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MOLECULAR AND CELLULAR MECHANISMS IN ASTROCYTE-T CELL CROSS-TALK

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ABSTRACT

Migration of encephalitogenic T cells into the brain parenchima through the blood–brain barrier (BBB) is a crucial feature for initiating tissue injury in different neuroinflammatory diseases [1] [2]. The BBB is comprised of astrocyte processes and endothelial cells, which form the lumen of the brain microvasculature and help in maintaining immune quiescence through contact-dependent mechanisms as well as release of soluble factors [3] [4-6]. Activated CD4+ T cells may establish physical contacts with astrocytes, thereby reciprocally influencing cellular activity and functions. In addition, astrocytes and CD4 T cells may communicate through the secretion of soluble signaling molecules during contact. Among molecules secreted by astrocyte, ATP is a key messenger which can also signal to CD4 T cell through purinergic P2 receptors. Our results show that activated CD4+ T cells inhibit calcium oscillations in astrocytes through direct modulation of extracellular ATP levels. This effect correlated with the expression of plasma membrane ectonucleoside triphosphate diphosphohydrolase CD39, which is induced by contact of the activated T cell with astrocyte. In addition, T cell contact with astrocyte results in the upregulation of the ecto-5'-nucleotidase CD73, which converts AMP to adenosine. This effect was peculiar of T cell contact with astrocyte since it did not occur with microglia or peritoneal macrophages. Pharmacological inhibition of Ca2+ oscillations in astrocyte completely prevented CD73 induction on T cell, thus suggesting that a gliostrasmitter released by astrocyte in a Ca2+-dependent fashion might be responsible of this effect. Since degradation of ATP to adenosine by CD73 regulates BBB permeability and leukocytes infiltration into the brain [7] [8] this regulatory circuit might have important pathogenetic implications in multiple sclerosis and other neuroinflammatory conditions. Finally, functional characterization of T cell upon contact with astrocyte allowed us to assess a proinflammatory phenotype and Th17 skewing albeit with important differences, such as CD39 and CD73 expression, with respect to conventionally activated cells. Thus, we characterized an astrocyte specific signature of T cell activation, which might be important in the pathogenesis of neuroinflammatory disorders.

INTRODUCTION

1.1 Adaptive immunity. A general overview

Adaptive immunity, also called acquired immunity, can be divided into humoral and cell-mediated immunity. The effector phase of humoral immunity, which is characterized by B lymphocytes as the main players, is triggered by the recognition of antigen by secreted antibodies; therefore, humoral immunity neutralizes and eliminates extracellular microbes and toxins that are accessible to antibodies, but it is not effective against microbes inside cells. In contrast, in cell-mediated immunity, the effector phase is initiated by the recognition of antigens by T cells. T lymphocytes recognize protein antigens as pathogens-derived peptides on the surfaces of infected cells in the context of major histocompatibility complex (MHC). Differently from innate immunity, adaptive immunity is characterized by somatic-rearrangement of antigen receptor genes, which confers clonality, high specificity and memory to the immune response [9]. In health, both humoral and cell-mediated immunity play important roles in immune surveillance and maintenance of tissue homeostasis, while in pathologic conditions, they work cooperatively to clear pathogens and form immunological memory [9]. It is well established that T lymphocytes with multiple specificities are generated in the thymus before antigen exposure. Naive T lymphocytes circulate throughout the body in a resting state, and only after T cell receptor (TCR) triggered activation, they acquire powerful effector potential. Naive T lymphocytes activation occurs in specialized lymphoid organs, where the naive lymphocytes and antigen presenting cells (APCs) are brought together. Protein antigens that cross epithelial barriers or derived from tissues are captured by dendritic cells (DC) (the most efficient APCs) and transported to lymph nodes. Antigens that enter the circulation may be captured by dendritic cells in the spleen. When a naive T cell is stimulated through its TCR by the cognate antigens bound to self MHC on an APCs, it receives concomitant costimulatory signals indispensable for full activation. After activation, a T cell proliferates, expands, and elicits effector functions either by cell-cell contact or cytokine release [9]. Whereas naive cells are activated mainly in lymphoid organs, differentiated effector cells may function also peripherally in tissues. The process of differentiation from naive to effector cells is associated with acquisition of the capacity to perform specialized functions and

competence to migrate to the site of infection or inflammation. At these sites, effectors cells respond to eliminate the source of the pathogen-derived antigens or provide immunregulatory functions to limit inflammation and consequent tissue damage.

