FLIP-Expressing Myeloid Cells as Driver of Systemic Immune Disorders

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Received date: May 31, 2022, Accepted date: June 23, 2022


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Abstract

The role of FLIP as a moonlighting protein is becoming progressively evident since this protein is often involved in various processes correlated to aberrant immunological responses independently from its function as master anti-apoptotic regulator. It has been uncovered that FLIP drives the acquisition of immunosuppression and inflammation-associated pathways in myeloid cells. The clinical picture raised during SARS-CoV-2 pandemic has given the possibility to deeply investigate FLIP involvement in releasing a systemic cytokine storm, also linked to a chronic inflammatory syndrome associated with immune suppression and cancer progression. Indeed, a FLIP/STAT3 axis orchestrates an aberrant inflammatory program in myeloid cells of COVID-19 patients and SARS-CoV-2 infected hACE2 transgenic mice. Moreover, the same activated FLIP/STAT3 axis was confirmed in a chimeric vFLIP mouse model, where vFLIP overexpression was restricted exclusively in myeloid cells by using a tissue-specific CRE-driver (e.g., LysMCre mice), validating this model as a feasible platform to study the late phase of COVID-19 disease. The STAT3 pro-inflammatory pathway triggered by the aberrant expression of FLIP in myeloid cells well correlates to the outcome of the cytokine release syndrome (CRS) that is the latest and most severe phase in COVID-19 disease confirming FLIP-mediated myeloid reprogramming as a cornerstone of systemic immune disorders.

Keywords: c-FLIP (Cellular FLICE [FADD-like IL-1β-converting enzyme]-inhibitory protein), CRS (Cytokine Release Syndrome), COVID-19, Inflammation, Myeloid cells

Introduction

Cellular and viral FLICE (FADD-like IL-1β-converting enzyme)-inhibitory proteins (c-FLIP and vFLIP respectively) are traditionally recognized for their relevance in the anti-apoptotic activity, regulating cell survival and proliferation by inhibiting caspase-mediated cell death [1]. c-FLIP is encoded by CFLAR gene and it is translated into 3 different protein isoforms derived from 13 different variants of splicing. Among these isoforms, produced in diverse rates, it is possible to define the 55 kDa long form (c-FLIPₖₙ), the 26 kDa short form (c-FLIPₛ), and the 24kDa form of c-FLIP (c-FLIPᵣ) [1,2]. These isoforms differ on the base of their primary structure and can differently exert their functions [3]. All of them contain 2 tandem N-terminal death effector domains (DEDs) responsible for protein-protein interaction properties, associated with c-FLIP ability in being recruited to the death signaling complex during apoptosis, and differ at the C-terminal domain. In particular, the long isoform includes a caspase-8 cleavage site that, cleaved upon heterodimerization with caspase 8, generates a cleaved-long isoform defined as p43FLIP [4]. This cleaved protein promotes the activation of nuclear factor kappa B (NF-κB) through their association with receptor-interacting protein 1 (RIP1) and tumor-necrosis-factor-receptor-associated factor 2 (TRAF2) [5,6]. Indeed, this proteolytic fragment has been reported to interact with NEMO (also known as IKKγ) by either a transient association with the ubiquitin binding domain of IKKγ [7] or a direct physical interaction when c-FLIP is overexpressed [8,9]. The vFLIP protein, which was initially identified in molluscum contagiosum virus and in several γ-herpesviruses...

