ECRONICON OPEN ACCESS

EC PULMONOLOGY AND RESPIRATORY MEDICINE

Research Article

Genetic Switches between Cancer and Emphysema Resolution of Cigarette-Smoke Induced Inflammation

Sandra Grumelli[1,2]*, Victor Pinto-Plata[2] and Bartolome Celli[2,3]

[1]*Center of Investigation in Medicine of Respiration, (CIMeR), Cordoba, Argentina*

[2]*Saint Elizabeth Hospital, Boston, United States*

[3]*Brigham and Woman's Hospital, Boston, United States*

***Corresponding Author:** Sandra Grumelli, Center of Investigation in Medicine of Respiration, (CIMeR), Cordoba, Argentina.

Received: September 26, 2019; **Published:**November 01, 2019

Abstract

Cigarette smoke initiates an inflammatory response that has aftermath long after quitting. We segregated former smokers, according to their lung function and their co-founding diseases, in 3 groups: Cancer, Emphysema and COPD. Then we searched for outlier genes in intersections of Venn diagrams where we identified 6 subsets and 23 genes that may be responsible for disease outcome. Genes expressed in the cancer patients with or without emphysema (PPA subset) were BHLH, FPRL2, CD49D, DEADH, NRs4A3, MBLL, GNS, BE675435, ISGF-3, and FLJ23462. Patients with emphysema as co-founding disease, with or without cancer (APP), had only ANXA2 in common. Genes expressed only in non-cancer patients (AAP subset) of COPD group were IL-1A, SOX13, RPP38; TBXA2R, NPEPL1, CFLAR, TFEB, PRKCBP1, IGF1R, DDX11, and KCNAB1. HIV-1Rev was the gene expressed in cancer patients with emphysema (APA subset). Then, we also looked at out-layers genes significantly expressed in all patients (PPP subset with 5066 genes), the down-regulated in Emphysema were MMP9, PLUNC, CEACAM5, and NR4A1 while the up-regulated were F2R, COL15A1, PDE4C, and BGN. We chose genes and checked them at the protein level on immune cells, this showed that neutrophils from Cancer group had increased expression of CD49d, and their total number was also increased in bronchial-alveolar lavage (154%). Macrophages in the lung of patients with emphysema were associated with a significant increase of adhesion molecule CD58 and to significant CD95 decrease, indicating they do not die. Besides, macrophages downregulated MMP9 in the lung compared to blood macrophages. Overall, we find that cancer progression requires a stickier and greater number of neutrophils in the lung while emphysema requires stickier and longevous macrophages to lead matrix destruction, and together with higher expression of SOX13 and RPP38, may promote autoimmunity. We also identified two genes, ANXA2 and HIV1-rev, that may be a pivot between cancer and emphysema outcome of inflammation.

Keywords: *Lung; COPD; Immune Response; Cancer; Genes; BHLH; FPRL2; DEADH; Nrs4a3; MBLL; GNS; BE675435; ISGF-3; FLJ23462; HIV-1Rev; ANXA2; IL-1A; SOX13; RPP38; TBXA2R; NPEPL1; CFLAR; TFEB; PRKCBP1; IGF1R; DDX11; KCNAB1; MMP9; PLUNC; CEACAM5; NR4A1 F2R; COL15A1; PDE4C; BGN; CD95; CD58*

Introduction

A healthy inflammatory response recruits to the site of injury all cells needed to clear the infection, damaged tissue, repair the matrix and stimulate epithelial cell growth; after this process is completed, the function is restored. When inflammatory cells leave the tissue immune homeostasis is attained. However, when the injury is prolonged over time the inflammation is sustained and immune homeostasis is not achieved. This can activate a cluster of genes that lead to excessive tolerance, with the proclivity to cancer, or another cluster that leads to excessive tissue destruction, which inclines the balance toward autoimmunity, playing a pivotal role in disease progression, that

may be the case with cigarette smoke-induced inflammation. Cigarette smoke triggers an inflammatory process that in 10% of ex-smokers resolves in severe emphysema and end-stage COPD [1] while 50 to 70% of them develops cancer [2,5], it seems reasonable then to expect that a genetic component will determine the inflammation outcome.

Translocation of inflammatory cells to the site of injury is mediated by the cell adhesion to endothelial cells from the blood via the lung [3]. This process is mediated by different sets of adhesion molecules that might not only preselect the types of leukocytes and monocytes that enter the injured tissue but also activate them to proliferate and adhere to the extracellular matrix (ECM) [4]. Thus, the type of inflammatory cells recruited may facilitate cancerous cells attachment to the ECM, favoring cancer progression [5], or conversely, it may destroy the ECM producing cell detachment and emphysema, which favors autoimmunity [6,7].

The adhesion molecules (AM) on monocytes are endothelial vascular cell adhesion molecule-1, very late antigen 4 (CD49d), CD58 and leukocyte function antigen-1 [3]. It has been shown that these AM are also essential to T cells recruitment and have a regulatory role because the loss of CD49d impairs T cell infiltration into tumors [8], while high expression of CD49d on T cells is associated with increased tissue destruction [9,10]. T helper 1 (Th1) and Th17 cells use CD49 to translocate at the inflammation site [5,8]. Also, T effectors cells (CD8+ T) recruitment is CD49d dependent given that its infiltration is abrogated by *in vivo* neutralization of CD49 [9]. Besides its homing capacity, CD49d has a regulatory function since it has been shown that regulatory T cells (Treg) CD49d+ are significantly more effective in suppressing T effectors cells then Tregs CD49d- [8]. Functional investigations suggested that CD58 up-regulates the expression of the transcription factor FOXP3 in Tregs [11], participates in co-stimulation of T-cells by endothelial cells [12] and in the cell to cell cross-talks with natural killer (NK) cells [13,14].

To discover genes that may play a pivotal role between cancer and emphysema we used Venn diagrams to segregate genes from mRNA microarrays, searching for outliers genes that when they are turned-on may trigger cancer or emphysema outcome of inflammation. We identified two genes, ANXA2 and HIV1-rev that may play a pivotal role in CD49 and CD58 differential expression on inflammatory cell from patients with Cancer compared to COPD.

Methods

Patients demographics

All tissue-related experiments and analysis were conducted according to the principle expressed in the Declaration of Helsinki. Written informed consent was obtained from all patients recruited from the Veteran Affair Hospital (VA) from Houston, TX and Saint Elizabeth Hospital from Boston, MA. The mRNA microarray was the same described in [7]. The study protocols and consent forms were approved by Saint Elizabeth Hospital Review Board for human studies in compliance with HIPAA regulations. Informed consent was obtained from all patients participating. Table 1 summarizes the clinical information and grouping criteria. All patients were former smokers, grouped according to their severity of COPD graded according to the stages of the disease as defined by the Global Initiative for Chronic Obstructive Lung Disease (GOLD). Cancer grouped non-emphysema patients in GOLD stage 0 to 1 that had cancer removal surgery. In Emphysema group were cancer patients in GOLD stage 2 and 3 that also had emphysema, as defined based on their computed scanning (CT). The COPD group was non-cancer patients in GOLD stage 4 with severe emphysema, that had lung volume reduction surgery (LVRS).