1.2 CD4+ T cell subsets

According to different phenotypes and functions, T cells are divided into two main populations, the CD4+ T helper (Th) and CD8+ cytotoxic T cells, which recognize antigens in the context of class II and class I MHC, respectively. CD4+ T cell-mediated immune reactions are essentially elicited by dendritic cells. Microbial antigens are internalized into vesicles by the DCs, processed, and presented in association with class II MHC molecules to CD4+ T cells. As mentioned above, the complete activation of naive T cells requires costimulatory signals. The best characterized costimulatory pathway in T cell activation involves the T cell surface receptor CD28, which binds the costimulatory molecules B7-1 (CD80) and B7-2 (CD86) expressed on activated APCs. Cytokines also play critical roles in adaptive immune responses; the most important cytokine produced by T cells early after activation, is interleukin-2 (IL-2), a growth, survival and differentiation factor for T lymphocytes.

CD4+ T cells can be subdivided into conventional and regulatory T (Tregs) cells, this second group comprises natural and adaptively induced Tregs and provide immunosuppressive signals to limit inflammation [10]. Depending on the microenvironment and the functional status of the APC, naïve CD4+ T cells (Th0) can expand and differentiate into different subtypes, which are classified according to the cytokine profile and effector functions. As shown in figure 1, the three major subsets of conventional effector CD4+ T cells are Th1 (expressing IL-2, IFN-γ, and TNF-α), Th2 (expressing IL-4, IL-5, and IL-13), and Th17 (expressing IL-17 and IL-22) cells [11, 12] [13]. The relatively recent discovery of the IL-17 producing Th17 lineage, has changed our previous understanding of T cell responses based on the Th1/Th2 paradigm, which considered the Th1 and Th2 lineages as mutually exclusive and regulated by the antagonistic activity of Th1 and Th2 cell-derived cytokines [14]. Th17 cells are important in the pathogenesis of many inflammatory diseases, such as psoriasis, inflammatory bowel disease, rheumatoid arthritis, and multiple sclerosis (MS). Th1 and Th17 cells may both be present in the lesions of these diseases, and their respective contribution

to the development and propagation of the pathologic condition is an area of intense research. Conversely, CD4+ Tregs characterized by expression of the alpha chain of IL-2 receptor (CD25) and the transcription factor Foxp3, are pivotal in controlling peripheral tolerance to self antigens. [15].