[10], also activates IKK by forming a stable complex with the regulatory subunit IKKγ [7] leading to phenotypical features and the cytokine secretion of Kaposi sarcoma cells [11]. Last years, c-FLIP involvement in cellular processes apparently autonomous from its original role has raised more attention towards its alternative properties, and its recent description as a moonlighting protein[12] well attests the attention that such an unpredictable protein deserves. In fact, c-FLIP has been reported to be up-regulated in several cancer types, including pancreatic cancer [13] and lung cancer [14], stimulating tumor progression not only by enhancing cancer cells’ resistance to apoptosis [15] but also by impacting on immune cells regulatory activity favoring the development of a local immunosuppressive tumor microenvironment [3]. In this context, the enforced c-FLIP expression in human monocytes has been related to the marked regulation of genes encoding for proteins typical of immunosuppressive mechanisms, such as signal transducer and activator of transcription (STAT3), interleukin (IL)-6, and programmed death-ligand 1 (PD-L1), possibly depending on the nuclear translocation of a protein complex formed by c-FLIP and nuclear factor kappa B p50 (NF-κB p50) [4] (Figure 1). On the contrary, FLIP genetic deletion completely abrogate the generation of monocytic-myeloid derived suppressor cells MDSCs [16], highlighting FLIP as a key regulator of this cell subset [17-19].

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**Figure 1: The c-FLIP/NF-κB role in STAT3 activation.** Stimulation by different pathogens or proinflammatory cytokines (e.g., TNF) causes phosphorylation and degradation of inhibitor of NF-κBα (IκBα), allowing NF-κB to translocate inside the nucleus and to exert its function as a transcription factor. Previous data [4] describe the nuclear colocalization of a c-FLIP/NF-κB p50 protein complex, which is associated with up-regulation of pro-inflammatory genes such as STAT3 and IL6. In addition, the JAK-STAT3 signaling pathway can be also activated by IL-6 stimulation of IL-6 receptor binding gp130, promoting together with NF-κB the regulation of a transcriptional program favoring the expression of several inflammatory mediators and an immunosuppressive state.
The Role of FLIP/STAT3 Axis in COVID-19 Patients

Traditionally, c-FLIP is studied as an anti-apoptotic protein, since it is capable of blocking caspase-mediated cell death [1]. During infection, this mechanism is exploited by several viruses to prevent host cell death supporting viral replication [20]. The SARS-CoV-2 outcome has focused the attention on viral-induced cellular alterations able to favor viral latency and, specifically, the correlation between SARS-CoV-2 infection and c-FLIP alteration was analyzed in lung autopsy samples by Musiu and colleagues [21]. Pulmonary immunohistochemistry has revealed a heterogeneous composition of myeloid cells including alveolar macrophages, monocytes/interstitial macrophages, and histiocytes, identified as CD68-expressing cells, that overexpressed c-FLIP and pSTAT3. Interestingly, COVID-19 patients, who present high frequency of CD68⁺ FLIP⁺ pSTAT3⁺ myeloid cells in lungs, had a significantly reduced survival rate. To strengthen the correlation between an aberrant STAT3 activation and c-FLIP expression, the same myeloid infiltrate was detected in lung autopsy samples isolated from SARS-CoV-2-infected mice transgenic for hACE2. Interestingly, circulating CD14⁺ c-FLIP⁺ cells isolated from COVID-19 patients showed an enforced PD-L1 expression, similar to what was displayed by monocytes isolated from PDAC patients with worst clinical outcome [4] as well as pSTAT3⁺ monocytes isolated from PDAC patients were identified as MDSCs since they displayed arginase-1-dependent T cell inhibition [23]. Besides, STAT3-mediated inflammatory pathway stimulated by FLIP plays a central role in inducing a fatal cytokine release syndrome (CRS), one of the latest and most severe hallmarks of SARS-CoV-2 infection [24] and generally associated with dramatic clinical aspects [25] (Figure 2).

**Figure 2: CRS pathophysiological progression.** Cytokine storm can be caused by different sources, including iatrogenic or pathogen-induced causes, cancer, and monogenic/autoimmune disorders. The failure of physiological negative regulatory mechanisms allows a deleterious hyper-activation of the immune system leading to the uncontrolled release of proinflammatory cytokines, implying the unbalanced activation of signaling pathways such as NF-κB and JAK-STAT3, which can in turn fuel a positive loop overstressing the inflammatory response. As a consequence, CRS-induced pathological condition could evolve towards a dramatic multi-organ failure and, in the worst case, patient’s death.
**CRS Origins and Pathophysiology**

