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Molecular characterization of the differential outcome of Rai modulation in astrocytes and T lymphocytes leading to context-specific cellular dysfunction

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Index

Introduction 3

1. **Molecular adaptors: a general overview** 3
2. **Shc adaptor proteins: expert factotum of the cells** 5
3. **The molecular adaptor Rai as a paradigm of a cytosolic protein playing opposite role in lymphocyte and astrocytes in response to external stimuli** 7

3.1. Functions of Rai in lymphocytes 9

3.1.1. Rai negatively regulates T cell receptor signaling 10

3.1.2. Rai negatively regulates Th17/Th1 cell differentiation and autoimmunity 11

3.1.3. Rai promotes adaptation of T lymphocytes to hypoxia in the absence of antigenic stimulation 12

3.2. Functions of Rai in astrocytes 14

3.2.1. Rai promotes the astrocytic reaction to the proinflammatory cytokines IL-17 and IFNγ 15

3.2.2. Rai positively regulates TrkB receptor signaling 17

Aim of the thesis 19

Materials and methods 21

Results 29

1. **Part 1: HIF-1α-induced overexpression of Rai drives PD-1 upregulation by impairing CD28/TCR-dependent phospho-inactivation of GSK-3 in T cells.** 29

1.1. HIF-1α binds to Rai promoter and induces Rai overexpression in T cells 30

1.2. Rai promotes PD-1 expression 31

1.3. Antigen-dependent inactivation of GSK-3 is prevented by Rai 34

1.4 Rai impairs antigen-dependent degranulation of $CD8^+$ T cells 38

2. **Part 2: Rai drives the pro-inflammatory response of astrocytes induced by encephalitogenic T cells** **39**

2.1. Differential proteome profile of control and Rai$^{-/-}$ astrocytes and astrocytes derived extracellular vesicles 40

2.2. Rai participates in the response of astrocytes to oxidative stress by a NRF2-independent mechanism 44

2.3. IL-17-induced activation of Rai/NF-κB/HIF axis drives detrimental response of astrocytes 49

2.4. Modulation of Rai expression during experimental autoimmune encephalomyelitis (EAE) 52

Discussion **54**

Conclusions **61**

References **62**

Introduction

1. Molecular adaptors: a general overview

The appropriate cellular response to environmental cues depends on the activation of different surface receptors and on the proper transduction of the incoming signals through tightly regulated and interconnected signaling pathways that ultimately lead to the activation/inactivation of transcription factors. Therefore, cells respond to environmental stimuli by changing gene expression pattern [1]. The signaling cascades activated by the triggering of surface receptors are able to modulate gene transcription thanks to a series of events including the transient alteration of proteins involved in the intracellular signaling pathways, such as post-translational modifications and/or conformational changes, that result in the formation of larger protein complexes that help to propagate the signals [1]. In this context, a class of proteins playing a crucial role in coupling surface receptors to intracellular signaling pathways, and in integrating different signaling pathways, are molecular adaptors.

Molecular adaptors are defined as proteins lacking enzymatic and transcriptional activities but endowed with protein-binding modules that confer to them the ability to act as scaffold and to build protein complexes in a reversible, dynamic, and inducible manner [1], [2]. The binding modules characterizing molecular adaptors can be divided into interaction domains and binding motifs. The interaction domains are usually conserved regions with high specificity that define the pathway in which the molecular adaptor is involved [3]. For instance, Src homology 2 (SH2) domains are crucial in protein tyrosine phosphorylation pathways, since these domains make proteins able to bind to specific phosphorylated tyrosine residues on other proteins [4]. The binding motifs are instead part of the so-called intrinsically disordered regions. These regions are a significant part of a molecular adaptors' sequence (48%-63%) [5] characterized by the absence of a stable 3D structure [6] but containing well-conserved linear motifs that can be recognized by other domains, allowing the binding to other proteins with high specificity but low affinity, which makes them ideal signaling

hubs [7]. Disordered regions are also very important since they confer structural flexibility to adaptor proteins which contribute to make them multifunctional proteins able to exert different roles depending on signaling pathways or cell types, this phenomenon is known as moonlighting [1].

The most important function of molecular adaptors is to ensure the propagation and amplification of a certain signaling cascade maintaining the correct order of events. To do that adaptor proteins rely on both their ability to act as scaffold for the recruitment of the molecules involved in a given signaling pathway regulating their subcellular localization, and on their ability to properly interact with effector proteins controlling their activities. Thereby, by bringing into proximity and promoting the binding of signaling proteins, molecular adaptors are able to properly propagate signaling pathways regulating them in space and time [2], [3]. One of the best examples in this sense is the Linker for Activation of T cells (LAT), an integral membrane associated molecular adaptor that is essential for proper activation of T cells. Following antigen-dependent activation of the T cell receptor (TCR), LAT is phosphorylated by ZAP-70 on its cytoplasmatic tail containing nine tyrosine residues; once phosphorylated it becomes a molecular hub recruiting SH2-containing proteins thereby leading to the formation of the so-called LAT signalosome which regulates many signaling pathways [8]. Phosphorylation of LAT on Tyr171 is associated with the activation of PI-3K/Akt pathway which promotes cell proliferation and survival, while LAT phosphorylated on Tyr191 and Tyr226 recruits VAV1 promoting the MAP kinase pathway and cytoskeleton reorganization. PLCγ is instead recruited by LAT following phosphorylation on Tyr132, and through the generation of inositol trisphosphate (I_3P) and diacylglycerol (DAG) promotes the intracellular release of Ca^{2+} and the activation of PKC and the Ras family of small GTPases. All these pathways initiated by LAT leads to the activation of transcription factors such as activator protein 1 (AP-1), the nuclear factor-κB (NF-κB) and nuclear factor of activated T cells (NFAT) which are essential for a proper T cell activation [9].

However, molecular adaptors are not only implicated in transducing and amplifying the signals, but they may also attenuate a stimulus-dependent signaling pathway [1]. For example, in the context of

the TCR signaling pathway it has been demonstrated that the molecular adaptor TSAd plays a negative role in the cascade, since the absence of this adaptor is frequently associated with dysregulated activation of T cells and autoimmunity [10].

2. Shc adaptor proteins: expert factotum of the cells

The Src homologous and collagen (Shc) family of molecular adaptors is constituted by four members, known as Shc/ShcA, ShcB/Sli, ShcC/Rai and ShcD/RaLP, which can be expressed, dependent on the cell type, as multiple isoforms, and participate to signaling pathways activated by a wide variety of surface receptors [11].

Shc proteins are small cytoplasmatic adaptors that share a unique and highly conserved modular organization, characterized by an N-terminal phosphotyrosine-binding (PTB) domain, a central collagen homology (CH1) domain rich in proline and glycine, and a C-terminal Src homology 2 (SH2) domain. A second N-terminal collagen homology domain (CH2) is present in the p66Shc, p64Rai and p69RaLP isoforms [11]. The CH1 domain is very important for the signaling functions of these proteins since it is characterized by the presence of three tyrosine residues (Tyr239, Tyr240 and Tyr317) that when phosphorylated become docking sites for proteins containing SH2 domain, whereas the proline-rich region allows the binding of SH3 domain-containing proteins. The function of the CH2 domain, is instead still poor known. It is known that, differently from CH1 it is not phosphorylated on tyrosine but on serine residues, and the phosphorylation on Ser36 of p66Shc isoform has been demonstrated to be essential for cell response to oxidative stress. In addition, a second motif involved in the ROS regulation has been described in the CH2 region of p66Shc. Despite this, whether the corresponding regions on others Shc proteins containing this domain can be phosphorylated and functionally analogous is not known [12], [13]. Finally, the SH2 and PTB domains are both able to bind phosphotyrosine containing sequences, thus allowing the interaction of Shc proteins with surface receptors or other proteins phosphorylated on tyrosine residues following

activation. The presence of both PTB and SH2 domains is a unique feature of the Shc adaptor proteins [14].

Despite the highly conserved structure, there is evidence that Shc proteins are not functionally redundant [15]. ShcA has been the first member of the family to be identified, and it is the most prevalent, since it is expressed in all tissues in adults with the exception of CNS [16]. ShcA is phosphorylated on tyrosine residues following engagement of many surface receptors such as growth factors receptors, hormone receptors, antigen receptors, cytokine receptors, G-protein coupled receptors and integrins, and plays an important role in mitogenic signaling by promoting the activation of the Ras/MAPK pathway [14]. Once phosphorylated ShcA interacts with Grb2, which subsequently interacts with the Ras guanine nucleotide exchange factor Sos. The ShcA-Grb2-Sos complex is then recruited to the membrane thanks to the interaction of the SH2 domain of ShcA with the phosphorylated receptor, an event leading to Ras activation [14], [17]. Furthermore, it has been shown that during mouse embryogenesis ShcA expression is restricted to the cardiovascular system, it is indeed involved in the cardiovascular development both by sensitizing cells to MAPK activation induced by growth factor, and by regulating cytoskeleton rearrangements secondary to integrin-extracellular matrix interaction [11]. Different from ShcA, Sli and Rai have the highest expression in the CNS [18], [19], and they appear to have both unique and overlapping functions in regulating neural development and in promoting survival of post-mitotic neurons [11], [20]. Finally, RaLP is expressed in adult brain, skeletal muscle, and melanocytes. It seems to be involved in the activation of acetylcholine receptors required for synaptic transmission at the level of the neuromuscular junction [21]. Moreover, RaLP expression has been correlated with melanoma progression, with a particular contribution of this adaptor in the acquisition of the migratory phenotype by melanoma cells [22].

Shc proteins are implicated in many cellular processes (growth, apoptosis, life span), and the crucial relevance of these proteins is further highlighted by the dramatic phenotypic alterations resulting from their deletion. Knockout of ShcA indeed resulted in embryonic lethality due to a defective

cardiovascular system development [11], while ablation of Sli and Rai results in a significant loss of neurons in the cervical ganglia [20]. Deletion of Rai is also the cause of neurological deficits and higher mortality in mice subjected to ischemia/reperfusion injury [15].

3. The molecular adaptor Rai as a paradigm of a cytosolic protein playing opposite role in lymphocyte and astrocytes in response to external stimuli

Rai is a member of the Shc family initially considered as a brain-specific adaptor, since it was identified, expressed as 64 and 52 kDa isoforms (Fig. 1), in neural stem cells and mature neurons [17], [18], where it contributes to propagate the pro-survival signaling pathway initiated by the neurotrophin receptors Trk and Ret [17].

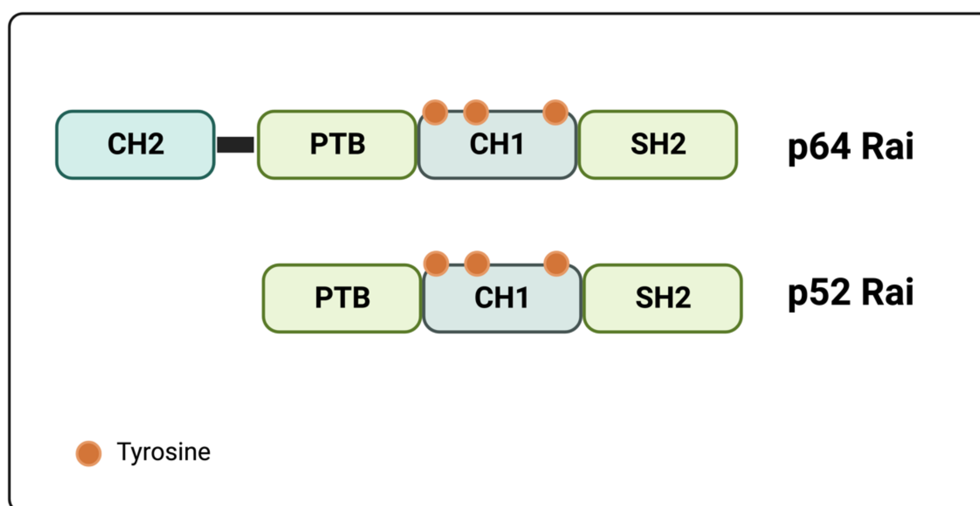


Figure 1. The molecular adaptor Rai. The conserved modular organization of Rai isoforms shown together with the phosphorylatable tyrosine residues on CH1 domain.

In neuronal cells Rai is constitutively associated with p85, the regulatory subunit of PI3K, through its SH2 domain. The stability of the Rai-p85 complex is further increased following glial cell line-derived neurotrophic factor (GDNF)-dependent Ret receptors triggering, which in turns induces phosphorylation of Rai on tyrosine residues. Moreover, activated Ret auto-phosphorylates itself on Tyr1062, which becomes a docking site for multiple proteins including Rai-p85 complex which is

recruited through the PTB domain of Rai to phosphorylated Ret and propagate neuronal survival by coupling Ret to the PI3K/Akt signaling pathway [17] (Fig. 2). The same happens following brain-derived neurotrophic factor-induced activation of TrkB. Neurotrophin stimulation induces the phosphorylation of this receptor on Tyr515 and the consequent interactions with Rai through its PTB domain resulting in activation of the pro-survival PI3K/Akt signaling pathways [23] (Fig. 2).

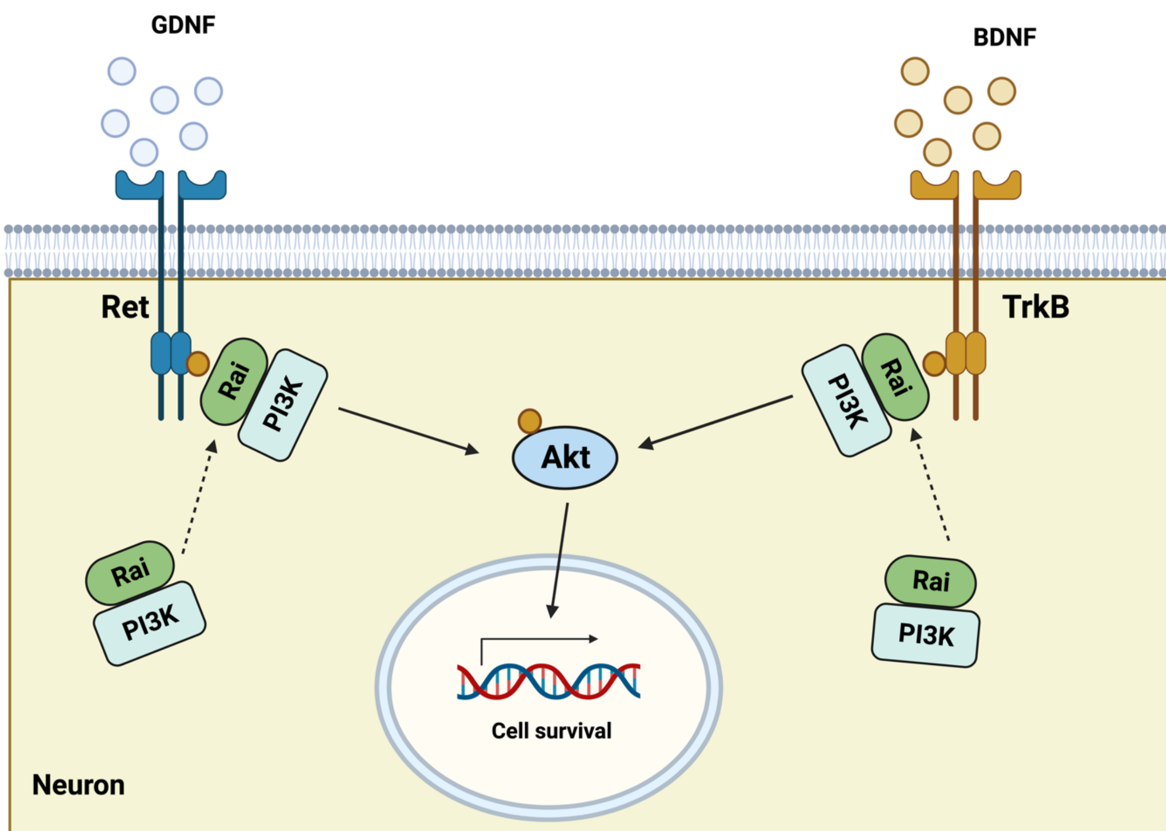


Figure 2. Positive regulation of neurotrophin receptors signaling by Rai in neurons. Rai promotes the pro-survival signaling pathways by coupling the activated receptors Ret and TrkB with the PI3K/Akt signaling pathway.

In addition, further studies have also shown a pro-survival effect of Rai in the regulation of the neuronal response to environmental stresses. Rai indeed plays a protective role, by activating the PI3K/Akt signaling pathway, in a mouse model of ischemia/reperfusion brain injury, as indicated by the more severe neurological deficits, increased apoptosis, and size of infarct area, associated with significantly higher mortality, displayed by Rai$^{-/-}$ mice compared to control mice [15].

The observation that Rai has a crucial role in promoting neuronal cells survival is consistent with its expression pattern during embryonic development which is complementary with the one of ShcA [24]. Rai, as opposite to ShcA, is indeed expressed at low levels in the developing CNS and highly expressed in adult brain, indicating that while ShcA allows proliferation of progenitor cells, Rai is crucial for their differentiation and survival [11].

Recent evidence showed that the expression of Rai is not confined to neuronal cells in the CNS, as expression of both isoforms of Rai has been found in astrocytes [25]. In addition, Rai expression has been detected, albeit at very low levels, also in cells outside the CNS like endothelial cells, smooth muscle cells of the gastrointestinal tract, and in T and B lymphocytes where only the p52 isoform was found [26], [27]. The discovery that Rai is expressed in different cell types where it subserves opposite functions depending on the cell type and the environmental context, as exemplified by its role as a positive or a negative regulator of the same signaling cascade, for example the PI3K/Akt signaling pathway, which is promoted by Rai in neurons [17] and impaired in T cells in response to proliferating stimuli, GDNF and antigen respectively [27] makes this cytosolic adaptor proteins an interesting target to modulate different cell population.