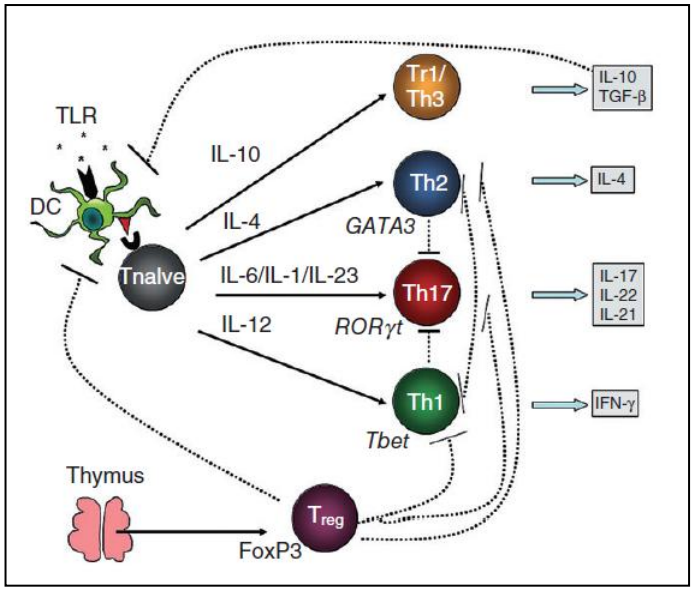


Figure 1: **The differentiation and regulation of CD4+ T cell subsets.** Naive T cells primed by antigen-presenting cells (APCs) such as dendritic cells (DCs) can differentiate into T regulatory-1 (Tr1)/T helper type 3 (Th3), Th1, Th2 or Th17 cells depending upon the cytokine environment. Priming in the presence of interleukin (IL)-10 /transforming growth factor (TGF)-b, IL-12, IL-4 or combinations of IL-6/IL-1/IL-23 promotes the differentiation of Tr1/Th3, Th1, Th2 or Th17 cells, respectively. Th17 cells can be negatively regulated by Th1 or Th2 cells. Natural regulatory T cells (Treg) cells are derived from the thymus (although they may also be converted in the periphery) and can suppress effector T cell responses directly or via the APC [16]

1.3.1 Blood brain barrier: the "immune privilege" point of view

In the late 19th century Paul Ehrlich observed that water-soluble vital dyes injected into the peripheral circulation would stain all organs except the brain, this experiment provided the first indication that the central nervous system (CNS) was anatomically separated from the rest of the body. Subsequent studies by Edwin Goldmann, showing that dye injected into the spinal fluid did not stain peripheral tissues, confirmed the idea that the brain was a unique anatomical compartment. Consequently, the CNS was

regarded as an “immune privileged” organ. The main reason for this definition was attributable to the existence of the blood-brain barrier (BBB) surrounding the brain parenchima. The lack of an obvious lymphatic system, low constitutive levels of MHC class I and II molecules, local CNS production of suppressive factors and, in the normal state, limited numbers of APCs reinforced the concept of the CNS as an “immune-privileged” site [17, 18]. Structurally, the BBB is composed of specialized endothelial cells (ECs) held together by multiprotein complexes known as junctional proteins [19] [3]. BBB limits the transport of specific factors and solutes, including >98% of antibodies and small molecules, into the brain parenchyma, while ensuring the efflux of others [16] [20]. Astrocytes, which are closely apposed to the CNS vasculature (figure 2), also help in maintaining BBB integrity and immune quiescence through contact-dependent mechanisms and by releasing soluble factors [3] [4-6].

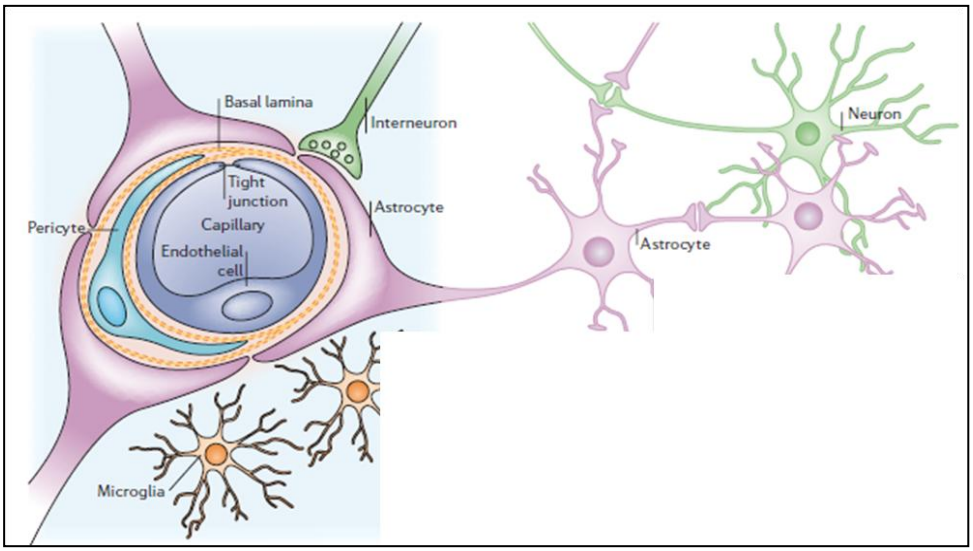


Figure 2: Cellular constituents of the blood–brain barrier. The barrier is formed by capillary endothelial cells, surrounded by basal lamina and astrocytic perivascular endfeet. The figure also shows pericytes and microglial cells