Samples collection

Lung tissues were collected at the time of the surgery at the VA and were used for mRNA extraction and cell extraction for flow cytometry as previously described [6]. Patients that had to go through bronchial-alveolar lavage (BAL) by medical prescription were recruited at St. Elizabeth Hospital after written informed consent was obtained; all samples were used for total inflammatory cells count and differential cell count by standard clinical laboratory procedures and flow cytometry.

Microarrays analyses

Data from microarrays generated as described in Grumelli., *et al.* [7]; identifier GSE119040, ID: 200119040, were further analyzed using Venn diagrams to detect outlier genes, expressed in patients with the same co-founding disease, that may have pivoted the immune response to a given disease. mRNA extracted from thirteen ex-smokers, with similar smoking history, were tested using individual Affymetrix Human Genome U133A chip, as previously described [7]. Patients were segregated in the 3 groups: Four in Cancer group, with cancer,

age 78 (6), average (SD); without obstruction determined by their forced expiratory volume in the one-second percentage of predicted (FEV1%) of 80 (24), nor emphysema as shown by their Ct scans. Emphysema group, with five patients of 71 (7) years old with cancer and emphysema, with mild obstruction of 65 (15) FEV1% and the COPD group were four non-cancer patients, 65 (5) years old, with severe emphysema by their Ct scan and severe obstruction, 34 (9) FEV1%. COPD was diagnosed according to the criteria recommended by the NIH/WHO workshop [15].

Venn Diagrams analyses

Probes with p values below 0.05 in the 13 patients were considered present (P) and probes with p-value(s) more than 0.05 were considered absent (A). To consider a gene absent within a group, it was required that at least 1 patient had p-value(s) more than 0.05 in that particular gene and p-value(s) not more than 0.05 to be included within the present group (Figure 1A). To identify outliers we further segregated genes present in each group (cancer, emphysema or COPD) and identified genes expressed in the intersections (∩) of each group, for example in the PPA subset, first letter of the subset signifies presence (PPA) in Cancer group, second letter (PPA) in Emphysema group and third (PPA) in COPD and searched their co-founding disease, cancer in this case. We also searched for those genes not expressed in another group e.g. AAP subset. Table 2 summarizes al the equations applied to identify outliers in the different subsets and the role they may play in disease progression.

The graph at the intersection of the three groups (PPP) was obtained by plotting the logarithm of 10 of the average intensity of expression of Emphysema relative to Cancer (E/C) versus the COPD relative to Cancer (COPD/C) of the 5066 probes. This was further narrowed down for those genes that were significantly different, Figure 2A and used for clustering analysis.

Hierarchical clusters analyses

Unsupervised hierarchical clustering analyses (HCA) were done using an exploratory multivariate statistical method to identify 'natural' groupings of patients considered in an analysis. The least distance, D(r,s), between two r and s (arrays r and s) consisting of nr and ns elements were first identified based on either the Euclidean distance ($0 \leq D(r,s) < + \infty$). The logarithm of 2 was used to transform raw data to determine Euclidean distance for the hierarchical analysis presented in the heat map [16].

The role of the different genes products within the cell was illustrated using Motfolio, Scientific Illustration Toolkits for Presentations and Publications.

Gene validation

Gene expression was validated using the same mRNA samples for quantitative real-time polymer chain reaction (qRT-PCR); probes were Assay-On-Demand primers and probes from A&B Applied Biosystems. Protein expression of the selected genes was quantified in isolated cells [7], the concentration of 107 cells/ml of RPMI medium; different cells populations were gated using side and forward scatter plot and labeled with fluorescent dyes conjugated to anti-CD3, -CD14, CD66, for lymphocytes, macrophages and neutrophils, respectively. The protein content of selected genes was quantified using CD49, CD58 and CD95 antibodies monoclonal, purchased from BD Biosciences Pharmingen (San Diego, California, United States). Data generated by the flow cytometer was analyzed using flowJo software 8.1.1 (Standford University 1995-96).

Zymogram

Macrophages from blood or lung tissue from the same patient were cultured in RPMI 1640 media with 10% fetal calf serum and 5% L-glutamate, all cell culture reagents were purchased from Invitrogen. Cells were stimulated with 50, 250 and 500 ng/ml of INFγ, IL4 and IP10, from Prepotech and supernatant (5 µg of protein) were dissolved in a non-denaturing buffer and loaded in 10% SDS-polyacrylamide gels containing 0.02% gelatin and then separated. SDS was then removed with 2.5% Triton X-100 washes, 20 min three times and incubated for 24 hours at 37°C in developing buffer (50 mM Tris-HCl pH 8, 5 mM $CaCl_2$, 0.02% NaN_3). Gels were fixed and stained with 50% methanol and 10% acetic acid containing 0.3% w/v Coomassie blue and used to measure MMP-9 (gelatinase B) activity, quantifying absorbance of the clear bands, visualized with Chemi-Doc gel documentation system (Bio-Rad) and the intensity measured with Quantity One software (Bio-Rad). Results are shown as mean and standard deviation. Statistical analysis was performed using the unpaired student's t-test, with significant differences considered as $P < 0.05$.

Results and Discussion

Venn diagrams analyses

Whenever there is sustained injury of a tissue the inflammation prolongs and immune homeostasis is not achieved this can lead to excessive tolerance, with the proclivity to cancer, or excessive tissue destruction, which inclines the balance toward autoimmunity, these are the cases of smokers. Cigarette smoke initiates an inflammatory response that has sequels long after they quit smoking, it is not well known why but some ex-smokers develop cancer and others autoimmunity. Thus, we thought that if some genes are always expressed, we can consider them as structural genes that may vary their expression level with the disease progression but genes that are expressed only when there is one particular disease may be key to decide the outcome of the inflammation towards cancer or emphysema. Therefore, we searched for outliers genes within each group of patients (Table 1) and using Venn diagrams (Table 2) we identified 6 subsets (Figure 1) and 23 genes that may be responsible for inflammation outcome. The PPA subset had 10 genes expressed in patients with cancer as a co-founding disease, which were BHLH, FPRL2, CD49D, DEADH, NRs4A3, MBLL, GNS, BE675435, ISGF-3 and FLJ23462 that may be a cluster of genes under the same regulation. We chose CD49d and NRs4A3 for validation by qRT-PCR on the same patients and CD49 at the protein level by flow cytometry in different patients.

Table 1: Clinical and Demographic Characteristics of Participants.