3.1 Functions of Rai in lymphocytes

Peripheral T and B lymphocytes from $Rai^{-/-}$ mice survived longer than their wild-type counterpart when cultured *ex vivo*, indicating that in these cells Rai does not promote cell survival, but instead antagonizes this process [27]. In addition, the absence of Rai in lymphocytes results in their hyper-activation following TCR or BCR engagement, as demonstrated by the increase of Akt phosphorylation to levels significatively higher than those observed in WT lymphocytes [27]. This observation suggests that Rai participates to the PI3K/Akt signaling pathway in lymphocytes, but here it exerts an opposite role compared with neurons, since it is involved in the attenuation of the antigen-dependent activation of Akt. Consistent with the reported activity of Rai as a negative regulator of TCR and BCR signaling, it has been observed that $Rai^{-/-}$ mice are characterized by

splenomegaly and the spontaneous activation, proliferation and survival of T and B cells. Accordingly, Rai$^{-/-}$ mice develop a spontaneous autoimmune glomerulonephritis [27].

3.1.1 Rai negatively regulates T cell receptor signaling

The mechanism by which Rai modulates the TCR signaling cascade has been dissected in a Jurkat T cell overexpression model [28]. The inhibitory effect of Rai on TCR signaling has been tracked to a key early step of the cascade, the recruitment of the kinase ZAP-70 to phosphorylated CD3. Indeed, in response to TCR engagement Rai is recruited to phosphorylated CD3, thereby preventing the recruitment of ZAP-70 and as such dampening of the downstream events [28] (Fig. 3). In addition, Rai is involved in the formation of complexes with both ZAP-70 and the p85 subunit of PI3K in quiescent cells, independently from TCR engagement (Fig. 3). These complexes are not recruited to activated TCR, indicating that Rai also acts by sequestering the two key signaling mediators in an inactive state in the cytosol, further contributing to prevent the full triggering of the TCR signaling cascade [28].

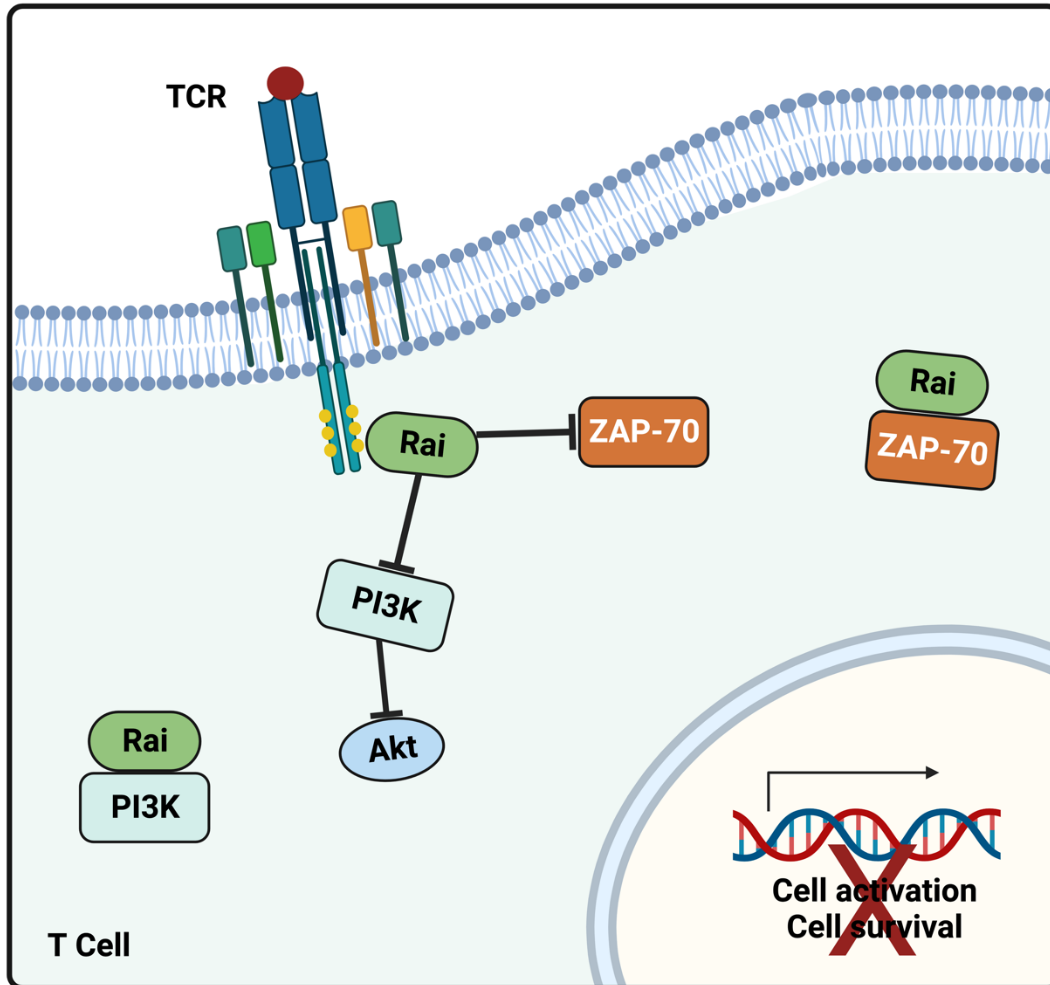


Figure 3. Negative regulation of T cell receptor signaling by Rai. Rai attenuates TCR signaling by preventing the activation of the initiating kinases ZAP-70 and PI3K. This results in the uncoupling of TCR from MAPKs and Akt activation, which finally leads to defective T-cell activation and impaired survival.

3.1.2 Rai negatively regulates Th17/Th1 cell differentiation and autoimmunity

The ability of Rai to fine-tune the antigen receptor signaling pathway also results in a role for this molecular adaptor in the regulation of T cell differentiation toward the pro-inflammatory subsets Th17 and Th1, as confirmed by the lupus-like syndrome spontaneously developed by Rai$^{-/-}$ mice [27]. An increase in circulating Th17 cells has indeed been associated with the development of lupus nephritis in several mouse models of lupus-like disease [29]. Consistent with this, it has been found that Rai deficiency in mice favors the generation and expansion of Th17 and Th1 cell subsets, which by infiltrating in the kidney promote the development of lupus nephritis and are considered the main

cause of renal damage in Rai$^{-/-}$ mice [27], [30]. Indicating that Rai plays a crucial role in limiting autoimmunity not only attenuating lymphocytes activation, but also negatively regulating the development and expansion of pro-inflammatory effector T cells.

In agreement with this, a reduced expression of Rai has been found in peripheral blood lymphocytes (PBLs) of patients with systemic lupus erythematosus (SLE), an autoimmune disease characterized by accumulation of Th17 and Th1 cells [30].

3.1.3 Rai promotes adaptation of T lymphocytes to hypoxia in the absence of antigenic stimulation

To further highlight the multifaceted and context-dependent function of Rai it has been demonstrated that this molecular adaptor, as opposed to the negative regulation of TCR signaling, can play a protective role in T cells, in the absence of antigenic stimulation, by promoting their survival under hypoxia [31].

Hypoxia is a condition characterizing tissues that are deprived of an adequate oxygen supply. It usually arises when the oxygen demand exceeded the amount of oxygen delivered, as happens during rapid cell turnover, immune cell infiltration and vascular disruption [32].

Cellular responses to hypoxic conditions are all mediated by the transcription factor hypoxia inducible factor (HIF)-1 [33], a heterodimer constituted by a constitutively expressed HIF-1β nuclear subunit and an oxygen-labile HIF-1α subunit. The latter in presence of oxygen is hydroxylated by prolyl hydroxylase-domain protein 1-3 (PHD1-3), ubiquitinated by von Hippel-Lindau (VHL) proteins and then directed to proteasome degradation. Conversely, in hypoxic conditions, HIF-1α is not targeted for degradation and can translocate to the nucleus, where dimerizes with HIF-1β and binds to hypoxia-responsive elements (HRE) on the promoter of target genes [33], [34].

Under hypoxia overexpression of Rai correlates with an increase in Akt phosphorylation and a decrease of pro-apoptotic markers, including caspase activities, leading to increased cell survival of T cells [31], resembling the same protective mechanisms activated by Rai in neurons exposed to

ischemic insults [15]. Interestingly, when Rai is overexpressed as in the case of the overexpressing Jurkat T cell transfectants, it promotes T cell adaptation to a hypoxic environment by increasing glucose uptake through the enhancement of GLUT-1 [31].

Severe hypoxia is a hallmark of many pathological conditions, such as inflammation, infection, and cancer, in which it can modulate the immune cells leading to a dysfunctional immune response and to disease development [35].

T lymphocytes are the major players in the adaptive immune system, as such following the encounter with antigen presenting cells (APCs) a large number of activated antigen-specific T cells migrate toward the site of the disease (i.e., tumoral mass, infected area, etc.) in order to exert their defensive functions [36]. However, it has been demonstrated in chronic infections and cancer that effector T cells migrated into the diseased area enter in a dysfunctional state defined T cell exhaustion [37].

This state of T cell dysfunction has been described for the first time in a murine model of chronic lymphocytic choriomeningitis virus (LCMV) infection [37], and it is characterized by the progressive loss of effector functions as well as transcriptional and metabolic changes and resistance to reactivation [38]. The main marker used to distinguish exhausted T cells is the overexpression of the inhibitory receptor PD-1 on their surface [37], but exhausted T cells also show increased expression of other inhibitory receptors such as cytotoxic T lymphocyte antigen-4 (CTLA-4), lymphocyte activation gene-3 (LAG-3), T cell immunoglobulin and mucin domain 3 (Tim-3), T-cell immunoglobulin and immunoreceptor tyrosine-based inhibitory motif domain (TIGIT) [38].

To date T cell exhaustion has been casually associated with the prolonged activation of T cells occurring during cancer or infections that leads to a sustained expression of inhibitory receptors [38]. However, emerging concepts indicate that the main cause of T cell exhaustion may be the metabolic stress to which T cells are subjected in a pathological environment. In particular, hypoxia seems to be one of the major drivers of T cell exhaustion, since it has been demonstrated that in TME drives the upregulation of inhibitory receptors such as CTLA-4 and LAG-3 [39] and impairs the ability of T cells to gain energy from oxidative phosphorylation [40]. On these bases, the role played by Rai in

promoting T cell adaptation to hypoxia, together with the inhibitory effect on TCR signaling pathway, raise the possibility for a potential role of this adaptor in promoting T cell exhaustion.

3.2 Functions of Rai in astrocytes

Astrocytes are the predominant glial cells in the CNS which perform important functions for the maintenance of CNS homeostasis [41]. Recently they have been also emerged as key regulators of CNS diseases [42]. They populate the entire brain and undergo different maturation processes depending on their specific localization which results in the acquisition of a high functional specialization, on which rely their multifaceted functions [43].

Astrocytes contribute to CNS homeostasis by providing trophic support to neurons through the release of neurotrophic factors such as BDNF and GDNF which promote neuronal survival. In addition, they regulate synaptogenesis and modulate synaptic transmission through the release and clearance of neurotransmitters, such as GABA, glutamate and D-serine, as well as the regulation of extracellular ion concentration [44],[45]. Furthermore, astrocytes end-feet constitute the glia limitans thereby contributing to the formation and maintenance of the blood brain barrier (BBB) [46] and regulating the diffusion of soluble factors across the BBB itself [47].

It has been demonstrated that astrocytes contribute to the pathogenesis of multiple sclerosis (MS) [42]. During MS, inflammation of the CNS is associated with the breakdown of the BBB and infiltration of peripheral immune cells. Infiltrating self-reactive immune cells, as well as CNS-resident cells start to release pro-inflammatory factors to which astrocytes respond by activating complex signaling pathways that drive the modulation of their phenotype in a highly heterogeneous manner, a profound transformation known as “reactive astrocytosis” [48].

Reactive astrocytes are characterized by thicker end-feet, enlargement of the cellular body and increased expression of glial fibrillary acidic protein (GFAP) [49], [50]. Despite these common features, reactive astrocytes modulate their phenotype in a highly heterogeneous manner, reflecting the existence of different subpopulation of astrocytes that can either promote the disease by releasing

pro-inflammatory mediators, or limit the disease and support neuroprotection [48], [51], [52]. To date, the signaling pathways driving astrocyte reactivity are still largely unknown, but activation of the transcription factor NF-κB has been casually linked to the neurotoxic activity of reactive astrocytes, since the blockade of NF-κB activation in mice during the experimental autoimmune encephalomyelitis (EAE) improves the clinical outcome and is associated with a reduction of pro-inflammatory cytokines levels and oxidative stress [53]. The transcription factor associated with neuroprotective functions is instead STAT3, as demonstrated by the fact that conditional knockout of STAT3 in astrocytes is associated with an impairment in remyelination process and worsening of EAE [54]–[56].

In this context, astrocytic Rai plays a key role in promoting disease development. It has been indeed found that $Rai^{-/-}$ mice developed a milder EAE, characterized by a lower clinical score, delayed onset and lower incidence compared to control mice, despite the enhanced generation of myelin-specific Th17 cells able to infiltrate into the CNS ensured by Rai deficiency [25], [27]. Thus, indicating that the lack of Rai in astrocytes dampens their pro-inflammatory response to infiltrated encephalitogenic T cells.

3.2.1 Rai promotes the astrocytic reaction to the proinflammatory cytokines IL-17 and IFNγ

Multiple sclerosis is an autoimmune neurodegenerative disease characterized by the presence of demyelinated lesions in the brain and spinal cord and in periphery by an abnormal polarization of $CD4^+$ T cells toward the pro-inflammatory subsets Th17 and Th1, associated with a defective activation of regulatory T cells (Treg), which are involved in the suppression of effector activities of T cells [57]. The pathological mechanism that drives neurodegeneration is believed to be primarily mediated by auto-reactive $CD4^+$ T cells that infiltrate into the brain and spinal cord by crossing the BBB and recognize CNS antigens [42].

As components of the BBB, astrocytes are the first CNS-resident cells encountered by infiltrating encephalitogenic T cells [46]. Soluble factors released by infiltrating T cells, as well as by other CNS

cells during MS, drive astrocytes reactivity, which can respond to pro-inflammatory cues released by immune cells either by propagating neuroinflammation or counteracting effector functions of infiltrating immune cells. For instance, the Th1 signature cytokine IFNγ induces the expression of IFNγ receptor 1 (IFNGR1) and MHCII on astrocytes, allowing them to promote T cell activation by acting as non-professional antigen presenting cells (APCs) [58], [59]. In addition, IFNγ can also sustain inflammation by inducing the secretion of CCL2 and CXCR10 from astrocytes with a mechanism controlled by NF-κB, which contribute to recruit peripheral immune cells to the CNS [60], [61].

Evidences obtained in MS patients and EAE mice highlighted the Th17 subset as the $CD4^+$ T cells subpopulation playing the most important role in disease pathogenesis [62]. Self-reactive Th17 cells express CCR6, so they are recruited to the BBB thanks to the local production of their ligand CCL20 and then infiltrate into the CNS where promote neuroinflammation by releasing pro-inflammatory cytokines such as IL-17A [63], [64].

Astrocytes express the IL-17A receptor (IL-17AR), and as such are target of Th17 cells into the CNS [65]. IL-17A signaling cascade promotes a pro-inflammatory response of astrocytes, and a crucial component for the propagation of this signaling pathway, the molecular adaptor Act1, which couple IL-17AR to NF-κB has been identified [66]. Moreover, in response to pro-inflammatory IL-17 and GM-CSF, astrocytes can activate transcriptional programs leading to production of pro-inflammatory cytokines including IL-1β, IL-6, iNOS, TNF and BAFF [43], [50].

Interestingly, it has been demonstrated that in astrocytes lacking Rai there was a strong impairment in the IL-17-dependent activation of the NF-κB pathway, which is essential for IL-6 production, a pro-inflammatory cytokine centrally implicated in neurodegeneration [67], indicating that Rai is component of the signaling cascade triggered by IL-17 [25]. Furthermore, astrocytes lacking Rai showed an impairment in iNOS expression and nitric oxide (NO) production in response to both endogenous factors (BDNF) and T cell-derived pro-inflammatory cytokines (IL-17 and IFNγ) [25].

The expression of iNOS, the enzyme responsible for the generation of NO [68], is induced in astrocytes by pro-inflammatory signals, such as IFN-γ and IL-17 [69], therefore Rai is supposed to be a positive regulator of all these signaling pathways leading to iNOS expression and consequent NO production.

Accordingly, astrocytic Rai has been demonstrated to promote the Th17-dependent production of pro-inflammatory mediators IL-6 and NO through the activation of NF-κB [25].

In addition, beside positively regulating the production of pro-inflammatory mediators (IL-6, NO) induced by Th17 cells, astrocytic Rai controls the pathogenic potential of autoreactive T cells by impairing the astrocyte-dependent conversion of extracellular ATP into the immunosuppressive molecule adenosine through the enhancement of CD39 activity [70] (Fig. 4). In agreement with a role of Rai as a critical mediator of the lymphocyte-dependent astrocyte reaction it has been found that soluble factors released by MOG-specific encephalitogenic T cells induced higher expression of two transcripts known to be upregulated in neuroprotective astrocytes, Emp1 and S100a10, in $Rai^{-/-}$ astrocytes compared with control astrocytes [70].

3.2.2 Rai positively regulates TrkB receptor signaling

In addition to attenuate the inflammatory response to factors released by infiltrating self-reactive T cells, Rai deficiency in astrocytes also attenuates the pro-inflammatory response of these cells to endogenous factors like BDNF [25] (Fig. 4).

It has been demonstrated that the BDNF-dependent activation of TrkB receptor in astrocytes is one of the main causes leading to neurodegeneration [71]. An enhanced expression of this receptor in astrocytes has been found in EAE and MS lesions, in addition it has been observed that mice lacking astrocytic TrkB were protected from neurodegeneration associated with EAE [71]. Based on this evidence, it has been demonstrated that TrkB engagement in astrocytes activates a signaling pathway leading to a strong production of the neurotoxic mediator NO which in turn induces neuronal degeneration and apoptosis [71]. In this context, consistent with the reported ability of astrocytic Rai

to promote the inflammatory response following immune infiltration in the CNS, it has been found that the BDNF-induced production of NO was significantly reduced in Rai deficient-astrocytes [25]. The diminished production of NO was paralleled by a reduction of CREB phosphorylation in these cells suggesting that Rai couples TrkB receptors to CREB [25]. CREB is indeed the transcription factor regulating the expression of many TrkB-induced genes, including the gene encoding iNOS. Of note, Rai subserves the same function in neuron and astrocytes respect to the TrkB-dependent activation of the TrkB signaling pathway leading to the activation of Ras/MAPK pathway [17], [23], [25].

