PhD thesis

Early leukocyte activation receptor CD69: a novel player in the maintenance of the Th17/Treg balance in peritoneal fibrosis

Georgios Liappas, MSc

Thesis Director:
Dr. Manuel López Cabrera

Departamento de Inmunología, Centro de Biología Molecular “Severo Ochoa” (CBMSO), Consejo Superior de Investigaciones Científicas (CSIC)

Madrid 2015
Introduction ................................................................................................................................. 21

1. Early leukocyte activation antigen CD69 .................................................................................. 23
   1.1. Genomic organisation and chromosomal localisation of the CD69-encoding gene .......... 23
   1.2. CD69 expression .................................................................................................................. 23
   1.3. Functional characteristics of hematopoietic cell lineages in CD69 deficient mice ......... 24
   1.4. Immunoregulatory role of CD69 in experimental pathologies ........................................ 24
   1.5. CD69 in human pathologies ............................................................................................... 25
   1.6. CD69+ Treg cells .................................................................................................................. 27

2. Th17 and Treg cell differentiation ............................................................................................... 27
   2.1. Th17 in fibrotic diseases ...................................................................................................... 28
   2.2. Treg in fibrotic diseases ...................................................................................................... 29

3. CD69 regulates the Th17 and Treg cell differentiation ............................................................... 30
   3.1. CD69 negatively regulates Th17 cell differentiation .......................................................... 30
   3.2. CD69 positively regulates Treg cell differentiation ............................................................. 31

4. PD as an alternative therapy of renal failure diseases ............................................................. 32
   4.1. Peritoneal cavity and peritoneal membrane .................................................................... 33
   4.2. PD technique ...................................................................................................................... 34
   4.3. Inflammatory stages and fibrosis ....................................................................................... 34
   4.4. Episodes of peritonitis ....................................................................................................... 35
   4.5. Pathogenesis of peritoneal membrane dysfunction .......................................................... 36
   4.6. MMT as a factor triggering peritoneal fibrosis ................................................................. 39
   4.7. Bio-incompatible PD solutions as a source of inflammation ......................................... 39

Objectives ....................................................................................................................................... 43
Materials and Methods ................................................................. 47
1. Human samples analysis .................................................................. 49
  1.1. Samples .................................................................................. 49
  1.2. CD69 expression in human peritoneum ....................................... 49
    1.2.1. CD69 expression in peritoneal effluent cells ......................... 49
2. Mice experiments ........................................................................... 50
  2.1. Mice ...................................................................................... 50
  2.2. Peritoneal exposure model ....................................................... 50
    2.2.1. Catheter installation ......................................................... 50
    2.2.2. Procedure ...................................................................... 50
  2.3. Bone marrow chimeric mice ................................................... 51
  2.4. IL-17 blocking experiments ..................................................... 52
  2.5. CD69 blocking experiments ..................................................... 52
  2.6. FACs analysis ........................................................................ 54
  2.7. Real-time quantitative PCR ..................................................... 54
  2.8. Immunoblotting ..................................................................... 55
  2.9. FlowCytomix ..................................................................... 56
  2.10. Trichrome Massons staining .................................................. 56
  2.11. Immunofluorescence ............................................................ 56
  2.12. Statistical analysis ............................................................... 57

Results ............................................................................................ 59
1. CD69 expression in T lymphocytes from PD patients .................... 61
2. CD69 in PDF treatment-induced fibrosis in mice ............................ 62
  2.1. 10-day PDF treatment ............................................................ 63
  2.2. 20-day PDF treatment ............................................................ 63
    2.2.1. Fibrotic and cellular response ........................................... 63
    2.2.2. Inflammatory cells in the peritoneal cavity ......................... 64
    2.2.3. Pro-inflammatory cytokines and chemokines in the peritoneal cavity ........................................................................... 66
    2.2.4. T cells in peritoneal effluents ............................................ 67
  2.3. 40-day treatment ..................................................................... 68
    2.3.1. CD69 deficiency promotes fibrosis and increases peritoneal cells number .......................................................... 68
    2.3.2. CD69 deficiency leads to an increase in expression of fibrotic markers .......................................................... 69
    2.3.3. CD69 protects against mesothelial to mesenchymal transition .................................................................................. 70
    2.3.4. CD69 deficiency promotes expression of Th17-related cytokines .......................................................... 72
2.3.5. CD69−/− mice display increased numbers of CD4- and CD8-positive T cells .............. 74
2.3.6. CD69 deficiency induces the Th17 response but not the Th1 and Th2 responses .......... 75
2.3.7. CD69 deficiency promotes the recruitment of myeloid cells into the peritoneal cavity.... 76
2.3.8. CD69 deficiency impairs the balance between Th17 and Treg................................. 77

3. CD69 in lymphoid cell-mediated fibrosis in bone marrow chimeric mice............................ 78
   3.1. Chimeric mice generation ........................................................................................ 78
   3.2. Transfer of Rag2γ−/−CD69−/− cells into WT increases peritoneal fibrosis in response to PD..... 80
   3.3. Transfer of Rag2γ−/−CD69−/− cells into WT induces the Th17, but not Th1-/Th2-responses..... 81

4. Implication of IL-17 in PDF-induced fibrosis in the CD69-deficient mice............................ 82
   4.1. Blocking of IL-17 results in a diminished fibrotic response........................................... 82
   4.2. Blocking of IL-17 results in a decreased Th17 response .............................................. 84
   4.3. Effect of IL-17 blockade on the number of monocytes and neutrophils.......................... 85

5. Blockade of CD69 exacerbates the response to PDF and reproduces the CD69−/− phenotype .... 86
   5.1. Fibrosis .................................................................................................................. 87
   5.2. Th17/Treg balance ................................................................................................... 87
   5.3. Analysis of neutrophils and monocytes in the peritoneal cavity................................. 89

Discussion ..................................................................................................................... 91
Conclusions/Conclusiones .............................................................................................. 103
References ..................................................................................................................... 107
Annex ............................................................................................................................ 131
ABBREVIATIONS
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>AGE</td>
<td>Advanced glycation end product</td>
</tr>
<tr>
<td>AP-1</td>
<td>Activator protein-1</td>
</tr>
<tr>
<td>BM</td>
<td>Bone marrow</td>
</tr>
<tr>
<td>CIA</td>
<td>Collagen-induced arthritis</td>
</tr>
<tr>
<td>DC</td>
<td>Dendritic cell</td>
</tr>
<tr>
<td>ECM</td>
<td>Extracellular matrix</td>
</tr>
<tr>
<td>EPS</td>
<td>Encapsulating peritoneal sclerosis</td>
</tr>
<tr>
<td>ERG-1</td>
<td>Erythroblast transformation-specific related gene-1</td>
</tr>
<tr>
<td>FGF</td>
<td>Fibroblast growth factor</td>
</tr>
<tr>
<td>FoxP3</td>
<td>Forkhead box P3</td>
</tr>
<tr>
<td>FSP</td>
<td>Fibroblast specific protein</td>
</tr>
<tr>
<td>GDP</td>
<td>Glucose degradation product</td>
</tr>
<tr>
<td>HMC</td>
<td>Human mesothelial cell</td>
</tr>
<tr>
<td>IFN</td>
<td>Interferon</td>
</tr>
<tr>
<td>IL</td>
<td>Interleukin</td>
</tr>
<tr>
<td>KO</td>
<td>Knock-out</td>
</tr>
<tr>
<td>MCs</td>
<td>Mesothelial cells</td>
</tr>
<tr>
<td>MMT</td>
<td>Mesothelial to mesenchymal transition</td>
</tr>
<tr>
<td>MS</td>
<td>Multiple sclerosis</td>
</tr>
<tr>
<td>NF-κB</td>
<td>Nuclear factor κB</td>
</tr>
<tr>
<td>NK</td>
<td>Natural killer</td>
</tr>
<tr>
<td>NOS</td>
<td>Nitric oxide synthase</td>
</tr>
<tr>
<td>PD</td>
<td>Peritoneal dialysis</td>
</tr>
<tr>
<td>PDF</td>
<td>Peritoneal dialysis fluid</td>
</tr>
<tr>
<td>PM</td>
<td>Peritoneal membrane</td>
</tr>
<tr>
<td>PMA</td>
<td>Phorbol myristate acetate</td>
</tr>
<tr>
<td>RA</td>
<td>Rheumatoid arthritis</td>
</tr>
<tr>
<td>RoR(γ)t</td>
<td>Retinoic acid receptor-related orphan receptor γt</td>
</tr>
<tr>
<td>S1P</td>
<td>Sphingosine 1-phosphate</td>
</tr>
<tr>
<td>Stat</td>
<td>Signal transducer and activator of transcription</td>
</tr>
<tr>
<td>TCR</td>
<td>T cell receptor</td>
</tr>
<tr>
<td>TGF</td>
<td>Transforming growth factor</td>
</tr>
<tr>
<td>Th</td>
<td>T helper</td>
</tr>
<tr>
<td>TLR</td>
<td>Toll-like receptor</td>
</tr>
<tr>
<td>TNF</td>
<td>Tumour necrosis factor</td>
</tr>
<tr>
<td>Treg</td>
<td>Regulatory T</td>
</tr>
<tr>
<td>VEGF</td>
<td>Vascular endothelial growth factor</td>
</tr>
<tr>
<td>WT</td>
<td>Wild type</td>
</tr>
<tr>
<td>---------</td>
<td>---------------------------------</td>
</tr>
<tr>
<td>α-SMA</td>
<td>α-smooth muscle actin</td>
</tr>
</tbody>
</table>
LIST OF FIGURES
Introduction

Figure 1: Genomic localization of CD69 in human and mouse. CD69 region promotors.
Figure 2: Th17 and regulatory T cell differentiation.
Figure 3: CD69 regulates the Th17 and Treg cells differentiation.
Figure 4: Representation of a normal peritoneal membrane in a patient in very early PD stage.
Figure 5: Schematic representation of the progressive alterations of the peritoneal membrane in the time course of PD.
Figure 6: Schematic representation of all the main chemokine and chemokine and T helper subsets with their main cytokines released in the peritoneal cavity during PD exposure.
Figure 7: Realistic representation of a healthy peritoneal membrane and a peritoneal membrane after exposure to PD fluid.

Materials and Methods

Figure 8: Catheter installation and peritoneal dialysis fluid instillation in mice.
Figure 9: Schematic representation of generation of bone marrow (BM) chimeric mice.
Figure 10: Summary of the experimental procedure and the treatment methods followed throughout the whole project.

Results

Figure 11: CD69 is expressed in peritoneal effluent cells. Flow-cytometry analysis of human PD peritoneal cells displays CD69⁺CD3⁺ and CD69⁺CD4⁺ lymphocyte populations.
Figure 12: PDF treatment of WT and CD69⁻/⁻ mice (10 days).
Figure 13: Fibrotic response after 20 days of treatment with PDF.
Figure 14: Inflammatory response after 20 days of treatment with PDF.
Figure 15: Cytokine and chemokine analysis of the peritoneal cytokines at day 20.
Figure 16: Th17, Th1 and Th2 responses after 20 days of treatment.
Figure 17: Fibrotic and cellular response after 40 days of treatment with PDF.
Figure 18: Collagen I and fibronectin expression in PM biopsies after 40 days of treatment.
Figure 19: Mesothelial to Mesenchymal Transition (MMT) after 40 days of treatment.
Figure 20: Peritoneal effluents cytokine analysis after 40 days of treatment with PDF.
Figure 21: Analysis of total cells infiltrating the peritoneal cavity after 40 days of treatment.
Figure 22: Th17, Th1 and Th2 responses after 40 days of treatment with PDF.
Figure 23: Inflammatory response after 40 days with PDF.

Figure 24: Regulatory T response and ratio between total Th17 and total regulatory T cells in the peritoneal cavity after 40 days of treatment.

Figure 25: Cell analysis of chimeric mice after 40 days of treatment with PDF.

Figure 26: Fibrotic response of reconstituted mice after 40 days of treatment with PDF.

Figure 27: Th17, Th1 and Th2 responses of reconstituted mice after 40 days of treatment with PDF.

Figure 28: Fibrotic and cellular response after 40 days of treatment with PDF when blocking with an anti-IL-17 Ab.

Figure 29: Th17, Th1, Th2 and Treg response after 40 days of treatment with PDF when blocking with an anti-IL-17 Ab.

Figure 30: Inflammatory response after 40 days of treatment when blocking with an anti-IL-17 Ab.

Figure 31: Fibrotic response in CD69+/+ mice when blocking with an anti-CD69 mAb-2.2.

Figure 32: Th17, Th1, Th2 and Treg response in CD69+/+ mice when blocking with an anti-CD69 mAb-2.2.

Figure 33: Inflammatory response in CD69+/+ mice when blocking with an anti-CD69 mAb-2.2.

Discussion

Figure 34: Mechanisms of Th17/Treg balance in the peritoneal cavity during PD. The Th17 predominance.

Figure 35: Schematic representation of the experimental procedure implicating nCD69 in peritoneal fibrosis.

Figure 36: Immunoblotting analysis of total, phosphorylated Stat3 and α-SMA expression in PM biopsies.

Figure 37: Regulatory role of CD69 in peritoneal fibrosis through the regulation of the Th17 and Treg cells in the peritoneal cavity of CD69−/− mice.

Figure 38: Schematic representation of the phenomena occurred of the blockade of Th17 response in CD69−/− mice.

Figure 39: Schematic representation of the phenomena occurred after blockade of CD69 receptor in WT mice.

Figure 40: General scheme summarizing the possible regulatory role of CD69 and all the main possible pathways implicated in peritoneal fibrosis.
LIST OF TABLES
Introduction

Table 1: CD69 regulatory role in animal model diseases.
Table 2: CD69 regulatory role in human pathologies.
Table 3: General function of cytokine and chemokine populations present in the peritoneal effluents of the peritoneal cavity.

Materials and methods

Table 4: PBS buffer (10X) components.
Table 5: Sequence of oligonucleotides used in the present work.
Table 6: Loading buffer (1X) components.
Table 7: Running buffer (1X) components.
Table 8: List of primary antibodies used for western blotting and immunofluorescence related to this work.
Table 9: List of secondary antibodies used for western blotting and immunofluorescence related to this work.

Results

Table 10: Composition of immune cells in the peritoneal membrane tissue after 40-day treatment.
ABSTRACT

PRESENTACIÓN
CD69 is induced after activation of leukocytes at inflammatory sites. Mice lacking CD69 develop exacerbated forms of inflammatory diseases, which are largely mediated by misbalanced responses of T helper (Th) 17 cells and regulatory T cells (Tregs), indicating that CD69 modulates Th17 differentiation and plays a role in regulatory T cell function. However, the pathophysiological role of CD69 in fibrotic diseases remains largely unknown.

Renal patients subjected to peritoneal dialysis (PD) develop progressive peritoneal fibrosis, which may lead to technique failure. Herein, we observed that infiltrating T lymphocytes of PD patients expressed high levels of CD69.

Thus, we explored the role of CD69 in fibro-proliferative responses by analyzing a model of peritoneal fibrosis induced by dialysis fluid exposure in WT and CD69-deficient mice. CD69−/− mice showed enhanced peritoneal thickness and myofibroblasts accumulation as well as higher incidence of mesothelial to mesenchymal transition (MMT). In parallel, CD69−/− mice showed exacerbated inflammatory infiltrates, a marked increase of Th17 cells and IL-17 cytokine and reduction of Tregs. Transplantation of a mixture of bone marrows from CD69−/− and Rag2−/− mice into WT recipients reproduced the severity of the disease upon PD fluid exposure, demonstrating that CD69 exerts its function within the lymphocyte compartment. Blockade of IL-17 in CD69−/− mice reduced Th17 response and inflammatory infiltrates and resulted in decreased peritoneal fibrosis induced by PD fluid. Conversely, blockade of CD69 in WT mice mimicked the exacerbated response of CD69−/− mice to PD fluid exposure.

Our findings indicate that CD69 modulates Th17-mediated inflammatory responses in the peritoneal cavity and negatively regulates peritoneal fibrosis induced by dialysis fluid exposure.
El receptor CD69 se induce tras la activación leucocitaria en los infiltrados inflamatorios. Los ratones deficientes en CD69 desarrollan formas exacerbadas de enfermedades inflamatorias que están mediadas por una respuesta desbalanceada entre linfocitos T “helper” Th17 y células T reguladora (Tregs), lo que indica que CD69 modula la diferenciación de Th17 y que juega un papel en la función de las células Tregs. Sin embargo, el papel fisiopatológico de CD69 en enfermedades fóbéticas es desconocido.

Los pacientes renales sometidos a diálisis peritoneal (DP) desarrollan fibrosis peritoneal progresiva, la cual puede dar lugar al fallo de la técnica. En este estudio, hemos observado altos niveles de expresión de CD69 en los linfocitos infiltrantes de los pacientes en DP.

Por lo tanto, decidimos explorar el papel de CD69 en la respuesta fibroproliferativa mediante el análisis de un modelo de fibrosis peritoneal inducido por líquidos de diálisis en ratones silvestres y en ratones deficientes para CD69. Los ratones CD69$^{-/-}$ mostraron un mayor engrosamiento peritoneal y una mayor acumulación de miofibroblastos, así como una mayor incidencia de transición mesotelio mesenquimal (TMM). En paralelo, los ratones CD69$^{-/-}$ mostraron infiltrados inflamatorios exacerbados, un marcado aumento de células Th17 y de la citoquina IL-17 y una reducción de células Tregs. El trasplante de una mezcla de medulas óseas procedentes de ratones CD69$^{-/-}$ y Rag2$^{-/-}$ en ratones silvestres reprodujo la severidad de la enfermedad inducida por la exposición al líquido de DP, demostrando así que CD69 ejerce su función en el compartimento de linfocitos. El bloqueo de IL-17 en ratones CD69$^{-/-}$ redujo la respuesta Th17 y el infiltrado inflamatorio y dio lugar a una reducción de la fibrosis inducida por el líquido de DP. Por otro lado, el bloqueo de CD69 en ratones silvestres mimetizó la respuesta exacerbada a la exposición del líquido de diáisis de los ratones CD69$^{-/-}$.