	Cancer (n = 36)	Emphy (n = 17)	COPD (n = 34)
Cancer	Yes	Yes	No
Emphysema[a]	No	Yes	Yes
GOLD stage	0 - 1	2 - 3	4
LVRS[b] (n)	0	0	12[f]
Small peripheral Cancer (n)	9[f]	17[f]	0
Age (Average ± SD)	65 ± 12	68 ± 45	60 ± 6
Percent FEV1[c] (Average ± SD)	84 ± 15	63 ± 15	28 ± 6*
Pack-years smoking[d] (Average ± SD)	55 ± 37	60 ± 24	52 ± 17
Ex-smokers (n)	23	17	30
QT[e] (Years)	9 ± 10	4 ± 6	11 ± 11

*[a]: Determined by Ct scan. [b]: Lung Volume Reduction Surgery (LVRS). [c]: Percentage of Forced expiratory volume (FEV1) in one second is *significantly different (p < 0.05) for the end-stage group compared to the control group. [d]: Packs per years (PPY), quitting time (QT) and age values are similar among the three groups. [e]: Quitting time: continue time without smoking since quitting. [f]: The patients had no history of allergy or asthma and had not received oral/systemic or inhaled corticosteroids during the last six months. At the time of the study, all patients were free of acute symptoms suggestive of upper or lower respiratory tract infection in the 6 weeks preceding the study.*

Table 2: Venn diagrams analyses for outliers' genes.

Subset	Operation	Cancer (*n=4*)	Emphysema (*n=5*)	COPD (*n=4*)	Co-founding Disease *(gene role)*
PPA	$\{(C \cap E) - (C \cap E \cap COPD)\}$	Yes	Yes	No	Cancer
PPP	$\{C \cap E \cap COPD\}$	Yes	Yes	Yes	(Structural genes)
APA	$\{E - (C \cup COPD)\}$	No	Yes	No	(Pivot) cancer°
APP	$\{(E \cap COPD - (C \cap E \cap COPD)\}$	No	Yes	Yes	(Pivot) Emphysema
AAP	$\{COPD - (C \cup E)\}$	No	No	Yes	Autoimmunity

Symbols are, C for cancer, E for emphysema, ∩ intersection, ∪ union, $\{\}$ subset, - minus.

The 11 genes (AAP) expressed in non-cancer patients that only had emphysema, in COPD group, were IL-1A, SOX13, RPP38; TBXA2R, NPEPL1, CFLAR, TFEB, PRKCBP1, IGF1R, DDX11 and KCNAB1 that are related to emphysematous inflammation. 2 genes may play a pivotal role (Figure 1A and 1B) between cancer and emphysema, ANXA2 in APP subset and HIV1-Rev in APA subset.

HIV1-Rev is a nuclear-exporting protein that binds gC1q, the first classical complement protein [17]. The essential mediators of the nuclear proteins exit containing nuclear export signals are HIV1-Rev type that increases the levels of the p53, tumor suppressor protein [18]; this indicates that HIV1-Rev may be involved not only in promoting emphysema by activation of the classical pathway (C1q) that leads to autoimmunity, which we previously demonstrated is activated in patients with autoimmune COPD [7], but also it may lead to tumor suppression due to p53 overexpression, (Figure 1C) that can pivot between both diseases promoting the expression of AAP subset, breaking the tolerance on a cancerous inflammatory process, recruiting, activating or differentiating Th1 cells, this makes it a possible switch between cancer and emphysema (Figure 1B).

Lipocortin-2 may be a switch between cancer and autoimmunity

Patients with emphysema as a common disease (APP subset) had only (Figure 1A, Table 2), lipocortin-2 or annexin 2 gene (ANXA2). ANXA2 has a PDZ motif located at the carboxylate terminus, a structural short domain found in signaling proteins that forms a complex to enhance the phosphorylation of cystic fibrosis transmembrane regulator (CFTR) that activate and deactivate the chloride ion channel by a mechanism of phosphorylation/dephosphorylation on the nuclear binding domain (NBD). NBD is activated by a cAMP product that activates protein kinase (PKA), acting as negative effector, phosphorylating the NBD of CFTR and reducing the chloride ion transport [19,20]. The annexins are a family of phospholipid-binding proteins of largely unknown function; ANXA2 is involved in airway smooth muscle cell proliferation induced by plasminogen because knockdown of ANX2 by siRNA produce inhibition of plasmin-stimulated ERK1/2 or PI3K/Akt signaling and attenuated plasmin-stimulated increases in airway smooth muscle proliferation [21]. Lung fibroblast secretes cathepsin B that induces the upregulation of stearoyl-CoA desaturase 1 through ANXA2 and PI3K/Akt/mTOR pathway (Figure 1C) that promoted metastatic colonization of the lung [22]. These reports indicate that ANXA2, upstream of PI3K, regulates lung cell proliferation by attaching the cells to the ECM trough collagen VI (Col6), secreted by bronchial epithelial cells. ANXA2 (-/-) knock out mice have reduced exercise tolerance with impaired lung tissue elasticity, which was phenocopied in Col6a1(-/-) mice. *In vivo*, ANXA2 (-/-) bronchial epithelial cells underwent apoptosis and detachment [23]. Also, ANXA2 associates with S100A to activate the chloride flux through CFTR in a cAMP/PKA-dependent manner [24,25]. Thus, the ANXA2 gene may be key to deviate the immune response toward cancer or autoimmunity regulating the ionic balance of Cl- and Ca++, which is essential for cell attachment to the matrix; because it may prompt expression of the cluster of 10 PPA genes tipping the balance toward attachment of cancerous cells to the ECM permitting excessive cell proliferation while its down-regulation may prompt the expression of 11 genes in AAP inducing excessive cell detachment, cell death, intolerance and autoimmunity (Figure 1B).

Among the genes highly expressed in Cancer were CD49d and N-formyl peptide receptors (FPRL2) an innate immunity receptor expressed in a nasal epithelial cell (Figure 1C) that upon binding to its ligands secrete VEGF-α and TGF-β, the key to tissue remodeling [26].

We further searched for genes that were significantly different from Cancer (Figure 2A), those up-regulated in both Emphysema group (E and COPD) are likely to contribute to emphysema progression, while those that are down-regulated for COPD but up-regulated for Emphysema (top left quadrant) may be contributing to cancer, like CD58 and MMP10. Gene clustering and hierarchical analysis show that they segregated according to emphysema progression, demonstrated by the similar color in the heat map (Figure 2B), hollow arrow show genes up-regulated by emphysema or cancer. Representative genes from microarrays results were validated using qRT-PCR; for PPA group we chose CD49d and nuclear receptor subfamily 4 (NRs4A2, Figure s 2C and 1A), which showed similar results. CD49d was also validated at the protein level since it may be of crucial importance to recruit and activate inflammatory cells for death/proliferation or autoimmunity/cancer.