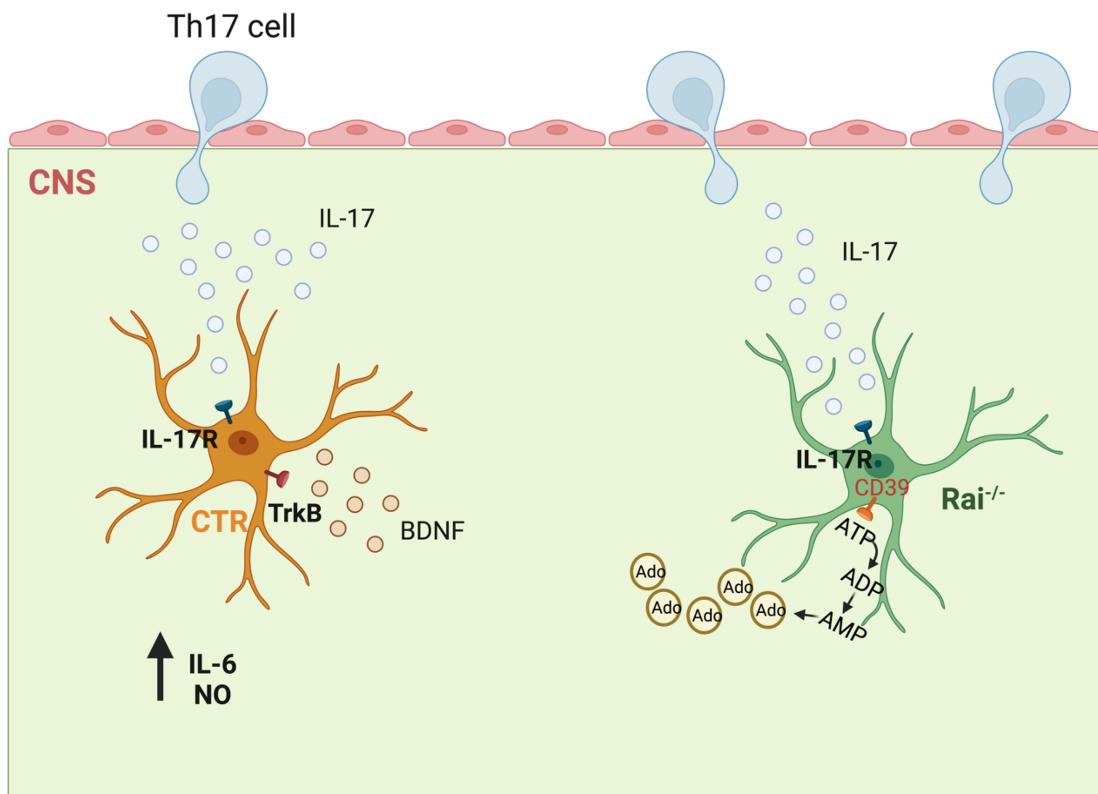


Figure 4. Differential Rai-dependent response of astrocytes to pro-inflammatory stimuli. Rai promotes the detrimental response of astrocytes to IL-17 by enhancing the production of IL-6 and NO. Conversely, astrocytes lacking Rai promotes the generation of an immunosuppressive environment by increasing the levels of extracellular adenosine.

Aim of the thesis

Molecular adaptors are proteins lacking enzymatic and transcriptional activities that play crucial functions in the regulation of cellular response to environmental cues. They can indeed act as positive regulators by propagating and amplifying an incoming signal, as well as they can act as negative regulators attenuating or inhibiting a stimulus-dependent intracellular signaling pathway. Hence, modulation of expression levels of molecular adaptors both under physiological and pathological conditions may result in the alteration of cellular functions including the adaptation of cells to a specific microenvironment.

Emerging evidences highlighted that molecular adaptor Rai, first identified in neuron where it exerts a pro-survival function mediated by the PI3K/Akt signaling pathway [17], supports the detrimental reaction of astrocytes in the EAE mouse model of multiple sclerosis in response to encephalitogenic T cell by positively regulating the production of pro-inflammatory mediators (IL-6 and NO) and by impairing the astrocyte-mediated conversion of extracellular ATP to the T cell suppressive molecule adenosine through the inhibition of CD39 activity [25], [70].

In this work we aimed to characterize in depth the role of the Rai-dependent signaling pathway in astrocyte reaction by investigating not only the potential outcomes of Rai modulation/deletion in astrocytes, but also its potential involvement in the composition of the protein cargo of extracellular vesicles, the nano-sized membrane vesicles secreted by all cells which, with their cargo of bioactive molecules (proteins, nucleic acids and lipids), actively shape the CNS microenvironment [72].

Beside central nervous system, Rai is involved in T cell function by controlling the TCR-dependent activation and proliferation [27], [28], [30]. While opposite to neuron and astrocytes, Rai is expressed at low levels in circulating T cells from healthy donors, downregulation of its expression in T cells from patients with lupus systemic erythematosus autoimmune disease has been reported suggesting an important biological output of reduced Rai expression in T cells [30]. Transcriptional regulators

of *rai* in T cells are at present unknown. Hence, the second aim of the thesis was to investigate whether enhanced expression of Rai in primary human T cells may account for their unresponsiveness, focusing on hypoxia, a novel driver of tumor microenvironment-induced T cell exhaustion, and to identify transcriptional regulators of Rai in T cells.

Results will provide new insights on the different context-dependent functions played by Rai associated with disease development and on the molecular mechanisms impacted by this molecular adaptor, thereby opening the possibility to identify novel therapeutic targets in T cells and astrocytes including Rai as well.

Matherials and methods

Mice

C57BL/6J Rai$^{-/-}$ mice and C57BL/6J controls were used. Mice were housed in the animal facility at the University of Siena in pathogen-free and climate-controlled (20 ± 2◦C, relative humidity 55 ± 10%) conditions. The cages were provided with mouse houses and nesting material as environmental enrichment, and mice were provided with water and pelleted diet *ad libitum*. Procedures and experimentation were carried out in accordance with the 2010/63/EU Directive and approved by the Italian Ministry of Health.

Induction of EAE

Anesthetized WT and Rai$^{-/-}$ female mice, 8- to 10-week old, received a subcutaneous injection of 200 g MOG3555 peptide (MEVGWYRSPFSRVVHLYRNGK, purity > 85%, Espikem) emulsified in an equal volume *Mycobacterium tuberculosis* H37Ra (Difco Laboratories) in PBS. On day 0 and 2 mice were i.p. injected with 300 ng of Bordetella pertussis toxin (Calbiochem) in PBS. Mice were monitored daily by two independent observers for neurological signs of EAE and clinical score was assigned according to a standard 0 to 5 scale, as previously described [25]. Another group of age matched Rai$^{-/-}$ and C57BL/6J female mice was injected with the same volume of CFA/PBS, as negative control. Brains were collected from EAE mice 20 days and 30 days post immunization for RNA extraction.

Primary astrocyte culture and treatments

Astrocytes were purified from newborn (2-day-old) Rai$^{-/-}$ and C57BL/6J mice as described by using the Neural Tissue Dissociation kit (T) (Miltenyi Biotec). Glial cells were cultured in flasks and maintained in Dulbecco's Modified Eagles Medium (DMEM) supplemented with 10% BCS and 20 U/ml penicillin. After two weeks the microglia-containing supernatant were collected and plated, while adherent astrocytes were trypsinized and replated. Purity of astrocytes and microglia was

assessed by flow cytometry using anti-GFAP mAb (clone GA5, eBioscence) and anti-CD11b mAb (clone M1/70, BD-Biosciences) respectively (% GFAP+ > 95%, %CD11b+ > 95%).

Astrocytes were treated with IL-17 for 24 h for qPCR analysis of HIF-1α transcripts, and for immunoblot analysis of HSP70 expression. To detect the degree of NF-κB phosphorylation cells were instead treated with IL-17 for 15 min. To assess the modulation of Rai expression in WT astrocytes by qPCR cells were exposed for 24 h either to a treatment with a combination of IL-17 and IFNγ or to conditioned media from IL-2- stimulated MOG-T cells.

Viability assay and *tert*-butylhydroquinone treatment of astrocytes

To evaluate astrocytes viability following oxidative stress WT and Rai$^{-/-}$ astrocytes were seeded in 6 well-plate (0.5 x 10^6 astrocytes/well) and treated with 1mM H_2O_2 in serum free medium for 24h. After treatment cells were washed two time with PBS and harvested by using trypsin/EDTA solution (Sigma). Cell pellets were resuspended in fresh PBS. The disruption of membrane integrity was determined by adding Propidium Iodide (50 mg/ml) for 1 min. Samples were acquired on Guava Easy Cyte cytometer (Millipore) and the percentage of viable cells (PI-negative) was measured and analyzed with FlowJo software (TreeStar Inc.). To assess the effect of *tert*-butylhydroquinone (tBHQ) on the viability of cells exposed to oxidative stress the same assay was performed, but in this case cells were first pretreated with tBHQ or ethanol for 24h, and then exposed to H_2O_2 treatment.

Human Primary T cell isolation and culture

The collection of peripheral blood samples from anonymous healthy donors was approved by the local ethics committee (Siena University Hospital) and performed after receiving signed informed consent according to institutional guidelines. Total T cells were purified by negative selection using RosetteSep™ Human T cell enrichment cocktail (STEMCELL Technologies) following manufacturer's instructions. Cells were then cultured at 37°C, 5% CO_2 in RPMI-1640-sodium

bicarbonate medium (Merck), supplemented with 10% bovine calf serum (GE Healthcare HyClone) and penicillin 50 IU/ml.

Hypoxic treatment

Human primary T cells were cultured for 24 hours at 2% O_2, 5% CO_2 and 94% N_2, in a hypoxia glove box (Coy Lab Products). Normoxic controls were kept at atmospheric O_2 levels in a cell culture incubator.

T cell nucleofection and treatments

$5x10^6$ freshly isolated T cells were co-transfected with 0,5 μg of pMAX-GFP and 1 μg of either pcDNA3.1-Rai or empty plasmid using homemade buffer 1M (5 mM KCl, 15 mM $MgCl_2$, 120 mM Na_2HPO_4/NaH_2PO_4 pH 7.2, and 50 mM mannitol) [73] and program V-024 of an Amaxa Nucleofector II (Lonza). T cells were allowed to recover in complete RPMI-1640-sodium bicarbonate medium and transfection efficiency was evaluated after 24h by flowcytometry and/or western blotting. Transfected cells were then stimulated for 24h at 37°C with plate bound 1μg/ml of anti-CD3 (clone UCHT1, BioLegend #300402) and 2μg/ml of anti-CD28 (clone CD28.2, BioLegend #302902) for qPCR analysis and for FACS analysis of PD-1 expression. The anti-CD3 + anti-CD28 coating was prepared in PBS, added to 12 or 24 well plates (Sarstedt) and then incubated for 5h at 37°C. After incubation coating was removed by PBS washing before cells were added. For FACS analysis of GSK-3 α/β and Akt phosphorylation T cells were stimulated for 5 minutes at 37°C in serum-free RPMI-1640-sodium bicarbonate with anti-CD3 (1μg/ml) + anti-CD28 (2μg/ml) antibodies.

siRNA transfection

Silencing experiments were carried out using a specific small interfering RNA (siRNA) targeting HIF-1α (Silencer®Select Validated siRNA, siRNA ID #s6541) and a control siRNA (Silencer®Select Negative Control #1 siRNA Cat. n° 4390843), purchased from Ambion. Briefly, cells were seeded in

a 6 well plate (Sarstedt,) at a concentration of 2.000.00 cells/well in RPMI 1640 without antibiotics, then transfection of 46 nM siRNAs was performed by using lipofectamine RNAi MAX (Invitrogen) diluted in OPTI-MEM® (1X) (Gibco, Thermo Fisher Scientific) according to the manufacturer's instructions. Cells were incubated for 24 h at 37°C under normoxic conditions (20,9% O_2 and 5% CO_2). Then the growth medium was replaced and supplemented with antibiotics, and cells were transferred for 24 h to hypoxic conditions (2% O_2 and 5% CO_2) or maintained in a normoxic environment. Silencing efficiency was evaluated by Western Blotting, using HIF-1α primary antibody (BD Biosciences) and β-actin (Sigma-Aldrich). Anti-mouse IgG HRP (1:5000; Cell signaling) was used as secondary antibody.

ChIP assay

Freshly isolated primary T cells ($2x10^6$) from healthy donors were kept for 24 hours under hypoxic conditions and then were used to perform ChIP assay, according to MAGnify Chromatin Immunoprecipitation System protocol (Thermo Fisher Scientific), in order to assess HIF-1α binding to the two identified putative HRE regions on *rai* promoter. In brief, cells were fixed with 1% formaldehyde for 10 minutes at room temperature then were lysed and exposed to 10 cycles of sonication (10 seconds on at 20% of power and 10 seconds off for each cycle) using SONOPULS Ultrasonic Homogenizers HD 2070 (BANDELIN) to obtain chromatin fragments of 200-500bp. Immunoprecipitation were performed using 5μg of anti-HIF1α (clone PA3-16521, Invitrogen) or rabbit IgG as a control. The immunoprecipitated chromatin fragments were quantitated using qPCR. The primer pairs used to amplify HRE1 and HRE2 regions are listed in table below.

Cell lysis and Immunoblots

Cells were lysed in 1% (v/v) Triton X-100 in 20 mM Tris-HCl (pH 8), 150 mM NaCl in the presence of Protease Inhibitor Cocktail Set III (Cal BioChem) and 0.2 mg Na orthovanadate/ml. Proteins were resolved by SDS-PAGE and transferred to nitrocellulose membrane (GE Healthcare Life Sciences)

for immunoblotting analysis. Immunoblots were carried out using the following primary antibodies: anti-HSP70 (clone EP1531Y, Abcam), anti-PhosphoNF-kBp65 (clone 93H1, Cell Signaling Technology), anti-Rai (clone 23. BD), anti-Actin (clone C4, Millipore) and peroxidase-labeled secondary antibodies. Labeled Abs were detected using the ECL kit (SuperSignal® West Pico Chemiluminescent Substrate, Thermo Scientific) and scanned immunoblots were quantified using the ImageJ software.

Flow cytometry

Surface expression of PD-1 was assessed on transfected T cells following 24h of treatment by labeling the cells with anti-PD-1 (Genetex) primary antibody and Alexa Fluor-647 anti-mouse secondary antibody (Thermo Fisher Scientific). Pospho-GSK-3α/β and pospho-Akt were quantitated following 5 minutes stimulation in transfected T cells fixed and permeabilized using fixation and permeabilization buffers (Biolegend) and stained with anti-pospho-GSK-3α/β (R&D systems) or anti-pospho-Akt (Cell Signaling) primary antibodies and Alexa Fluor-647 anti-rabbit secondary antibody (Thermo Fisher Scientific). Surface staining to evaluate the percentage of $CD8^+$ $CD57^+$ population was performed on freshly isolated T cells by using PE-conjugated anti-CD8 (clone RPA-T8, Biolegend) and PerCP-conjugated anti-CD57 (clone HNK-, Biolegend) antibodies. Samples were acquired on Guava Easy Cyte cytometer (Millipore) and analyzed with FlowJo software (TreeStar Inc.)

For the degranulation assay were used T cells with a percentage of $CD8^+$ $CD57^+$ (effector T cells) population higher than 10% of total. Raji B cells were loaded with a mixture of SEA, SEB and SEE peptides (2μg/ml) (Toxin Technology) or 1% BSA for controls, and incubated for 1h at 37°C. Then, T cells transfected with pMAX-GFP and either pcDNA3.1-Rai or empty vector were added in a 10:1 ratio and incubated for 4 hours in presence of APC anti-LAMP1 antibody (clone H4A3, Biolegend #328620). Monensin (2mM, Biolegend #420701) was added 1h after the incubation start to block vesicular recycling. At the end of the incubation cells were also stained with PE anti-CD8 antibody

(clone RPA-T8, Biolegend #301008) and the degranulation extent was measured among the GFP+ population as the percentage of $CD8^+$ T cells expressing LAMP-1 on their surface by flow cytometry analysis.

Sample preparation for proteomic analysis

$40x10^6$ astrocytes/sample were seeded in $175cm^2$ flasks (7×10^6 cells /flask) and treated or not with IL-17 (50 ng/ml) in a serum free medium for 24 h. After the treatments, cells and conditioned media were collected. Astrocyte derived extracellular vesicles (ADEVs) were purified from the cell media by differential ultracentrifugation. Briefly, free cells and cellular debris were removed by two centrifugation steps, the first at 300 x g for 10 min and the second at 100.000 x g for 30 min at 4°C. The resulting supernatants were centrifuged at 100.000 x g for 1.30 h at 4°C to pellet ADEVs. The pellets were resuspended in PBS and washed by an additional centrifugation at 100.000 x g for 1.30 h at 4°C. Then the pellets were solubilized in a denaturation solution composed of 8 M Urea, 2 M Thiourea, 4% w/v 3-[(3-cholamidopropyl) dimethylammonia]-1-propanesulfonate hydrate (CHAPS) and 1% w/v dithioerythritol (DTE). To prepare cells for proteomic analysis, adherent cells were washed two times with PBS. Cells were collected and washed once in PBS. The resulting pellets were solubilized in a denaturation solution composed of 8 M Urea, 2 M Thiourea, 4% w/v 3-[(3-cholamidopropyl) dimethylammonia]-1-propanesulfonate hydrate (CHAPS) and 1% w/v dithioerythritol (DTE). Eventually, denaturation solution and traces of bromophenol blue were added to samples carrying the same protein amount in 350 µl solution for the analytical run and 700 µg in average in 450 µl solution for the preparative run. After estimation of protein concentration by Bradford's assay, protein extracts were running on the Immobiline-polyacrylamide system to perform 2-D Electrophoresis as previously described [74]. Immobilized nonlinear pH 3-10 gradient on strips 18 cm in length (GE Healthcare) were employed in the first dimensional run.