Nuestros resultados indican que CD69 modula la respuesta inflamatoria mediada por Th17 en la cavidad peritoneal y regula negativamente la fibrosis peritoneal inducida por la exposición a líquido de diálisis.
INTRODUCTION
1. Early leukocyte activation antigen CD69

1.1. Genomic organization and chromosomal localization of the CD69-encoding gene

The cloning of the human early lymphocyte activation antigen CD69 was performed for the first time in 1993 by Lopez-Cabrera. CD69 is the first activated cell surface receptor on leukocytes and is a C-type lectin, disulfide-linked homo-dimer (24 kDa) type II protein, as well as a member of the natural killer (NK) receptor family. Its genes are located in a genomic region known as the NK gene cluster on mouse chromosome 6 and human chromosome 12. The genetic and molecular characteristics of mouse CD69 are very similar to its human homologue.

![Genomic localization of CD69 gene in human and mouse](image)

*Figure 1. Genomic localization of CD69 gene in human and mouse. Promoter region is highlighted (González-Amaro et al, 2013).*

1.2. CD69 expression

CD69 is detected on small subsets of T and B cells in peripheral lymphoid tissues from healthy subjects but is undetectable on peripheral blood lymphocytes. Furthermore, CD69 is persistently expressed by platelets, mature thymocytes, bone marrow and lymphoid precursors and generally in leukocytes infiltrates of various chronic inflammatory diseases, suggesting that inflammatory cytokines might be involved in CD69 expression. The genomic organization, promoters regions and transcriptional activity of the human CD69 gene have been described.
The transcription of CD69 gene appears to be tightly regulated in vivo, as it is almost exclusively expressed at sites where inflammation takes place, suggesting that inflammatory cytokines might participate in the control of the CD69 expression. The sequence upstream from the gene functions as a phorbol myristate acetate (PMA)-inducible promoter element. It has been confirmed by the observation that the expression of CD69-encoding mRNA in leukocytes is induced by in vitro treatment with a wide range of stimuli, including PMA. Specifically, the CD69 promoter region contains binding sites for transcription factors such as nuclear factor (NF)-κB, activator protein (AP)-1 and erythroid differentiation factor. The sequence of CD69 promoter region it has been shown that it also mediates the tumor necrosis factor (TNF)-α-induced expression of CD69. This could explain the expression of CD69 in sites of chronic inflammation, characterised by abundant TNF-α expression. Similarly, ERG-1, whose expression is induced by various agents, including phorbol esters, has been implicated in the activation of lymphocytes B and T.

1.3. Functional characteristics of hematopoietic cell lineages in CD69 deficient mice

To investigate the physiological relevance of CD69, the first CD69-deficient (CD69<sup>−/−</sup>) mouse model was generated in 2000 by Lauzurica et al. This group showed that CD69<sup>−/−</sup> mice presented normal hematopoietic and T-cell development in the thymus and periphery and also similar NK cytotoxic activity as wild type (WT) mice. In contrast, B-cell development in CD69<sup>−/−</sup> was affected as a specific bone marrow pre-B cell compartment (B220<sup>hi</sup>IgM<sup>hi</sup>) was induced in the absence of CD69. The knockout mice presented besides a small increase in immunoglobulin (Ig) G2a- and IgM-responses to immunization with T-dependent and T-independent antigens. Apart from the observation on the role of CD69 in B-cell development, CD69-deficient mice displayed an enhanced susceptibility to several inflammatory diseases, mediated by T helper (Th) 17 cells.

1.4. Immunoregulatory role of CD69 in experimental pathologies

In vivo studies using CD69<sup>−/−</sup> mice uncovered a key role of CD69 as an immunoregulatory player during immune responses. It has been proposed as a negative regulator of autoimmune reactivity for conditions including collagen induced arthritis (CIA), anti-tumor responses, allergic asthma and skin contact hypersensitivity, autoimmune myocarditis as summarized in Table 1. In the case of CIA, allergic asthma and cardiomyopathy, CD69<sup>−/−</sup> mice displayed an exacerbated inflammatory response. However, in another study in which murine arthritis was induced by anti-type II collagen murine antibody (mAb), the CD69-deficient mice showed a remarkably attenuated neutrophil-mediated inflammation compared with WT mice. This could be due to the fact that a different experimental procedure has been applied to the study or because the strain of used CD69-deficient
mice varied depending on the study. In addition, the chemical stimulus that is used each time in order to provoke or control an inflammatory response in a designed study could vary, causing the role of CD69 in immunoregulation to vary as well. The collective data above indicate a dual role of CD69 in inflammation: on the one hand as a negative regulator in a lymphocyte- and NK-dependent manner, and as a positive regulator in acute inflammatory responses mediated by neutrophils²², on the other.

Table 1. CD69’s regulatory role in animal model diseases (adapted from González-Amaro et al, 2013).

<table>
<thead>
<tr>
<th>Disease</th>
<th>Mouse model</th>
<th>Mediated by</th>
<th>Tissue</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Collagen-induced arthritis</td>
<td>C57BL/6J CD69/-</td>
<td>T and B cells</td>
<td>Synovial leukocytes</td>
<td>18</td>
</tr>
<tr>
<td>Anti-type II collagen-induced arthritis</td>
<td>C57BL/6J CD69/-</td>
<td>Neutrophils</td>
<td>Joints</td>
<td>22, 23</td>
</tr>
<tr>
<td>Ovalbumin-induced allergic airway inflammation</td>
<td>Balb/c CD69/-</td>
<td>Th2/Th17</td>
<td>Lungs, airways</td>
<td>20, 24</td>
</tr>
<tr>
<td>Lung inflammation induced by bleomycin</td>
<td>C57BL/6J CD69/-</td>
<td>Macrophages, neutrophils, lymphocytes</td>
<td>Lungs</td>
<td>25</td>
</tr>
<tr>
<td>Cigarette smoke-induced inflammation</td>
<td>C57BL/6J CD69/-</td>
<td>Macrophages, neutrophils</td>
<td>Lungs</td>
<td>26</td>
</tr>
<tr>
<td>Contact hypersensitivity to oxazolone</td>
<td>C57BL/6J CD69/-</td>
<td>Th1/Th17</td>
<td>Skin</td>
<td>20</td>
</tr>
<tr>
<td>Experimental autoimmune myocarditis</td>
<td>Balb/c CD69/-</td>
<td>Th17</td>
<td>Myocardium</td>
<td>16</td>
</tr>
<tr>
<td>Systemic lupus erythematosus</td>
<td>NZBxNZW mice</td>
<td>CD4+CD69 + T cells</td>
<td>Peripheral lymphoid tissues, kidney, lungs</td>
<td>27</td>
</tr>
<tr>
<td>Listeria monocytogenes infection</td>
<td>C57BL/6J CD69/-</td>
<td>Th1</td>
<td>Liver, spleen</td>
<td>28</td>
</tr>
<tr>
<td>CD4 T cell transfer colitis</td>
<td>C57BL/6J CD69/-</td>
<td>Th1</td>
<td>Bowel</td>
<td>15</td>
</tr>
</tbody>
</table>

1.5. CD69 in human pathologies

Beside animal-based data, early studies suggested that CD69 might also play an important role in human diseases and tissue damage-associated inflammation through the activation of leukocytes², 4, 17, as summarized in Table 2.

Biopsies from patients suffering from rheumatic diseases (rheumatoid arthritis (RA) and osteoarthritis) have shown that CD69 is overexpressed in the joints and synovial fluid, and it is apparently connected with the pathogenesis and/or progression of these pathologies⁹.
Table 2. Implication of different cells in CD69-mediated regulation in selected human pathologies (adapted from González-Amaro et al, 2013).

<table>
<thead>
<tr>
<th>Disease</th>
<th>Cells</th>
<th>Tissue</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rheumatic diseases</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rheumatoid arthritis</td>
<td>T and NK</td>
<td>Synovial fluid</td>
<td>9, 29, 30</td>
</tr>
<tr>
<td>Osteoarthritis</td>
<td>T cells</td>
<td>Synovial membrane and fluid</td>
<td>30</td>
</tr>
<tr>
<td>Autoimmune diseases</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Multiple sclerosis</td>
<td>Th17</td>
<td>Cerebrospinal fluid</td>
<td>31, 32</td>
</tr>
<tr>
<td>Inflammatory diseases</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Chronic bronchitis</td>
<td>CD4+ T cells</td>
<td>Peripheral blood, lung</td>
<td>33, 34</td>
</tr>
<tr>
<td>Other diseases</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Asthma</td>
<td>T cells, eosinophils</td>
<td>Peripheral blood, lung</td>
<td>35-37</td>
</tr>
<tr>
<td>Kidney transplant</td>
<td>Treg</td>
<td>Peripheral blood, kidney</td>
<td>38, 39</td>
</tr>
</tbody>
</table>

Furthermore, in patients with RA CD69 is constitutively overexpressed in most of the T and NK cells from the synovial fluid and it participates in the expression induction of the pro-inflammatory mediator TNF-α in macrophages. Similarly to RA, high number of CD69-expressing (CD69⁺) leukocytes has been observed in the synovial fluid from osteoarthritis patients.

Furthermore, increased levels of CD69⁺ cells have been linked with an autoimmune disease, multiple sclerosis (MS); the cerebrospinal fluid of MS patients contains an abundant population of CD4⁺CD69⁺ lymphocytes and is characterised by increased pro-inflammatory interferon (IFN)-γ and TNF-α production. It has also been demonstrated that these patients displayed an increased Th17 response; thus suggesting that CD69⁺Th17⁺ cells might have a potent pathogenic role.

Regarding inflammatory diseases, it appears that some CD69⁺ cells are able to stimulate an inflammatory environment. For example in the case of atherosclerosis, the disease outcome is correlated with the percentage of the CD69⁺ lymphocytes in atheroma plaques according to a recent study. Finally, although CD69-expressing regulatory T cells (Treg), which are a subset of lymphocytes T, had an immunosuppressive role in asthma and kidney transplantation, the majority of CD69-positive T cells exerted a pathologic role in the progression of these diseases.
Although CD69 was first studied primarily in human pathologies and was believed to be a positive immune regulator, further studies on CD69-deficient mice provided evidence that this molecule exerts a negative regulatory role in immune response. The fact that high expression of CD69 is observed in different human biopsies does not necessarily mean that this molecule participates exclusively in the activation of the inflammatory response. Instead, it rather indicates that CD69 is necessary to modulate inflammation in order to protect from an exacerbated response, and is therefore highly expressed.

1.6. CD69+ Treg cells

Regulatory T cells are important in the control of self-reactivity and in the pathogenesis of autoimmune inflammatory conditions, in which they might display an immunosuppressive role. In particular, the CD69+ Treg subset has been linked with anti-inflammatory functions. Besides, CD69 molecule plays role in the differentiation of Tregs from the human thymus, as described by Martin-Gayo et al. They reported that CD69 expression induces the differentiation of CD4+CD8+CD69high thymocytes into CD4+CD25highFoxp3+ Treg cells. It was supported by the observation that CD69+ mice presented a reduced capacity of CD4+CD69+ lymphocytes to differentiate into Treg cells.

The existence of another Treg cell subset, i.e. the CD69+CD4+CD25− population, suppressing T cell proliferation and regulated by membrane-bound TGFβ, has also been reported in mice. Accordingly, in the peripheral blood from patients with thyroid disease, two subpopulations of CD69-expressing Treg lymphocytes, CD69−TGFβ+ and CD69−IL-10+, have been found. Furthermore, Gandhi et al. described the TGFβ+ group, pointing out to their strong immunosuppression. In this regard, patients with systemic lupus erythematosus presented an increased CD69+CD4+ lymphocyte population, probably due to their immunosuppressive functions. Overall, these data confirm that CD69, expressed in different Tregs, including highly immunosuppressive populations, is important for their development and cellular activity.

2. Th17 and Treg cell differentiation

T cells can differentiate, among others, into Th17 and regulatory Tregs. Th17 cells, a subset of T helper cells, primarily secrete interleukin (IL)-17 in addition to other pro-inflammatory cytokines, related to many autoimmune and chronic inflammatory diseases. There is a balance between Th17 and Treg cells that highly depends on the activation of the transcription factors: retinoic acid receptor–related orphan receptor (RoR) (γ) and signal transducer and activator of transcription (Stat) 3, or forkhead box (Fox) P3 and Stat5, respectively, which regulate the immune response through the
secretion of pro- and anti-inflammatory cytokines\textsuperscript{49-52} (Figure 2). On the other hand, the importance of Treg cells in the maintenance of peripheral tolerance in non-inflammatory conditions throughout life has also been confirmed. In fact, mice lacking Treg cells demonstrated a fatal inflammatory response\textsuperscript{53,54}.

The primary cytokines involved in Th17 and Treg cell differentiation are transforming growth factor (TGF) $\beta$ and IL-6\textsuperscript{55-57} (Figure 2). IL-6 is strongly induced in cells of the innate immune system upon stimulation of pattern recognition receptors such as toll-like receptors (TLR) and C-type receptors. It has been shown that mice lacking IL-6 exhibit a deficiency in the differentiation of effector T cells\textsuperscript{58,59}. TGF$\beta$ induces Foxp3 in the absence of IL-6, thus pushing T-cell differentiation away from the Th17 transcriptional program and toward the Treg lineage\textsuperscript{52,60}. In contrast, the pro-inflammatory cytokine IL-6 in the absence of TGF$\beta$ activates Stat3 by phosphorylation, which overcomes Foxp3 inhibition of RoR(\gamma)t transcriptional activity. This process leads to the up-regulation of the IL-23 receptor (IL-23R), promoting the T-cell differentiation toward the Th17 fate\textsuperscript{52}. Therefore, the cytokine environment is essential for the predominance of either Th17 or regulatory T cell response.

**Figure 2.** The role of TGF$\beta$ and IL-6 as key elements involved in pushing T cell differentiation into Treg or Th17 cells.

2.1. Th17 in fibrotic diseases

Fibro-proliferative diseases such as idiopathic pulmonary, liver, cardiovascular and renal fibrosis are usually associated with chronic inflammation, as previously reported\textsuperscript{61-65}. When an inflammatory response becomes chronic, the accumulation of extracellular matrix (ECM) is more extensive and the function of the organ becomes compromised. A number of studies have highlighted the role of Th17/Treg/Th1/Th2 responses in the pathogenesis of tissue fibrosis\textsuperscript{66,67}. 
Among these responses, Th17 cells mediate strong inflammation by producing a cocktail of cytokines such as IL-6 and IL-17, among which IL-17 has been recently characterized as a major effector cytokine in causing a sustained inflammatory response. Also, recent studies have revealed the role of Th17 response in fibrosis. Specifically, it has been reported that administration of IL-17A in vitro into alveolar epithelial cells increased the synthesis and secretion of collagen. Moreover, all IL-17-associated signalling pathways were activated in fibrotic lung biopsies in a pulmonary fibrosis model; on the other hand, a blockade of IL-17 attenuated tissue injury, inflammation and fibrosis in that protocol. Furthermore, IL-17 is involved in the pathogenesis of chronic liver fibrosis. IL-17-dependent mechanism of liver damage was confirmed in chronic hepatitis B patients, who presented an elevated Th17 cells population. As for Th17 lymphocytes, they also play a crucial role in autoimmune myocarditis, showed by in vitro and in vivo experiments that linked IL-17 induced cardiac fibrosis with the activation of the protein kinase C-β/Erk1/2/NFκB pathway. Similarly to previous data, the regulatory role of CD69 was associated with the modulation of Th17 responses, resulting in attenuated myocardial inflammation, fibrosis and heart failure.

Recent studies have shown the importance of Th17 cells and its hallmark cytokine IL-17 in immune-mediated kidney disorders called glomerulonephritis and lupus nephritis. Th17 cells participate in renal damage; mice receiving Th17 cell injections displayed albuminuria and neutrophils infiltration into kidney. Elevated renal production of IL-17 by Th17 cells was also found in non-immune experimental renal diseases, including a model of unilateral ureteral obstruction. In experimental ischaemia reperfusion, neutrophils, but not Th17 cells, were the main sources of IL-17 and contributed to renal injury by T cells activation by NK and IL-12 and IFN-γ production. In renal damage and allograft rejection model, positive staining for IL-17 has been detected in tubular cells, suggesting that renal cells could produce this cytokine as well and thus contribute to the kidney failure. Using the same model it was demonstrated that a blockade of IL-17 diminished renal inflammation. Based on the above and other studies, IL-17 has been proposed as a target for treatment of fibrotic diseases.

### 2.2. Treg in fibrotic diseases

Although an induced Th17 response is connected to fibrogenesis, a relative decrease in the number of Treg cells might also be involved in the pathogenesis of inflammatory and fibrotic diseases. The evaluation of these cells in the context of experimentally induced fibrosis has been challenging, and Tregs depletion in mice has been demonstrated to promote the development of lung fibrosis. In an experimental animal model of cardiac fibrosis, the adoptive transfer of isolated Tregs ameliorated cardiac fibrosis, indicating a protective role of regulatory T cell in tissue fibrosis. It has recently been reported that Treg cells are essential for preventing the accumulation of fibrocytes and collagen in a
pulmonary disease animal model. In this study, it was shown that a blockade of Treg cells increased the accumulation of solid collagen and progression of the disease. Finally, in another study (Keloid fibrotic disease) the potential role of Treg cells in attenuating collagen synthesis was investigated. This group found the imbalance of Tregs might contribute to the development of this fibrotic disease and the correction of this imbalance could be of therapeutic value.