CRS definition covers a wide immune pathologies embodying acute systemic inflammation and immune dysfunction resulting in multi-organ failure. Since CRS definition still represents a debated question, Fajgenbaum et al. proposed 3 specific criteria for CRS's characterization: elevated circulating cytokine levels, acute systemic inflammatory symptoms, and secondary organ dysfunction [26]. Regarding the clinical symptoms of CRS early phase, patients display a high grade of fever in several cases, as well as headache, fatigue, diarrhea, and myalgia [27]. Worsening condition can lead to a second phase of CRS symptoms including catastrophic hemorrhages, hypotension, vasodilatory shock, and vascular occlusion, culminating in acute respiratory distress syndrome (ARDS) in lungs, renal failure, liver injury, and death at worst. Notably, pre-existing pathological chronic conditions (e.g., hypertension, obesity, diabetes) were found to exacerbate CRS development. At root of CRS rising, it is possible to define 3 main sources. Largotenic cytokine storm finds its bases on specific drug treatments like immunotherapy that can alter the homeostatic cytokine levels. Differently, pathogen-induced cytokine storm is strictly correlated to pathogen infection, in which sepsis condition can be correlated to many cytokine productions that can cause undesirable effects more serious than the pathogen itself. Finally, the last source of CRS could be monogenic or autoimmune [26] (Figure 2). Among the various markers useful to identify CRS, high levels of C-reactive protein (CRP), blood count abnormalities, anemia, leukopenia, thrombocytopenia, and elevated ferritin and D-dimer levels are the most relevant ones, together with increased levels of circulating inflammatory cytokines such as interferon (IFN)-γ, IL-1, IL-6, IL-18, and TNF [28,29]. Consequently, cytokines play the main role in CRS progression since they can finely regulate the entire immunological framework driving acute inflammation. Indeed, abnormal levels of pro-inflammatory cytokines like IL-1 can initiate the NF-κB intracellular signaling pathway resulting in hyper-activation of inflammatory genes transcription, whereas IL-6, upon IL-6 receptor binding gp130, triggers the JAK-STAT3 signaling pathway leading to systemic hyper-inflammation [30] (Figure 1). Inside the myeloid cell population implicated in innate immunological response, neutrophils, monocytes, and macrophages are the main source of cytokine production. Specifically, during netosis, neutrophils can generate a neutrophil extracellular trap (NET) that can participate in thrombi formation and magnify the intensity of cytokine storm. On the other hand, during CRS, over-stimulated monocytes and macrophages secrete an atypical amount of pro-inflammatory cytokines causing immune imbalance. Treating CRS by targeting all the involved pro-inflammatory pathways represent the main therapeutic goal, even if to discriminating between physiological and aberrant inflammation still remains an open challenge.

**vFLIP Mouse Model as an Optimal Platform to Develop CRS-Targeting Approach to Restrain Severe COVID-19 Disease**

In order to get an in vivo model suitable to mimic the pathophysiological characteristics of CRS in COVID-19 disease, a chimeric mouse model expressing v-FLIP in myeloid cells was generated. As detected in COVID-19 patients, an analogous lung myeloid infiltrate composition, characterized by the presence of pSTAT3 in mononuclear phagocytes (defined by CD68+, F4/80+ or Ly6C+) and neutrophils (defined by CD68+, NE+ or Ly6G+), was found in vFLIP mice displaying diffused lung tissue damage. Remarkably, an increasing number of myeloid cells expressing pSTAT3 and, more specifically, a significant number of cytokine-producing monocytes was detected in spleen of vFLIP mice, supporting a SARS-CoV-2-like inflammatory landscape. Deepening further the structural organization of vFLIP lung myeloid infiltrate, single-cell RNA sequencing (scRNA-seq) data analysis unveiled lymphocytopenia in parallel with an increasing number of neutrophils in vFLIP model compared to wild type (WT) mice. Moreover, a gene set enrichment analysis (GSEA) underlined a significant upregulation of genes involved in inflammatory response, in accordance with STAT3 upregulation. These findings well correlate with the late phase of COVID-19 disease [31].