Parametric and non-parametric , Mann-Whitney, Wilcoxon, Kruskal-Wallis, FDR and ANOVA by IBM SPSS® software platform were used to compare the percentage of relative volume (%V: Vol of a single spot divided by the total volume of spots computed over the whole image and expressed in percentage) of the 2DE protein spots among the groups. Particularly, only differentially abundant spots between two conditions with a p- -fold change in the ratio of the %V means were considered statistically significant. In order to visualize the behaviour of the differentially abundant spots in the considered conditions a heatmap analysis using the %V values of the statistically significative abundant spots was performed. In particular, the clustering of protein spots was performed using Ward ́s clustering method and Euclidean distance. Spots of interest were identified by peptide mass fingerprinting (PMF) on an UltrafleXtreme™ MALDI-ToF/ToF instrument (Bruker Corporation). Identified protein species were used to perform functional and pathways analysis by STRING software.

Network and pathway analysis

Network and pathway analysis were performed submitting the accession number of the identified proteins to the MetaCore 6.8 network building tool (Clarivate Analytics). This software shows a network of protein interactions, graphically represented by "nodes" (proteins) and "arches" (interactions), by the "shortest-path" algorithm. This algorithm builds a hypothetical network connecting two experimental proteins directly or indirectly using one MetaCore database protein, based on information from scientific literature data and annotated databases of protein interactions and metabolic reactions. The relevant pathway maps were then prioritized according to their statistical significance ($p \leq 0.001$).

RNA Purification, Reverse Transcription and qPCR

For all the experiments RNA was extracted from samples by using the RNeasy Plus Mini Kit (Qiagen) according to the manufacturer's instructions, and RNA purity and concentration were measured using QIAxpert (Qiagen). Single-strand cDNAs were generated using the iScriptTM cDNA Synthesis Kit

(Bio-Rad), and qPCR was performed using the SsoFastTM EvaGreen® supermix kit (Bio-Rad) and specific pairs of primers listed in the table below (h = human, m = murine). Samples were run in triplicate on 96-well optical PCR plates (Sarstedt AG). Values are expressed as ΔΔCT relative to housekeeping gene HPRT (for human T cells) or GAPDH (for mouse astrocytes) expression.

Gene	Forward 5'-3'	Reverse 5'-3'
hRai	TTACCAGGGAAGCCATCAG	TTGCTCTTTCCCAAGATGCT
HRE1	TGTTAGACACTTTTACAGGCTCAC	TCCTTCAAATCGTCAAATGAACTT
HRE2	CGCTCCATCAGAGGCAACTA	ACCGTTAAAAGCCAGCACAG
hT-bet	TAATAACCCCTTTGCCAAAGG	TCCCCCAAGGAATTGACAGT
hPD-1	GTGTCACACAACTGCCCAAC	CTGCCCTTCTCTCTGTCACC
hHPRT	GTAGCCCTCTGTGTGCTCAA	TCACTATTTCTATTCAGTGCTTTGATG
mHIF-1α	TGCTTACACACAGAAATGGCCC	TATGGCCCGTGCAGTGAAGC
mRai	TGTGCCAGGTGCCAAAGG	GCGGTGGAGATGGTCAGG
mGAPDH	AACGACCCCTTCATTGAC	TCCACGACATACTCAGCAC

Statistical analysis

Two-way ANOVA with post hoc Bonferroni's test was used for all the other experiments in which multiple groups were compared. Either paired or unpaired *t*-test were used to determine the statistical significance of differences between two groups. GraphPad Prism Software (Version 8.4.2) was used for statistical analyses. A $p < 0.05$ was considered as statistically significant.

Results

Part 1: HIF-1α-induced overexpression of Rai drives PD-1 upregulation by impairing CD28/TCR-dependent phospho-inactivation of GSK-3 in T cells.

Hypoxia is the main feature of many pathological conditions, such as infection and cancer, which has been demonstrated to impact on immune cell functions, thus shaping innate and adaptive immune responses [35]. Recently, it has been shown that hypoxia can promote the acquisition of an exhausted phenotype by T cells [40]. T cell exhaustion is a state of dysfunction characterized by the progressive loss of effector functions by T cells and the increased expression of co-inhibitory receptors on their surface. According to this, a direct involvement of the transcription factor HIF in the induction of inhibitory CTLA-4 and LAG-3 receptors expression has been demonstrated [39]. In addition, inhibition of HIF-1α, as well as reduction of hypoxia by oxygenation, have been shown to reactivate T cells and restore their effector functions [75], [76].

Despite the evidence reported above and showing a key role of HIF-1α and hypoxia in the acquisition of an exhausted phenotype of T cells, molecular mechanisms underlying this process are largely unknown, including the signaling pathway controlling the upregulation of PD-1, the main marker of exhausted T cells.

It has been previously demonstrated that under physiological condition, the molecular adaptor Rai is expressed in T cells even if at lower levels compared with neurons [27]. Despite the constitutive low amount of this adaptor in T cells, data obtained in murine Rai-deficient T cells demonstrate that it is required to maintain a fine balance of signals regulating T cell activation and differentiation [27], [30]. Mechanistically, it negatively regulates T cell activation and proliferation, by preventing antigen-dependent activation of ZAP-70 and PI3K, two key mediators of TCR signaling[27], [28].

In agreement with an important biological output of aberrant Rai expression in T cells, reduced expression of Rai in human peripheral blood lymphocytes (PBLs) from systemic lupus erythematosus

(SLE) and multiple sclerosis (MS) patients, characterized by hyperactivation of T cells and peripheral accumulation of Th1/Th17 subsets, has been documented [30], and unpublished results.

To date overexpression of Rai in primary T cells, both humans and murine, has not been reported and the regulatory mechanism of Rai expression in T cells is largely unknown. Based on the results obtained in Rai overexpressing Jurkat cells, showing that Rai restrains TCR-dependent T cell activation and proliferation by impairing ZAP-70 recruitment to CD3 and PI3K/Akt activation, here we have investigated whether the dysregulated expression of this molecular adaptor may drive T cell exhaustion focusing on the impact of hypoxia.

1.1 HIF-1α binds to Rai promoter and induces Rai overexpression in T cells

Emerging evidence indicate that T cell exhaustion is not only due to the persistent T cell exposure to antigenic stimulation, but this dysfunctional state is the result of metabolic stress to which T cells are subjected in pathological conditions as cancer. It is known that cancer cells consume a huge amount of glucose to gain energy for rapid cell division through glycolysis, that lead to hypoglycemia whitin the tumor microenvironment (TME). In addition, the rapid growth of the tumor does not allow a proper vascularization, leading to hypoxic areas inside the tumoral mass [40]. Thus, when T cells infiltrate solid tumors have to face the situation in which the lack of glucose and oxygen prevents them to produce energy, respectively through glycolysis and oxidative phosphorylation, necessary for their full activation [40]. The incomplete activation of T cells thus leads them to an anergic and hyporesponsive state [77].

Moreover, hypoxia induces a series of responses in T cells which try to adapt to this condition. All the cellular responses to hypoxic stress are mediated by HIF-1, the master regulator of hypoxia, that by enhancing expression of genes with HRE [78], controls metabolic and angiogenic processes as well as cell survival, differentiation, and apoptosis [31], [34]. Based on this evidence, and on the fact that Rai overexpression in the Jurkat T cell line, promotes cell survival under hypoxia [31], we asked whether HIF-1 might control Rai expression in T cells exposed to hypoxic conditions.

We therefore used JASPAR software to analyse *in silico* the promoter region of *rai* gene (0.9-kb, encompassing nucleotides -958 to + 1) looking for putative binding sequences for HIF-1α. The analysis revealed two putative HRE regions, named HRE 1 (from -616 to -606) and HRE 2 (from -239 to -229), with a high relative profile score (> 80%) on *rai* promoter (Fig. 5A). To validate the results of the *in silico* analysis, $CD3^+$ T cells from healthy donors (HD) were exposed to hypoxic conditions, and Rai mRNA levels were analyzed by qPCR. Results show that Rai mRNA levels were significantly enhanced in $CD3^+$ T under hypoxia compared with normoxic controls (Fig. 5B), suggesting that HIF-1α promotes Rai expression in T cells. Consistently, knock down of HIF-1α in $CD3^+$ T cells using HIF-1α-specific siRNA showed significantly reduced Rai mRNA levels under hypoxia, compared with those transfected with siRNA control (Fig. 5C).

To demonstrate the direct binding of HIF-1α to the identified HRE regions, we performed chromatin immunoprecipitation (ChIP) assays on hypoxic $CD3^+$ T cells using anti-HIF-1α antibody or anti-IgG as control. The results showed that HIF-1α was bound to both HRE 1 and HRE 2 regions in T cells under hypoxic conditions (Fig.5D). Collectively, these data demonstrated that HIF-1α is recruited to *rai* promoter and activates *rai* transcription under hypoxia.

1.2 Rai upregulation promotes PD-1 expression

PD-1 receptor is upregulated on the surface of T cells upon antigen-mediated activation to attenuate the immune response following antigen clearance and to restore immune tolerance and homeostasis. Indeed, PD-1 expression levels on T cell surface decreases to basal levels in resting T cells [79]. Accordingly, perturbations in PD-1 pathway leading to its highly and sustained expression, are associated with the exhausted phenotype of T cells characterized by the loss of effector functions [37]. To date the transcription factors known to regulate PD-1 expression, including nuclear factor of activated T cells (NFAT), Forkhead box protein O1 (FoxO1), Notch, activator protein 1 (AP1), B-lymphocyte maturation protein 1 (Blimp1) and T-bet have been indentified [80]. Nevertheless, the

molecular characterization of the intracellular signaling pathways controlling PD-1 expression following TCR engagement is still poorly known [80]. Here, we decided to investigate whether Rai upregulation in T cells may impacts on signaling pathways controlling PD-1 expression. To dissect the role of Rai in this process, avoiding the global metabolic and transcriptional changes induced by hypoxia, we decided to mimic the hypoxia-dependent Rai upregulation by transiently co-transfecting CD3$^+$ T cells from healthy donors with a plasmid encoding Rai and a plasmid encoding GFP (Fig. 6A).

Transfected cells were then stimulated by plate bound anti-CD3 and anti-CD28 antibodies or left untreated, and the frequency of PD-1 positive cells was measured on the GFP positive population (considered as transfected cells population) by flow cytometry (Fig. 6A). We found that Rai-transfected cells showed a significant increase in the percentage of PD-1$^+$ cells compared with those transfected with control plasmid following CD3+CD28 stimulation (Fig. 6B). Conversely, overexpression of Rai does not affect the level of PD-1 in basal condition suggesting that Rai promotes PD-1 upregulation following antigen-dependent T cell activation (Fig. 6B).

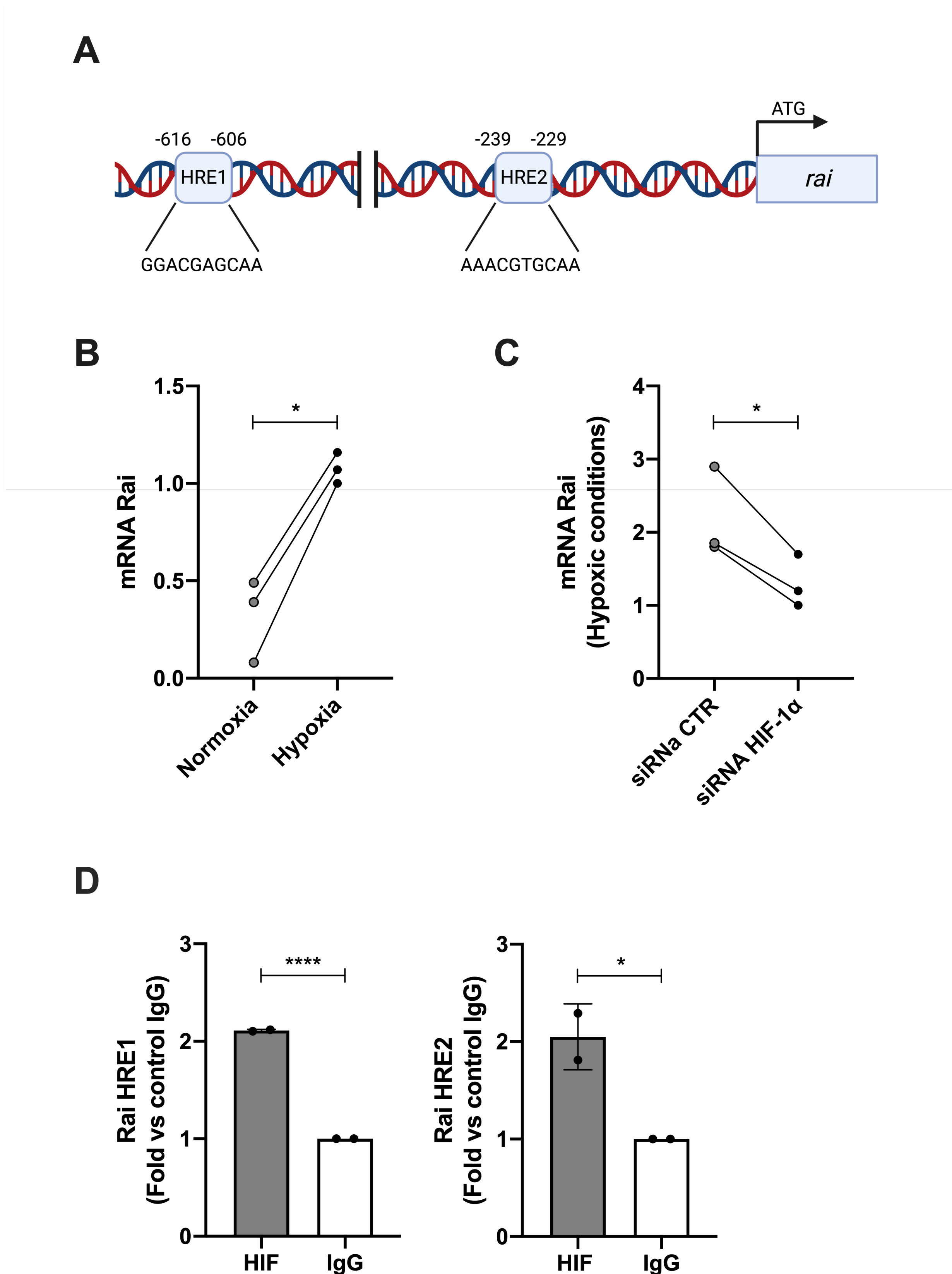


Figure 5. HIF-1α induces Rai upregulation in T lymphocytes. **(A)** Schematic representation indicating the position of the hypoxia-response elements (HRE) predicted by Jaspar analysis on human rai promoter. **(B)** qPCR analysis of Rai transcripts in human T lymphocytes cultured under normoxic or hypoxic conditions for 24 hours (n = 3). **(C)** qPCR analysis of Rai transcripts in T lymphocytes silenced (siRNA HIF-1α) or not (siRNA CTR) for HIF-1α and cultured under hypoxic conditions for 24 hours (n = 3). **(D)** ChIP assays of nuclear extracts from T

lymphocytes cultured under hypoxic conditions for 24 hours using either anti-HIF-1α or control unspecific Rabbit IgG antibodies. Regions of rai promoter containing putative binding sites for HIF-1α showed in **(A)** were amplified by qPCR. Data are presented as fold enrichment ($n = 2$). **(B, C)** paired t test. **(D)** unpaired t test. **** $p < 0.0001$, * $p < 0.05$.

1.3 Antigen-dependent inactivation of GSK-3 is prevented by Rai

The serine threonine kinase glycogen synthase kinase (GSK)-3 has been suggested as the key regulator of PD-1 expression in T cells [81], [82].

This kinase is constitutively active in resting T cells [83], and it is inactivated through phosphorylation of specific serine residues following stimulation of TCR/CD28 [83]. GSK-3 plays a crucial role as a negative regulator of T cell activation. Active GSK-3 is indeed able to phosphorylate NFAT facilitating its exit from the nucleus that results in the inhibition of T cell proliferation and IL-2 production [84]. Stimulation of TCR/CD28 instead induces the inactivation of GSK-3 through the Akt-dependent phosphorylation of its inhibitory serine residues leading to T cell activation and proliferation [85].

Two isoforms of GSK-3 are expressed in mammals, the 51 kDa GSK-3α and the 47 kDa GSK-3β isoform. Although a non redundant role of GSK-3α and GSK3-β has been recently demonstrated in T cells, with the β isoform playing a dominant role over the α isoform by a still unknown mechanism, it has been demonstrated that the inhibition of both isoforms is required to reduce PD-1 expression [83].