1.3.2 Trafficking of CD4+ T lymphocytes into the brain

Only very recently the concept of the brain as “immune privileged” site has been revised [18] [21]. Indeed, it is becoming more and more clear that whereas few T lymphocytes patrol the brain for immune surveillance and are indispensable for brain homeostasis [21] under injurious or disease conditions, more T but not B cells infiltrate the brain at site of injury [1] [2]. Cells of immune system have access to three distinct brain anatomical compartments (i.e., cerebrospinal fluid [CSF], meninges, and parenchyma). The migration of leukocytes to the CSF is thought to occur through the choroid plexus and the subarachnoid space (which contains the CSF), while the direct migration from the blood into the brain parenchyma occurs across the BBB via the perivascular space (figure 3). It is important to note that leukocyte recruitment is cell- and site-specific, thus infiltration is not likely due to massive disruption of the BBB [1] [2]. Following injury to the CNS in fact, the activation of endothelial cells and associated cells as astrocytes lead to reduced tight junction integrity and formation of transendothelial cell channels [22-24], which facilitate the migration of leukocytes across the BBB [20, 25, 26].

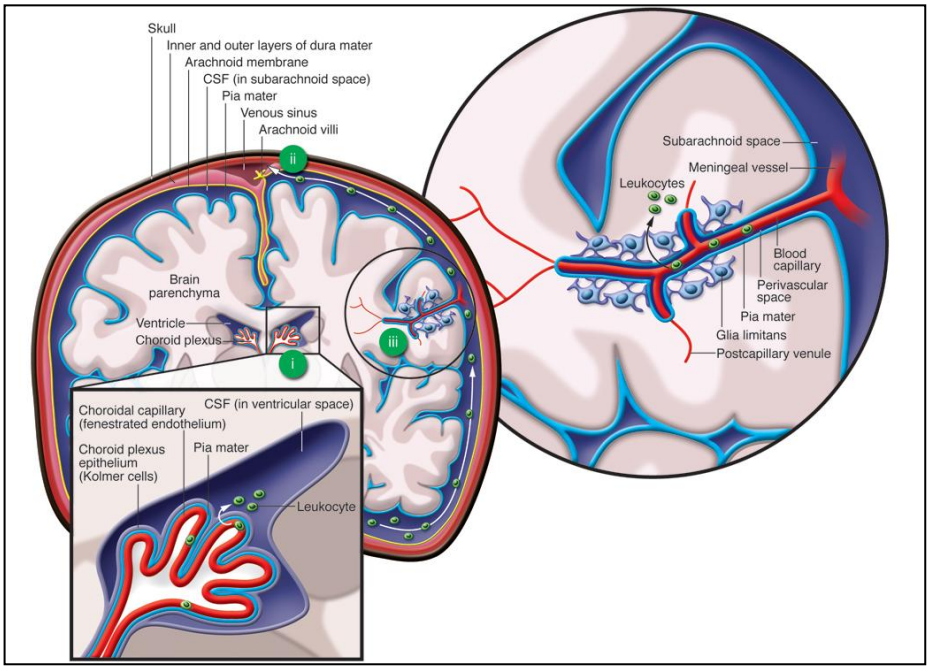


Figure 3: **The structure of the brain and routes of leukocyte entry.** Beneath the skull lie three membranes that enclose the parenchyma of the brain: the dura mater, the arachnoid membrane, and the pia mater. The latter two enclose the subarachnoid space. (i) Leukocytes can enter across the choroid plexus, where CSF is produced by the choroid plexus epithelium in the ventricles. CSF containing leukocytes then enters the subarachnoid space, circulates around the brain, and (ii) exits via the venous

sinus to be resorbed by the blood via the arachnoid villi. (iii) Blood supply to the brain enters in the subarachnoid space over the pia mater, generating the perivascular space (or Virchow-Robin space). Main arterial branches divide into capillaries, which terminate deep within the brain, supplying the parenchyma with blood. Leukocytes can potentially enter from the blood (iii), which requires them to cross the tightly regulated vascular endothelium (i.e., the BBB: the glia limitans, the subarachnoid space, and the pia mater). Cells can adhere to the endothelium and arrest at any point during this process.[27]