3. CD69 regulates the Th17 and Treg cell differentiation

It has been reported that combined treatment with low dose of phorbol ester (mitogenic factor, activator of protein kinase C) and monoclonal antibody against CD69 (causing CD69 activation) triggered Ca2+ influx and the ERK1/2 pathway induction. Subsequently, expression of IL-2, IFN-γ and increased Treg cell proliferation were observed. These data were the first to suggest that the CD69 receptor could be involved in the category of pro-inflammatory receptors that induce leukocyte activation. The group of Martín et al. (2011) reported that the cytoplasmic tail of the CD69 receptor associates with Jak3 kinase, which positively interacts with a transcription factor Stat5, regulating in turn another transcription factor FoxP3, important for T cell differentiation into Treg. On the other hand, CD69 might inhibit Th17 differentiation: (i) by phosphorylating Stat5, which blocks the phosphorylation of Stat3 (positive regulator of RoR(γ)t and Th17) and (ii) by antagonizing Stat3-mediated activation through FoxP3 activation on Stat5 activation

3.1. CD69 negatively regulates Th17 differentiation

The importance of CD69 in different pathological models (such as contact dermatitis, allergic asthma and autoimmune myocarditis) was highlighted by in vivo studies, using CD69-deficient mice. The genetic ablation of the protein resulted in exacerbated reactions in these knockout mice (e.g. high susceptibility to developing asthma). Interestingly, a blockade of the CD69 expression, by introducing a neutralizing antibody into WT mice, also showed an increased inflammatory response in the lungs in allergic asthma model. Importantly, this altered response in CD69 knockout mice was linked with augmented T cell activity in damage sites. Specifically, CD69-deficient mice displayed enhanced T cell responses in the lungs. These observations suggested CD69 as a candidate molecule regulating immune allergic responses by decreasing T cell effector responses. Moreover, in a model of experimental autoimmune myocarditis CD69-deficient mice presented induced Th17 cells in the heart followed by cardiac inflammation.

A number of in vitro studies further described the CD69-dependent reactions as negative modulators of inflammatory responses, mediated among others by IL-17-secreting T (Th17) lymphocytes. It was
shown, that CD69 regulates the generation and functions of Th17 cells. For instance, the absence of CD69 enhanced the differentiation of T cells into Th17 cells *in vitro* and was associated with T cells activation and transcriptional induction of molecules required for this process, such as RoR(γ)t, Stat3 and IL-2349,51 (Figure 3).

**Figure 3.** CD69 regulates Th17 and regulatory T cell differentiation. MHC II: major histocompatibility complex II; P: phosphorylation. Dashed lines indicate indirect effects or interactions, and multiple arrows indicate multistep processes (adapted from Martin *et al.*, 2011).

Th17 cells from CD69-deficient mice also produced lower amounts of IL-2 and showed decreased activation of Stat5, compared with the cells from WT mice86. The affected Stat5 phosphorylation, which is a negative regulator of Stat3 activation, led, in turn, to higher activation of Stat3 in CD69-deficient Th17 cells, an event that favors their differentiation87, 88. Further evidence of CD69 implication in Th17 differentiation was delivered by introducing exogenous IL-2 into CD69-deficient T cells. The exogenous IL-2 restored Stat5 activation and, thereby, blocked Th17 differentiation. Moreover, in contrast to WT cells where blocking of IL-2 inhibited Stat5 activation and promoted differentiation, CD69-deficient cells displayed no effect to this IL-2 inhibition. CD69 thus promotes Stat5-mediated inhibition of Th17 cell differentiation independently of the IL-2 pathway86 (Figure 3).

In conclusion, the above evidence points to CD69 as a key molecule in negatively regulating Th17 differentiation.
3.2. CD69 positively regulates Treg cell differentiation

It has been observed that plasma of patients suffering from systemic sclerosis, which contained low numbers of Treg cells, simultaneously, had diminished expression of the CD69 receptor on the surface of blood cells\(^9\). It was therefore postulated that CD69 might influence the Treg-dependent immune response.

It has been shown that CD69 impairs the function of sphingosine 1-phosphate receptor 1 (S\(_1\)P\(_1\)), normally involved in the migration of lymphocytes from lymphoid tissues to organs during so called recirculation, a process essential for immune surveillance\(^9\). Accordingly, in the absence of CD69 there were increased levels of S\(_1\)P\(_1\) and increased migration of T lymphocytes into lymph nodes or inflamed tissues\(^9\). A high expression of S\(_1\)P\(_1\) in CD4\(^+\) T cells was associated with abundant IL-17 secretion, and, consequently, enhanced Th17 differentiation\(^9\). Additionally, the absence of CD69 and low concentration of S\(_1\)P\(_1\) led to the differentiation of CD4\(^+\)CD25\(^-\)FoxP3\(^+\) expressing cells, thus pointing to Treg cell fate\(^9\). In regard to this, in a mouse model of contact sensitization, S\(_1\)P\(_1\) promotes migration of dendritic cells\(^9\) and the CD69 deficiency increases the abundance of S\(_1\)P\(_1\), enhancing the ability of these cells to migrate in vivo and in vitro\(^8\). Therefore, CD69 might modulate the balance between the Th17 and Treg cells, thus contributing to a resolution of inflammatory response.

4. PD as an alternative therapy in renal failure diseases

When the function of the kidneys diminishes to 10 – 15\%, the individual needs therapy to replace renal function to sustain life, which is why techniques that clean the blood or kidney transplantation have been introduced. Patient with impaired renal function suffer from uremia, a term that was described for the first time by Piorry et al. in 1840. Uremia was defined as set of symptoms at the systemic level, produced as a result of the accumulation of toxic products in the blood that are retained in the kidney with dysfunction, instead of being eliminated.

Peritoneal dialysis (PD) is a form of renal replacement therapy that has become an alternative to hemodialysis\(^9\). It has been used for approximately three decades as a treatment for end-stage renal diseases. However, the two first decades were marked by frequent improving catheter designs, technical aspects and peritonitis control\(^9\). During the last 10 to 15 years, great effort was made to improve the biocompatibility of the solutions with the hope of diminishing their adverse effects on peritoneal morphology and function\(^9\). PD offers major advantages in terms of quality of life, cost and home-based treatment opportunities compared with the haemodialysis. Undoubtedly, the increase of PD programs could also be attributed to improvements in the PD technique, especially in terms of
peritonitis prevention, and to the biocompatibility of the dialysis solutions. At present, PD is a successful treatment for end-stage renal disease, and several studies have confirmed equivalent adequacy, mortality, and fluid balance status when compared with haemodialysis, at least for the first 4-5 years of treatment\textsuperscript{103-106}. However, the growth of PD continues being limited by the membrane’s incapacity to perform adequate diffusive and/or convective transports at long-term\textsuperscript{97, 107}. Ultrafiltration failure and fibrosis of the peritoneum are the primarily complications during PD treatment.

4.1. Peritoneal cavity and peritoneal membrane

The peritoneal cavity is the largest cavity of the body and harbours the major internal organs belonging to the \textit{tractus digestivus}, including the spleen, pancreas and liver. In physiological conditions, the most abundant cell type in the abdominal (peritoneal) fluid are macrophages (80%), known as the first line of defence against pathogens\textsuperscript{108}. The remaining cell population consists of peritoneal T lymphocytes, eosinophils, dendritic cells, mast cells and mesothelial cells.

The peritoneal membrane (PM), which covers the entire peritoneal cavity, is divided into the parietal peritoneum and the visceral peritoneum. The parietal peritoneum covers the inner surface of the abdominal wall, whereas the visceral peritoneum covers the surface of the intraperitoneal organs. The total surface area of the peritoneum of adult subjects is approximately 1 m\textsuperscript{2} composing to 10-15\% of the parietal peritoneum and 85-90\% of either the visceral or hepatic. Structurally, the peritoneal membrane consists of two layers: the mesothelium and the interstitium, supported on the muscles rich in peritoneal vessels\textsuperscript{109, 110} as represented in Figure 4.

Mesothelial cells (MCs), connected to each other by intercellular junctions\textsuperscript{111}, form a compact monolayer covering the peritoneal membrane and are the most numerous cells in the peritoneal cavity. Under normal condition MCs play a crucial role in peritoneal homeostasis, including anti-adhesive and fibrolytic mechanisms, regulation of local blood circulation and vessel permeability, regulation of matrix formation and local defense mechanisms\textsuperscript{112, 113}. They produce prostaglandins\textsuperscript{114}, cytokines\textsuperscript{115}, chemokines\textsuperscript{116} and factors modulating local hemodynamic such as nitric oxide\textsuperscript{117}. MCs also orchestrate the local inflammatory response during peritoneal injury, as described below.

Mesothelium covers the layer of interstitium and a compact zone of muscles from within\textsuperscript{118} (Figure 4). The former is built up by loose connective tissue composed of collagen fibers, proteoglycans and other extracellular matrix (ECM) compounds and, in healthy individuals, contains small numbers of fibroblasts, macrophages, and mast cells. Some vessels are also present in the interstitium\textsuperscript{119}.
4.2. PD technique

The aims of PD technique are the elimination of the small toxic molecules from the blood when these molecules cannot be eliminated in a normal situation and the elimination of excess of water. It requires the instillation and periodical renovation of a hyper-osmotic dialysate liquid, peritoneal dialysis fluid (PDF) through a permanently installed catheter in the peritoneal cavity. The instilled PD fluid creates an ideal environment for the exchange of the toxic molecules from the blood to the peritoneal cavity through the PM.

![Figure 4. Schematic representation of healthy peritoneal membrane (insert on the right) in a patient undergoing PD therapy.](image)

4.3. Inflammatory stages and fibrosis

During the PD treatment, the peritoneal cavity is exposed to an average of 2200-7000 liters of PD fluids per year\textsuperscript{120} and the patient can remain on this treatment for years. During peritoneal dialysis, patients’ biopsies change, revealing the disappearance of mesothelial cells\textsuperscript{121} or their conversion to fibroblastic cells (in a process of mesothelial to mesenchymal transition or MMT)\textsuperscript{122}, angiogenesis\textsuperscript{123} (formation of new vessels) and fibrosis of the PM (accumulation of ECM and collagen in the peritoneum)\textsuperscript{124}.

The non-physiological nature of PD fluids and episodes of peritonitis appear to be the primary etiologic factors leading to the functional decline of the peritoneal membrane\textsuperscript{97, 102, 125} and eventually fibrosis. Such alterations are considered the major cause of ultrafiltration failure and the loss of dialytic capacity of the peritoneum during peritoneal dialysis. All these phenomena during PD lead to an incapacity of the peritoneal membrane to discharge the toxic molecules from the blood, which is called ultrafiltration failure\textsuperscript{96} (Figure 5).
Figure 5. Schematic representation of the progressive alterations of the peritoneal membrane during evolution of PD. Angiogenesis, vasculopathy, mesothelial to mesenchymal transition and fibrosis are the primary alterations that deregulate the function of the peritoneum during PD (from López-Cabrera, 2014).

The primary alteration in the peritoneal membrane in patients undergoing PD is the development of fibrosis. A variable degree of diffuse peritoneal fibrosis has been documented in these patients. It might have only a moderately detrimental effect on peritoneal transport kinetics, or might be manifested by a progressive, sclerosing, encapsulating peritonitis, which can lead to cessation of peritoneal dialysis and to death. An accompanying ECM expansion is a combination of increased ECM production, as a consequence of increased proliferation of fibroblasts, biochemical modification of the matrix by advanced glycation end product (AGE) formation and reduction in ECM degradation.

4.4 Episodes of peritonitis

Episodes of peritonitis which involve inflammation of the peritoneum, are one of the primary causes for functional decline of the peritoneal membrane during PD. Peritoneal infections are frequently caused by *Staphylococcus epidermidis, Staphylococcus aureus* or *Escherichia coli*.

During these episodes, the immune cells are attracted to the peritoneal cavity, causing a strong inflammatory response attracting T cells and the macrophages arrive to the peritoneal cavity. As for CD4+ T cells (Th), they can be classified according to their phenotype: Th1 and Th2. In general, Th1 lymphocytes produce increased IFN-γ essential for the activation of macrophages, whereas Th2 lymphocytes secrete IL-4, activating the function of B cells. Studies performed during episodes of peritonitis on patients undergoing PD, revealed a very strong induction of the Th1 response and increased expression of cytokines such as IL-1β and IFN-γ, with a concurrent increase of other Th1-related...
cytokines. If the inflammatory response is persistent or a peritonitis episode recurs, the risk for peritoneal damage increases, ultimately resulting in a loss of peritoneal function. Therefore, episodes of peritonitis are crucial to the outcome of PD treatment.

4.5 Pathogenesis of peritoneal membrane dysfunction

It is generally believed that uremic status can affect, to some extent, the architecture of the peritoneal membrane and its transport characteristics. It was observed that the peritoneal membrane of partially nephrectomised PD-treated rats increased its permeability, which was associated to up-regulation of nitric oxide synthase (NOS) isoforms, vascular endothelial growth factor (VEGF), and fibroblast growth factor (FGF)-2, and accumulation of AGEs.132 Despite these findings in animal models, the effect of uremia itself on the human peritoneum is debated. Two human peritoneal biopsy studies have shown a modest compact zone thickening and vasculopathy in pre-dialysis renal patients.123, 133 In contrast, other studies found no significant fibrosis or vasculopathy in uremic non-PD patients.134

The bio-incompatible nature of PDF together with the episodes of bacterial infections (peritonitis) mentioned above are being considered as the main factors guiding to the peritoneal damage and peritoneal dysfunction. These events can produce an inflammatory environment and damage the peritoneal membrane.97, 102, 125, 135, 136 The peritoneal immune response to these factors involves MCs and resident macrophages that work in a coordinated manner to recruit other inflammatory cells, including mononuclear phagocytes (macrophages and monocytes), lymphocytes and neutrophils. Furthermore, MCs and infiltrating immune cells produce a wide range of cytokines, growth factors and chemokines to establish a complex network that results in acute or chronic inflammation as summarised in Table 3 and Figure 6.

In 2010, Zhang et al.139 found that the plasma of uremic patients undergoing haemodialysis contained increased numbers of Th17 cells and decreased Treg cells, and, accordingly, increased expression levels of Th17-related cytokines (IL-17, IL-6 and IL-23) and decreased levels of Treg-related cytokines (e.g. IL-10 and TGFβ), when compared with healthy individuals. Another study reported that IL-17 was overexpressed in peritoneal membrane in human biopsies from PD patients and in PM from PD-treated mice.140 Recently, our group revealed that WT mice treated with PD fluid were characterised by increased IL-17 level in the peritoneal cavity fluid.141

Previous studies on PD in clinic have demonstrated that in the absence of peritonitis episodes, the patients also had a considerable percentage of inflammatory cells in the peritoneal cavity compared with healthy individuals. Along these lines, Lewis et al.142 suggested there was a certain degree of cell activation in the peritoneal cavity during PD, including the cases where the peritonitis episodes were
absent. This statement was based on previous studies, in which the percentage of lymphocytes was elevated in the peritoneal cavity during PD, primarily in the first month of treatment\textsuperscript{142}. On the other hand, other authors found different proportions between CD4 and CD8 cells in the peritoneum during PD compared to the healthy individuals\textsuperscript{143, 144}. Moreover, a role for Th2 cells in patients under PD treatment has also been proposed\textsuperscript{145}. However, other studies refer to Th1 cells and their primary secreted cytokine IFN-γ, as major participants of the immune response during PD\textsuperscript{146} (Figure 6).

**Figure 6.** Schematic representation of the immune cells and their corresponding factors released in the peritoneal cavity, upon exposure of the peritoneum to PD fluids.

Furthermore, regarding B cells and their function during PD, there are studies that reported their decrease during the first two years of PD\textsuperscript{144, 147}. B1 cells (the primary subpopulation of B cells in the peritoneal cavity\textsuperscript{148}) might modify the phagocytic activity of macrophages *in vitro* due to secretion of IL-10\textsuperscript{149}.
Macrophages are the most abundant cell population during PD. In a study carried out by Ganguly et al. in 1980, they observed that in stable PD patients, the macrophages in drained PD fluids were approximately 72%, whereas the lymphocytes comprised only 14%.

The concentration of cytokines and chemokines secreted from specific cell populations during PD are also increased. For example, the level of intraperitoneal IL-6 is increased during dialysis treatment, compared with healthy individuals.

A sustained inflammation manifested by accumulation of pro-inflammatory cells, secretion of related cytokines might trigger the fibrogenic and angiogenic processes associated with ultrafiltration failure.

**Table 3.** Functions of cytokines and chemokines present in the peritoneal cavity. GMCSF: granulocyte macrophage colony-stimulating factor; MCP: monocyte chemoattractant protein; RANTES: regulated on activation, normal T cell expressed and secreted; MIP: macrophage inflammatory protein.
The bio-incompatible nature of PDF appears to be a factor triggering the inflammatory response. However, another phenomenon takes place during PD known as mesothelial to mesenchymal transition (MMT), which is implicated in the complex scenario of peritoneal damage. During PD the MCs of the peritoneal membrane gradually lose their polarity and convert into myofibroblasts. Myofibroblasts were first described by Gabianni et al. in 1972; they play an important role in fibrosis. This is in line with their capacity to synthesize ECM, cytokines and chemokines and promote an inflammatory response.