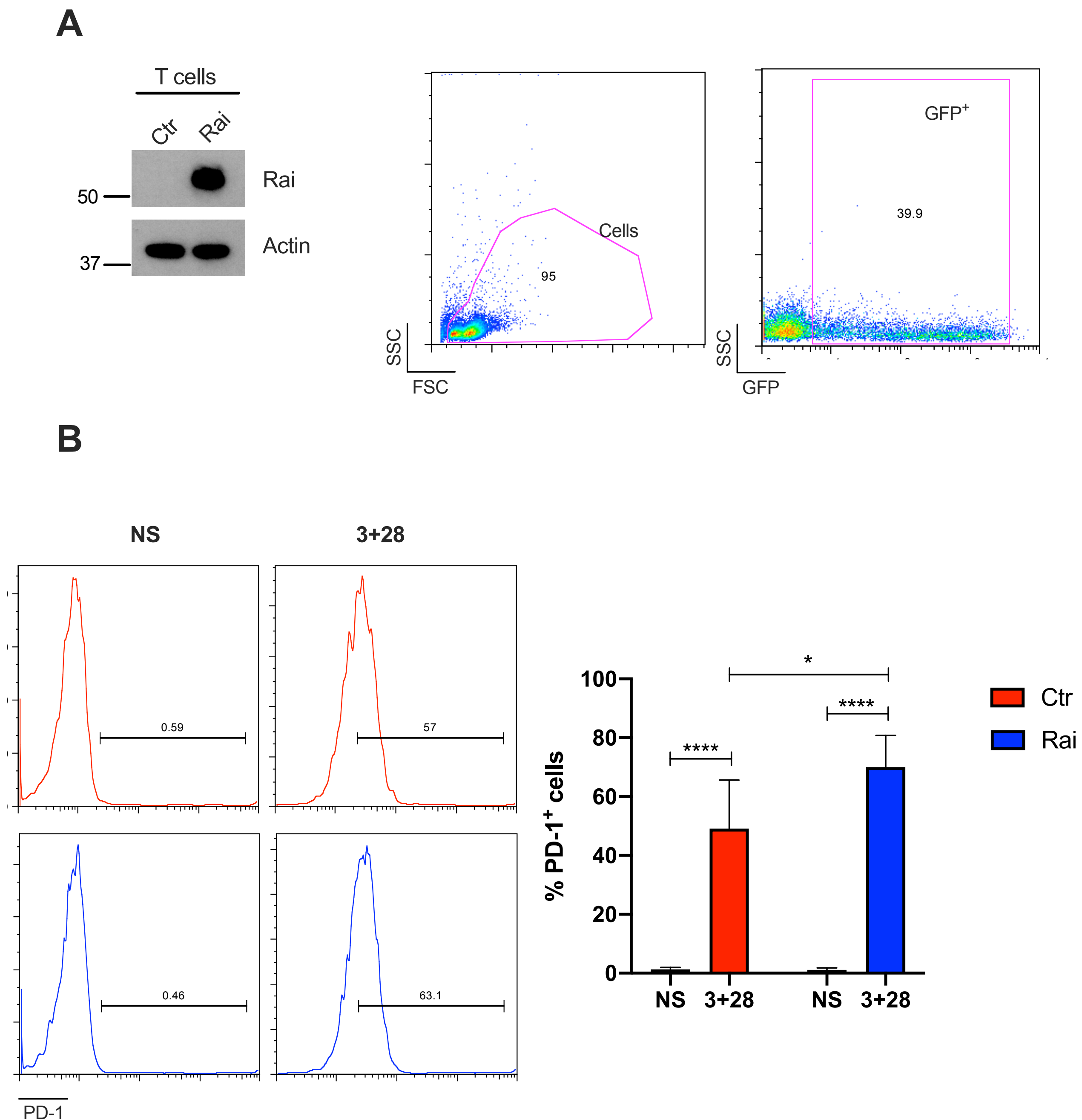


Figure 6. Rai upregulation in T lymphocytes increases the frequency of PD-1⁺ cells following anti-CD3 plus anti-CD28 stimulation. (A) Immunoblot of exogenous Rai protein expression (left) and evaluation by FACS of the percentage of GFP^+ cells (transfected cells) (right) in T lymphocytes transiently co-transfected with either pcDNA3.1-empty plasmid (Ctr) or pcDNA3.1-Rai plasmid (Rai) and pmax-GFP plasmid. **(B)** Flow cytometric analysis of the percentage of PD-1^+ cells in transfected T cells (GFP^+) expressing or not Rai either untreated (NS) or treated with anti-CD3 plus anti-CD28 (3+28) antibodies for 24hours. Flow cytometric histograms (left) are shown. Data are presented as mean value ± SD (n = 4, 4 independent transfections for both Ctr and Rai). Two-way ANOVA, Tukey's multiple comparison test. **** $p < 0.0001$, * $p < 0.05$.

Since Rai overexpression was found to inhibit antigen-dependent Akt activation in Jurkat T cells we reasoned that the molecular mechanism by which Rai sustains PD-1 upregulation in primary T cells involves its ability to modulate GSK3 activation. First, we evaluated whether Rai-dependent AKT inhibition occurs also in primary T cells. To this end human primary T cells, overexpressing or not Rai, were treated with anti CD3+CD28 antibodies and the phosphorylation status of AKT quantified by flow cytometry. Results show that AKT phosphorylation is significantly impaired in primary human T cells overexpressing Rai compared with control cells following CD3+CD28 stimulation, in agreement with the data obtained in Jurkat cells [28] (Fig 7A). We therefore examined by flow cytometry the phosphorylation status of the inhibitory residues on GSK3α/β (Ser21 and Ser9 respectively) in human primary T cells, overexpressing or not Rai, following CD3+CD28 stimulation. We observed that in Rai overexpressing T cells phosphorylation of both the inhibitory serine residues on GSK-3 was reduced compared to cells transfected with control plasmid (Fig. 7B). Hence, Rai prevents GSK-3 inactivation by impairing AKT activation.

In agreement with the role played by Rai in preventing GSK-3 inactivation, together with the reported ability of inactive GSK-3 to downregulate PD-1 expression in $CD8^+$ T cells and TILs through the upregulation of the transcription factor T-bet [85], T cells overexpressing Rai significantly increased PD-1 mRNA levels compared with mock-transfected control cells. Consistent, PD-1 upregulation in these cells was paralleled by a significant reduction of T-bet transcript (Fig. 7C). Collectively, these results demonstrated that Rai promotes PD-1 upregulation by preventing AKT-dependent inactivation of GSK-3.

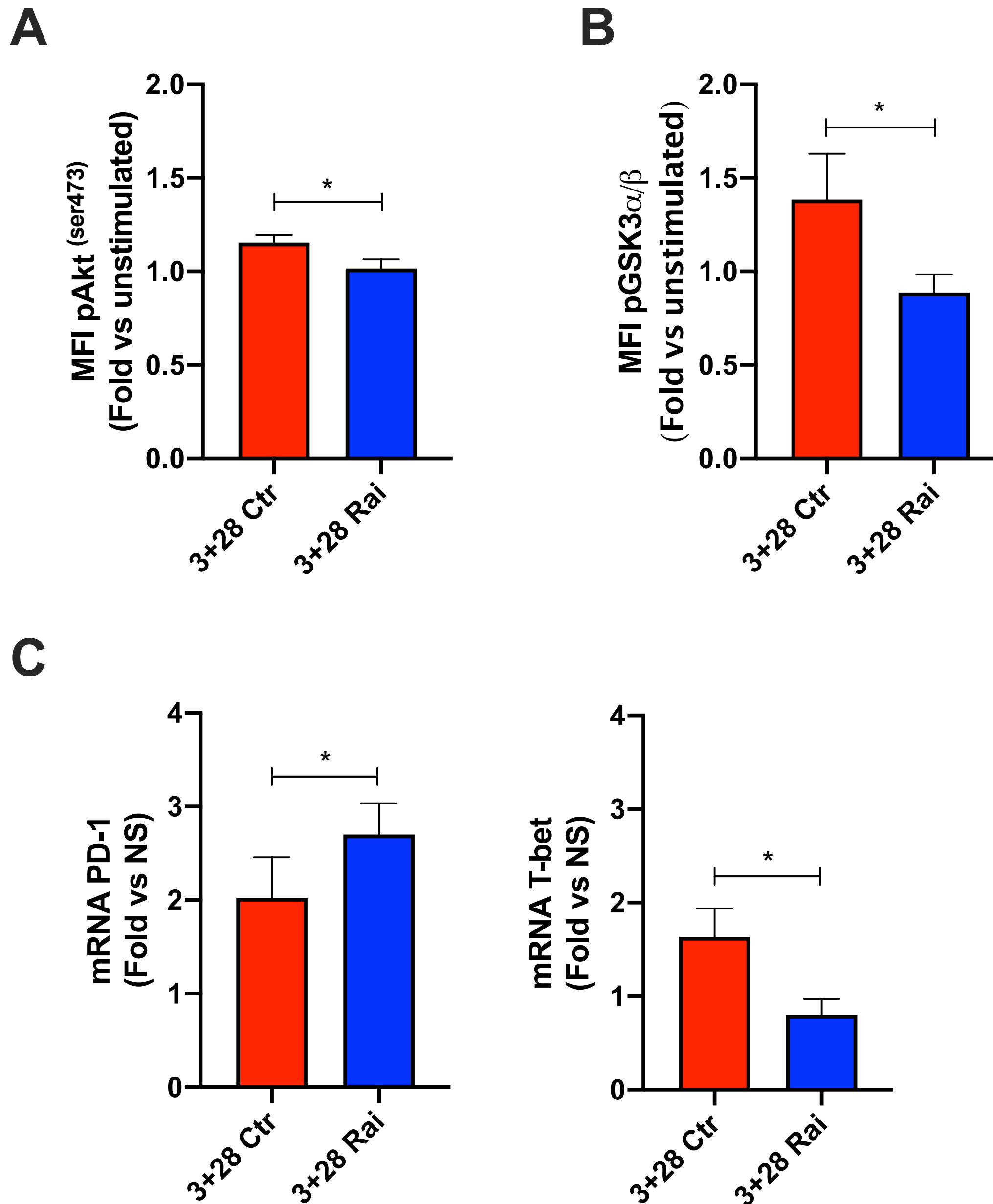


Figure 7. Rai promotes PD-1 upregulation in T cells by preventing antigen-dependent GSK3 phospho-inactivation. (A) Flow cytometric analysis of Akt phosphorylation (serin 473) in transfected T cells (GFP^+), expressing or not Rai, unstimulated (NS) or stimulated with anti-CD3 plus anti-CD28 (3+28) antibodies for 5 minutes (n = 3) Data are reported as fold increase *vs* unstimulated conditions. **(B)** Flow cytometric analysis of inactive GSK3α/β (phosphorylated on serin 21 and serin 9 respectively). Experimental conditions are the same reported in (A) (n = 3). Data are reported as fold increase *vs* unstimulated conditions. **(C)** qPCR analysis of PD-1 (n = 4) and T-bet (n = 4) transcripts in Rai-transfected and Ctr cells either untreated (NS) or treated with anti-CD3 plus anti-CD28 (3+28) antibodies for 24 hours. For each experiment independent transfections were performed for both Rai and Ctr. (**A**, **B)** Two-way ANOVA, Tukey's multiple comparison test. (**C)** unpaired t test. ** $p < 0.01$ * $p < 0.05$.

1.4 Rai impairs antigen-dependent degranulation of $CD8^+$ T cells

To examine the functional outcome of Rai overexpression in $CD8^+$ T cells we evaluated the ability of these cells to release cytotoxic granules in response to antigen stimulation. We performed a flow cytometry-based assay of degranulation, in which the degranulation extent was assessed by measuring, within the GFP^+ population, the percentage of $CD8^+$ T cells showing lysosome-associated membrane protein 1 (LAMP1) on their surface following incubation with bacterial superantigen (SE)-loaded Raji B cells. LAMP-1 can indeed be detected on the T cell surface only during granule release [86].

We found that Rai overexpression led to a significant decrease of $CD8^+$ degranulation when compared with control $CD8^+$ T cells (Fig. 8), consistent with the reduction of effector functions caused by PD-1 upregulation, and with the well-documented effect of GSK-3 inhibition on the enhanced expression of LAMP-1 and granzyme B in $CD8^+$ T cells [85].

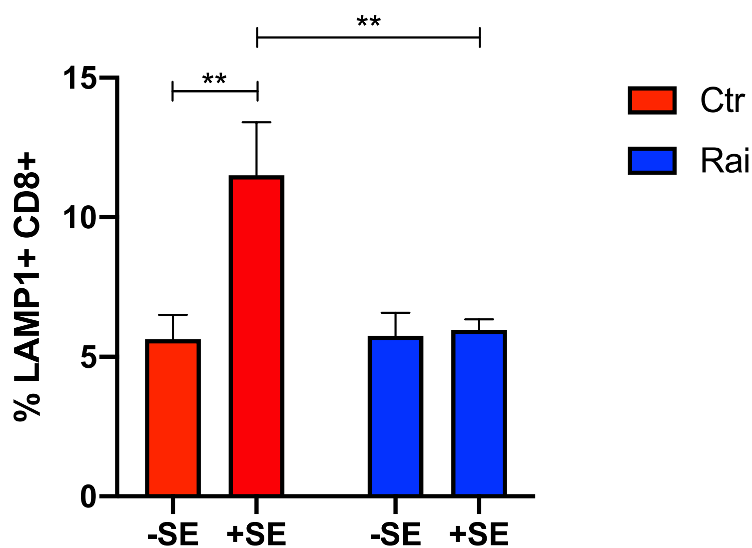


Figure 8. Rai overexpression reduces the frequency of $LAMP1^+$ cytotoxic T lymphocytes following antigen stimulation. Quantification of degranulation extent of Rai-transfected and mock-transfected $CD8^+$ T cells incubated with Raji B cells loaded with an SAg mixture (+SE) or left unloaded (-SE). T cells were incubated with Raji cells at a 10:1 ratio. Histogram show the percentage of $LAMP1^+$ cells in $CD8^+$ T cells ($LAMP1^+CD8^+$) among transfected T cells (gated on GFP^+). Data are presented as mean value ± SD (n = 3, 3 independent transfections for both Ctr and Rai). Two-way ANOVA, Tukey's multiple comparison test. ** $p < 0.001$.

Part 2: Rai drives the pro-inflammatory response of astrocytes induced by encephalitogenic T cells

As part of the BBB, they are the first cells encountered by infiltrating T cells and respond to immune cells infiltration by modulating their phenotype in a highly heterogeneous manner, which can be associated with protective or pathogenic activities [48], [51]. Astrocytes are indeed able to react either by producing pro-inflammatory mediators which amplify the local immune response and promote neurodegeneration [50], [87], or releasing immunosuppressive cytokines [88].

Previous data showed that Rai, a member of the Shc family of protein adaptors, is expressed in astrocytes where it promotes the production of pro-inflammatory IL-6 and nitric oxide (NO) and the activation of transcription factor NF-κB in response to T cell infiltration [25]. In addition, Rai deficiency in astrocytes has been shown to promote their polarization toward a neuroprotective phenotype following treatment with conditioned media from MOG-specific T cells and to affect the pathogenic potential of encephalitogenic T cells by increasing the extracellular levels of the immunosuppressive molecule adenosine through the enhanced activity of CD39 [70]. Notably, Rai deficiency in mice impairs the CNS response to infiltrating autoreactive T cells, consistent with this, EAE mice lacking this molecular adaptor showed amelioration of disease severity [25].

Collectively, these data highlighted Rai as a driver of the detrimental response of astrocytes induced by auto-reactive T cells and, in addition, provided evidence of the neuroprotective effects of Rai deficiency under the same conditions. Nevertheless, whether astrocytes lacking this molecular adaptor gain some neuroprotective functions contributing to cell-autonomous neuroprotection is still an open question.

Here we investigated whether Rai has an impact on astrocyte and astrocyte-derived extracellular vesicles proteome both in basal conditions and following exposure to pro-inflammatory IL-17, to identify the neuroprotective molecular pathways restrained by Rai.

2.1 Differential proteome profile of control and $Rai^{-/-}$ astrocytes and astrocytes derived extracellular vesicles

At the present, proteomics, along with all the other "omics" approaches, represents the golden method to understand cell-intrinsic characteristics and the impact of external stimuli on astrocyte response. To deepen the role of Rai in astrocytes and to further dissect the signaling pathways in which this protein is involved during the response to inflammatory stimuli we performed a large-scale comparison of the proteome profiles of control and $Rai^{-/-}$ astrocytes. Using this approach, we assessed the impact of Rai on astrocyte proteome both under basal conditions and following activation with IL-17, the signature cytokine secreted by Th17 cells responsible for astrogliosis during EAE [89], whose signaling is impaired by Rai deficiency [25]. In both conditions heatmap analysis of differentially abundant protein spots showed two major clusters of protein spots with opposite Rai-dependent behavior (Fig. 9).

First, we determined whether Rai deficiency impacts on astrocytes' proteome in homeostatic conditions. 2D-Electrophoresis (2DE) and image analysis revealed 22 differentially abundant spots between control and $Rai^{-/-}$ astrocytes, 9 of which have been identified by mass spectrometry. Proteasome subunit beta type 2 (PSB2), ubiquitin-conjugating enzyme E2 N (UBE2N), superoxide dismutase (SODC) and terminal nucleotidyl transferase 5C (TET5C) were the identified differential spots we found upregulated in $Rai^{-/-}$ astrocytes, while among the downregulated spots we found alpha enolase (ENOA), heat shock cognate 71 kDa protein (HSP7C), NADH dehydrogenase iron-sulfur protein 2 mitochondrial (NDUS2), tubulin beta-2A chain (TBB2A) and astrocytic phosphoprotein PEA-15 (PEA15) (Fig. 9A). The molecular pathways associated with the identified differential proteins were revealed by enrichment analysis performed using MetaCore 6.8 network building tool. Proteins that were differentially expressed under basal conditions were involved in the putative ubiquitin pathway, the HSP70-dependent folding pathway, the HSP70/TLR signaling pathway, the negative regulation of HIF-1α function and TGFβ induction of epithelial-mesenchymal transition (EMT) via reactive oxygen species (ROS) (Fig. 9A).

Then, we investigated the global changes induced by IL-17 in protein composition and the impact of Rai expression on these changes by comparing the proteome of IL-17-treated control and Rai$^{-/-}$ astrocytes. From the analysis we found 5 differential spots, but only glial fibrillary acidic protein (GFAP), which was upregulated in Rai$^{-/-}$ astrocytes, was identified (Fig. 9B).