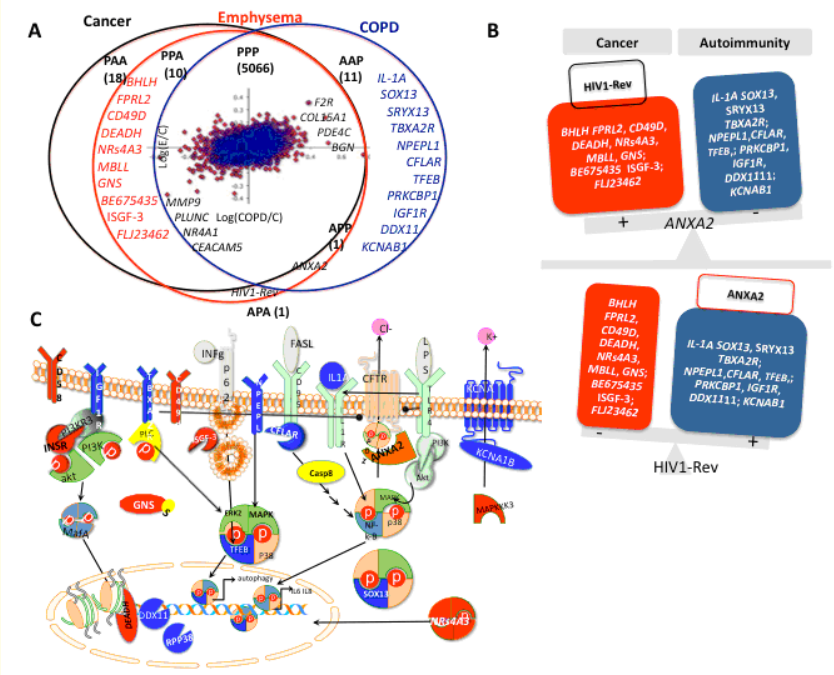

Figure 1: *Genes segregated according to emphysema progression.*

(A) Venn diagram showing presence (P, p < 0.05) or absence (A, p > 0.05) of genes expressed among groups of: Cancer (n = 4), cases of non-emphysema ex-smokers with lung cancer; Emphysema (n = 5), cases of ex-smokers with lung cancer and emphysema; COPD (n = 4), non-cancer ex-smoker with emphysema. (PPA) Genes uniquely expressed in Cancer and Emphysema group are symbolized by BHLH, basic helix-loop-helix domain containing, class B; FPRL2, formyl peptide receptor-like 2; CD49D, alpha 4 subunit of VLA-4 receptor; DEADH, RNA helicase A, nuclear DNA helicase II; NRs4A3, nuclear receptor subfamily 4, group A, member 3; MBLL, melanogaster muscle blind B protein; GNS, glucosamine (N-acetyl)-6-sulfatase; hypothetical proteins BE675435 and FLJ23462. Gene reliably expressed only in Emphysema (APA) was HIV-1 Rev, HIV rev binding protein. The only gene expressed at Emphysema and COPD intersection (APP) was lipocortin-2 or ANXA2, annexin II. (AAP) Genes highly expressed only in the COPD group were IL-1A, interleukin 1; SOX13, SRY (sex-determining region Y)-box 13; TBXA2R, thromboxane A2 receptor; NPEPL1, aminopeptidase-like 1; CFLAR, CASP8 and FADD-like apoptosis regulator; TFEB, transcription factor EB; PRKCBP1, protein kinase C binding protein 1; IGF1R, insulin-like growth factor 1 receptor; DDX11, I(Asp-Glu-Ala-Asp/His) box polypeptide 11; KCNAB1, potassium voltage-gated channel. Insert Plot in intersection of genes expressed in all patients (PPP), represent data log_10 transformed of fold changes, relative to Cancer group; insert show out-layers genes significantly up or down-regulated, symbolized by F2R, coagulation factor II (thrombin) receptor; COL15A1, collagen, type XV, alpha 1; PDE4C, phosphodiesterase 4C, cAMP-specific; BGN, biglycan; MMP9, matrix metalloproteinase 9 (gelatinase B, 92kD gelatinase, 92kD type IV collagenase; PLUNC, palate lung and nasal epithelium clone; CEACAM5, carcinoembryonic antigen-related cell adhesion molecule 5; NR4A1, nuclear receptor subfamily 4, group A, member 1. (B) Schematic representation showing the proposed role of the different genetic sub-group in the immune process that leads to cancer or autoimmunity. (C) Role of the different gene at the cellular level.