Although myofibroblasts are present neither in normal peritoneum, nor in peritoneum of uremic-non PD patients they are present in patients undergoing PD. It was demonstrated for the first time in 2003 that the conversion of mesothelial cells into myofibroblasts (MMT) is present during PD. The authors reported that soon after PD was initiated, peritoneal MCs showed a progressive loss of epithelial phenotype and acquired myofibroblast characteristics. However, definitive proof demonstrating that the PD-induced MC phenotype changes were related to a MMT process came from the analysis of the expression of several mesenchymal markers including transcription factor Snail, N-cadherin, fibronectin, collagen I, α-smooth-muscle actin (α-SMA) and fibroblast specific protein-1 (FSP-1), which were gradually up-regulated in effluent MCs with epithelioid and non-epithelioid phenotypes.

Therefore, MMT is an important inducer of peritoneal damage and fibrosis due to the conversion of MCs into myofibroblasts producing large amounts of extracellular matrix components (fibronectin, collagens) and wide range of inflammatory, pro-fibrotic and angiogenic factors (VEGF, TGFβ, IL-8, IL-6). The overall effect of these changes is the structural and functional deterioration of the peritoneal membrane.

### 4.6. MMT as a factor triggering peritoneal fibrosis

<table>
<thead>
<tr>
<th>IFN-γ</th>
<th>T and NK cells 154</th>
<th>Activate macrophages, epithelial and endothelial cells 154</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Promote migration of the leukocytes 155</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Inhibit collagen production in vitro and α-SMA expression 155</td>
</tr>
<tr>
<td>GMCSF</td>
<td>Activated T cells &amp; macrophages 154</td>
<td>Proliferation and differentiation of granulocytes/macrophages progenitors</td>
</tr>
<tr>
<td>TNF-α</td>
<td>Macrophages, fibroblasts</td>
<td>Neutrophil recruitment</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Th1 cytokine 155</td>
</tr>
<tr>
<td>TGFβ</td>
<td>Fibroblasts, immunologic cells, macrophages 63, 175</td>
<td>Regulate ECM formation, migration and apoptosis 176, 177</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Pro- or anti-inflammatory action 178</td>
</tr>
</tbody>
</table>

### 4.7 Bio-incompatible PD solutions as a source of inflammation
The principal cause of inflammation is the bio-incompatibility of the PD fluids used during PD. The primary factor contributing to this bio-incompatibility is the high concentration of glucose-derivatives present in PD fluids. An appropriate osmotic capacity of the PD fluid is necessary for elimination of toxic molecules from the blood and their transport towards the peritoneal cavity. It is achieved by high glucose concentration which is 15 – 40 times as concentrated as in normal abdominal fluid\(^2\). The mesothelial layer of the peritoneum is the first line of contact with the PD fluid. It has been noted that high concentrations of PD fluid in human peritoneal mesothelial cells (HPMCs) induce the synthesis of TGFβ, which is implicated in fibronectin production (forming ECM) and fibrosis\(^3\). Furthermore, the exposure of HPMCs to glucose products can provoke apoptosis\(^4\).

The sterilization of PD fluids is primarily performed by heating. This might give rise to the appearance of high glucose degradation products (GDPs). The GDPs activate inflammatory cells and induce apoptosis of mesothelial cells\(^5\). For example the exposure of HPMCs to GDPs induces the secretion of high levels of IL-6 and pro-angiogenic factor, VEGF from these cells\(^6, 7\) (Figure 7).

Another factor affecting the biocompatibility of the PD fluids are AGEs. These products can react with collagen fibers (component of the ECM). Solubility of these fibers increases, making them more resistant to degradation, thus favoring the deposition of collagen and provoking fibrosis during PD\(^8\). Moreover, the receptor for AGEs (RAGE) is mainly present on mesothelial cells, endothelial cells, macrophages and fibroblasts\(^9, 10\), all capable of triggering an inflammatory response. There is evidence that the conversion of mesothelial to mesenchymal cells is mediated, at least in part, by RAGE\(^11\). For this reason upon exposure to PD fluid the mesothelial cells might acquire fibroblastoid characteristics and modify the expression of molecules implicated in the generation of ECM and could secrete many pro-inflammatory cytokines and chemokines such as MCP-1, IL-1, IL-6, IL-10, TGFβ and RANTES (Figure 6 and 7). Fibroblasts can also secrete these pro-inflammatory molecules in response to AGEs\(^12, 13\) (Figure 7).

Furthermore, the binding of AGEs to their receptor might lead to production of free oxygen species and induce pro-inflammatory processes. Schwenger et al., using RAGE-deficient mice, suggested that T cells could be activated in response to binding a RAGE ligand\(^14\). This is in line with the observation that the binding to RAGE can also activate signalling pathways, including mitogen-activated protein kinases\(^15\) and transcription factor NF-κB pathway\(^16\).

Finally, the low pH of the PDFs used to prevent the degradation of PD fluids during sterilization process could be a source of an irritation. This hypothesis, however, remains without verification\(^17\).
Figure 7. The PD fluid itself is capable of releasing a wide range of pro-inflammatory cytokines and eventually leads to an inflammatory response. (A) The normal peritoneum in a non-PD patient and (B) the peritoneum on PD treatment.
OBJECTIVES
The main goal of this thesis was to gain insights into the involvement of CD69 in peritoneal fibrosis by using CD69 deficient mice in an established peritoneal dialysis mouse model.

In order to achieve this goal we had the following aims:

1. To examine the *in vivo* contribution of CD69 in peritoneal fibroproliferative responses induced by dialysis fluid by studying the expression of inflammatory and fibrotic markers, measuring the peritoneal thickness and analyzing the Th1/Th2/Th17 and Treg responses in the peritoneal cavity.

2. To analyze the role of CD69 in the regulation of the Th17/Tregs balance in experimental PD.

3. To demonstrate that CD69 exerts its function within the lymphocyte compartment by generating chimeric mice.

4. To confirm the involvement Th17 response in peritoneal fibrosis by blocking IL-17 in CD69−/− mice.

5. To validate the involvement of CD69 in peritoneal fibrosis by blocking the CD69 molecule in WT mice.
MATERIALS AND METHODS
1. Human sample analysis

1.1. Samples

Non-PD and PD patients biopsies were gathered from the Department of Pathology Unit (Hospital de la Princesa). Similarly peritoneal effluent cells were collected from the peritoneal dialysis unit, Nephrology Department (Hospital Universitario La Paz). Peritoneal effluent cells were obtained from two different randomized patients. One was a six-month ongoing PD patient with an episode of peritonitis and the other was a normal 6-month PD patient without episode of peritonitis (free-infection).

1.2. CD69 expression in human peritoneum

1.2.1. CD69 expression in peritoneal effluent cells

Cell suspensions obtained from peritoneal lavage from PD patients were stained with fluorochrome-conjugated mouse-specific antibodies against CD3 (Miltenyi 130-094-965), CD4 (Miltenyi 130-091-231), and CD69 (Table 8). Samples were analysed in a BD FACS Canto II (BD Biosciences) flow cytometer and further analyses were performed with FloJo software.

![Table 4. PBS buffer (10X) components.](image)

2. Mice experiments

2.1. Mice

CD69-deficient mice were generated in the 129-Sv background as described\(^1\) and were backcrossed to C57BL/6 for more than 12 generations. CD69\(^{-/-}\) and control wild type mice used for experiments were 8 to 12 week-old females, and were either littermates or age-matched offspring of these littermates in the C57BL/6 background. Rag2\(^{-/-}\);γc\(^{-/-}\) double knockout were kindly provided by Dr. M.L. Toribio (Centro de Biología Molecular, CSIC, and Spain). All mice were maintained and used under pathogen-free conditions at the Animal Facility of CNIC (Centro de Investigaciones...
Cardiovasculatres, Spain). All animal procedures were approved by the Ethics Committee of CNIC and Comunidad Autónoma de Madrid, and were conducted in accordance with institutional guidelines that comply with the National Institutes of Health’s Guide for the Care and Use of Laboratory Animals.

2.2. Peritoneal exposure model

2.2.1. Catheter installation

A customized vascular access port (ROP; Access Technologies, USA) was implanted into the mice. The catheters had 10 holes located within 1 cm of the tip to help the fluid exit and to prevent obstructions (Figure 8A). The animals were anaesthetized with intraperitoneal 100 mg/kg isofluoran. Afterwards, we performed an incision in the skin in the right flank of the animal. The skin was separated from the muscle layer below. Through another incision in this second layer, we introduced the end of the catheter into the peritoneal cavity (Figure 8B). The port was displaced at the subcutaneous space of the mouse's back (Figure 8C).

2.2.2. Procedure

During the first week post-surgery, 0.2 mL saline was instilled in order to prevent catheter trapping. Thereafter, during the experimental procedure, 2 mL standard PD fluid (PDF, lactate with 4.25% glucose; Fresenius Medical Care, Bad Homburg, Germany) or physiologic saline (Salina Fisiologica Grifols 0,9%, Cloruro de Sodio reference: 610667) was instilled daily for 40 days. This volume was chosen because it is almost equivalent to a single exchange in PD patients, relative to body weight, and does not affect mouse respiration. The state of the animals’ health was checked daily by a veterinary doctor, and mice presenting any sign of illness were excluded from the experiment. We used 4 groups of mice in the 1st part of the project. The control groups WT and CD69−/− had a catheter and received 2 ml of physiologic saline (n=10/group). The treated WT and CD69−/− groups received an instillation of 2 ml of PDF daily through the catheter (n=10/group). In order to study the mechanism by which CD69 receptor is involved in peritoneal fibrosis, we performed the comparison between WT and CD69−/− PDF treated groups respectively. The experiment was repeated at least two times. The experimental procedure was performed according to NIH guidelines for the care and use of laboratory animals. The experimental procedure was approved by the institutional ethics committee. The whole experimental procedure and treatment protocol is summarized in figure 10.
Figure 8. Catheter installation and PDF instillation in mice. (A) The catheter contains 10 holes located within 1 cm of the tip to help the fluid exit and to prevent obstructions. (B) The catheter installation surgical procedure consists of a small incision in the right flank, where the catheter is sutured. (C) At the end of the procedure, the catheter port is located in the back of the mouse in the subcutaneous space.

2.3. Bone marrow chimeric mice

10-12 week old CD69<sup>+/+</sup> and CD69<sup>−/−</sup> females were lethally γ-irradiated with 2 doses of 6.5 Gy and transferred i.v. with a mixture of 4×10<sup>6</sup> BM cells from Rag2<sup>−/−</sup>γ<sup>−/−</sup> and CD69<sup>−/−</sup> or Rag2<sup>−/−</sup>γ<sup>−/−</sup> and WT cells in a proportion of 3:1 respectively. The Rag2<sup>−/−</sup>gamma c-double mutant mice are completely alymphoid (lack T-, B- and NK- subsets) which gives specificity to our assay: myeloid cell subset will belong to the Rag2<sup>−/−</sup> gamma c-double mutants whereas lymphocyte compartment will belong only to WT or CD69<sup>−/−</sup> donors (Figure 9). Reconstitution of the immune system by the donors was analysed 6 weeks after bone marrow transplant and reconstituted mice were subsequently analysed 40 days after PD treatment.

Figure 9. Schematic representation of the generation of bone marrow chimeric mice.
2.4. IL-17 blocking experiments

PDF-instilled CD69−/− mice were treated with a recombinant neutralizing antibody against IL-17A (eBioscience, affymetrix, reference: 16-7173) or its corresponding control (mouse IgG1K, isotype control Ab). Anti-IL-17A and IgG1-K antibodies (100µg / mouse) were instilled in the peritoneal cavity of the mice thought an installed catheter together with PDF solution every 5 days for a total period of 40 days.

2.5. CD69 blocking experiments

Murine Ab CD69-2.2 (IgG1-k), specific for mouse CD69, was generated as described19,200,201. It was purified from concentrated supernatants obtained in an INTEGRA CL 350 flask (Integra Biosciences AG, Switzerland) using a protein G column (Pharmacia- Biotech, Uppsala, Sweden). Purified mAb was dialyzed extensively against PBS, tested for endotoxin (levels were < 0.1 ng/ml) and stored at -20 degrees. As a control the murine IgG1 anti-human CD69 (mAb-2.8) purified in the same way was used. PDF-instilled WT mice were treated with a neutralizing antibody (mAb-2.2) against CD69 (Materials and methods 2.5.1 section). CD69 neutralizing antibody can reduce dramatically the expression of CD69 in all compartments of the mouse. A dose of (100µg / mouse) was instilled in the peritoneal cavity through the catheter together with PDF every 5 days for a total of 40 days. As control group WT PDF-instilled mice were treated with the murine IgG1 anti-human CD69 (mAb-2.8) (isotype control Ab).
Figure 10. Summary of the experimental procedure and the treatment methods followed throughout the whole project.
2.6. FACs analysis

Cell suspensions obtained from peritoneal lavage and peritoneal membranes from PDF-treated and untreated mice were sequentially filtered through 70-µm (BD Falcon) and 40-µm (Miltenyi Biotec) cell strainers. Cells were stained with fluorochrome-conjugated mouse-specific antibodies against CD45.1, CD45.2, CD4, CD8α, B220, CD11b, Ly-6C, CD25, Gr-1, IFN-gamma, IL-4 and IL-17, purchased from BD Pharmingen and FoxP3 (eBiosciences). Before intracellular staining, cells were re-stimulated for 4 h with 50ng/ml PMA and 500ng/ml ionomycin in the presence of 1µg/ml Brefeldin A. Samples were analysed in a BD FACs Canto II (BD Biosciences) flow cytometer and further analyses were performed with FloJo software.

2.7. Real-time quantitative (q) PCR

cDNA for real-time quantitative PCR (qPCR) was generated from 0.5 µg of total RNA using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems) in a 10 µl of final reaction volume. The qPCR reactions were performed in triplicate using 10µl of each cDNA 1/40 dilutions, 10 µM of each oligonucleotide and HOT FIREPol qPCR mix (Solis Biodyne) in a total volume of 8 µl in MicroAmp Optical384-well plates (Applied Biosystems). PCR reactions were carried out in an ABI PRISM7900HT (Applied Biosystems). The amount of amplified DNA was measured through the emission of light by the SYBR green dye, intercalating in synthesized double stranded DNA. All samples were measured in triplicates. SDS v2.2 software was used to analyze results. Specific amplification was controlled by melting-curve analysis. The data were exported, processed to Microsoft excel and analyzed by the comparative Ct Method (DDCt). X-fold change in mRNA expression was quantified relative to control WT samples from the same experiment. β-actin mRNA was used as a housekeeping gene. The primers that were used in this study were as follows:

Table 5. Sequences of oligonucleotides used in the experiments.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward Sequence (5' → 3')</th>
<th>Reverse Sequence (5' → 3')</th>
</tr>
</thead>
<tbody>
<tr>
<td>Collagen I</td>
<td>CCAGAGTGGAACACGCATTAC</td>
<td>GCAGGGAGATGGCTTATTT</td>
</tr>
<tr>
<td>Fibronectin</td>
<td>GCAACCTATAGCTGAGAAGTC</td>
<td>CAAGTACAGTCACCACATC</td>
</tr>
<tr>
<td>IL-1β</td>
<td>TGGTGTGACGTCCATT</td>
<td>CAGCAGGCTTTTGTGTC</td>
</tr>
<tr>
<td>IL-6</td>
<td>GAGGATACCACCTCCAACAGACC</td>
<td>AAGTGCATCATCGTTGTTGATAC</td>
</tr>
<tr>
<td>TGFβ</td>
<td>GGAATCTACCCAGAAATAGCAACATTC</td>
<td>TGTAATCCTCCTGCTCTGCA</td>
</tr>
<tr>
<td>β-actin</td>
<td>AAGGAGATTACTGCTGCTCCTA</td>
<td>ACTCATCGTACTCCGCTGCTGAT</td>
</tr>
</tbody>
</table>
2.8. Immunoblotting

Proteins were denatured using standard loading buffer, containing 5% of β-mercaptoethanol (Table 6), for 10 minutes at 99°C. Polyacrylamide gel electrophoresis (PAGE) was performed according to a common procedure. Denatured protein samples, at volumes corresponding to equal protein amounts, were loaded into 12.5% polyacrylamide gel wells and proteins were separated in a running buffer (Table 7) connected to a source of electrical field (120 V, for 2 hours, at RT).

Separated proteins were electro-blotted onto a nitrocellulose membrane using a transfer buffer (Table 4). The membrane was later incubated with a blocking solution, containing 5% skimmed milk dissolved in a TBS buffer (Table 5) with 0.1% of Tween 20, at RT. The blocked membrane was incubated overnight with a primary antibody (Table 8) diluted in a blocking solution at 4°C. Protein expression was addressed using fluorescent secondary antibodies (Table 9) and LI-COR Odyssey infrared scanning.

Table 8. List of primary antibodies used in the experiments. (*) WB - western blot (immunoblotting); IF- immunofluorescence, (***) Rb - rabbit; Ms - mouse;

<table>
<thead>
<tr>
<th>Antibody Name</th>
<th>Company and Reference No.</th>
<th>Dilution Used *</th>
<th>Specificity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phospho-Stat3 (D3A7) (Tyr705)</td>
<td>Cell Signalling, #9145</td>
<td>1:1000 (WB)</td>
<td>Rb</td>
</tr>
<tr>
<td>Stat3</td>
<td>Santa Cruz Biotechnology, sc-482</td>
<td>1:1000 (WB)</td>
<td>Rb</td>
</tr>
<tr>
<td>β-actin</td>
<td>Millipore, MAB2081</td>
<td>1:20000 (WB)</td>
<td>Ms</td>
</tr>
<tr>
<td>CD69 (TP1/55.3.1)</td>
<td>R&amp;D Systems, MAB23591</td>
<td>1:50 (IF)</td>
<td>Ms</td>
</tr>
<tr>
<td>α-SMA</td>
<td>Sigma A2547</td>
<td>1:50 (IF)</td>
<td>Ms</td>
</tr>
<tr>
<td>Cytokeratin</td>
<td>Novocastra 5D3</td>
<td>1:50 (IF)</td>
<td>Ms</td>
</tr>
</tbody>
</table>
**Table 9.** List of secondary antibodies used in the experiments. (*) WB - western blot (immunoblotting); IF - immunofluorescence.