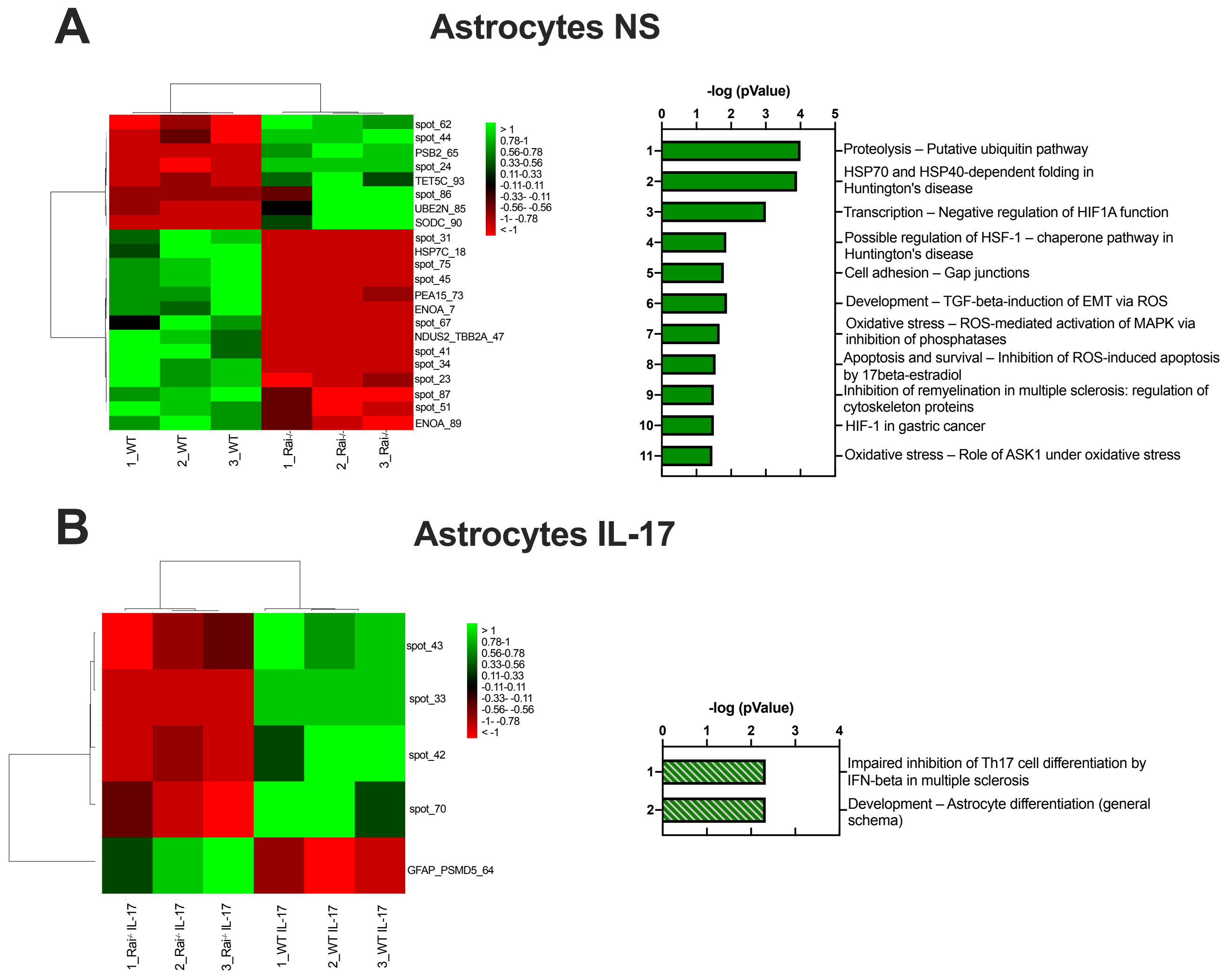


Figure 9. Differential proteomic analysis of control and Rai$^{-/-}$ astrocytes. Supervised hierarchical clustering heat map (left) of differentially abundant proteins found in untreated **(A)** and IL-17-treated **(B)** astrocytes from WT and Rai$^{-/-}$ mice (n = 3). Heat maps: columns correspond to individual preparation of astrocytes and row to spot identity (protein name, or spot number for not identified spots) Color scale (from high value in green to low value in red) illustrates % volume values of the statistically significant differentially abundant spots. The most relevant pathway maps reported by MetaCore, based on all differentially expressed proteins and prioritized according to their statistical significance ($p \leq 0.001$), are shown for each condition (right).

Since astrocytes have shown the ability to shape the CNS microenvironment through the release of ADEVs [72], and it has been recently shown that the cargo of these vesicles is regulated by external

stimuli, as witnessed by the protective functions exerted by ADEVs released following ATP or IL-10 stimulation and, conversely, the enrichment in proteins supporting the immune cell infiltration found in ADEVs following stimulation with pro-inflammatory IL-1β [90], we decided to analyze whether Rai impacts on ADEVs cargo both under basal conditions and in response to IL-17.

Heatmap analysis demonstrated difference between $Rai^{-/-}$ and control samples indicating that the protein cargo of extracellular vesicles is shaped by this molecular adaptor (Fig. 10). Under basal conditions the quantitative comparison revealed 14 differentially abundant spots, 8 of which have been identified by mass spectrometry. Rab3 GTPase-activating protein non-catalytic subunit (RBGPR), protein disulfide-isomerase A3 (PDIA3), thiosulfate sulfurtransferase/rhodanese-like domain-containing protein 2 (TSTD2), alpha-enolase (ENOA), glutamine synthetase (GLNA), kelch-like protein 9 (KLHL9) and microtubule-associated protein 4 (MAP4) were all down-regulated in $Rai^{-/-}$ ADEVs compared with the WT counterpart, while S10A6 was present only in WT ADEVs (Fig. 10A). Pathway analysis of these identified differentially abundant proteins highlighted their involvement in beta-catenin dependent transcription regulation, nitrogen metabolism, Wnt signaling of oligodendrocytes differentiation, GABAergic neurotransmission, HIF-1-dependent transcription, glycolysis and gluconeogenesis, histidine-glutamate-glutamine metabolism and plasmin signaling (Fig. 10A).

Following IL-17 treatment 7 differentially abundant proteins were extrapolated by comparative analysis and 6 of which were identified. Most of the identified proteins, namely actin cytoplasmic 1 (ACTB), peroxiredoxin-6 (PRDX6), heat shock factor-binding protein 1 (HSBP1), actin aortic smooth muscle (ACTA) and MAP4, were enriched in IL-17-$Rai^{-/-}$ ADEVs compared with IL-17-WT ADEVs, whereas endoplasmic reticulum resident protein 44 (ERP44) was found only in IL-17-$Rai^{-/-}$ ADEVs. These differentially abundant proteins are involved in pathways like HSF-1/chaperone pathway, dysregulation of adiponectin secretion, regulation of cytoskeleton proteins in oligodendrocytes differentiation, plasmalogen biosynthesis and oxidative stress (Fig. 10B).

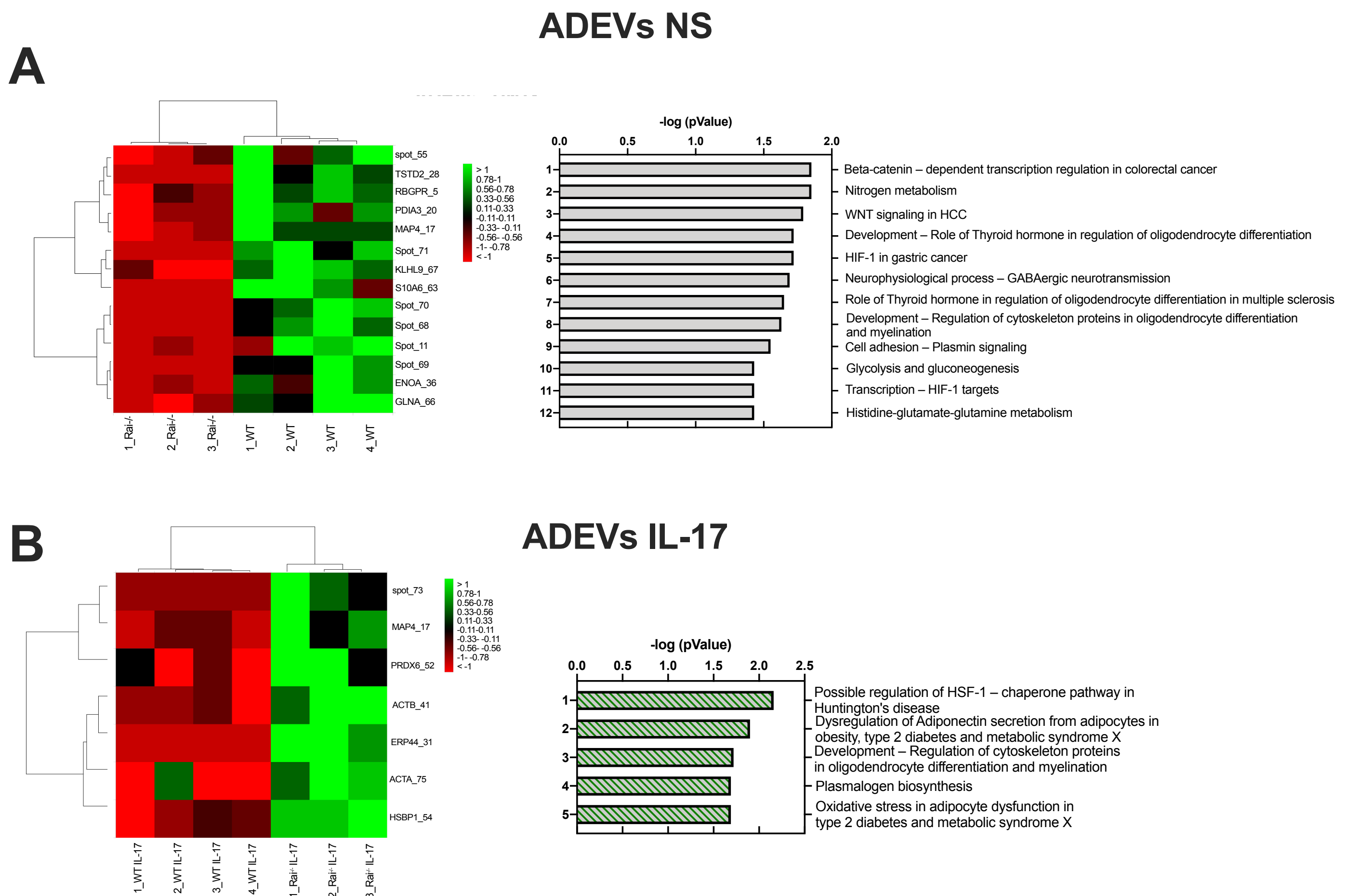


Figure 10. Differential proteomic analysis of control and Rai$^{-/-}$ ADEVs. Supervised hierarchical clustering heat map (left) of differentially abundant proteins found in ADEVs purified from the cell media of WT and Rai$^{-/-}$ astrocytes untreated **(A)** and treated with IL-17 **(B)** (n = 4 WT, 3 Rai$^{-/-}$), and the most relevant pathway maps (right) reported by MetaCore are shown for each condition as in Fig. 9.

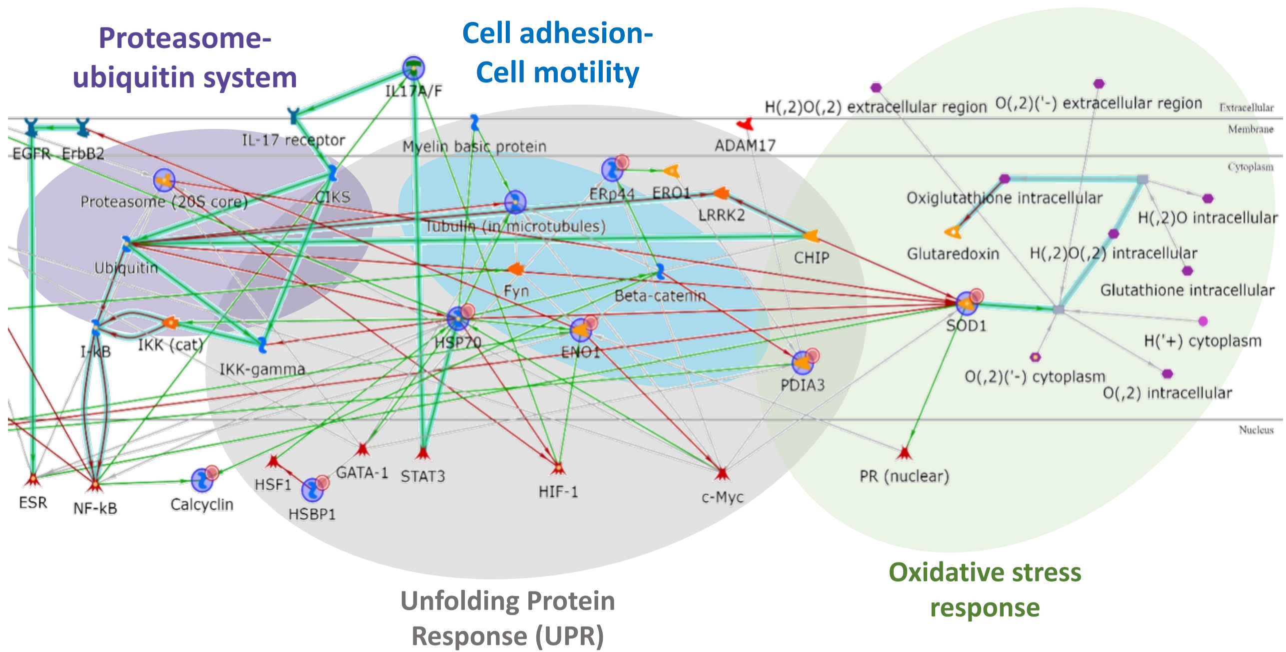
Proteomic data obtained from ADEVs showed that in IL-17-stimulated conditions the differentially expressed proteins were more abundant in Rai$^{-/-}$ ADEVs with respect to wild-type counterpart, while under basal conditions we observed an opposite behavior. Worth of note, MAP4, the only protein shared in both conditions totally reflected this scenario, as it was found to be enriched in IL-17-Rai$^{-/-}$ ADEVs, while under basal conditions it was less abundant in Rai$^{-/-}$ compared to WT ADEVs. Taken together these results indicate that Rai impacts on ADEVs proteome in both conditions.

Since the ADEVs proteome may reflect the cellular changes induced by Rai, we performed a pathway analysis comparison including all differential proteins identified in astrocytes and ADEVs in both

basal and IL-17-stimulated condition. Among the most relevant shared molecular pathways we found HIF-1α pathway, glycolysis and gluconeogenesis, and regulation of cytoskeleton proteins in oligodendrocytes differentiation and myelination. In particular, ENO1 dysregulation is related to HIF-1 pathway, glycolysis and gluconeogenesis and plasmin signaling, while MAP4 and tubulin beta influenced the regulation of cytoskeleton proteins in oligodendrocytes differentiation and myelination. In addition, we also performed a protein network analysis which identified proteasome ubiquitin system, unfolding protein response, ECM remodeling/cell adhesions, and oxidative stress response as the most relevant pathways influenced by Rai. Central functional hubs of this pathways include protein proteasome (20S core), HSP70, ENO1 and SOD1 (Fig. 11).

2.2 Rai participates in the response of astrocytes to oxidative stress by a NRF2-independent mechanism

Oxidative stress is the deleterious complex of events induced by the accumulation of highly reactive molecules, such as reactive oxygen species (ROS) and reactive nitrogen species (RNS), that occurs during CNS inflammation [50]. In MS and EAE, infiltrating peripheral immune cells (i.e., lymphocytes and macrophages) as well as CNS-resident astrocytes and microglia produce high levels of ROS and RNS, that are indeed found in both MS and EAE lesions and are responsible for neurodegeneration [91].



Protein network	Central hub
Proteasome-Ubiquitin System	Proteasome (20S core)
Cell Adhesion- Cell Motility	ENO1
Unfolding Protein Response	HSP70
Oxidative Stress Response	SOD1

Figure 11. Protein network of pathways influenced by Rai in astrocytes and ADEVs
Protein network analysis by using all the proteomic data obtained in wild type astrocytes and ADEVs and those obtained in Rai$^{-/-}$ astrocytes and Rai$^{-/-}$ ADEVs. Proteasome (20S core), HSP70, ENOA and SOD1 are central functional hubs.

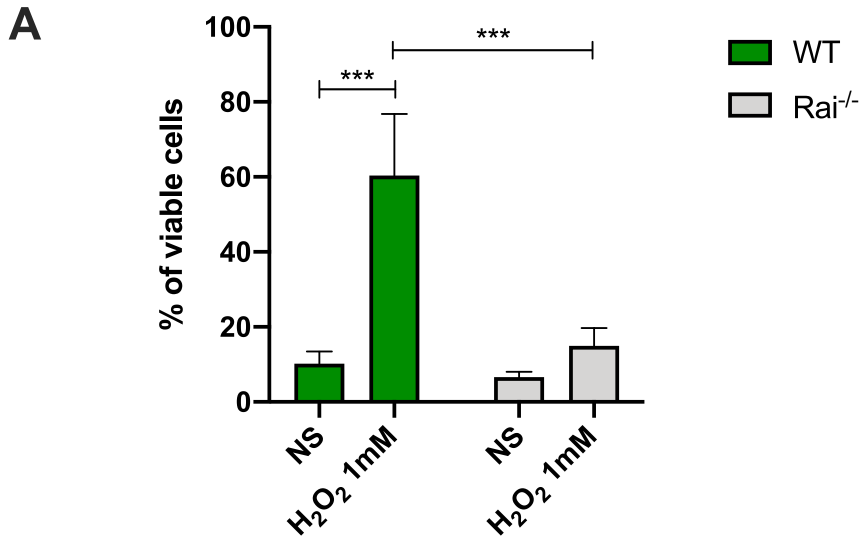
Astrocytes are considered as the major NO producers during EAE [71], [92], and the BDNF-dependent stimulation of TrkB, which expression is induced in astrocytes in CNS lesions, has been identified as one of the signals driving NO production during the disease [71]. In addition, iNOS expression in astrocytes can be induced also by pro-inflammatory cytokines such as the combination of IFNγ and IL-17 [69]. Interestingly, despite their role in NO production in response to pro-inflammatory stimuli, astrocytes are also considered the major players in the antioxidative system of the CNS, since the loss of their antioxidant functions occurring in a pathological context leads to accumulation of high levels of ROS and RNS [50]. Accordingly, in active lesions of MS patients a strong upregulation of antioxidant enzymes in astrocytes has been reported and it has been correlated with an adaptive defense mechanism to reduce ROS-mediated neurodegeneration [93].