During CNS entry, activated T cells upregulate many integrins and adhesion molecules, enabling their rolling and adhesion to vessel walls. On the other side, circulating and CNS-resident cells express and upregulate multiple integrins, chemokines and adhesion molecules which participate to infiltration process. Among them, the adhesion molecule P-selectin, the vascular cell adhesion molecule 1 (VCAM1) and the intercellular adhesion molecule (ICAM1), which bind to very late antigen-4 (VLA-4, also known as α4β1 integrin) and lymphocyte function–associated-1 (LFA-1), respectively [28, 29]; and the chemokines CCL19 and CCL20 [29-31]. Interestingly, blockade of VLA-4/VCAM1 interactions delayed the onset and/or decreased the severity of experimental autoimmune encephalomyelitis (EAE), a model of the neuroinflammatory disease multiple sclerosis [32]. The observation led to the clinical development of a monoclonal antibody (known as natalizumab) that targets α4 integrin (a component of VLA-4). Natalizumab has been successfully used in clinical trials to manage the disease [33]. After rolling along, adhering to, and finally crossing the endothelial cells of the BBB and their associated basement membrane, the migrating leukocytes reach their next barrier, the glia limitans. Figure 4 shows that this structure surrounds the blood vessel, and is composed of astrocytic foot processes. Only in the past few years a dialogue between peripheral immunity and the CNS has been appreciated [34] [1, 35]. It is now clear that the two systems interplay during physiological condition and in pathological situation. The focus of my project was to characterize the molecular basis and functional significance underlying the interaction between astrocytes and CD4+ T lymphocytes, two of the main critical players belonging to nervous and immune system.

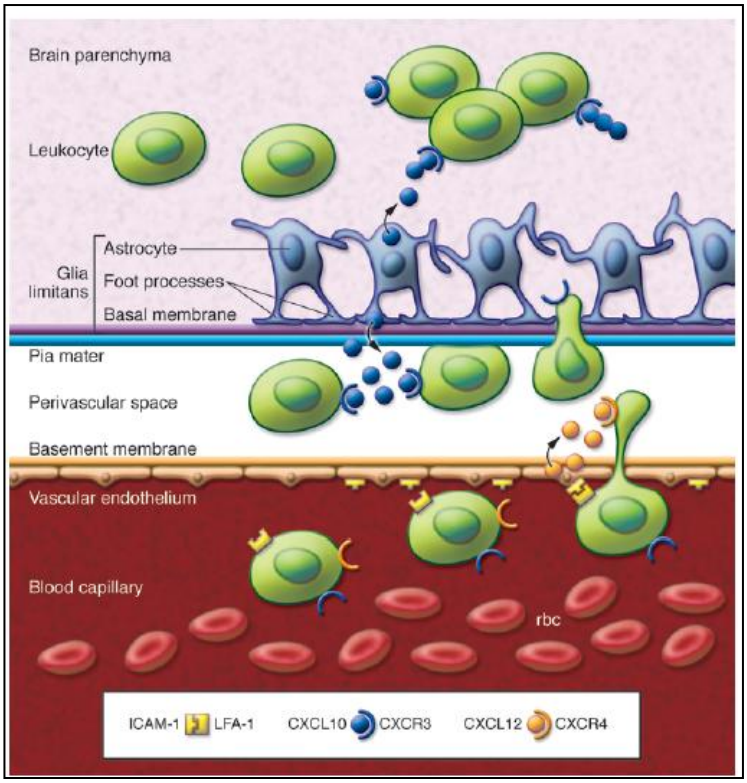


Figure 4: **Leukocyte trafficking across the glia limitans into the parenchyma of the brain.** Activated leukocytes expressing adhesion molecules and integrins roll and attach to the vascular endothelium. Successful diapedesis requires appropriate ligation of adhesion molecules, selectins, and integrins, signaling to both the infiltrating leukocyte and the brain endothelium. Expression of CXCL12 on the basolateral surface of endothelial cells recruits CXCR4+ T cells. However, retention of cells in the perivascular space occurs in the presence of high concentrations of CXCL10. Continued migration puts cells in contact with the glia limitans, which is composed of a highly structured wall of astrocytes. Further positive migratory signals, including chemokines, from these and surrounding cells may allow leukocyte migration into the parenchyma. [27].