<table>
<thead>
<tr>
<th>Antibody Name and Specificity</th>
<th>Company and Reference No.</th>
<th>Dilution Used *</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alexa Fluor 680 Goat Anti-Rabbit</td>
<td>Molecular Probes, A-21109</td>
<td>1:5000 (WB)</td>
</tr>
<tr>
<td>Alexa Fluor555 Mouse IgG1Anti-Mouse</td>
<td>Molecular Probes, Z-25005</td>
<td>1:500 (IF)</td>
</tr>
<tr>
<td>Alexa Fluor 647 Donkey Anti-Rabbit</td>
<td>Molecular Probes, A-31573</td>
<td>1:500 (IF)</td>
</tr>
<tr>
<td>FITC Polyclonal Rabbit Anti-Mouse</td>
<td>DakoF0313</td>
<td>1:50 (IF)</td>
</tr>
</tbody>
</table>

2.9. FlowCytomix

To determine the quantity of different cytokines and chemokines in the peritoneal effluents, the peritoneal cavity was washed with 2 ml of saline immediately after the mice were euthanatized. The solution was centrifuged and the supernatant was analyzed for cytokine and chemokine production using the FlowCytomix technique (Bender MedSystems GMBH).

2.10. Trichrome Massons staining

Parietal peritoneal biopsies were collected from the opposite side of the catheter installation. The biopsies were fixed in Bouin’s solution, embedded in paraffin, cut into 5 µm sections and stained with Trichrome Massons. The peritoneal membrane thickness was determined using a microscope (Leica CTR6000, with a Leica Microsystems LAS-AF6000). Microphotographs were obtained using an Olympus BX41® clinical microscope and an Olympus DP20® digital camera using cell Acquisition software. The peritoneal thickness of each mouse was calculated by the median of measurement taken every 50 µm from one extreme to the other of biopsy. The result was used to calculate the group thickness.

2.11. Immunofluorescence

Biopsies were frozen in OCT and cut into 5 µm sections. To block unspecific proteins the sections were incubated, for 1 hour at 37°C, in 20% of goat serum in 3% of bovine serum albumin (BSA) in distilled water. To identify mesothelial cells, we used mouse anti-cytokeratin (Table 8). Fibroblasts were stained with α-SMA antibody (Table 8). Both antibodies were diluted in blocking solution and then added onto the tissue sections that were placed in a humid chamber for an overnight incubation at 4°C. As secondary antibodies Alexa Fluor Zanon Fab fragments were used in each case (Table 9). Finally, the preparations were mounted with a 4,6-diamidino-2-phenylindole (DAPI) nuclear stain.
(Vectashield; Vector Laboratories). Negative controls for immunofluorescent staining were conducted using 10% horse serum instead of primary antibody. Micrography was performed with a fluorescence microscope (Leica CTR6000, Leica Microsystems Heidelberg, Germany, with LAS-AF6000 software) or with a confocal microscope (Leica TCS SPE with LAS-AF software, version 2.0.1 build 2043).

2.12. Statistical analysis

Data were analyzed by paired or unpaired Student’s t-test, depending on the type of data, using GraphPad Prism 5.03. P values \( \leq 0.05 \) were considered significant different. Results are means ± SD. Correlations were assessed using Spearman’s correlation tests (GraphPad Prism 5.03).
RESULTS
1. CD69 expression in T lymphocytes from PD patients

In order to support the clinical relevance of our PD animal model using CD69-deficient mice, we first wanted to confirm the expression of CD69 in human samples of patients undergoing PD.

Since CD69 is expressed in activated T cells, including lymphocytes in peripheral lymphoid tissues, we collected cells from peritoneal effluents from two randomized patients and performed T cell stainings using specific CD3, CD4 and CD69 antibodies, according to the procedure described in Materials and Methods. Two samples were taken from a patient suffering from an episode of peritonitis (day 1 and one week later counting from the beginning of infection) and the other was collected from a 6-month PD patient free of infection.

Analysing flow-cytometry results, we found that CD69 was highly expressed in CD3-positive T cells from both patients (Figure 11A, B). Interestingly, in the peritonitis patient CD69 was expressed on about 24% of CD3-positive cells at day 1 of the infection episode, and this fraction was even higher at day 7, reaching almost 50%. Similarly, the analysis of the second non-infected patient, treated for 6 months with PD, showed that CD69 was also expressed in a big fraction of the CD3-positive T cells of the peritoneal cavity. The percentage of CD4⁺CD69⁺ cells was approximately a half of the CD3⁺CD69⁺ sub-population (Figure 11B), showing that half of CD69 expressing lymphocytes belong to the CD4 subset of peritoneal cells from PD patients.

![Figure 11](image-url)
2. CD69 in PDF-induced fibrosis in mice

CD69−/− mice have been reported to be sensitive to exogenous stimuli and they produce an induced inflammatory response in different models. In order to evaluate the role of CD69 receptor in peritoneal fibrosis, we performed 3 independent in vivo experiments using WT and CD69−/− mice and PD protocol (as described in Materials and Methods). It has been demonstrated that WT mice, after exposure to PDF for at least 21 days, develop a remarkable fibrosis following inflammation compared to controls. We treated mice for different periods of time (10, 20 and 40 days) with PD fluid and analysed the samples (peritoneal effluent cells and peritoneal membrane (PM) biopsies).

The PD catheter implantation was followed by daily treatments with physiologic saline (control group) or with the standard PDF (lactate with 4.25% glucose; Fresenius Medical Care) at a total volume of 2 ml each time. This volume was chosen because it is almost equivalent to a single exchange in PD patients, relative to body weight, and does not affect mouse respiration.

2.1. 10-day PDF treatment

Following the 10-day exposure to PDF, we observed no increase of the PM thickness in either WT or CD69−/− mice, compared with saline-treated controls, using a specific Trichrome Massons staining of the paraffin-embedded sections (Figure 12A,B). Also the numbers of total cells (Figure 12C) and percentages of T cells, including Th17 cells (Figure 12D), from the peritoneal cavity, measured by flow-cytometry, were mostly similar in all untreated and treated mice. We only observed a decreased number of Treg (CD4+FoxP3+) cells in PDF-treated CD69−/− mice compared to all remaining groups, although this difference was statistically significant only between the treated groups (Figure 12E). It is known that during inflammation the number of Tregs is usually decreased. We hypothesised, therefore, that this decrease could be a sign of inflammation possibly starting at day 10 in PDF-instilled CD69−/− mice. In addition, the levels of pro-inflammatory cytokines: IL-17, IL-6, IL-1β and TGFβ in peritoneal effluent protein remained unchanged independently of the treatment in both genotypes (data not shown).

2.2. 20-day PDF treatment

2.2.1. Fibrotic and cellular response

In order to evaluate fibrotic response in both genotypes at longer times after initiation of the PDF treatment, we exposed WT and CD69−/− mice to PDF for 20 days. After mice sacrifice we performed Trichrome Massons staining to measure the peritoneal thickness (Figure 13A).
Figure 12. PDF treatment of WT and CD69⁻/⁻ mice (10 days). (A) Representative pictures of PM stained with Trichrome Massons. Black arrows indicate PM. (B) Peritoneal membrane thickness. (C) Quantification of the total peritoneal cells in the peritoneal cavity. (D,E) Percentage of Th17 (D) and Treg (E) cells in the peritoneal cavity measured by flow-cytometry. Values in (B-E) are means ± SD (n≥5). *p<0.05 was considered statistically significant. Student t-test was used.

Figure 13. PDF treatment of WT and CD69⁻/⁻ mice (20 days). (A) Representative pictures of PM stained with Trichrome Massons. (B) Peritoneal membrane thickness. (C) Quantification of the total peritoneal cells in the
peritoneal cavity. Values in (B,C) are means ± SD (n≥5). p<0.05 was considered statistically significant. *p<0.05, **p<0.01. Student t-test was used. MCs: mesothelial cells.

PDF-instilled WT mice presented only a small increase in fibrosis. However, we observed that PDF-instilled CD69−/− mice developed a significant fibrotic response compared with tCD69−/− and WT controls and also with PDF-instilled WT group (Figure 13B). This demonstrates that instillation of PDF in CD69−/− mice induces morphological alterations in PM, coinciding with fibrotic changes observed in clinics in PD patients. Moreover, after the sacrifice of the mice, the peritoneal cavity was washed extensively in order to collect peritoneal cells. We found that PDF-instilled CD69−/− mice displayed a high number of total peritoneal cells in the peritoneal cavity compared both to a control group and to PDF-instilled WT mice. We suspected that this cell abundance was an indicator of an inflammatory response (Figure 13C).

2.2.2. Inflammatory cells in the peritoneal cavity

Inflammation is one of the adverse effects in patients under PD treatment and it can lead to the interruption of the therapy\textsuperscript{204}. After we evaluated the thickening of the PM in both genotypes we wanted to further study inflammatory processes, such as monocyte and neutrophil accumulation in the peritoneal cavity in mice undergoing PD.

![Figure 14](image)

**Figure 14.** Flow-cytometry analysis of (A,B) CD11b\(^{+}\)Ly6G\(^{+}\) (monocytes), (A,C) CD11b\(^{+}\)Ly6G\(^{\text{high}}\) (neutrophils) and (D) CD45\(^{+}\) cells in the peritoneal cavity of WT and CD69−/− mice treated or not with PDF for 20 days. Numbers in quadrants indicate percentages of cells. Values in (B-D) are means ± SD (n≥5). p<0.05 was considered statistically different. *p<0.05, **p<0.01. Student t-test was used.
Figure 15. Different (A) chemokines and (B) cytokines released in the peritoneal cavity after 20 days of the PDF treatment. Data are expressed as means ± SD (n≥5). p<0.05 was considered statistically significant. *p<0.05, **p<0.01. Student t-test was used.

Using flow-cytometry analysis, we found that both monocytes (CD11b^+Ly6G^+) and neutrophils (CD11b^+Ly6G^{high}) were increased in the PDF-instilled CD69^-/^- mice, whereas in PDF-instilled WT
mice we did not observe this induction (Figure 14A-C). Moreover, we found that CD69−/− mice treated with PDF displayed high numbers of infiltrating CD45+ cells (Figure 14D), which is probably due to the recruitment of inflammatory cells, including neutrophils and monocytes, to the peritoneal cavity.

2.2.3. Pro-inflammatory cytokines and chemokines in the peritoneal cavity

Pro-inflammatory chemokines and cytokines are molecules released during a sustained inflammatory response. In this regard, we wanted to examine their levels in the peritoneal effluents from mice of both genotypes after 20 days of PDF treatment.

After the mice sacrifice we collected the peritoneal effluents and using the FlowCytomix technique (see Materials and Methods) we analysed a number of cytokines and chemokines (Figure 15). We found that chemokines GMCSF, MIP1α, MIP1β and RANTES were significantly up-regulated in the PDF-instilled CD69−/− group compared with control and were, in general, higher than treated WT mice, also in the case of MCP3, but not MCP1 (Figure 15A). Noteworthy, the above-mentioned chemokines are released mainly by macrophages and T cells when a sustained inflammation takes place.

It has been described that inflammatory cytokines, such as IL-6, TNF-α and IL-17 are highly expressed in the peritoneal cavity during PD140,205. Since we found that the PDF-treated CD69−/− mice expressed significantly higher levels of IL-2, IL-6, TNF-α and IL-17 compared to WT (Figure 15B), we concluded that this induced cytokine expression in the knockout mice contributes, at least in part, to the fibrosis of the PM and to immune cells accumulation in the cavity, previously described in sections 2.2.1 and 2.2.2 of Results.
2.2.4. T cells in the peritoneal effluents

CD69 is involved in experimental and human inflammatory diseases and Th17 cells mediate many of these inflammatory responses. We were interested, therefore, to analyse different Th cell populations from the peritoneal cavity of mice undergoing PD. We quantified the numbers of Th1, Th2, Th17 and Treg cells from peritoneal effluents, using flow-cytometry (Figure 16).

![Images of flow-cytometry graphs showing Th17, Th1, Th2, Treg populations, and the ratio Th17/Tregs.](image)

**Figure 16.** The total numbers of (A) CD4^+IL-17^+ (Th17), (B) CD4^+IFNγ^+ (Th1), (C) CD4^+IL-4^+ (Th2) and (D) the percentage of CD4^+FoxP3^+ (Treg) cells collected from the peritoneal cavity of WT and CD69^-/-_ mice treated or not with PDF for 20 days. (E) Ratio between Th17 and Tregs. Data are expressed as means ± SD (n=5). p<0.05 was considered statistically significant. **p<0.01. Student t-test was used.

We observed that the PDF-instilled CD69^-/-_ group presented elevated number of total Th17 (CD4^+IL-17^+) cells compared with the PDF-instilled WT mice and control group (Figure 16A). However, we did not detect any significant differences for the remaining T cells between WT and CD69^-/-_ PDF-treated groups (Figure 16B-D). In addition and accordingly to these results, the ratio Th17 / Tregs in the peritoneal effluent from treated knockout mice was significantly higher than in WT (Figure 16F).
2.3. 40-day PDF treatment

2.3.1. CD69 deficiency promotes fibrosis and increases peritoneal cells number

Previously described results reveal that the CD69<sup>-/-</sup> mice are more prone to PM thickening (fibrosis) than WT during 20-day peritoneal dialysis (Figure 13A-C). In order to further confirm our data and to address the influence of the CD69 deficiency on the PDF-treatment outcome in mice during a longer exposure to PD, we performed the similar procedure by treating mice for 40 days with saline (controls) or PD fluid.

![Figure 17. PDF treatment of WT and CD69<sup>-/-</sup> mice (40 days). (A) Trichrome Massons staining of PM sections in WT and CD69<sup>-/-</sup> mice treated or not with PDF. Representative pictures are shown. Arrows shows the peritoneal mesothelial cells layer in controls and solid lines show thickness of PM in the PDF treated groups. Representative photos from necropsies with PM images are represented below for each group. (B) Peritoneal membrane thickness. (C) Quantification of the total peritoneal cells in the peritoneal cavity. In (B,C) data are means ± SD (n≥9). P values <0.05 were considered statistically significant: *p<0.05, **p<0.001. Student t-test was used. (D) Correlation between total number of cells and PM thickness (Spearman regression, p<0.0001; r² =0.694; N=36).](image-url)
The fibrotic response was checked using Trichrome Massons staining of PM sections from WT and CD69<sup>−/−</sup> mice. We observed that after 40 days of PDF treatment both genotypes showed an increased thickness of PM comparing with control (Figure 17A,B). Of note, CD69-deficient mice were characterised by a more pronounced fibrotic response than PDF-instilled WT mice and the difference between the two groups was statistically significant (Figure 17B). The thickened PM in treated mice observed in tissue sections coincides with a phenotypical image of the peritoneum during necropsy, manifested by reduced transparency of the membrane and its whitening corresponding to the fibrotic process (photograph inserts in Figure 17A).

Furthermore, total cells from the peritoneal cavity, extensively washed with physiologic saline, were higher in number upon PDF treatment and this increase was more induced in CD69<sup>−/−</sup> mice comparing with WT (Figure 17C). Moreover, we found a very strong correlation between the thickening of the PM and the total number of cells found in the peritoneal cavity with a p value <0.0001 (Figure 17D).

2.3.2. CD69 deficiency leads to an increase in expression of fibrotic markers.

One of the primary changes observed in biopsies from patients undergoing PD is the thickening of the peritoneum, which is due to the deposition of collagen and fibronectin. The main sources of collagen in fibrotic tissues are fibroblasts. Fibronectin is a glycoprotein of the extracellular matrix and is a very important protein for the differentiation and embryonic development. Both have been previously described as key markers for the evaluation of a fibrotic response during PD<sup>140,206,207</sup>.

In order to evaluate a possible involvement of the CD69 deficiency on the expression of these two markers during PM fibrosis, we measured the mRNA levels of the corresponding genes from total RNA extracted from PM biopsies from controls and PDF-treated WT and CD69<sup>−/−</sup> mice. Using RT-qPCR we found that both collagen and fibronectin were up-regulated after PDF at the mRNA level, but only in CD69<sup>−/−</sup> mice; this induction was absent in WT (Figure 18).
Figure 18. Quantitative RT-PCR of collagen I and fibronectin mRNA in total RNA from PM tissue from WT and CD69<sup>-/-</sup> mice treated or not with PDF for 40 days. Results were normalised to β-actin mRNA and represented in arbitrary units as means ± SEM. One corresponds to a WT control (n=3). p<0.05 was considered statistically significant. *p<0.05, ***p<0.001. Student t-test was used.

2.3.3. CD69 protects against mesothelial to mesenchymal transition

One of the typical changes that take place during PD is the conversion of mesothelial cells into mesenchymal cells displaying acquired fibroblastic phenotype<sup>185</sup>. This process is one of the main etiologic factors of the functional decline of the peritoneum during PD.

In our CD69<sup>-/-</sup> model we found that the deficiency of CD69 induces the MMT process. In order to visualise mesothelial cells that acquired a specific fibroblastic phenotype after exposure to PDF, we stained PM biopsies with antibodies against cytokeratin (mesothelial marker) and α-smooth muscle actin (α-SMA) (fibroblastic marker) as shown in Figure 19. We observed that PDF-instilled CD69<sup>-/-</sup> mice presented significantly higher number of fibroblasts (red cells) compared to PDF-instilled WT mice and controls (Figure 19A). Moreover, PDF-instilled CD69<sup>-/-</sup> mice displayed an increased number of double positive cells for cytokeratin and α-SMA cells, which corresponded to mesothelial cell acquiring fibroblastic character (Figure 19A-B).