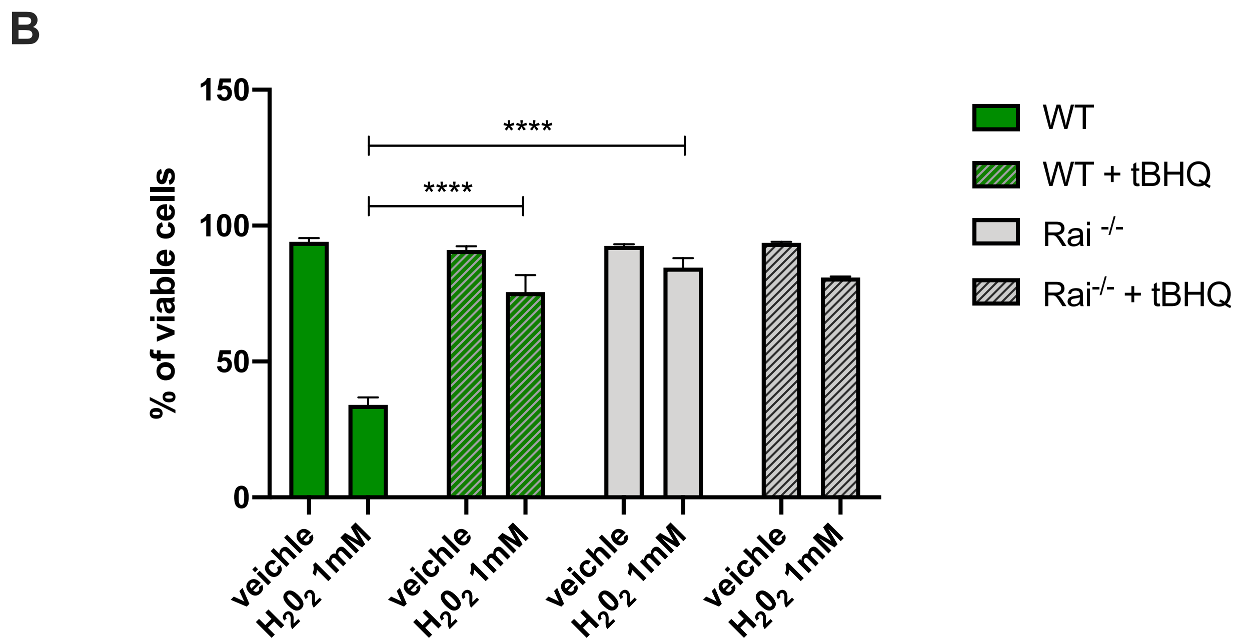
Since our proteomic analysis revealed that Rai might influence the oxidative stress response in astrocytes, control and $Rai^{-/-}$ astrocytes were treated with H_2O_2 and cell viability quantified using PI staining by flow cytometry. Following 24 h of treatment using 1mM H_2O_2, the lowest concentration showing effect on cell viability (data not shown), we observed a significant reduction in cell viability of control astrocytes compared to $Rai^{-/-}$ (Fig. 12A), indicating that Rai deletion protects astrocytes from extracellular ROS.

The major regulator of the oxidative stress response is the transcription factor nuclear factor (erythroid-derived 2)-like 2 (NRF2), which plays a central role in maintaining cellular homeostasis upon exposure to oxidative stress by inducing expression of antioxidant proteins and detoxification enzymes [94]. Under basal conditions NRF2 is bound to Kelch ECH associating protein 1 (Keap1), which prevents NRF2 translocation to the nucleus and promotes its ubiquitination and degradation [94]. In response to oxidative stress, Keap1 undergoes conformational changes resulting in the release of NRF2, which subsequently translocates into the nucleus and binds to antioxidant response elements (AREs) in the promoter of target genes, thus activating cellular responses that limit oxidative stress [94]. Consistent with this, a decreased expression of *nfe2l2* has been documented in the most expanded astrocyte subpopulation during EAE, and conditional knockout mice for the astrocytic *nfe2l2* showed a more serious EAE course and an increased activation of pro-inflammatory pathways, indicating that NRF2 signaling limits detrimental responses of astrocytes that promote EAE pathogenesis [51].

Since astrocytes lacking Rai are protected from H_2O_2-induced killing compared to control astrocytes we evaluate whether Rai modulate NRF2 activity. To this end we analyzed the effect of *tert*-butylhydroquinone (tBHQ), a well-characterized activator of NRF2 [95] on astrocytes viability following H_2O_2 treatment. The mechanism of action of tBHQ relies on its ability to interact with thiol groups of cysteine molecules on the Keap1 protein, thereby preventing the inhibitory binding of Keap1 to NRF2, and allowing NRF2 translocation to the nucleus [96]. Control and $Rai^{-/-}$ astrocytes were treated either with tBHQ or vehicle (ethanol) for 24h prior to subject cells to oxidative stress by

incubation with 1mM H_2O_2 for other 24 h. Cell viability was measured by FACS as the percentage of PI^- cells. Results showed that while the tBHQ-dependent activation of NRF2 protects control astrocytes from H_2O_2 treatment, it has no effect on the viability of Rai deficient astrocytes following H_2O_2 treatment (Fig. 12B). In this context, a constitutive activation of NRF2 in $Rai^{-/-}$ astrocytes might explain their intrinsic protection against oxidative stress and the unresponsiveness to tBHQ treatment. To address this point, we decided to evaluate by qPCR analysis the expression levels of known NRF2 target genes, namely *sod2, catalase* and *gstm1* [51], [97], [98], in both control and $Rai^{-/-}$ astrocytes under basal conditions. No difference was found in the expression levels of these genes neither in *nfe2l2* expression (Fig. 12C), suggesting that $Rai^{-/-}$ astrocytes were protected from oxidative stress by a NRF2-independent mechanism.


A
% of viable cells
100
80
60
40
20
0
***
***
NS
H_2O_2 1mM
NS
H_2O_2 1mM
WT
Rai-/-


B
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150
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veichle
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WT
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Rai -/-
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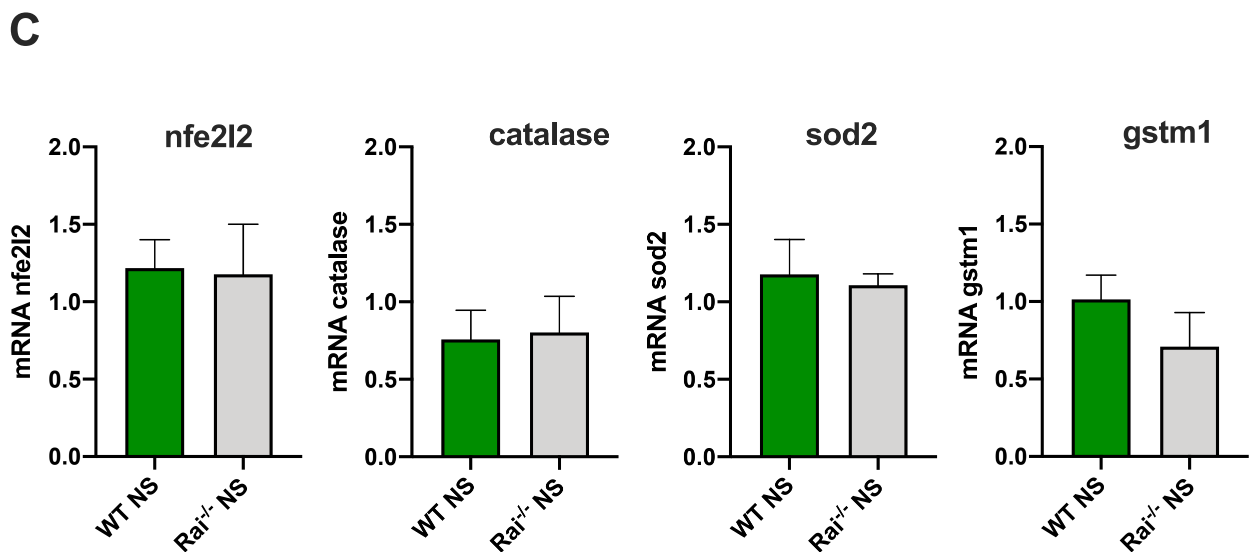
C
nfe2l2
catalase
sod2
gstm1
mRNA nfe2l2
mRNA catalase
mRNA sod2
mRNA gstm1
2.0
1.5
1.0
0.5
0.0
WT NS
Rai-/- NS

Figure 12. Rai$^{-/-}$ astrocytes survive to oxidative stress through a NRF2-independent mechanism. **(A)** Flow cytometric analysis of WT and Rai$^{-/-}$astrocyte viability following treatment with 1 mM H_2O_2 for 24 hours at 37 °C and stained with PI immediately before the acquisition. Histogram reports the percentage of viable cells (PI negative). Data are presented as mean value ± SD of the percentage of cells (viable cells) (n = 3). **(B)** Flow cytometric assay of cell viability as in (A). Before the H_2O_2 treatment cells were pretreated with ethanol (vehicle) or the NRF2 activator *tert*-butylhydroquinone (tBHQ) for 24 hours at 37°C. Data are presented as mean value ± SD of the percentage of cells (viable cells) (n = 2). **(C)** qPCR analysis of expression levels of NRF2-target genes in WT and Rai$^{-/-}$astrocytes under unstimulated conditions (nfe2l2, catalase, gstm1 n = 4) (sod2 n = 3). Data are presented as mean value ± SD. **(A, B)** Two-way ANOVA, Tukey's multiple comparison test. **** $p < 0.0001$, *** $p < 0.001$

2.3 IL-17-induced activation of Rai/NF-κB/HIF axis drives detrimental response of astrocytes

Our proteomic analysis revealed a contribution of Rai in the unfolded protein response (UPR), which has been identified as one of the main pathways implicated in the pathogenic activities of astrocytes in EAE and MS [51]. The abnormal accumulation of misfolded proteins is indeed a common pathological trait of all the neurodegenerative diseases [99]. Under physiological conditions molecular chaperones exert housekeeping functions controlling the proper folding of newly synthesized proteins and play an important role in the identification and degradation of misfolded proteins, thus maintaining cellular homeostasis [99]. Oxidative stress and pro-inflammatory signals, instead, lead to an accumulation of misfolded or unfolded proteins which results in the activation of the UPR, a biochemical response that aims to restore cellular homeostasis [100].

In this context, the major players are the molecular chaperones heat shock proteins (HSP), which are responsible for the proper folding of proteins and for driving the misfolded ones to degradation under basal conditions. Following cellular stress, such as exposure to inflammatory cytokines and oxidative stress, expression of HSP is induced with the purpose to face the increased number of misfolded proteins, trying to refold them and/or prevent their aggregation, or promoting their degradation [101]. HSP70, a member of the HSP family, has been identified by our proteomic analysis as the central functional hub of the UPR pathway. This protein is worth of note since its overexpression in astrocytes, in addition to the role played in protein refolding, has been associated with neuroprotection thanks to its inhibitory effect on NF-κB [102]. With respect to our results showing that Rai promotes

IL-17-dependent activation of NF-κB [25] it might be possible that this molecular adaptor controls the expression of HSP70.

Analysis of HSP70 expression and NF-κB activation in astrocytes expressing or not Rai following IL-17 treatment, showed that a significant upregulation of HSP70 was paralleled by NF-κB inhibition in astrocytes lacking Rai compared to control astrocytes (Fig. 13A), suggesting that Rai negatively modulate the IL-17-dependent UPR. In addition, upregulation of HSP70 has been found in MS lesions, and is considered as a mechanism to counteract the oxidative and inflammatory microenvironment characteristic of the disease [101], [103], for this reason the enhanced ability of $Rai^{-/-}$ astrocytes to survive oxidative stress compared with control astrocytes further underline the role of Rai in promoting neurodegeneration by restraining the upregulation of HSP70.

Pathway analysis highlighted hypoxia inducible factor 1 (HIF-1) as a crucial transcription factor linked to HSP70. HIF-1 is the transcription factor that controls cellular responses to hypoxia. In particular, HIF-1α is the HIF-1 subunit that under hypoxic conditions escapes from degradation and dimerizes with the constitutive HIF-1β subunit promoting the transcription of a wide variety of genes [34]. Besides hypoxia, upregulation of HIF-1α can be induced by pro-inflammatory cytokines through NF-κB [104], and it has been demonstrated that in astrocytes this leads to the upregulation of IL-6 and iNOS [105], [106]. Given that Rai deficiency in astrocytes resulted in a reduced production of IL-6 and iNOS in response to IL-17 treatment [25], the expression levels of HIF-1α in control and $Rai^{-/-}$ astrocytes following treatment with IL-17 was measured by qPCR. Results showed that opposite to control astrocytes, $Rai^{-/-}$ astrocytes did not upregulate mRNA levels of HIF-1α (Fig. 13B), consistent with the reduced activation of NF-κB found in these cells. Together these results suggest that Rai drives the IL-17-dependent detrimental response of astrocytes *via* Rai/NF-κB/HIF-1α pathway.

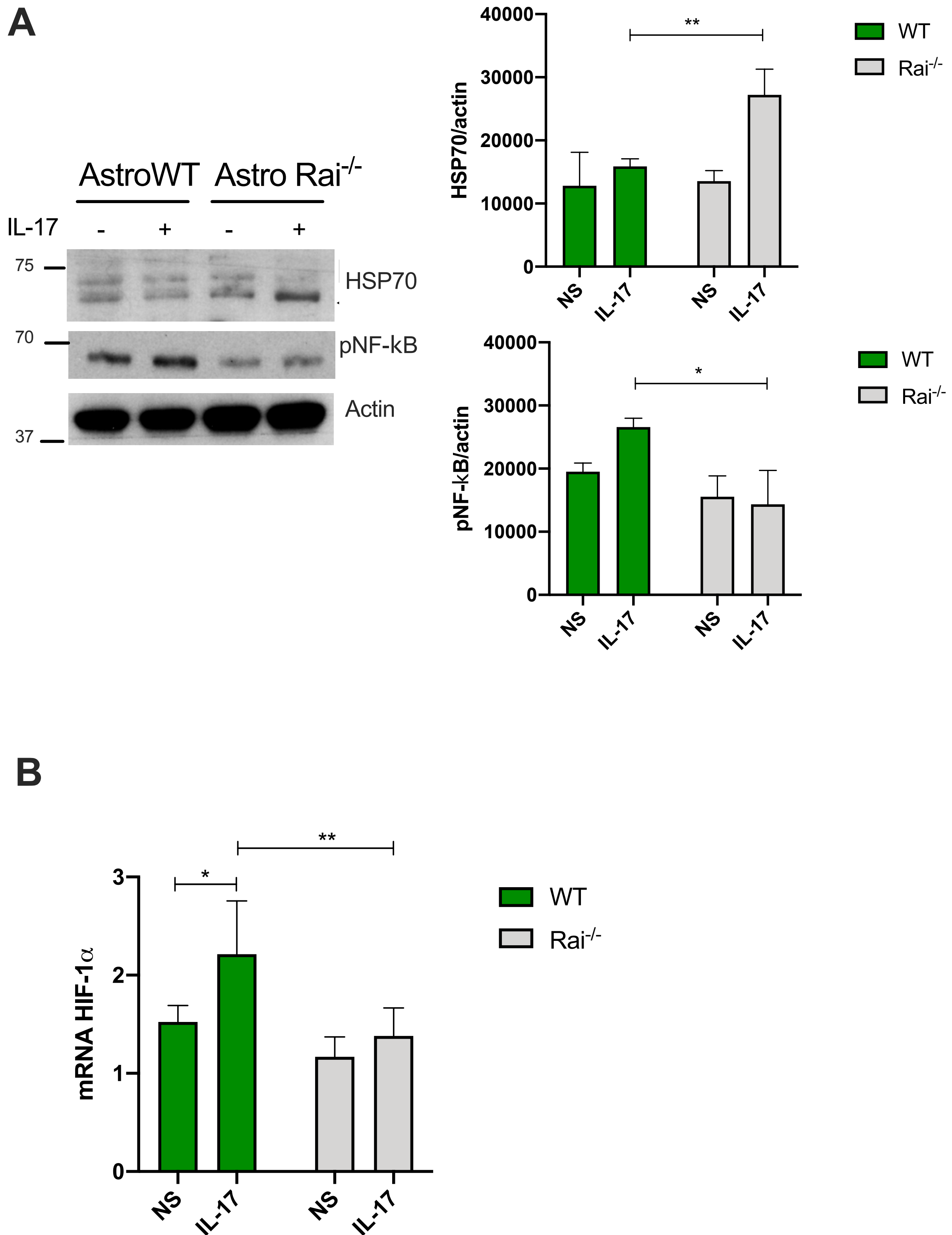

Figure 13. HSP70 upregulation in Rai$^{-/-}$ astrocytes inhibits NF-κB/HIF-1α pro-inflammatory axis. **(A)** Immunoblot analysis of HSP70 and NF-kB phosphorylation (pNF-κB) in lysates of WT and Rai$^{-/-}$ astrocytes untreated (NS) or treated with IL-17 for 24 hours (HSP70) (n = 3) or 15 minutes (pNF-κB) (n = 3). Histograms showed in represent the quantification by densitometric analysis of the levels of the indicated proteins relative to actin. **(B)** qPCR analysis of HIF-1α transcript in WT and Rai$^{-/-}$ astrocytes untreated (NS) or treated with

IL-17 for 24 hours at 37°C (n = 4). Data are presented as mean value ± SD. Two-way ANOVA, Sidak's multiple comparison test. ** $p < 0.01$; * $p < 0.05$.

2.4 Modulation of Rai expression during EAE

Based on our previous studies, and on the results discussed above, Rai appears as a previously unknown driver of the Th17-induced pro-inflammatory response of astrocytes [25], [70]. Since molecular adaptors can exert different functions depending on their amount within the cells, we decided to investigate whether Rai expression is modulated during the course of EAE, looking for a correlation between Rai expression and disease severity.