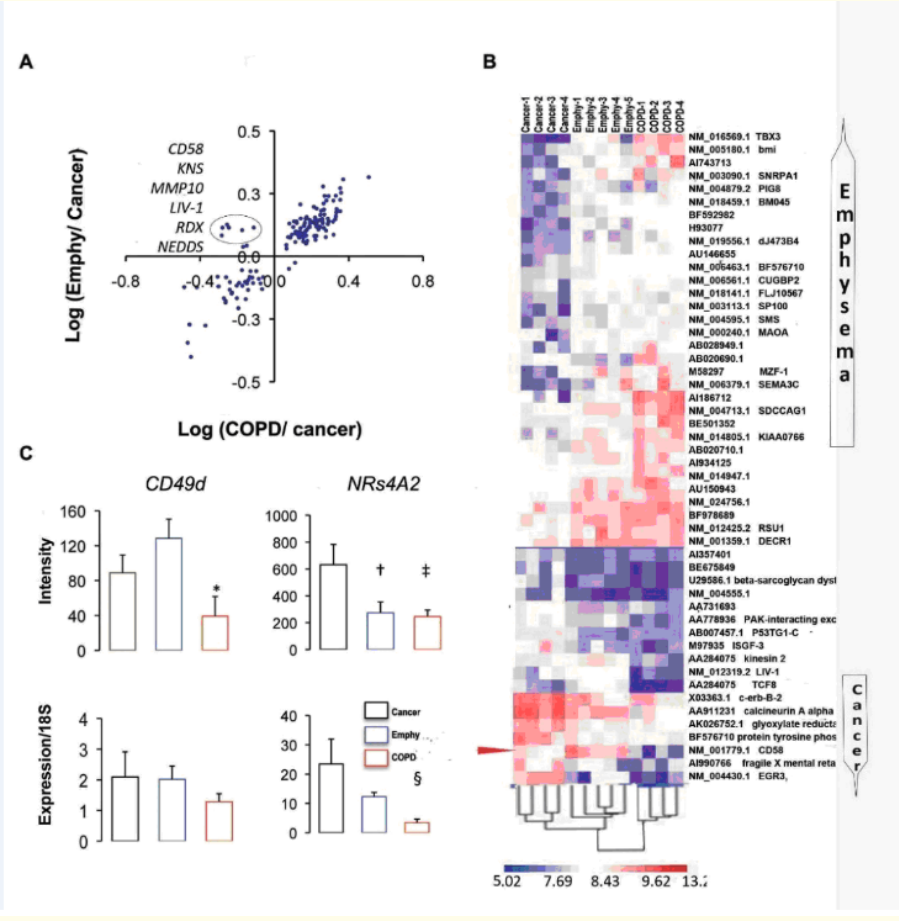

Figure 2: *Heat map of genes significantly expressed in all subjects, PPP intersection. (A) Representation of log_10 transformed fold changes, relative cancer group, show the 143 genes significantly different from cancer group reliably expressed in all patients (PPP). Insert details the genes up-regulated by cancer in patients with emphysema. KNS, kinesin 2; p115, vesicle docking protein p115; LIV1, LIV-1 protein, estrogen-regulated; MMP10, disintegrin and metalloproteinase domain 10; CD58, lymphocyte function-associated antigen 3; RDX, Homo sapiens radixin; NEDD5, neural precursor cell expressed, developmentally down-regulated 5. (B) Heat map of clustered genes. Color-based on sorted, Log_2 transformed raw data, the distance is a function of the Euclidean distance as a metric for the dissimilarity of significantly different genes present in all patients (PPP). Cancer group (n = 4), ex-smokers with lung cancer cases. Emphysema group (n = 5), ex-smokers with lung cancer and emphysema. Ex-smokers with severe emphysema and without cancer are COPD (n = 4). (C) Validation of the gene expression from microarray data, quantified by q RT-PCR on the same mRNA samples, for chosen genes. Values determined by unpaired T student test, *0.01, †0.04, ‡ 0.026, § 0.0006.*

CD49 expression positively correlates with lung function

The protein expression of selected genes was quantified on inflammatory cells obtained from lung tissue (Figure 3A), CD49d showed stronger association (r = 0.40) with lung function (FEV1%) than CD58 (r = 0.29); because both are cell adhesion molecules expressed in a wide variety of cells and required for ECM attachment, recruitment to the injured site and cell survival, we further quantified its expression on lymphocytes, macrophages and neutrophils with the objective to determine how they may be contributing to inflammation and immune homeostasis (Figure 3B).

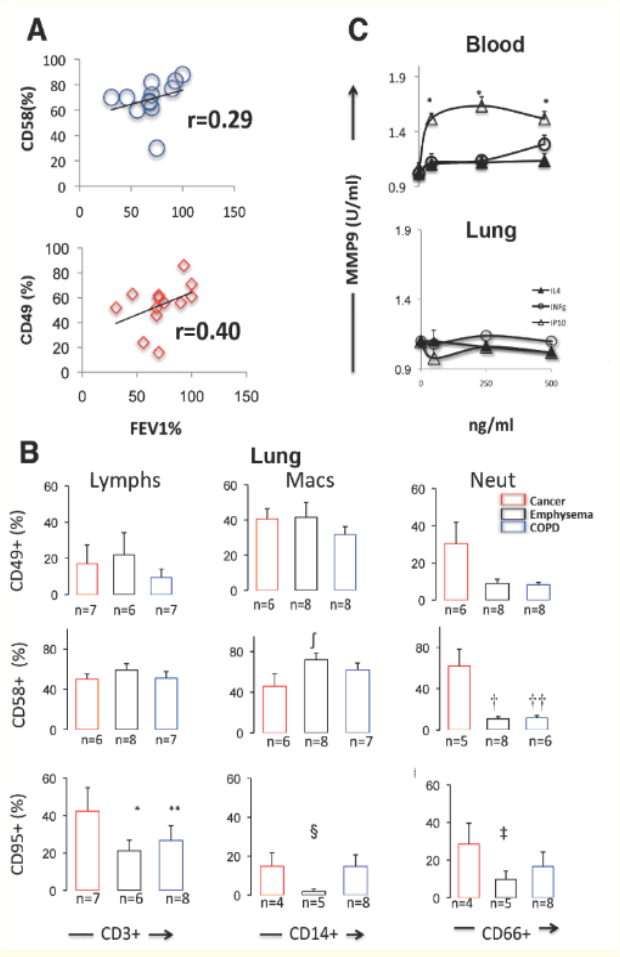

Figure 3: *Quantification of protein expression for selected genes.*

*(A) A linear regression of the forced expiratory volume in one second, % of predicted (FEV%), versus the total cells surface content of CD58, CD49 and CD95 that were determined using flow cytometry on live lung cells. (B) Plots showing the percentage of double-positive cells for CD58, CD49 or CD95 with CD3, CD14 or CD66 utilized to identify the populations of lymphocytes, macrophages and neutrophils. P values are *0.02; **0.009; ∫ 0.026; † 0.002; ††1.2 10-5, § 0.05 and ‡0.0005. (C) MMP9 activity measured by gelatin zymogram, units determined by the intensity of absorbance relative to control. The macrophages from blood and lung tissue of the same patient with emphysema were stimulated with INFγ, IL,4 and IP10.*

Lymphocytes survive longer in emphysematous lung

CD58 is an adhesion molecule that also serves as a co-stimulatory protein for T cells to induce FOXP3 in Tregs [11]. Lymphocytes from emphysematous lung tissue expressed similar adhesion molecules CD58 and CD49d in Figure 3B, indicating their recruitment to the lung may not be hampered by the number of these AM. However, it coincided with a significant decrease of CD95 on lymphocytes of Emphysema and COPD groups; this receptor is coupled to caspase-mediated apoptosis [27] and involved in memory formation [28]. Increased survival of lymphocyte in emphysematous groups (E and COPD), may help to maintain the total number lymphocytes in tissue and BAL while their numbers decrease in blood from vein (-19.2%, r = 0.64, Figure 4B) or artery (-16.8%, r = 0.36), yet remaining unchanged in bronchial-alveolar lavage (r = 0).