In addition, the immunofluorescent analysis revealed a very strong correlation between the double positive (cytokeratin/α-SMA-positive) cells and thickening of the peritoneum (Figure 19C). In conclusion, under these conditions the CD69 deficiency exacerbates the mesothelial to mesenchymal transition after exposure to PDF.
Figure 19. (A) Immunofluorescent microscopy analysis of parietal peritoneal tissue sections stained for α-SMA (red) and cytokeratin (green) with a 4,6-diamidino-2-phenylindole (DAPI) counterstaining. (B) Number of double positive cells for α-SMA and cytokeratin as per field. 10 randomized calculations per mouse were performed. Data are means ± SD (n=9). p<0.05 were considered statistically significant. *p<0.05, ***p<0.001. Student t-test was used. (C) Correlation between cytokeratin/α-SMA-positive cells of peritoneal tissue and thickness of the PM (Spearman regression, P<0.0001; r²=0.811; N=36).
2.3.4. CD69 deficiency promotes expression of Th17-related cytokines

The inflammatory response is orchestrated by a wide range of growth factors and cytokines and their altered expression may have a major impact on immune response. TGFβ and IL-6 are important for the differentiation of Th17 cells. Their presence creates an environment that forces T cells to differentiate towards Th17 lineage, what is mediated by the activation of RoR(γ)t transcription factor. Moreover, Martin et al. (2011) reported that in activated T cells deficient in CD69, the lack of CD69 promotes induction of RoR(γ)t transcription factor and, subsequently, Th17 differentiation.

We analysed whether the enhanced fibrotic response in PDF-instilled CD69−/− mice could be influenced by altered cytokine expression. We checked the expression of a panel of pro-inflammatory cytokines at the protein level in peritoneal effluents, by FlowCytomix, and at the mRNA level in peritoneal membrane, by quantitative RT-PCR.

IL-17, IFN-γ and IL-4 are the most representative cytokines released from Th17, Th1 and Th2 cells, respectively. At 40 days after the PDF treatment initiation, there was an increase of IL-17 in the effluent of PD fluid-treated CD69−/− mice, whereas the remaining cytokines (INF-γ and IL-4) did not show significant differences among groups (Figures 20A-C).

The analysis of cytokines implicated in Th17 differentiation revealed that only TGFβ increased significantly in PDF-treated CD69−/− mice, whereas IL-6 did not show differences among groups and IL-1β was increased in the effluent of both control and PDF-treated CD69−/− mice (Figure 20D,F,H). In contrast, when the expression of TGFβ, IL-6 and IL-1β was analysed at the mRNA level in the peritoneal tissue, all three cytokine-encoding transcripts increased significantly in PDF-treated CD69−/− mice (Figure 20E,G,I). Taken together, these data indicate that TGFβ, but not IL-6 or IL-1β, was mostly locally produced and that local Th17 differentiation was mainly TGF-β-dependent.
Figure 20. Quantitative analyses of cytokines from WT and CD69−/− mice after 40-day treatment with PDF. (A-C) Bars represent the concentration of IL-17, IFN-γ and IL-4. (D, F, H) The quantification of pro-inflammatory cytokines TGFβ, IL-6 and IL-1β in the peritoneal effluents by FlowCytomix (n≥8). (E,G,I) mRNA expression levels of TGFβ, IL-6 and IL-1β assessed by quantitative RT-PCR (n≥3). Data are means ± SD. p<0.05 was considered statistically significant, using Student t-test. *p<0.05, **p<0.01, ***p<0.001.
2.3.5. CD69−/− mice display increased numbers of CD4- and CD8-positive T cells

In order to further characterise cell populations infiltrating the peritoneal cavity in response to PDF-induced inflammation and to check a possible influence of the CD69 deficiency on this process, we calculated the CD4- and CD8-positive cells in the peritoneal cavity, using flow-cytometry. The results are presented in Figure 21.

**Figure 21.** Flow-cytometry analysis of CD8⁺ and CD4⁺ lymphocytes recruited in the peritoneal cavity of WT and CD69−/− mice treated or not with PDF for 40 days. (A) Density blots with corresponding percentages of the CD4/CD8 positive cells. (B, C) Total numbers of CD8⁺ (B) and CD4⁺ (C) cells in the peritoneal effluents. Data are means ± SD (n>6). p<0.05 was considered statistically significant. *p<0.05, **p<0.01, ***p<0.001. Student t-test was used.

PDF treatment resulted in an increase of both T cell populations in the two genotypes compared with saline-treated mice (Figure 21). However, in comparison with WT, CD69−/− mice presented higher total numbers of CD4 and CD8-positive cells after PDF (Figure 21B,C), indicating their involvement in PDF-provoked peritoneal inflammation.

2.3.6. CD69 deficiency induces the Th17 response but not the Th1 and Th2 responses

As mentioned above, CD69−/− mice exposed to PDF were characterised by a pronounced induction of IL-17 expression in the effluents. Flow-cytometry results in Figure 22 are in line with these data; we have observed that peritoneal cavity of PDF-treated knockout mice was highly enriched by CD4⁺IL-17⁺ (Th17) cells compared to control and also to PDF-challenged WT mice (Figure 22A,B). However,
and with one exception, we did not observe any differences in Th1 and Th2 numbers between groups (Figure 22C,D). We only found that CD4^+IFNγ^+ (Th1) cells were increased in the CD69^+ PDF-treated mice compared with control (Figure 22C).

Furthermore, Th17 cells were strongly correlated with peritoneal thickness (Figure 22E), suggesting that Th17 cells play an important role in the development of peritoneal fibrosis and that this role is regulated by CD69. In conclusion, our results unequivocally demonstrate that CD69 exerts a regulatory role in Th17 differentiation in the PD model.

**Figure 22.** (A) Density plot of flow-cytometry analysis of CD4^+IL-17^+ (Th17) and CD4^+IFNγ^+ (Th1) cells. Numbers in quadrants indicate percentages of corresponding cells. (B-D) Total numbers of CD4^+IL-17^+ (Th17), CD4^+ IFNγ^+ (Th1) and CD4^+ IL-4^+ (Th2) cells collected from the peritoneal cavity. Data are means ± SD (n≥6). p<0.05 was considered statistically significant. *p<0.05, **p<0.01. Student t-test was used. (E) Correlation between Th17 cell numbers and thickness of PM (Spearman regression, P<0.0001; r²=0.777; N=33).
2.3.7. *CD69 deficiency impairs the balance between Th17 and Treg*

Using the PD model we wanted to check a possible importance of the presence or absence of CD69 in regulation of Treg cells during the exposure to PDF.

Peritoneal cells were collected from control and treated WT and CD69<sup>−/−</sup> mice and stained with specific antibodies for CD4 and FoxP3 markers, and analysed by flow-cytometry. In both genotypes the PDF treatment resulted in a significantly decreased percentage of Tregs compared to corresponding controls in the levels for each condition were similar regardless of the CD69 status (Figure 23A,B).

The elevated number of Th17 cells (Figure 22B) in PDF-treated mice, much higher particularly in the knockout mice explains the differences in an observed misbalance between Th17 and Tregs between the two genotypes (Figure 23C). Specifically, in PDF-treated CD69<sup>−/−</sup> mice the Th17/Treg ratio was higher than in WT; the difference was, however, insignificant between the two genotypes (Figure 23C). This result coincides with the data obtained from 20-day protocol in which we observed a significantly higher Th17/Tregs ratio in treated CD69<sup>−/−</sup> mice compared with WT (Figure 16E).

![Figure 23](image)

**Figure 23.** (A) Density plot analysis of flow-cytometry of CD4<sup>+</sup> gated FoxP3<sup>+</sup> (Treg) cells in WT and CD69<sup>−/−</sup> mice treated or not with PDF. Numbers in quadrants indicate a percentage of cells corresponding to the quadrant. (B) Percentage of regulatory T cells in the peritoneal cavity. (C) The ratio between the total numbers...
of Th17 and regulatory T cells present in the peritoneal cavity after 40 days of treatment with PDF or saline (control). Results are means ± SD (n≥6). Differences were considered statistically significant for p values <0.05: *p<0.05, **p<0.01. Student t-test analysis was performed.

2.3.8. CD69 deficiency promotes the recruitment of myeloid cells into the peritoneal cavity

PDF treatment in its acute phase is characterized by inflammatory cells infiltration into the peritoneal cavity, including monocytes and neutrophils. These cells are the primary signs of an inflammatory response.

To further demonstrate the role of CD69 in the inflammation after 40 days of exposure to PDF we stained peritoneal cells from WT and CD69−/− mice with Ly6G and CD11b specific antibodies and analysed them by flow-cytometry. We found a significantly higher number of monocytes and neutrophils in PDF-instilled CD69−/− mice compared to PDF-instilled WT and controls (Figure 2A-C). These results indicate that the deficiency of CD69 favours the accumulation of monocytes and neutrophils in the peritoneal cavity during PD and contributes to peritoneal inflammation mediated by these cells.

Figure 24. (A) Density plot of flow-cytometry analysis of CD11b+ and Ly6G+ cells. In each quadrant the upper square represent neutrophils and the bottom quadrant - monocytes. Numbers in quadrants indicate percentages of the corresponding cells. (B,C) Total numbers of CD11b+Ly6G+ (monocytes) (B) and CD11b+Ly6Ghigh (neutrophils) (C) in the peritoneal cavity. Data are as means ± SD (n≥6). Values between indicated groups were considered significantly different for p<0.05 (Student t-test): *p<0.05, **p<0.01, ***p<0.001.
We were also interested to check infiltrating inflammatory cells present in the PM. We found that in PDF-instilled CD69<sup>−/−</sup> mice both monocytes and neutrophils were elevated compared to CD69<sup>+/+</sup> control group. Moreover the number of CD4 and CD8 infiltrating cells were increased in the PDF-instilled CD69<sup>−/−</sup> mice compared to controls. However, PDF-instilled CD69<sup>−/−</sup> mice presented a diminished number of B cells compared to CD69<sup>+/+</sup> control and PDF-instilled WT group. This was probably due to the fact that during an inflammation B cells migrate to the lymph nodes (Table 10).

Table 10. Composition of immune cells in the PM in WT and CD69<sup>+/−</sup> mice after 40 days of treatment. *p<0.05, **p<0.01, ***p<0.001, #p<0.001 WT-PDF vs. CD69<sup>-/-</sup>-PDF.

<table>
<thead>
<tr>
<th></th>
<th>WT Control (%)</th>
<th>PDF Control (%)</th>
<th>WT PDF (%)</th>
<th>PDF PDF (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD11b&lt;sup&gt;+&lt;/sup&gt;B-&lt;sup&gt;+&lt;/sup&gt;Gr-&lt;sup&gt;1&lt;/sup&gt;-&lt;sup&gt;+&lt;/sup&gt; (Monocytes)</td>
<td>0.05 ± 0.01</td>
<td>0.18 ± 0.09</td>
<td>0.05 ± 0.01*</td>
<td>0.21 ± 0.05*</td>
</tr>
<tr>
<td>CD11b&lt;sup&gt;+&lt;/sup&gt;B-&lt;sup&gt;+&lt;/sup&gt;Gr-&lt;sup&gt;-1&lt;/sup&gt;TPP (Neutrophils)</td>
<td>0.04 ± 0.01</td>
<td>0.20 ± 0.11</td>
<td>0.03 ± 0.01***</td>
<td>0.34 ± 0.07***</td>
</tr>
<tr>
<td>B220&lt;sup&gt;+&lt;/sup&gt; (B cells)</td>
<td>8.12 ± 1.87***</td>
<td>20.0 ± 2.21****</td>
<td>0.26 ± 1.19*</td>
<td>4.99 ± 0.98***</td>
</tr>
<tr>
<td>CD&lt;sup&gt;+&lt;/sup&gt; (c dendritic cells)</td>
<td>0.04 ± 0.01*</td>
<td>0.12 ± 0.03*</td>
<td>0.03 ± 0.01*</td>
<td>0.10 ± 0.02*</td>
</tr>
<tr>
<td>pDCs (p dendritic cells)</td>
<td>0.57 ± 0.19</td>
<td>1.68 ± 0.60</td>
<td>0.32 ± 0.08**</td>
<td>1.97 ± 0.40**</td>
</tr>
<tr>
<td>CD4&lt;sup&gt;+&lt;/sup&gt; (T cells)</td>
<td>0.04 ± 0.02*</td>
<td>0.26 ± 0.08*</td>
<td>0.04 ± 0.01**</td>
<td>0.21 ± 0.04**</td>
</tr>
<tr>
<td>CD8&lt;sup&gt;+&lt;/sup&gt; (T cells)</td>
<td>0.04 ± 0.02**</td>
<td>0.18 ± 0.04**</td>
<td>0.05 ± 0.01**</td>
<td>0.14 ± 0.02**</td>
</tr>
</tbody>
</table>

3. CD69 in lymphoid cell-mediated fibrosis in bone marrow chimeric mice

3.1. Chimeric mice generation

To ascertain the role of CD69 expression in the lymphocyte compartment, we performed chimera experiments. In order to analyse the contribution of innate immunity to the enhanced fibrotic response observed in our previous experiments in PDF-instilled CD69<sup>−/−</sup> mice, irradiated WT (CD69<sup>+/+</sup>) mice received BM transplant of Rag2γ<sup>-/-</sup>CD69<sup>+/+</sup> (KO) BM cells and, in parallel, CD69<sup>-/-</sup> (KO) mice were reconstituted with Rag2γ<sup>-/-</sup>CD69<sup>−/−</sup> (WT) BM cells. Additionally, we added two control groups: WT recipients were reconstituted with Rag2γ<sup>-/-</sup>CD69<sup>−/−</sup> (WT) cells and CD69<sup>−/−</sup> mice reconstituted with CD69<sup>+/+</sup>Rag2γ<sup>-/-</sup> (KO) cells. All groups were treated equally with PDF for 40 days, according to the same protocol described before.

Prior to the PD protocol initiation, eight weeks after BM transplantation, we analysed B and T cell development in the chimeric mice. Using flow-cytometry we observed that the percentages of CD4<sup>+</sup>, CD8<sup>+</sup>, NKs, and B cells remained constant among all groups (Figure 25A-E). Moreover, we evaluated the percentages of CD69-expressing cells within CD4<sup>+</sup> T cell population in peripheral blood from chimeric mice; we confirmed a successful reconstitution (Figure 25F).
Figure 25. CD69\(^{+/+}\) (WT) and CD69\(^{-/-}\) (KO) recipients were lethally irradiated and reconstituted with a mix of BM cells from WT or KO cells from donors as described in Material and Methods. (A) Flow-cytometry density plot analysis of corresponding markers in peripheral blood cells (PBL) from chimeric mice. (B-E) Percentages of (B) CD4, (C) B (D) NKs and (E) CD8 cells in the four chimeric groups. (Right part of panel A and panel F) Scatter plot analysis and a corresponding quantification of CD4\(^{+}\)CD69\(^{+}\) cells in PBL in the four chimeric
groups. For determination of CD69 expression T cells from peripheral blood were activated with 50 ng/ml PMA and 500 ng/ml ionomycin for 4 h. Data are expressed as means ± SD (n≥6). P values <0.05 were considered statistically significant: *** p <0.001. Student t-test was used.

3.2. Transfer of Rag2γ−/−CD69−/− cells into WT increases peritoneal fibrosis in response to PD

We addressed the involvement of CD69-expressing lymphocytes in PDF-induced fibrosis using the reconstituted chimeric mice. We first analysed the thickening of the peritoneal membrane in the four groups using Trichrome Massons staining and measuring the PM thickness as shown in Figure 26.

![Figure 26](image)

Figure 26. Peritoneal thickness in reconstituted mice subjected to a 40-day exposure to PDF. (A) Trichrome Massons staining of parietal peritoneal tissue. Representative pictures are shown. (B) Thickness of PM. Data are means ± SD (n≥4). P values <0.05 were considered statistically significant using Student t-test: **p<0.01.

We found that WT mice reconstituted with donor KO cells and treated with PDF presented an exacerbated fibrotic response compared with PDF-treated WT mice reconstituted donor WT cells. It was manifested by an increased deposition of extracellular matrix (fibrosis) observed by light microscopy as appearance of bluish elements in PM. Contrarily, CD69−/− mice reconstituted with donor WT cells and subjected to PDF treatment presented a significantly lower fibrosis compared to PDF-treated CD69−/− mice reconstituted with KO cells. These data unequivocally demonstrate that
CD69 exerts an important panel in peritoneal fibrosis during PD and that the effect observed comes from the lymphoid and most probably not from the myeloid compartment.

3.3. Transfer of Rag2γ−/−CD69−/− cells into WT induces the Th17, but not Th1-/Th2-responses

Taking into consideration that PDF-treated CD69−/− mice presented an increased Th17 response, we wanted to check if this response would be reproduced in the CD69−/− group reconstituted with Rag2−/− CD69−/− cells and treated with PDF for 40 days. Once the PD protocol was completed, we stained peritoneal cells with markers specific to Th17, Th1 and Th2 cells and analysed them by flow-cytometry (Figure 27). The WT mice transplanted with KO cells displayed an increased fraction of Th17 cells compared with the WT/WT mice. Accordingly, CD69−/− mice reconstituted with WT cells presented a significantly reduced percentage of Th17 cells comparing with KO/KO. In the case of Th1 and Th2 responses and in contrast to Th17 response, we did not detect clear differences within recipient groups reconstituted with either CD69-positive or −negative donor cells.

Remarkably our data demonstrate that Rag2γ−/−CD69−/− cells transferred to WT BM recipients lead to an induced fibrotic response and Th17 differentiation in the peritoneal cavity under these conditions. These results support the notion that CD69 acts as a regulator of Th17 response in our PD model and that its activity is highly associated with lymphocytes during PD.