To do that, we immunized wild type mice with the myelin peptide MOG_{35-55} to induce EAE, and then we collected the whole brain from these mice during the peak of the disease (20 days after injection) or during the chronic phase (30 days after injection), to evaluate Rai expression levels in different stages. Results obtained from qPCR analysis showed that while during the chronic phase the levels of Rai were comparable to those detected in untreated mice, during the peak of EAE, there was a huge decrease in Rai expression (Fig.14A), indicating a modulation of this molecular adaptor during the disease course.

Astrocytes are the most abundant cell population in the brain [107], so the decreased expression of Rai detected in the whole brain of mice, may reflects the downregulation of astrocytic Rai. Since the EAE peak is the stage of the disease where the infiltrate of immune cells is at its maximum [108], we decided to investigate whether soluble factors released by infiltrating T cells are the responsible of the downregulation of Rai expression during this stage of the disease.

We treated wild type astrocytes with either conditioned media from MOG-specific T cells or the combination of IL-17 and IFNγ, the signature cytokines of encephalitogenic T cells. After 24 h of treatment Rai expression levels were measured by qPCR, and the results showed a downregulation of Rai in astrocytes, consistent with that observed in the brain of EAE mice (Fig. 14B). Collectively, the reduction of Rai expression found in astrocytes in response to dangerous signals from

encephalitogenic T cells, and the amelioration of disease severity in Rai$^{-/-}$ mice suggested that Rai modulation might be a protective mechanism used by astrocytes to face immune cells infiltration into the CNS. In addition, this view of reduced Rai expression as a defensive mechanism correlates with our previous results indicating that Rai deficiency promotes the acquisition of neuroprotective features by astrocytes [70].

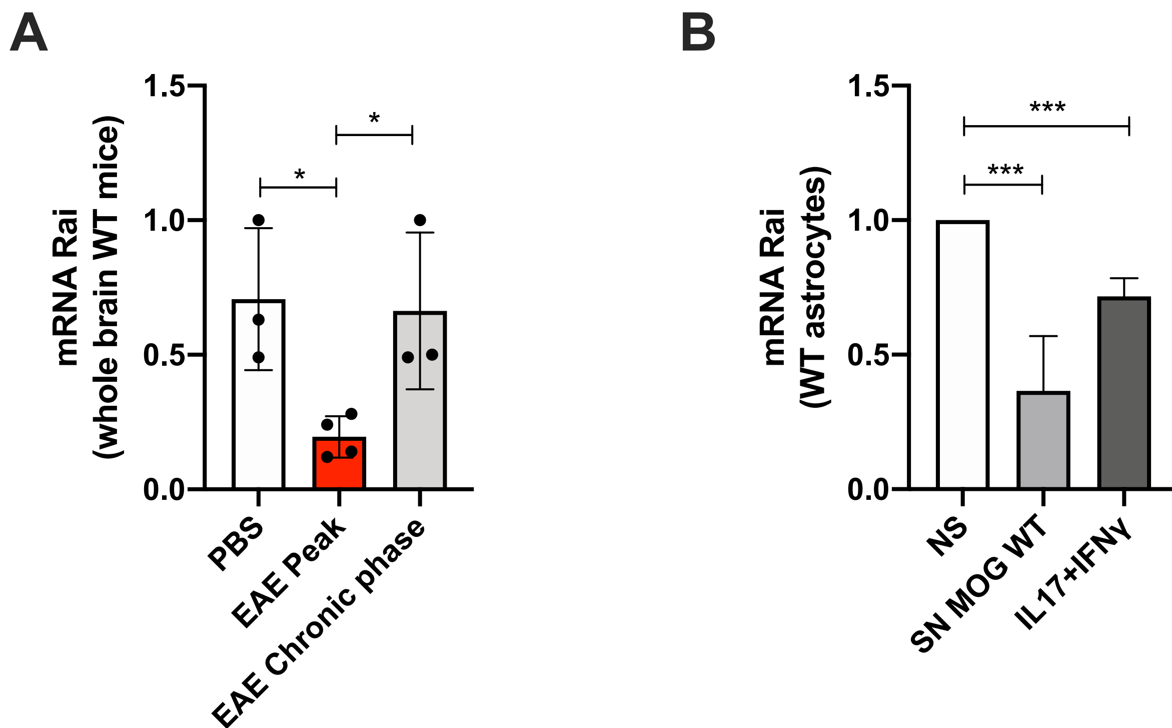

Figure 14. Modulation of Rai expression in astrocytes by encephalitogenic T cells. (A) Analysis of Rai expression levels in the whole brain of untreated mice (PBS) (n = 3) or mice at the peak (20 days post immunization) (n = 4) and at the chronic phase (30 days post immunization) (n = 3) of EAE. Data are presented as mean value ± SD. **(B)** qPCR analysis of Rai transcript in WT astrocytes untreated (NS) or treated with culture supernatants from MOG specific T cells (SN MOG WT) or a combination of IL-17 and IFNγ (IL17+IFNγ) for 24 hours at 37°C (n = 4). Data are presented as mean value ± SD. unpaired t test. *** $p < 0.001$, * $p < 0.05$

Discussion

The molecular adaptor Rai, has been for long time considered a brain specific adaptor expressed only in neurons as two isoforms, p52 and p64, where it promotes cell survival by triggering the PI3K/Akt signaling pathway following TrkB and Ret activation [17].

Recent reports have identified Rai expression also in other tissues [26], and previous studies from this laboratory have shown that the p52 isoform of Rai is expressed also in T and B lymphocytes [27], highlighting the multifaceted role of this molecular adaptor, that is involved in a wide variety of signaling pathways, acting either as a positive or a negative regulator of the cascade, to modulate the response of different cell types to external stimuli.

In murine lymphocytes, as opposed to neurons, Rai impairs TCR-dependent activation of the PI3K/Akt signaling pathway [27], underlying the differential role exerted by Rai on the same signaling pathway depending on the cell type. Indeed, while in neurons it positively regulates Akt activation, in lymphocytes it acts as an inhibitor of the same molecule.

To add further complexity to this scenario, it has been found that Rai when overexpressed in a human T cell line promotes the survival under hypoxic conditions through the PI3K/Akt signaling pathway that ultimately leads to NF-κB activation and HIF-1α expression [31]. This indicates that the opposite effect played by Rai on the PI3K/Akt signaling cascade is not only dependent on the cell type, but also depends on the stimulus received by the cells.

In primary murine T cells Rai is expressed at low levels, compared with neurons and astrocytes, however it is crucial to maintain lymphocyte homeostasis as demonstrated by the fact that Rai$^{-/-}$ mice spontaneously develop a lupus-like autoimmune syndrome [27] and that Rai negatively regulates murine Th17 differentiation and expansion [30].

Consistent with the importance of the proper amount of this protein in human primary T cells, reduced expression of Rai was found in human peripheral blood lymphocytes (PBLs) from systemic lupus erythematosus (SLE) patients, which at the cellular levels are characterized by hyperactivated

lymphocytes and a spontaneous skewing towards the Th17 subpopulation [30]. Here we demonstrate that Rai upregulation induced by hypoxia promotes the acquisition of an exhausted phenotype indicating that alteration of Rai expression correlates with T cell dysfunction in humans.

Hypoxia is a common feature of many pathological niches, such as tumors and inflamed tissues [109], which recent evidence indicate as one of the main drivers of T cell exhaustion [40]. Hence, the HIF-dependent upregulation of Rai found in this context may account for a defective activation of T cells. Interestingly, we also found that human T cells overexpressing Rai showed increased levels of the inhibitory receptor PD-1, the main marker of exhausted T cells [37], on their surface following TCR stimulation. To date, despite many transcription factors involved in the regulation of PD-1 expression have been identified, very little is known regarding the upstream signaling pathways driving PD-1 expression following TCR engagement [80]. Whether Rai is involved in these signaling pathways was unknown. In this work we characterized the molecular mechanism by which Rai when expressed at higher levels in T cells compared to homeostatic conditions, as occurs under hypoxia, prevents the Akt-dependent phospho-inactivation of GSK-3 and promotes PD-1 expression. GSK3 is phosphorylated on inhibitory serine residues following TCR engagement, and this leads to the activation of transcription factor T-bet which subsequently negatively regulates PD-1 expression [85]. The finding that Rai prevents GSK-3 inactivation and promotes a sustained PD-1 expression on T cells leading to the acquisition of the exhausted phenotype indicates that a pathological environment deprived of oxygen induces a dysfunctional state of T cells driven, among others, by Rai.

As discussed above, overexpression of Rai in Jurkat cells is also involved in T cell survival under hypoxic conditions by upregulating HIF-1α [31]. Surprisingly here, we demonstrated that HIF-1α is the transcription factor responsible for Rai upregulation in T cells under hypoxia. Together, these findings might suggest the existence of a feedback mechanism by which hypoxia by inducing upregulation of Rai in T cells promotes their survival, and in turn Rai by upregulating HIF-1α amplifies the adaptive responses to this condition. However, we can speculate that this mechanism becomes negative in a pathological context characterized by severe hypoxia, such as in the tumor

microenvironment, since the enhanced levels of Rai promotes the acquisition of the exhausted phenotype in antigen-activated T cells leading to the loss of their effector functions, thus favoring the progression of the disease.

In contrast to the inhibitory outcome of Rai overexpression in T cells, the lack of this molecular adaptor in lymphocytes dictate the spontaneous development of lupus-like syndrome in $Rai^{-/-}$ mice [27]. In agreement with this data, peripheral blood lymphocytes (PBLs) from patients with systemic lupus erythematosus (SLE) and from MS patients (unpublished results) show reduced levels of Rai expression compared with their healthy counterparts (Savino et al. 2013) [30], indicating that the still unknown mechanism promoting downregulation of Rai in T cells may crucially contribute to the development and the progression of autoimmune diseases.

Respect to the role played by Rai in T cells and astrocyte/neurons in the pathological setting of the EAE disease, it has been observed that while Rai deficiency in T cells resulted in higher encephalitogenic potential, Rai deficiency in CNS cells protects mice from the disease [25].

The first cells of the CNS encountered by infiltrating self-reactive T cells during EAE are astrocytes, which are able to respond to encephalitogenic T cell infiltration by activating several mechanisms leading to the activation of pathogenic or protective activities [48]. Accordingly, it has been found that Rai is expressed in astrocytes, as both the p52 and p64 isoforms, where it promotes the Th17-induced production of pro-inflammatory IL-6 and NO [25]. In addition, the lack of Rai in astrocytes has been associated with an enhanced activation of CD39 that leads to increased levels of extracellular adenosine, a T cell suppressive molecule which promotes the upregulation of the inhibitory receptor CTLA-4 and inhibition of TCR signaling [70]. Thus, the impaired ability of $Rai^{-/-}$ astrocytes to produce these pro-inflammatory mediators, along with the generation of an immunosuppressive environment, may account for the reduced ability of encephalitogenic T cells to promote neurodegeneration. Indicating that Rai has an opposite immunomodulatory function in Th17 cells and astrocytes, with the latter dominant over the former. This evidence indicates that in astrocytes, as

in neurons, Rai drives the response to a stimulus acting as a positive regulator of signaling, but worth of note is the opposite outcome on CNS integrity, since Rai in neurons is protective against ischemic injury [15] while in astrocytes promotes neurodegeneration, further highlighting the different functions exerted by Rai in different cell types and under different conditions.

Based on the different Rai-dependent astrocyte reaction induced by encephalitogenic T cells, we decided to investigate whether Rai deficiency confers protective functions to astrocytes, thus contributing to cell-autonomous neuroprotection. To do that, we assessed the impact of Rai on the proteome profile of astrocytes. Proteomic analysis identifies many neurotoxic pathways impacted by Rai such as the unfolded protein response (UPR), which has been identified as one of the main pathways linked to the pathogenic activities of astrocytes during EAE and MS [51]. Indeed, in their seminal work Wheeler and colleagues, identified UPR as one of the neurotoxic pathways associated with the so-called cluster 4 subpopulation of astrocytes, that they identified as the most expanded population during EAE by single cell-transcriptomics analysis [51]. In addition to UPR, cluster 4 astrocytes are also characterized by the activation of NF-κB and the upregulation of iNOS, two neurotoxic signaling pathways that have been found activated also in astrocytes expressing Rai following the encounter with encephalitogenic T cells [25], further demonstrating the involvement of Rai in the detrimental response of astrocytes during EAE.

Heat shock proteins (HSPs) are a group of molecular chaperones that in homeostatic conditions are implicated in the proper folding of newly synthetized proteins, and for this reason become very important in conditions of stress as key mediators of the UPR, a protective mechanism activated by the cells to counteract the dysregulated folding of proteins and eliminate the misfolded ones to restore cellular homeostasis [101]. In particular, during neuroinflammation, it has been described in astrocytes the upregulation of a specific member of this family, HSP70 [101], which in addition to act as a chaperon controlling protein folding, in astrocytes also plays protective functions by inhibiting NF-κB activation and, in turn, pro-inflammatory cytokines expression [102].

Consistent with the previously reported neuroprotective features of Rai$^{-/-}$ astrocytes, we found an upregulation of HSP70 in these cells following IL-17 treatment complemented by the inhibition of NF-κB. This finding highlights the role played by Rai in the modulation of IL-17-induced UPR, leading to neurodegeneration.

We have also reported an increased resistance to oxidative stress of Rai$^{-/-}$ astrocytes compared to control astrocytes. The major regulator of the oxidative stress response is the transcription factor NRF2 [94], which has been also indicated as a transcription factor limiting the detrimental response of astrocytes during EAE, as conditional knockout mice for the astrocytic *nfe2l2* showed a worsening in disease course [51]. On this basis we hypothesized that the protection showed by Rai$^{-/-}$ astrocytes may be due to a constitutive activation of NRF2 since astrocyte lacking Rai were insensitive to the treatment with the NRF2 activator tBHQ. Nevertheless, analysis of the expression levels of specific genes regulated by NRF2 revealed no differences between control and Rai$^{-/-}$ astrocytes in basal conditions, indicating that Rai deficiency promotes survival to oxidative stress through a NRF2-independent mechanism. In this context, the upregulation of HSP70 found in in astrocytes Rai$^{-/-}$ compared with control astrocytes may be the main mechanism by which astrocytes Rai$^{-/-}$ counteracts the oxidative stress. Interestingly, upregulation of HSP70 during EAE or MS has also been suggested as a mechanism to counteract the oxidative microenvironment, as it promotes the enhanced expression of scavenger enzymes like SOD [110].

A pro-inflammatory environment, through the activation of NF-κB, can also lead to the upregulation and stabilization of HIF-1α that has been demonstrated to contribute to the production of IL-6 and iNOS in astrocytes [104]–[106]. Here we show that IL-17-induced expression of HIF-1α is inhibited in Rai$^{-/-}$ astrocytes, consistent with the impairment of NF-κB activation and IL-6 and NO production in these cells, suggesting that IL-17 promotes the pro-inflammatory response of astrocytes by the Rai/NF-κB/HIF axis.

In addition, here we provided evidence that the Rai-dependent differential protein composition in astrocytes results in a different protein composition of ADEVs, and for the first time we evidenced that the protein cargo of vesicles can be further modified by IL-17. Interestingly, Rai$^{-/-}$ ADEVs were found to be enriched in Erp44 protein which has a role in promoting the correct folding of Adiponectin [111], a protein that limits CNS inflammation during EAE by restraining Th17 differentiation and limiting the pro-inflammatory phenotype of microglia [112], [113]. In addition, IL-17 stimulation increases the amount of MAP4 and PRDX6 in Rai$^{-/-}$ ADEVs, which are respectively involved in the regulation of the microtubule network during oligodendrocytes differentiation [114], and in the protection of the myelin sheet against oxidative stress [115], thus indicating that the cargo of astrocyte-derived extracellular vesicles (ADEVs), fine-tuned by Rai, may be involved in oligodendrocyte differentiation and myelination.

In this work we have highlighted astrocytic Rai as a crucial driver of the encephalitogenic T cell-induced neurodegeneration during EAE. However, we have also provided evidence that this molecular adaptor is modulated during the course of the disease, as witnessed by the huge decrease of Rai expression during the peak of EAE followed by the restoration of basal levels during the chronic phase. Since the different roles played by molecular adaptors depending on their amount [1], this observation suggest that Rai may subserve different functions in the different stages of the disease. The EAE peak is the stage characterized by the highest immune infiltrate [108]. Accordingly, *in vitro* treatment of astrocytes with soluble factors released by T cells induced the same downregulation of Rai expression found in EAE mice, indicating that astrocytes reduced Rai levels in response to T cell infiltration.

These observations suggest that, the reduction of Rai expression in CNS may be considered as one of the mechanisms activated by astrocytes to face the immune cell infiltration and protects the CNS environment during the peak phase. We can speculate that modulation of Rai expression, among others, within different astrocytes subpopulation in CNS during the disease might account for the reported heterogeneous response of astrocytes which can adopt a pro-inflammatory or a protective

phenotype according to Rai levels. Hence, the overall balance between these two subpopulations of astrocytes might be the discriminant of the disease severity.

Conclusions

Molecular adaptors are proteins crucially implicated in the regulation of the cellular responses to environmental stimuli or stresses, which can be involved in different signaling pathways depending on the cell type and context. As such, a full understanding of their functions, both positive and negative, could be very useful for the development of novel therapeutic strategies.

In this context, the molecular adaptor Rai has proved very interesting because of the wide variety of signaling pathways in which is involved, and the different outcomes induced by its different modulation. In this work we shed light on the opposite effects of Rai expression levels in two different cell types, astrocytes and T cells, focusing on pathological contexts where altered expression of this molecular adaptor underlying specific cell dysfunction.

We found that during EAE astrocytes respond to infiltrating self-reactive T cells by downregulating Rai expression levels as a protective mechanism since we demonstrated that the lack of Rai in astrocytes promotes the activation of neuroprotective responses, such as the upregulation of HSP70 which directly interacts and inhibits NF-κB, by shaping the CNS environment through the release of neuroprotective extracellular vesicles.

Conversely, hypoxic conditions, characterizing inflamed or tumoral environments, promote the upregulation of Rai in T cells that drives them to the acquisition of the dysfunctional exhausted phenotype characterized by the loss of their effector functions.

Collectively, these observations highlighted Rai as a potential pharmaceutical target, since its modulation may promote protective response in different cell types and in different pathological contexts.

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