1.3.3 Astrocytes cross-talk with CD4+ T cells at the BBB and into the parenchima

Independently from effects mediated by endothelial cells, astrocytes may establish direct physical contacts with immune cells within the BBB, thereby directly influencing T cell activity. Previous studies have investigated whether an immunological synapses (IS) may be formed between astrocytes and activated CD4+ T cells [36]. While surface expression of MHC class II molecules was unequivocally reported in astrocytes in

response to the cytokine IFN-gamma [37], conflicting results have been published about the expression of the co-stimulatory molecules ([38, 39] and our unpublished data), which are required to form an IS and to initiate Ag-specific immune response [40]. Thus, it is unlikely that a true IS can be established between astrocytes and activated CD4+ T cells, especially in vivo, where MHC class II expression is highly debated. However, other types of adhesive contacts may be formed between astrocytes and T cells during BBB entry, which may reciprocally influence the activity of the two cells. In addition, astrocytes and CD4+ T cells may reciprocally communicate through secretion of signaling molecules during contact. In this regard, notably, both T cells and astrocytes express proteins of the connexin and pannexin families (Cx32, Cx43, Cx30, pannexin1), which form membrane hemichannels that mediate release of signalling molecules into the pericellular space [41-44]. Through formation of intercellular channels, called gap junction, connexins also allow the direct transfer of signaling molecules between adjacent cells including cAMP, Ca2+, adenosine-5-triphosphate (ATP), inositol 1,4,5-trisphosphate and morphogens [45]. Finally it was discovered that beta1 integrin plays an important role not only in the epithelial/T-cell, but also in astrocyte/T cell interaction [46-49]. The group of Colombatti discovered that alfa3beta1 integrins are active components of the molecular complex mediating astrocyte / T-cell binding [48] and another group showed that the subunits of VLA-4 integrin, alfa4beta1, are necessary for T cell contact-induced generation of proinflammatory molecules in astroglia [46-49].

In conclusion astrocyte and T cell can interact hypothetically through multiple mechanisms, but the significance and the final effect underling their interplay is far to be completely understood.

2.1.1 Physiological functions of astrocytes

Astrocytes are the most abundant glial cells, within the CNS. They are of neuroectodermal origin and they owe their name to the star-like shape (ancient Greek αστρον: star, and κυτύος: cell). Indeed astrocytes display several thin processes, through which they contact neurons, blood vessels and other astrocytes. In the late nineteenth century and the early twentieth century Camillo Golgi and Ramon y Cajal had already noticed that although all astrocytes share a stellate shape (figure 5, left), their morphology is extremely diverse in different areas of the brain (Figure 5 A-B). Morphological diversity of astrocytes was subsequently confirmed by studies performed both *in vitro* and *in vivo* [50, 51].