**Figure 27.** Analysis of flow-cytometry data of Th17 (CD4+IL-17+), Th1 (CD4+IFNγ+) and Th2 (CD4+IL-4+) cells from the peritoneal cavity. Bars represent percentages of indicated cells in each chimeric group. Data are means ± SD (n≥7). p<0.05 was considered statistically significant using Student t-test: * p <0.05, ** p <0.01.
4. Implication of IL-17 in PDF-induced fibrosis in the CD69-deficient mice

The classical view of the immune system has been changed by the discovery of a novel T cell subset, Th17 cells. IL-17A, produced by these cells, participates in immune-mediated kidney pathologies, such as glomerulonephritis, and more recently it has been linked with renal injury in an experimental model. Moreover, biopsies from long- and short-term PD patients, immunostained with specific antibodies, were IL-17A-positive predominantly in inflammatory areas, but not in the healthy peritoneum. Besides, chronic exposure to dialysis fluids resulted in a peritoneal Th17 response, including elevated IL-17A gene and protein production, submesothelial cell infiltration of IL-17A-expressing cells, and up-regulation of Th17 differentiation factors and cytokines. This was the first result highlighting IL-17 implication in peritoneal inflammation and fibrosis.

We already demonstrated that PDF-instilled CD69−/− mice presented exacerbated peritoneal fibrosis after 40 days of treatment. However, PDF-instilled WT group was characterised by smaller level of fibrosis, compared with CD69−/− PDF treated mice. We wanted to check whether IL-17 is involved in this pronounced response and if blocking the IL-17 pathway in the PDF-instilled CD69−/− mice would rescue the increased fibrotic response.

In order to evaluate a possible contribution of IL-17 in peritoneal fibrosis in CD69−/− mice, PDF-instilled CD69−/− mice were treated with a neutralizing antibody (Ab) against IL-17 or its corresponding isotype control Ab. Both groups were equally treated for 40 days with PD fluid.

4.1. Blocking of IL-17 results in a diminished fibrotic response

After the sacrifice of mice we checked the fibrotic response in both PDF-instilled CD69−/− group treated or not with the anti-IL-17 neutralizing Ab. Surprisingly, we saw that while treatment of CD69−/− mice with PDF plus an isotype control presented an exacerbated fibrotic response, while this response was completely abolished in the CD69−/− group, simultaneously instilled with PDF and neutralizing anti-IL-17 Ab (Figure 28A,B). In addition, we observed a significant reduction in the total number of peritoneal cells in CD69−/− mice treated with PDF enriched with IL-17 Ab (Figure 28D). This was probably due to the fact that blocking of IL-17 pathway decreased the inflammatory response associated among others with infiltration of immune cells into the peritoneal cavity.

Moreover, we measured the levels of detectable IL-17 in the peritoneal effluents by FlowCytomix. We found that mice treated with anti-IL-17 Ab did not contain detectable IL-17 (Figure 28C). Also, total peritoneal cells, decreased in the Ab-treated mice compared with control, were significantly
correlated with peritoneal thickness (Figure 28E), which is in agreement with the fact that high number of peritoneal cells is linked with the development of peritoneal fibrosis in PDF-treated mice.

**Figure 28.** PDF-instilled CD69<sup>−/−</sup> mice were treated regularly with a neutralizing anti-IL-17 Ab or its corresponding control antibody (100 µg/mouse every 5 days via peritoneal catheter), starting with PDF treatment. (A) Trichrome Massons-stained sections show that administration of a neutralizing Ab against IL-17 dramatically decreased the thickness of the peritoneal membrane, compared with CD69<sup>−/−</sup> mice treated with the isotype control plus PDF. Representative pictures are shown. (B) Statistical analysis of the PM thickness in both groups (n≥5). Horizontal lines are means. Error bars represent SD. ***p<0.001. (C) FlowCytomix quantification of IL-17 in the peritoneal effluents at the end of the experiment. IL-17 is not detected (n.d) in the PDF-treated mice administered with the neutralizing anti-IL-17 Ab (n≥5). (D) Total numbers of peritoneal cells recovered from both groups after the end of experiment (n≥4). In (C,D) data are means ± SD. P values <0.05 were considered statistically significant using Student t-test: **p<0.01. (E) Correlation between total cell numbers and thickness of PM (Spearman regression, P<0.01; r<sup>2</sup>=0.772; N=10).

These results confirm that IL-17 pathway is important for peritoneal fibrosis development is the PD model.
4.2. Blocking of IL-17 results in a decreased Th17 response

We then were interested to check the CD4⁺IL-17⁺ (Th1), CD4⁺IFNγ⁺ (Th2) and CD4⁺IL-4⁺ (Th2) responses in the peritoneal effluent cells.

Figure 29. PDF-instilled CD69⁻/⁻ mice were treated with a neutralizing Ab against IL-17 or control isotype antibody (100 µg/mouse every 5 days, via the peritoneal catheter). (A) Density plot of flow-cytometry analysis of IL-4⁺, IL-17⁺ and IFN-γ⁺ cells. (B-E) Percentages of CD4⁺IL-17⁺ (Th17) (B), CD4⁺IFNγ⁺ (Th1) (C), CD4⁺IL-4⁺ (Th2) (D) and CD4⁺FoxP3⁺ (Treg) cells (E) in the peritoneal effluents from both groups. (F) Th17/Treg ratio. In (B-F) data are expressed as means ± SD (n=8). Values with p<0.05 were considered statistically different using Student t-test: **p<0.01, N.S – not significant.

After 40 days of the combined PDF/anti-IL-17 Ab treatment we analysed by flow-cytometry the above T cell peritoneal populations. We found that Th17 cells were dramatically decreased in the PDF-instilled CD69⁻/⁻ group that was enriched with the neutralizing antibody against IL-17 (Figure
29A,B). However, we did not observe any significant differences between the two groups regarding Th1 and Th2 responses (Figure 29A,C,D). The decreased percentage of Th17 cells is in accordance with our previous observation showing no IL-17 detection in the peritoneal effluents in the same group (Figure 28C). No difference was observed in the number of Tregs between the groups after blockade with the anti-IL-17Ab.

These data confirm that blocking of IL-17 activity in PDF-treated knockout mice rescues the Th17 cell increase in the peritoneal cavity undergoing fibrosis.

### 4.3. Effect of IL-17 blockade on the number of monocytes and neutrophils

After analyzing the T subsets we wanted to check a possible changes in numbers of monocytes and neutrophils in the total peritoneal cells in response to IL-17 blocking in dialysed mice. We collected the effluents and performed flow-cytometry of Ly6G- and CD11b-stained cells. The summary of the analysis is shown in Figure 30.

![Figure 30](image)

**Figure 30.** Percentages of (A) CD11b\(^+\)Ly6G\(^+\) (monocytes) and (B) CD11b\(^+\)Ly6G\(^{high}\) (neutrophils) in the peritoneal effluents from indicated groups. Data are expressed as means ± SD (n≥8). Values with p<0.05 were considered statistically different using Student t-test *p<0.05.

Interestingly, we found that PDF-instilled CD69\(^-\) mice that were administered with the anti-IL-17 Ab, presented significantly lower amounts of CD11b\(^+\)Ly6G\(^+\) (monocytes) cells compared to non-IL-17-treated group (Figure 30A). However, although CD11b\(^+\)Ly6G\(^{high}\) (neutrophils) showed a tendency to decrease, they failed to reach significant difference compared to control (Figure 30B). This finding suggests that blocking of IL-17 pathway in PDF-instilled CD69\(^-\) mice reduces the production of inflammatory cells such as monocytes and neutrophils.
5. Blockade of CD69 exacerbates the response to PDF and reproduces the CD69\(^{-/-}\) phenotype

It has been previously described that CD69 targeting by antibodies affects the course of many diseases in experimental mouse models. For example, treatment with anti-CD69 (mAb-2.2) reduced the severity of collagen induced arthritis (CIA) by partially depleting CD69-expressing T cells\(^{200}\). Another study showed that the administration of the same mAb-2.2 in vivo down-regulates CD69 and leads to an effective lack of CD69 expression on the cell membrane, without any depletory effect\(^{209}\).

To further confirm the importance of the CD69 receptor in vivo in attenuation of the PDF-induced peritoneal fibrosis and inflammation, we used anti-CD69 mAb (mAb-2.2, mouse IgG1) in order to block CD69 expression in mice subjected to PD. We hypothesised that targeting CD69 receptor in PDF-instilled WT mice would reproduce the fibrotic, Th17 and Treg responses observed in PDF-instilled CD69\(^{-/-}\) mice. We used WT mice treated with a combination of PDF and either isotype control Ab (mAb-2.8, control group) or a neutralizing Ab against CD69 (mAb-2.2). Both groups were treated for 40 days under the same conditions.

![Figure 3](image.png)

**Figure 3.** WT mice were treated with PDF combined with anti-CD69 mAb or control mAb (100 µg/mouse every 5 days) for 40 days. (A) Representative pictures of Trichrome Massons-stained sections. Administration of anti-CD69 mAb significantly increased the matrix deposition and the thickness of peritoneal membrane, compared to isotype control. (B) Analysis of the PM thickening in the two groups. Data are expressed as means ± SD (n=5). Values with p<0.05 were considered significantly different using Student t-test. *p<0.05.
5.1. Fibrosis

To determine whether the mAb-2.2-induced down-regulation of the CD69 expression enhanced the peritoneal fibrosis in PDF-treated WT mice, PM biopsies were analysed according to the previous protocol (Figure 31). Observation of the Trichrome Massons stainings of PM sections by light microscopy and measurements of the membrane thickness revealed that mAb-2.2 (anti-CD69 Ab)-treated mice displayed an increased fibrosis compared with mAb-2.8 (control)-treated mice. Administration of anti-CD69 mAb and CD69 expression blockade in PDF-instilled WT mice reproduced, therefore, the exacerbated fibrotic response, previously observed in PDF-treated CD69−/− mice at day 40. Our data coincide with other published animal model, in which the CD69−/− phenotype was restored in WT mice by mAb-2.8-mediated in vivo CD69 down-regulation200.

5.2. Th17/Treg balance

To gain an insight into a possible mechanism by which CD69 regulates peritoneal fibrosis through the regulation of the Th17 and Treg responses, we wanted to analyse the Th17, Th1, Th2 and Treg responses in the peritoneal cavity of WT mice treated with PDF supplemented with mAb-2.2 or mAb-2.8.

Flow-cytometry analysis of peritoneal cells from these mice showed an induced Th17 response in PDF-instilled WT treated with anti-CD69 antibody compared with control (Figure 32A,B). This finding suggests that blocking CD69 during PD results in an augmented Th17 response, similarly to the PDF treatment of CD69−/− mice as observed before. Accordingly to these previous results, we did not observe changes in the Th1/Th2 responses in the two groups, either (Figure 32C,D). In contrast, the Treg population was significantly decreased after CD69 blocking (Figure 32E,F). Expectedly, a high induction of the ratio between Th17 and Tregs was also observed in WT mice with down-regulated CD69 expression (Figure 32G).

Summarizing, our data present that the exogenous CD69 down-regulation in mice under the PDF treatment is a potent pro-fibrotic factor, which also causes an impaired balance between Th17 and Treg cells in the peritoneal cavity.
Figure 3. (A-D) Flow cytometry density plots (A,E) and data analysis of (B) Th17 (CD4^+IL-17^+), (C) Th1 (CD4^+IFN-γ^+), (D) Th2 (CD4^+IL-4^+), and (E,F) Treg (CD4^+FoxP3^+) cells in the peritoneal effluents. (B-D,F) The percentage of Th17, Th1, Th2 and Tregs cells is shown. (G) The ratio between the percentage of Th17 and Tregs is shown. Data are expressed as means ± SD (n≥5). Values with p<0.05 were considered statistically different using Student t-test: *p<0.05.
5.3. Analysis of neutrophils and monocytes in the peritoneal cavity

Monocytes and neutrophils are two populations that are mainly elevated during exposure of the peritoneum to PDF. Using specific stainings and flow-cytometry analysis we found that CD11b⁺Ly6G⁺ (monocytes) and CD11b⁺Ly6G⁺high (neutrophils) were augmented in the PDF-instilled WT groups administered with the mAb-2.2 compared to control (mAb-2.8) group (Figure 33). Thus, the blockade of CD69 in PDF-instilled mice induced an inflammatory phenotype similarly to the effect observed in the treated CD69⁻/⁻ mice (Figure 24). Therefore, CD69 is important for the preservation of the immune response during PD.

**Figure 33:** (A) Density plot analysis of (B) CD11b⁺Ly6G⁺ (monocytes) and (C) CD11b⁺Ly6G⁺high (neutrophils) in the peritoneal effluents. (B) Graphs represent percentages of indicated cell population in the peritoneal cavity. Data are means ± SD (n≥5). Values with *p<0.05 were considered statistically different, using Student t-test.
DISCUSSION
Published data highlight the importance of CD69 in regulating inflammatory responses\textsuperscript{19-21}. For instance, CD69 deficiency resulted in an enhanced inflammation in murine models of cardiac disease\textsuperscript{21}, thereby revealing a protective anti-inflammatory role of CD69 in these pathologies.

Recent data also suggest that Th17 and Treg cells, present in the peritoneal cavity during peritoneal dialysis (PD), are major factors influencing the outcome of the peritoneal functions. Local stimulation of Th17, during the dialysis, is mediated by exogenous and endogenous signals (Figure 34). The exogenous factors include bacteria and their derivatives and provoke episodes of peritonitis by entering into the peritoneal cavity through the PD-catheter or via intestinal translocation\textsuperscript{210}. On the other hand, endogenous factors, such as AGEs\textsuperscript{211} might be involved in the IL-17 induction by activating pro-inflammatory cytokines IL-6 and/or TGFβ, which are both promoters of Th17 differentiation. Thus, an induced Th17 activity during PD could be associated with a poor prognosis and peritoneal damage. Moreover, it has been demonstrated that effluents of the peritoneal cavity in PD patients, suffering from peritonitis episodes, contained increased levels of IL-17\textsuperscript{210}. Although IL-17-dependent peritoneal membrane (PM) deterioration, manifested by mesothelial to mesenchymal transition (MMT), has not been demonstrated, IL-17 is capable of inducing MMT in bronchial cells\textsuperscript{212}. We therefore hypothesised that IL-17 could contribute to the MMT induction in PM as well.

![Figure 34. Mechanism of the Th17/Treg balance in the peritoneal cavity during PD.](image-url)
It has been demonstrated that Treg cells are strongly connected with immune tolerance, as patients suffering from end-stage renal diseases had a suppressed immunity, partially due to impaired regulation of Tregs\(^{211}\). However, at present there is little information regarding the role of Treg cells in the peritoneal cavity during PD. There are only two studies on the functions of Treg cells in peritoneal damage, in which the authors demonstrated their protective role\(^{141,203}\).

In this thesis, we have defined, for the first time, a novel role of CD69 as a negative regulator of the fibrotic and inflammatory response during PD through the modulation of proportions of Th17 and Treg cells in the peritoneal cavity. We have established this connection by subjecting CD69\(^{-/-}\) mice to the PD procedure. We have also shown that a possible mechanism of this regulation is through the balance between Th17 and Treg cells, both contributing to peritoneal fibrosis.

Previous studies had reported that WT mice exposed to PD fluid (PDF) for 21 days presented induced fibrotic response in the peritoneal membrane when compared with controls\(^{203}\). However, we did not observe this phenomenon at day 20 and our WT mice displayed a phenotype rather similar to that observed in control group. This discrepancy could be explained, at least in part, by imperfect animal maintenance conditions and lower expertness level in the mentioned work and/or due to different origins of the mice used in both studies. Interestingly and contrary to the WT mice, the severity of PDF-induced peritoneal fibrosis was remarkably induced in CD69\(^{-/-}\) mice at this time point. Interestingly, an application of a longer procedure, by exposing both genotypes to PDF for 40 days, resulted in peritoneal fibrosis induction in each group, however to a different extent. Specifically, the loss of CD69 was again associated with a fibrosis exacerbation (Figure 35A).

In order to verify a possible role of CD69 in bone marrow (BM)-derived cells on the induction of the fibrotic response we performed experiments with chimeric mice using Rag2\(^{+/+}\), WT (CD69\(^{+/+}\)) and CD69\(^{-/-}\) mice. The transfers of Rag2\(^{+/+}\)/CD69\(^{+/+}\) BM into WT and Rag2\(^{+/+}\)/CD69\(^{+-}\) BM into CD69\(^{-/-}\) were performed to check if the fibrotic effect observed in the PDF-treated CD69\(^{-/-}\) mice was due to the lymphoid compartment. After a 40-day treatment of the reconstituted chimeras with PDF, the WT immunized recipients, with Rag2\(^{+/+}\)/CD69\(^{+/+}\) BM cells, displayed severe peritoneal fibrosis, mimicking the phenotype previously observed in PDF-treated CD69\(^{-/-}\) mice (Figure 35B). In contrast, the transfer of Rag2\(^{+/+}\)/CD69\(^{+-}\) BM cells into CD69\(^{-/-}\) mice and their exposure to PDF led to a significantly lower response when compared with the fibrosis of the CD69\(^{-/-}\) control chimeras. These data indicate that CD69 exerts its regulatory functions during the PD-dependent peritoneal damage through the lymphoid cells.