To make consistency between the mouse model and COVID-19 disease, scRNA-seq data obtained from lungs of vFLIP mice and bronchoalveolar lavage fluids (BAL) of patients affected by severe COVID-19 were clustered and consequently compared in order to generate two different maps useful to identify shared cell populations in lung myeloid infiltrate. The analysis revealed a conserved arrangement of myeloid cells between the two different species, and, among myeloid populations, monocytes presented a similar profile of active inflammatory pathways such as tumor necrosis factor (TNF)-α via NF-kB and JAK-STAT3 signaling. These data clearly support vFLIP mouse as a representative model of CRS in severe COVID-19 patients.

Since clear evidence correlate FLIP up-regulation with an enforced aberrant STAT3 activation, it is linear to consider STAT3 as an appealing target in attempt to control the immunosuppressive activity of myeloid cells and therefore, several drugs such as baricitinib or silibinin and short hairpin RNAs (e.g., shSTAT3) delivery [32,33] have been developed and tested on vFLIP mice. Firstly, STAT3 inhibitors seem to soften the inflammatory condition in localized tissue as well as systemically, though maintaining the physiological antiviral response. Secondly, recovery of lymphocyte proliferation was observed upon STAT3 inhibition by restraining the myeloid immunosuppressive phenotype triggered by SARS-CoV-2
infection. Finally, pointing to STAT3 as the most important player in FLIP-expressing myeloid cells during CRS, targeting this specific transcription factor might greatly contribute to preventing immunopathological disorders and tissue damage [21].

Conclusions

The evolution of c-FLIP protein towards a key regulator in immunosuppression in diverse contexts, apart from its physiological anti-apoptotic one, has raised the curtain for a better comprehension of immune molecular mechanisms in both tumor microenvironment and pro-inflammatory-induced pathologies, as CRS. Actually, FLIP was found to be involved in modulating some key immunoregulatory pathways, such as NF-κB and JAK-STAT3, and transcription of genes typically related to immunosuppressive mechanisms, such as IL6, and CD274 encoding for PD-L1. A specular FLIP activity was observed also in SARS-CoV-2 infected patients. Indeed, monocytes isolated from COVID-19 patients displayed immunoregulatory function that correlated with the expression of PD-L1. At the same time, FLIP over-expression in monocytes modulates pro-inflammatory pathways, conducing to aberrant cytokine production and severe CRS manifestation. Therefore, FLIP may be a crucial target to develop more effective targeting approaches to either improve cancer immunotherapy or control aberrant immune response that fuel CRS. For instance, FLIP promotes the up-regulation of indoleamine 2,3-dioxygenase 1 (IDO1) [4], which play a critical role on T cell inhibition by depleting L-tryptophan availability in tumor microenvironment [34,35], consequently the protumoral effect of immunosuppressive myeloid cells can be potentially mitigated by limiting specific FLIP-dependent products [36]. At the same time reprogramming myeloid cells by enforcing FLIP expression may be a critical step to establish innovative therapy based on immunosuppressive cell-based tools able to fight immunopathologies originating from enhanced immune reactivity against host-self antigens in the course of transplantation (e.g., graft-versus host disease, GvHD) and autoimmunity (e.g., multiple sclerosis, MS). FLIP-related knowledge needs further examination to clarify its untraditional features linked to the alteration of cellular transcriptional program, possibly by acting as a transcriptional factor or cofactor in collaboration with transcriptional machinery.

Author Contributions

All authors listed have made a substantial, direct, and intellectual contribution to the work and approved it for publication.

Disclosure of Potential Conflict of Interest

The authors disclose no potential conflict of interest.

Funding

This work was supported by the PRIN programs of the Italian Ministry of Education, University and Research (MIUR, CUP: B38D19000140006) Fondazione Associazione Italiana per la Ricerca sul Cancro (AIRC, Project: 21509) and Fondazione Cariverona (Enact project).

Acknowledgments

We thank all components of Immunology section of University of Verona (Bronte lab; #Immunologia1) for providing feedback on this manuscript. We would like to thank Biorender for the artwork used in the figure of this manuscript (https://biorender.com, accessed on 24 August 2021).

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