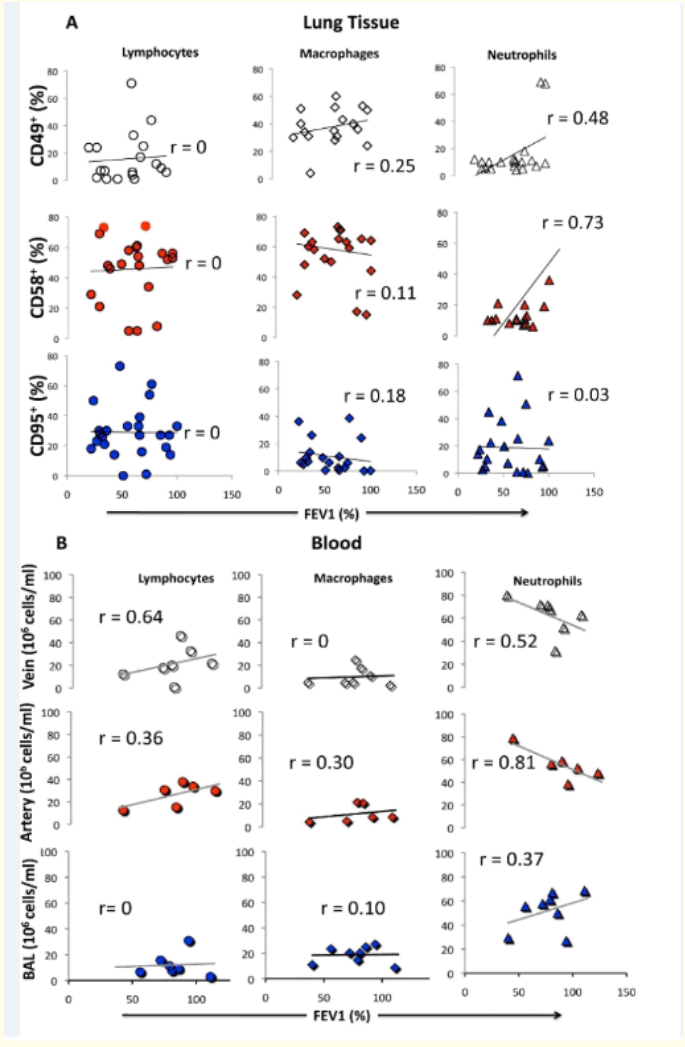

Figure 4: *Inflammatory cells traffic.*

(A) Linear regression of the forced expiratory volume in one second, % of predicted (FEV%), versus a percentage of CD58, CD49 or CD95 on lymphocytes, macrophages and neutrophils determined by flow cytometry on live lung cells. (B) Plots showing association values (r) between FEV1 (%) and a number of inflammatory cells in peripheral or arterial blood and lung tissue, represented by the bronchial-alveolar lavage (BAL).

Longevous macrophages from emphysema lungs are more adhesive

CD49 expression showed a slight decrease while CD58 was significantly increased 250% on macrophages of Emphysema group ($p = 0.026$, $n = 6 - 8$, Figure 3B). Coincident with this increase there was a significant decreased CD95 expression (6.1%, $p = 0.05$, Figure 3B), indicating these macrophages do not go through apoptosis, living longer then Cancer macrophages ($p = 0.026$). Figure 4B shows that circulating macrophages are recruited from arteries ($r = 0.3$) into the lung ($r = 0.10$, BAL), although their numbers are associated with the lung function, there are consistently more macrophages in the BAL (18.10^6 cells/ml) than in the venous (7.10^6 cells/ml) or arterial blood (4.10^6 cells/ml). There is only -0.1% decrease of macrophages in BAL from 100 to 30 FEV1% indicating they may be more adhesive to the ECM (6.7% more CD58) therefore they cannot be removed from the tissue.

Sticky neutrophils are predominant in lung cancer

Since CD58 expression was significantly increased in Emphysema group (Figure s 2A and 2B) we quantified its protein expression on inflammatory cells. CD58 was also significantly increased in neutrophils of cancer patients relative to COPD (Figure 3B, $p = 0.002$) and emphysema ($p = 1.2\ 10\text{-}5$) with a strong positive correlation with lung function (Figure 4A, $r = 0.73$), which was also seen for CD49, $r = 0.48$, although this increase was not significant for CD49, in cancer patients (Figure 3B).

Emphysema patients showed decrease expression of these adhesion molecules on the cell surface proteins (-61.4% for CD58 and -25.6% for CD49) strongly correlated with increased neutrophilia in arterial ($r = 0.81$) and venous blood ($r = 0.52$), which could not migrate into the lung, as shown by decreased neutrophils in BAL ($r = 0.37$, Figure 4B).

CD95 expression is also significantly decreased (-1.7%) with emphysema indicating decreased apoptosis. Note that neutrophils are decreased in BAL of emphysematous lungs supporting that they cannot be removed from the bloodstream, whereas they are increased the number and fewer are recruited into the lung tissue from blood and their decline is strongly associated with the lung function ($r = 0.81$, Figure 4B).

MMP9 is downregulated in lung macrophages

Enzymatic activity of MMP9, determined by collagenase activity on zymogram (Figure 3C), showed that blood monocytes are strongly stimulated by IP-10, a second messenger of INF-γ, a key element of the Th1 immune cross-talk. After arriving in the injured lung tissue, MMP9 activity is inhibited, as shown by tissue macrophages activity (Figure 3C) while MMP12 may assist ingress of monocytes into the tissue by degrading the ECM. MMP10 is significantly up-regulated in Emphysema group with cancer as a co-founding disease (Figure 2A) but it is down-regulated in emphysema patients without cancer (COPD group), indicating a role for this protease in cancer progression. Close inspection of macrophages (Figure 3B) show an increase in their adhesion molecules, CD58 and CD49d with a significant decrease of CD95, which suggest a role for macrophage-metalloprotease 10 (MMP10) cleavage of this cell surface death receptor (CD95), further studies in this direction should be pursued.

Neutrophils transfer into the lung is CD58 and CD49 dependent

We further studied the association of AM expression with the lung function, for lymphocytes, there was no association between CD58, CD49 and CD95 with their lung function (Figure 4A, $r = 0$). This was somehow corroborated by lymphocytes invariance in the BAL (Figure 4B, $r = 0$). In contrast neutrophils trafficking was associated to adhesion molecules expression on their surface as shown by their significant association with CD58 (Figure 4A, $r = 0.73$) and CD49 ($r = 0.48$) expression and their reduced presence in lung tissue represented by lower BAL neutrophils count ($r = 0.37$), downregulation of these molecules was also associated with a higher number of circulating neutrophils in arterial (Figure 4B, $r = 0.81$) and peripheral blood (Figure 4B, $r = 0.52$).

Conclusion

The analysis of outlier genes expressed in the different subsets and the co-founding disease allowed us to identify genes that may be under the same regulation, like clusters that are turned-on only in that disease process and several findings emerge.

First: CD49d in PPA subset, CFLAR an apoptosis regulator, in AAP subset and 2 genes that may play a pivotal role in disease progression. HIV1-Rev in APA subset indicates that viral infections may produce emphysema in patients with cancer while among patients with emphysema as a co-founding disease in APP subset there was only ANXA2, which is essential for the attachment of bronchial epithelial cells to the matrix by secretion of collagen. From the work of others [23] we learned that ANXA2 knock out mice fibroblasts retains collagen VI within intracellular vesicles and adhered poorly to their matrix unless ANXA2 expression is restored, indicating their incapacity to go through the membrane, perhaps due to deficient membrane potential, caused ionic deregulations. ANXA2(-/-) bronchial epithelial cells undergo apoptosis and detachment (Figure 1C). Immunoprecipitation and immunoelectron microscopy revealed that ANXA2 associates with COL6 to establish cell-matrix interactions that underlie normal pulmonary function and epithelial cell survival [24,25,29]. Herein we hypothesize that ANXA2 is a switch that when it is turned on favors cancer by facilitating cell attachment to the matrix while when it is turned off cells detach from the matrix and undergo apoptosis turning on emphysema genes, expressed in the AAP subset (Figure 1B);

while when HIV1-Rev in APA is turned on it breaks cancer-induced tolerance permitting emphysema progression and probably eliminating cancerous cells.

