimination by the infected host, the aim of this study was to assessment the participation of CCR4 in response to pulmonary infection with *M. tuberculosis*. **Methods and Results:** CCR4 deficient ($CCR4^{-/-}$) and wild type (WT) mice were infected with $1x10^5$ bacilli by intra-tracheal route. Fifteen, thirty and seventy days post-infection, Colony-Forming Unit (CFU) number in the lung and spleen, cell subsets and cytokine production were evaluated in the lungs of $CCR4^{-/-}$ and WT mice. After fifteen days of infection, $CCR4^{-/-}$ (n=12) mice compared with WT (n=11) mice showed similar CFU counts. The frequency and total number of $CD4^+$ cells were higher and the frequency of $CD4^+Foxp3^+$ and NK cells were lesser than those detected in WT mice ($p<0.05$). At thirty days of infection, $CCR4^{-/-}$ (n=9) mice had similar CFU number in the lung and lower number in the spleen ($p<0.05$); frequency and total number of $CD4^+$ cells and production of IFN-γ were higher compared to WT (n=10) mice. At the chronic phase (70 days), $CCR4^{-/-}$ (n=9) mice were more susceptible to *M. tuberculosis* infection compared to WT (n=9) mice ($p<0.05$). In addition, 70-day infected $CCR4^{-/-}$ mice showed an increase in the total number of $CD4^+$ cells and in the frequency of $CD8^+$ cells, followed by a decrease in the frequency of NK cells compared to WT mice. There was also a significant production of IFN-γ compared to WT mice. **Conclusion:** These data suggests that CCR4 participates of the immune response to *M. tuberculosis*. However, this receptor seems to have a key role at the chronic phase of the infection.

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THE INTERCELLULAR ADHESION MOLECULE 1 (ICAM-1) PLAYS AN IMPORTANT ROLE IN CONTROLLING *Mycobacterium avium* INFECTION

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Introduction: Intercellular adhesion molecule 1 (ICAM-1) is a type I transmembrane protein involved in the processes of leucocyte firm adhesion and transmigration through the endothelia, primarily upon binding to LFA-1 (CD11c/CD18) and Mac-1 (CD11b/CD18), present in leukocytes. ICAM-1 comprises a short intracellular region which lacks flag domains or domains with any known intrinsic kinase activity. However, this intracellular portion has a large number of positively charged amino acids and a tyrosine residue which points out to a possible role in intracellular signaling. *Mycobacterium avium* is an important opportunist pathogen. It belongs to the *Mycobacterium avium* complex (MAC) and can be ubiquitously found in the environment. This bacterium is of particular interest once it is capable of causing disseminated infection in immunocompromised individuals such as AIDS patients, being involved in increased mortality and morbidity among them. Little is known about ICAM-1 function during *M. avium* infection.

Methods and Results: Knockout mice for ICAM-1 ($ICAM^{-/-}$) and C57BL/6 mice were infected intravenously with $1x10^6$ CFU of virulent *M. avium* 2447. Animals were sacrificed 30 or 100 days *post infection* and CFU counting was determined in the spleen, liver and lung. Spleen cells from these animals were cultured *in vitro*, re-stimulated with *M. avium 2447* and analyzed for CCL2, CCL3, CCL5, TNF-α and IFN-γ production by ELISA assay. Moreover, after 1 or 2 weeks of infection animals were sacrificed and CD4+, CD8+, CD11b+ and CD11c+ cell populations were determined in the spleen by FACS. When compared to wild type, $ICAM^{-/-}$ mice presented higher CFU counting in all organs and time points, indicating increased susceptibility to *M. avium* infection in these animals. Splenocytes from $ICAM^{-/-}$ mice showed significant decrease in IFN-γ production at both 30 and 100 days *pi* whereas TNF-α production was unaffected. In addition, knockout mice showed significant CCL5 decrease at 30 days *pi* whilst the levels of CCL2 and CCL3 were increased at 100 days *pi*. The chemokine disbalance observed may indicate decreased

cell activation and migration in the earlier periods of infection followed by a compensation attempt later in the process. Finally, FACS results showed no difference in total cell count between knockout and wild type mice.

Conclusion: ICAM-1 participates in the immune response to *M. avium* being involved in control of infection and IFN-γ production.

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IMPORTANT ROLE OF CCL2 IN COGNITIVE DAMAGE ASSOCIATED WITH SEPSIS

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INTRODUCTION: The incidence of sepsis in ICUs is high and sepsis is a major death cause. The survival septic patients can develop many disorders, as well cognitive impairment. Some studies shown that patients from ICUs could to develop cognitive impairment until 6 years after discharged. Our group demonstrated that the CCL2 functioned as anti-inflammatory molecule, which promote a balance of inflammatory response and increase the survival rate of the septic animals. The proposal of this work was analyze the role of CCL2 in the cognitive damage associated with sepsis.

METHODS AND RESULTS: Initially the choice model was the CLP and we analyze the survival rate, the severity score and the glycemia of the animals during 144 hours and 15 days after the CLP, we analyze cognitive function in these animals. To analyze the contextual memory we submitted the animals to open field and the water maze tests. To evaluate the aversive memory we submitted the animals to passive avoidance. For this study, we used genetically deficient mice to CCR2 (CCR2-/-), the receptor to CCL2. In our results, the mortality rate of the CCR2-/- submitted to CLP was higher when we compare with the control group (80% vs 20%). The glycemia and the severity score was similar in both groups submitted to CLP. Interestingly, when we analyze the contextual memory by open field as well as water maze, we observed that cognitive impairment in CCR2-/- in CLP group, but surprisingly also observed cognitive impairment in CCR2-/- sham operated group. This results suggesting that CCR2-/- have cognitive impairment without the presence of the infectious process. To analyze this hypothesis, we submitted naive CCR2-/- and control mice to open field, water maze and to passive avoidance. These results showed that naive CCR2-/- mice present impairment of contextual memory and of aversive memory. The next question of this work was why the CCR2 participates of the memory consolidation. To answer this question, we evaluated the expression of BDNF by western blotting in hippocampus of these animals 6 hours after the passive avoidance task. We observed an important decrease of BDNF expression in hippocampus CCR2-/- mice when compared with control group.

CONCLUSION: These results suggest the important role of CCL2 signaling by CCR2 in memory formation.

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