In order to further confirm the protective role of CD69 during PD we used neutralizing anti-CD69 murine antibody (mAb)-2.2, to down-regulate the in vivo function of the protein\(^{200}\) without affecting
CD69-expressing cells in WT mice. The effect of this antibody consistently mimics the CD69− phenotype in different experimental models, as shown, for example, antitumour immune response19, CIA200 and in acute arthritis induced by anti-collagen type-II antibodies22. In each study the blockade of CD69 in WT mice with mAb-2.2 increased the severity of pro-inflammatory responses. In this regard, our results clearly showed that the down-regulation of CD69 in PDF-instilled WT mice increases severity and progression of peritoneal damage. We showed that combined instillation of WT mice with PDF and mAb-2.2 for 40 days provoked an exacerbated fibrosis comparing to no-mAb-2.2 controls and similar to the one observed in the PDF-instilled CD69− mice (Figure 35C). Taken together, our data indicate that the enhanced fibrotic response present in CD69-deficient mice is specifically result of the lack of CD69 expression. Noteworthy, this experiment establishes CD69 as a possible factor involved in the peritoneal fibrosis in PD patients.

![Figure 35. Effects of different in vivo procedures on the severity of the PDF-induced peritoneal fibrosis: (A) Induced fibrosis in CD69− mice treated with PDF alone. (B) Induced fibrosis of CD69− immunized bone marrow recipients injected with a mixture of Rag2γ−/−CD69− cells and treated with PDF alone. (C) Induced fibrosis in WT mice administered with a combination of PDF and anti-CD69 antibody.](image)

The involvement of IL-17 in the pathogenesis of peritoneal fibrosis has recently been documented140. The authors demonstrated that IL-17 was over-expressed in peritoneal biopsies from both mice and human. This was the first study to demonstrate that IL-17 participates in typical fibrotic changes occurring in peritoneal membrane during long-term PD (induced expression of fibronectin, α-SMA and FSP-1). The same study revealed besides that intraperitoneal injection of exogenous IL-17 into PDF-treated WT mice increased the damage to the peritoneum comparing with non-IL-17 PDF-
treated mice. Accordingly, an intraperitoneal injection with a neutralizing antibody against IL-17 into PDF-treated mice attenuated the phenotypical changes in the PM, including the progress of peritoneal fibrosis.

It has been described that CD69 deficient mice exacerbate Th17 and Th2 responses in a model of allergic asthma and contact hypersensitivity. In this study the authors also showed that inflammatory response in these mice was accompanied by elevated IL-17 levels. It is therefore conceivable that the increased Th17 response might be responsible, at least in part, for the increased inflammation in CD69-deficient mice.

Our study proposes that exposure of the peritoneal membrane of CD69-deficient mice to PDF for 20 and 40 days induces a local Th17 inflammatory response, which is significantly elevated compared to WT PDF-treated group at both time points. We demonstrated the importance of IL-17 in peritoneal dialysis. This is the first study showing that CD69 controls peritoneal fibrosis by regulating the IL-17 expression in the peritoneal cavity during PD. This conclusion was gathered from four different independent experiments including chimeric mice generation and in vivo neutralization of IL-17 and CD69. PDF exposure of CD69-deficient mice probably creates an ideal environment for T cells without CD69 receptor or with impaired CD69 function, to differentiate into Th17 and thereby to initiate an inflammatory process. Therefore, the increased inflammatory response observed after 40 days in the CD69-deficient mice was partially due to the high levels of the pro-inflammatory IL-17 cytokine found in the peritoneal effluents, which was one of the most abundant cytokine found in the peritoneal cavity.

Apart from CD4+ lymphocytes, including Th17, IL-17 is also released by other cells, such as CD8+ T cells, γδ T cells, natural killer cells, dendritic cells and, under special circumstances, by mast cells, as shown in chronic rheumatoid arthritis and in experimental models of renal damage. It has been described that IL-17 acts synergistically with IL-1β inducing a strong pro-inflammatory response. In our study IL-1β level was increased too, therefore it is possible that it has a prominent role in inflammation (or at least in its induction) together with the IL-17, both driving peritoneal inflammation and fibrosis. In contrast to WT, the deficiency of CD69 in PDF-treated mice led to a significant increase of TGFβ in peritoneal effluents measured at the protein level; it was not observed in the case of IL-6. We hypothesize, however, that the constant, relatively highly expressed IL-6 could be involved in a complex mechanism by which TGFβ, IL-6 and IL-1β all coordinate the induction of IL-17 and induce inflammation and fibrosis. In addition, it is described that IL-17 itself can induce fibroblastic, epithelial and endothelial cells to release IL-6 and IL-8 and therefore this could be another mechanism by which IL-17 directly activates fibroblasts to produce IL-6 and guide to
fibrosis. Moreover, when fibroblasts were cultured in the presence of human IL-17, they could sustain the proliferation of CD34 hematopoietic progenitors and their preferential maturation into neutrophils. These observations suggest that IL-17 might constitute an early initiation of T cell-dependent inflammatory response\textsuperscript{220}.

\begin{figure}
\centering
\includegraphics[width=\textwidth]{figure36.png}
\caption{Immunoblotting of phosphorylated Stat3, total Stat3 and α-SMA in protein lysates of PM tissue from WT and CD69\textsuperscript{-/-} mice treated or not with PDF for 40 days. β-Actin was used as loading control.}
\end{figure}

IL-17 is the most representative cytokine released from Th17 response during immune responses\textsuperscript{223}. CD69 has been described as an unexpected regulator of Th17 activity\textsuperscript{208}. It has been shown that the absence of CD69 receptor in the T cell membrane fails to phosphorylate Stat5 transcription factor (negative regulator of Th17 differentiation) and subsequently Stat3 is phosphorylated which is a positive regulator of Th17 response. Thus, RoR(γ)t transcription factor is activated and the fate of Th17 differentiation begins\textsuperscript{224}. In our study when CD69 deficient mice were treated with PDF, we observed a significant increase of total Stat3 protein measured by immunoblotting, in lysates of peritoneum (Figure 36). The induced phosphorylation of Stat3 in the PDF-treated CD69\textsuperscript{-/-} mice, compared with WT, was not significant and could be due to basal level of Stat3 activation, higher in Stat3-abundant samples (Figure 36). Supposing that Stat3 in the peritoneum is overexpressed in among others T cells, the partial induction of Stat3 could suggest, at least to some extent, its contribution to the T cell differentiation into Th17.

So far there has been little information regarding the role of Treg cells in the peritoneal cavity during PD. There are only two studies focusing on the function of Treg cells on peritoneal damage\textsuperscript{141,203}. Our study, for the first time, describes the regulatory role of CD69 in the balance between Th17 and Tregs in the peritoneal cavity during experimental PD and how this balance may affect the outcome of PD. We found that the 20– or 40–day treatment of WT and CD69\textsuperscript{-/-} mice with PD fluid gave rise to similar percentages of Treg cells in both groups. In contrast, we observed a higher ratio between total Th17 and Treg cells in CD69\textsuperscript{-/-} mice compared to WT group.
Figure 37. Regulatory role of CD69 in peritoneal fibrosis through the regulation of Th17 and Treg cells in the peritoneal cavity of CD69−/− mice during PD

This result identified and established the first mechanistic link between CD69 and peritoneal fibrosis. Th17 and Treg cells seem to be the major players in peritoneal fibrosis. Conversion of CD4+FoxP3+ Tregs into Th17 cells with arthritogenic properties establishes a new in vivo pathological importance of Tregs conversion to Th17 cells. Although CD69 deficiency diminishes the production of Tregs in the peritoneal cavity upon PDF exposure, it is possible that a fraction of Th17 cells comes from the conversion of Tregs into Th17. Thus the fate of Tregs might be a critical determinant of self-tolerance versus autoimmunity (Figure 37).

The described mechanistic role of CD69-dependent regulation of peritoneal fibrosis was confirmed by bonne marrow (BM) transplantation. The transfer of Rag2γ−/−CD69−/− cells into WT BM recipients, followed by PD procedure, reproduced the effect of the increased Th17 response, as observed in CD69−/− mice treated with PDF. In contrast, CD69 deficient mice, reconstituted with Rag2γ−/−CD69+/+ BM cells, displayed a decreased percentage of Th17 cells in the peritoneal cavity. Moreover, accordingly to our previous data, the severity of the induced Th17 response was associated with increased peritoneal fibrosis (Figure 35B) and was consistent with the role of Th17/Treg balance in this pathology.

We also evaluated the expression of key cytokines involved in Th17 and Treg differentiation. The differentiation of Th cell subsets is driven by cytokines and regulated by specific transcription factors. IL-6 promotes Th17 differentiation, whereas Th1/Th2 cytokines inhibit this process. TGFβ
participates in Th17 differentiation by inhibiting Th1/Th2 activation. We found that the levels of TGFβ and IL-6 were up-regulated in the PM of PDF-instilled CD69 deficient mice; it is in accordance with the fact that Th17 differentiation requires the co-operation of TGFβ and IL-6 cytokines. However, the hallmark Th1/Th2-related cytokines (IFNγ and IL-4) remained unchanged.

Moreover, the blockade of IL-17 with a neutralizing antibody in CD69−/− mice undergoing PDF treatment, according to the procedure previously described in WT mice, led to diminished peritoneal fibrosis (preservation of PM) and reduced inflammatory responses, manifested by lower Th17, monocytes and neutrophils (Figure 38). This confirms the already published data pointing to IL-17 as an important regulator of peritoneal fibrosis and inflammation.

![Schematic representation of the phenomena occurred after blockade of Th17 response in CD69−/− mice.](image)

**Figure 38.** Schematic representation of the phenomena occurred after blockade of Th17 response in CD69−/− mice.

Last but not least, our results with the anti-CD69 mAb-2.2 demonstrate that the enhanced Th17 and reduced Treg responses present in CD69 deficient mice is a result of the loss of CD69 functions. When we blocked CD69 receptor in WT mice and subjected them to PD, we reproduced the increased Th17 response and decreased Treg response that we had observed in PDF-instilled CD69−/− mice (Figure 39).

It has been described that mesothelial to mesenchymal transition (MMT) is one of the factors triggering peritoneal fibrosis. Many inflammatory mediators such as for example TNF-α, IL-1β, and TGFβ have a role in peritoneal fibrosis by stimulating resident fibroblasts proliferation and ECM
component deposition\textsuperscript{228}. They also induce MMT, turning mesothelial cells (MCs) of the membrane into fibroblasts whose number increases\textsuperscript{185}. MCs that acquire fibroblastic properties during MMT are characterized by the expression of $\alpha$-SMA and cytokeratin markers. Fibroblasts, which can be detected by immunofluorescence as $\alpha$-SMA-positive cells, have a capacity to synthesize ECM, growth factors, and cytokines, thus participating in typical inflammatory responses and fibrosis\textsuperscript{181}. CD69\textsuperscript{−/−} mice presented a significantly increased number of these cells in the peritoneum after exposure to PDF for 40 days. As expected there was a strong correlation between the thickness of PM from treated CD69\textsuperscript{−/−} mice and the number of double positive ($\alpha$-SMA$^+$Cyt$^+$) fibroblasts. This indicates a unique role of CD69 in the peritoneal deterioration. Taken together, these results indicate that the inflammation/MMT axis drives the fibrotic process of the PM induced by the PD fluid exposure (Figure 37).

**Figure 39.** Schematic representation of the phenomena occurred after blockade of CD69 receptor in WT mice.

Moreover, immunoblotting of protein lysates of PM from untreated and PDF-treated mice with an $\alpha$-SMA antibody revealed that this protein is more expressed in CD69 knockouts than in WT mice exposed to PD (Figure 36). Since CD69 deficiency exacerbates fibrosis and increases Th17 differentiation and MMT, it is possible that MMT is a part of this complex mechanism by which CD69 controls peritoneal fibrosis. Moreover, locally produced IL-17 could also activate mesothelial cells, which express the IL-17 receptor\textsuperscript{229}, to release pro-inflammatory factors, contributing to the recruitment of leukocytes into the damaged peritoneum. It is tempting to speculate that one proposed mechanism by which CD69 regulates fibrosis through MMT process could be that IL-17 itself induces
the MCs to convert into fibroblasts, subsequently producing ECM components and contributing to peritoneal fibrosis.

**Figure 40.** General scheme summarizing the possible regulatory role of CD69 and all the main possible pathways implicated in peritoneal fibrosis. I. Absence of CD69 induces the expression of Th17 cells and therefore release IL-17 which together with IL-6 and IL-1β may activate mesothelial cells to produce collagen and contribute to fibrosis. II) CD69 deficiency induces MMT and therefore forming fibroblasts and promoting collagen deposition. III) IL-17 produced from Th17 cells may interact with the IL-17 receptor located in mesothelial cells which give rise to collagen-secreting fibroblasts.
Inflammation is mediated, at least in part, by monocytes and neutrophils. Unresolved recurrent inflammation may contribute to fibrosis. In this study we show that CD69 deficient mice presented increased numbers of monocytes and neutrophils after a 20- and 40-day treatment. When we blocked IL-17 we significantly diminished the percentage of these cells. Similarly, by down-regulating CD69 expression in WT mice we observed higher percentage of these populations in the peritoneal cavity. Taken together, monocytes and neutrophils could be also involved in the chronic inflammation driving peritoneal fibrosis.

**Concluding remarks**

Our study supports a novel role for the leukocyte activation receptor CD69 as a brake on the progression and severity of peritoneal damage and development of peritoneal fibrosis. We found that the genetic ablation of CD69 affects the outcome of experimental PD by regulating the balance between Th17 and Treg cells. These findings are of great importance because they provide a cellular and molecular basis for the development of novel specific therapies targeting peritoneal fibrosis through the regulation of Th17 and Treg responses. For example, the results from phase II clinical trials, in which patients with psoriasis were treated with specific antibodies targeting IL-17A or its receptor, proved their beneficial effect in humans\(^{230}\).

In addition, our study paves the way to investigations into whether defects in CD69 expression or function influence the initiation and progression of peritoneal fibrosis in dialyzing PD patients. The recent discovery of the ligand of CD69 receptor (galectin-1) in DCs\(^{231}\) is a quite promising tool regarding its regulation of CD69 expression during PD. This ligand-receptor pair may represent in the future a novel regulatory pathway for the control of peritoneal fibrosis during PD.
CONCLUSIONS
CONCLUSIONES
1. CD69 has a modulatory role in peritoneal inflammation and fibrosis as shown by CD69-dependent negative regulation of immune and fibrotic responses during PD.

2. CD69 deficiency promotes exacerbated Th17 response and decreased the Treg response, leading to an increase of the ratio between the Th17 and Treg cells, whereas Th1 and Th2 responses remain almost unchanged.

3. CD69 limits peritoneal fibrosis by modulating the mesothelial to mesenchymal transition.

4. The induced fibrotic response in WT mice after bone marrow transplantation with Rag2γ−/−CD69−/− BM cells demonstrates that CD69 exerts its function within the lymphocyte compartment.

5. Blocking IL-17 in CD69-deficient mice abolishes peritoneal fibrosis.

6. Blockade of CD69 in PD fluid-treated WT mice mimics the effect in treated CD69−/− mice demonstrated by the induced fibrosis and Th17 response and the decreased Treg response.

7. These results point to possible new approaches for the therapy of peritoneal fibrosis by: (a) activating the early-leukocyte receptor CD69 in the human peritoneal cavity and (b) targeting the Th17/Treg-axis by decreasing Th17 response and/or increasing Treg response.
1. CD69 tiene un papel modulador en la inflamación peritoneal y la fibrosis del peritoneo asociado a una regulación negativa de la respuesta inmune y fibrótica durante la DP dependiente de CD69.

2. La ausencia de CD69 promueve una respuesta Th17 exacerbada y una reducción de la respuesta Treg, lo que lleva al aumento de la proporción entre las células Th17 y Treg, mientras que las respuestas Th1 y Th2 se mantienen inalteradas.

3. CD69 limita la fibrosis peritoneal mediante la modulación de la transición mesotelio mesenquimal.

4. El incremento de la respuesta fibrótica en los ratones silvestres, tras el trasplante de médula ósea con células Rag2\(\gamma^{-}\)CD69\(^{-}\) demuestra que CD69 ejerce su efecto de la población de linfocitos.

5. El bloqueo de IL-17 en los ratones deficientes para CD69 disminuye la fibrosis peritoneal.

6. El bloqueo de CD69 en los ratones silvestres tratados con el fluido DP imita el efecto observado en los ratones CD69\(^{-}\) bajo el mismo tratamiento, demostrando una fibrosis inducida, una activación de la respuesta Th17 y una disminuida respuesta Treg.

7. Estos resultados sugieren nuevas dianas para el tratamiento de la fibrosis peritoneal humana a través de: (a) la activación temprana del receptor CD69 en la cavidad peritoneal y (b) la regulación del equilibrio Treg/Th17 reduciendo la respuesta Th17 y/o aumentando la respuesta Treg.


35. Hartnell A, Robinson DS, Kay AB, Wardlaw AJ. CD69 is expressed by human eosinophils activated in vivo in asthma and in vitro by cytokines. *Immunology* 1993; **80**: 281-286.


82. Cao Y, Xu W, Xiong S. Adoptive transfer of regulatory T cells protects against Coxsackievirus B3-induced cardiac fibrosis. *PloS one* 2013; 8: e74955.


149. Popi AF, Lopes JD, Mariano M. Interleukin-10 secreted by B-1 cells modulates the phagocytic activity of murine macrophages in vitro. Immunology 2004; 113: 348-354.


156. Sobota RM, Muller PJ, Heinrich PC, Schaper F. Prostaglandin E1 inhibits IL-6-induced MCP-1 expression by interfering specifically in IL-6-dependent ERK1/2, but not STAT3, activation. *The Biochemical journal* 2008; **412**: 65-72.


176. Leask A. TGFbeta, cardiac fibroblasts, and the fibrotic response. *Cardiovascular research* 2007; **74**: 207-212.


197. Schwenger V, Morath C, Salava A, Amann K, Seregin Y, Deppisch R, Ritz E, Bierhaus A, Nawroth PP, Zeier M. Damage to the peritoneal membrane by glucose degradation products is mediated by the receptor for advanced glycation