Second: From genes that are expressed only in non-cancer patients with emphysema (COPD group) we find that AAP subset summarizes the genes driving the inflammation that leads to autoimmunity such as IL-1 interleukin, a well-described pleiotropy cytokine in COPD patients and used as a marker of systemic inflammation [30,31]. Mice mutant in SOX13 has impaired migration of Th17 to the inflammatory site [32,33], which indicates that increased activity of SOX13 is favoring Th17 migration to the lung and facilitating the development of autoimmunity, since it is also an autoantigen, as we previously proposed [7]. The NPEPL1 gene product is a direct target of regulation by miR-19a [34]. Micro RNA plays an essential role in cell proliferation and apoptosis [35], but miR-19 3b was shown to regulate estrogen receptor and other steroid hormones [36], up-regulation of NPEPL1 in COPD patients may be associated with corticosteroid resistance [37]. IGF1R, insulin-like growth factor-1 receptor mRNA is a target of miR-503 in glioblastoma multiforme cell lines that not only suppressed cell proliferation through cell cycle arrest and apoptosis but also inhibited cancer cell migration and tumor invasion. Mechanistic analysis revealed that forced expression of miR-503 inhibited AKT activation, suggesting the tumor-suppressive effect of miR-503 is partially mediated by phosphatidylinositol 3-kinase/AKT signaling. Taken together, miR-503 is a tumor suppressor for glioblastoma multiforme and a favorable factor against glioma progression through targeting IGF1R [36]. RPP38 is a subunit of the human ribonucleoprotein ribonuclease P38 which is an autoimmune antigen in scleroderma, an elastin rich tissue like the lung [37] and it is required for processing rRNA [38], These results suggest that further detection of RPP38 and SOX13 autoantibodies should be also tested in patients with severe COPD, as another source of autoimmunity.

From the genes that were validated at the protein level in different immune cells and compartments such as arterial or peripheral blood and lung BAL, we had several findings.

Third: CD49d is present in patients that had cancer as a co-founding disease and CD49 expression positively correlates with lung function. CD49d is expressed in T cell [28], neutrophils [39], basophils [40] and monocytes and it is responsible for adherence to vascular cells molecule 1 (VCAM-1) from the endothelium and ECM [41], it is essential for their recruitment to the site of inflammation. In addition, CD49 up-regulation is associated with migration of hematopoietic stem cells to the inflamed tissue [42], thus its up-regulation in PPA subset contributes to tissue repair but also lung cancer because it is essential for leukemic cells survival [9]. Lymphocytes have 3-fold increase expression of CD49d in cancer, compared to COPD patients, although it was not significantly different ($p = 0.13$ and 0.07, respectively) it shows a trend toward higher expression, which is essential for carcinoma elimination by NK CD8+ T cells [9], thus CD49+ lymphocytes favors tissue repair and tumor proliferation due to evasion of the immune system.

Fourth: Although neutrophils from carcinoma tissue had increased expression of CD49d, it was not significant (123% in emphysema and 70% in cancer group, relative to COPD) nevertheless the total number of neutrophils was increased in cancer BAL (154%). CD49 protein expression showed the same trend then array results (Figure 2B), which is required for neutrophils migration to the site of inflammation across the interstitium into the alveoli [39], in response to C5a, a C1q downstream protein of the classical complement pathway [35]. We also found that neutrophils transfer to the lung is CD58 dependent due to their significant association with CD58 (Figure 4A, $r = 0.73$) and their reduced number in lung tissue show by lower BAL neutrophil count ($r = 0.37$), downregulation of these molecules was associated with higher number of circulating neutrophils in arterial (Figure 4B, $r = 0.81$) and peripheral blood ($r = 0.52$).

Fifth: Macrophages in the lung of patients with emphysema are associated with a significant increase of adhesion molecule CD58 and to significant CD95 decrease indicating they do not die, further accumulating in the tissue. Indicating that long lived macrophages from emphysema lungs are more adhesive and they drive the lung destruction. Of note is the increased MMP10 expression that suggests it may play a role in shedding receptors from the cell surface thus, increasing their life span and promoting emphysema while MMP9 down-regulation on macrophages compared to monocytes (Figure 3C) suggest it is required for translocation to the tissue from the blood.

Acknowledgment

The author wants to thank Dr. Bartolome Celli and Dr. Victor Pinto-Plata for collecting the BAL and blood samples and Lizhen Song and Lin Song for their help with tissue collection and mRNA extraction.

Bibliography

1. Anthonisen NR., *et al*. "Smoking and lung function of Lung Health Study participants after 11 years". *American Journal of Respiratory and Critical Care Medicine* 166.5 (2002): 675-679.

2. Punturieri A., *et al*. "Lung cancer and chronic obstructive pulmonary disease: needs and opportunities for integrated research". *Journal of the National Cancer Institute* 101.8 (2009): 554-559.

3. Imhof B A and Aurrand-Lions M. "Adhesion mechanisms regulating the migration of monocytes". *Nature Reviews Immunology* 4.6 (2004): 432-444.

4. Popper HH., *et al*. "Expression of adhesion molecules in allergic lung diseases". *Virchows Archiv* 440.2 (2002): 172-180.

5. Garmy-Susini B., *et al*. "PI3K activates integrin a4b1 to establish a metastatic niche in lymph nodes". *Proceedings of the National Academy of Sciences of the United States of America* 110.22 (2013): 9042-9047.

6. Grumelli S., *et al*. "An immune basis for lung parenchymal destruction in chronic obstructive pulmonary disease and emphysema". *PLOS Medicine* 1.1 (2004): e8.

7. Grumelli S., *et al*. "CD46 Protects against Chronic Obstructive Pulmonary Disease". *PLOS ONE* 6.5 (2011): e18785.

8. Kraczyk B1., *et al*. "CD49d Treg cells with high suppressive capacity are remarkably less efficient on activated CD45RA- than on naive CD45RA+ Teg cells". *Cellular Physiology and Biochemistry* 34.2 (2014): 346-355.

9. Singh V., *et al*. "Cooperativity of CD44 and CD49d in Leukemia Cell Homing, Migration, and Survival Offers a Means for Therapeutic Attack". *Journal of Immunology* 191.10 (2013): 5304-5316.

10. Ifergan I., *et al*. "Central nervous system recruitment of effector memory CD8+ T lymphocytes during neuroinflammation is dependent on α4 integrin". *Brain* 134 (2011): 3560-3577.

11. De Jager PL., *et al*. "The role of the CD58 locus in multiple sclerosis". *Proceedings of the National Academy of Sciences of the United States of America* 106.13 (2009): 5264-5269.

12. Shaw S., *et al*. "Two antigen-independent adhesion pathways used by human cytotoxic T-cell clones". *Nature* 323.6085 (1986): 262-6084.

13. Selvaraj P., *et al*. "The T lymphocyte glycoprotein CD2 binds the cell surface ligand LFA-3". *Nature* 326.6111 (1987): 400-403.

14. Ariel O1., *et al*. "Distinct membrane localization and kinase association of the two isoforms of CD58". *Cell Signal* 16.6 (2004): 667-673.

15. Uppaluri R., *et al*. "Quantification of pulmonary emphysema from lung computed tomography images". *American Journal of Respiratory and Critical Care Medicine* 156.1 (1997): 248.

16. Peterson L. "Factor analysis of cluster-specific gene expression levels from cDNA microarrays". *Computer Methods and Programs in Biomedicine* 69.3 (2002): 179-188.

17. Das S1., *et al*. "Multifunctional activities of human fibroblast 34-kDa hyaluronic acid-binding protein". *Gene* 190.1 (1997): 223-225.

18. Laína S., *et al*. "An Inhibitor of Nuclear Export Activates the p53 Response and Induces the Localization of HDM2 and p53 to U1A-Positive Nuclear Bodies Associated with the PODs". *Experimental Cell Research* 248.2 (1999): 457-472.

19. Welting TJ1., *et al*. "Mutual interactions between subunits of the human RNase MRP ribonucleoprotein complex". *Nucleic Acids Research* 32.7 (2004): 2138-2146.

20. Van Eenennaam H., *et al*. "Autoantibodies against small nucleolar ribonucleoprotein complexes and their clinical associations". *Clinical and Experimental Immunology* 130.3 (2002): 532-540.

21. Stewart AG., *et al*. "Plasminogen-stimulated airway smooth muscle cell proliferation is mediated by urokinase and annexin A2, involving plasmin-activated cell signaling". *British Journal of Pharmacology* 170.7 (2013): 1421-1435.

22. Liu G Feng S., *et al*. "Lung fibroblasts promote metastatic colonization through upregulation of stearoyl-CoA desaturase 1 in tumor cells". *Oncogene* 37.11 (2018): 1519-1533.

23. Dassah M., *et al*. "Annexin A2 mediates secretion of collagen VI, pulmonary elasticity and apoptosis of bronchial epithelial cells". *Journal of Cell Science* 127 (2014): 828-844.

24. Borthwick LA., *et al*. "Defective formation of PKA/CnA-dependent annexin 2-S100A10/CFTR complex in DeltaF508 cystic fibrosis cells". *Cell Signal* 20.6 (2008): 1073-1083.

25. Muimo R. "Regulation of CFTR function by annexin A2-S100A10 complex in health and disease". *General Physiology and Biophysics* (2009): F14-19.

26. Prevete N., *et al*. "Role (s) of formyl-peptide receptors expressed in nasal epithelial cells". *Journal of Biological Regulators and Homeostatic Agents* 25.4 (2011): 553-564.

27. Nagata S. "Apoptosis by death factor". *Cell* 88.3 (1997): 355-365.

28. Cimo AM., *et al*. "CD25 and CD69 induction by α4β1 outside-in signalling require TCR early signalling complex proteins". *Biochemical Journal* 454.1 (2013): 109-121.

29. Beghé B., *et al*. "Echocardiography, Spirometry, and Systemic Acute-Phase Inflammatory Proteins in Smokers with COPD or CHF: An Observational Study". *PLOS One* 8.11 (2013): e80166.

30. Wang YX1., *et al*. "Upregulation of ICAM-1 and IL-1β protein expression promotes lung injury in chronic obstructive pulmonary disease". *Genetics and Molecular Research* 15.3 (2016).

31. Gray EE., *et al*. "Deficiency in IL-17-committed Vγ4 (+) γδ T cells in a spontaneous Sox13-mutant CD45.1 (+) congenic mouse substrain protection from dermatitis". *Nature Immunology* 14.6 (2013): 584-592.

32. Malhotra N., *et al*. "A network of high-mobility group box transcription factors programs innate interleukin-17 production". *Immunity* 38.4 (2013): 681-693.

33. Ouchida M1., *et al*. "Novel direct targets of miR-19a identified in breast cancer cells by a quantitative proteomic approach". *PLOS One* 7.8 (2012): e44095.

34. Zhang Y., *et al*. "MicroRNA-503 acts as a tumor suppressor in glioblastoma for multiple antitumor effects by targeting IGF-1R". *Oncology Reports* 31.3 (2013): 1445-1452.

35. Leivonen SK., *et al*. "Identification of miR-193b targets in breast cancer cells and systems biological analysis of their functional impact". *Molecular and Cellular Proteomics* 10.7 (2011).

36. Barnes PJ. "Chronic obstructive pulmonary disease: important advances". *The Lancet Respiratory Medicine* 1.1 (2013): e7-e8.

37. Guggino WB and Stanton BA. "New insights into cystic fibrosis: molecular switches that regulate CFTR. Nature reviews". *Molecular Cell Biology* 7.6 (2006): 426-436.

38. Borthwick LA., *et al*. "The formation of the cAMP/Protein Kinase A-dependent Annexin 2 S100A10 complex with cystic fibrosis conductance regulator protein (CFTR) regulates CFTR channel function". *Molecular Biology of the Cell* 18.9 (2007): 3388-3397.

39. Ridger V C., *et al*. "Differential Effects of CD18, CD29, and CD49 Integrin Subunit Inhibition on Neutrophil Migration in Pulmonary Inflammation". *Journal of Immunology* 166.5 (2001): 3484-3490.

40. Smaniotto S., *et al*. "Mouse Basophils Reside in Extracellular Matrix-Enriched Bone Marrow Niches Which Control Their Motility". *PLOS ONE* 8.9 (2013): e70292.

41. Trotta T., *et al*. "Effect of acute lung injury on VLA-4 and CXCR4 expression in resident and circulating hematopoietic stem/progenitor cells". *Respiration* 85.3 (2013): 252-264.

42. Zang Y., *et al*. "B Lymphocyte inhibition of anti-tumor response depends on expansion of Treg but is independent of B cell IL10 secretion". *Cancer Immunology, Immunotherapy* 62.1 (2013): 87-99.

Volume 8 Issue 12 December 2019