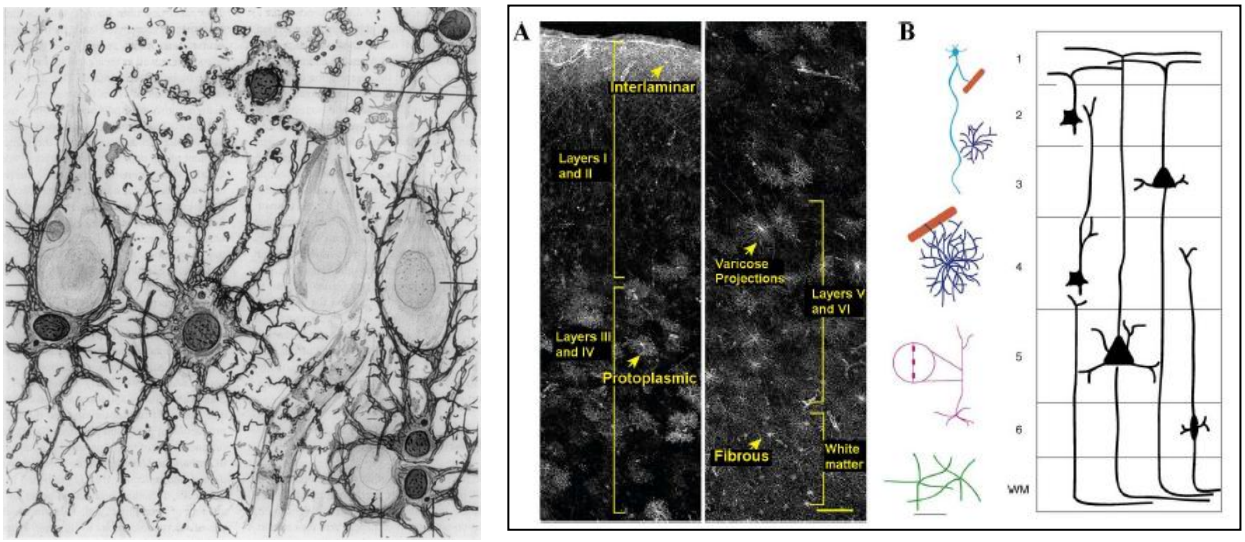


Figure 5: **Morphological heterogeneities in human cortical astrocytes.** (Left) Neuroglia from the hippocampal formation (stratum radiatum of the Ammon horn) of a human brain. In this drawing Cajal shows astrocytic processes embracing pyramidal neurons as well as astrocytic processes in contact with blood vessels. Form Legado Caljal- in: GarcAa Segura 2002. **(**A) Shows the GFAP immunolabelling of all layers of the human temporal cortex; different morphologies of astrocytes that reside at different depth in the brain are visible. Scale bar, 150 μm. (B) shows -on the left- schematic representations of classes of astrocytes located within the cortex; -on the right- the cortical layers. Primate-specific interlaminar astrocytes (light blue) located in layer 1 send long fibers throughout the cortex terminating in layers 3 and 4. Protoplasmic astrocytes (dark blue; shown here in layers 2 and 4) characteristically inhabit layers 2–6. These astrocytes, which vary markedly in size, are organized into domains associated with neurons and blood vessels (red). Polarized astrocytes (pink), found in layers 5–6 rather than near the pia, also extend

long, but various processes. Fibrous astrocytes (green) reside in the white matter (WM) and are not organized into domains. Scale bar 100 μm.

Astrocytes are distinguished by the expression of glial fibrillary acidic protein (GFAP), a protein which compose intermediate filaments in their cytoplasm and that is used today as an astrocytic marker [52]. Astrocytes are extensively coupled by gap junctions. The cellular network created by adjacent astrocytes is called the astrocytic 'syncytium' [53]. Although astrocytes form a highly interconnected network, they occupy separate domains of the parenchyma, with very limited or no overlap between the region occupied by one astrocyte and the regions occupied by its neighboring cells [54, 55].
Traditionally astrocytes were regarded as supportive cells with the main task to maintain the physiological homeostasis of neurons by taking up excess neurotransmitters and buffering the ionic content of the extracellular medium in the brain. However, studies of the last 20 years have shown that astrocytes exert a series of complex and different functions and are involved in almost everything the CNS does. In fact, astrocytes assist synapse formation and function, by stimulation of neurite growth and branching, modulation of synaptic transmission and plasticity [56, 57] [58, 59] [60] [61]. Furthermore astrocytes, regulate the BBB permeability, myelination of axons and participate in the development of the nervous system [62-67]. The relevance of astrocytes as active components of synaptic transmission is highlighted by the term "tripartite synapse" (figure 6), which indicates a synaptic structure composed of the astrocyte wrapped around the synapse as well as the presynaptic and postsynaptic terminals [68]. Given the crucial role of astrocytes in brain development, metabolism, and function, it is not surprising that astrocytes are involved in almost every disease affecting the CNS.

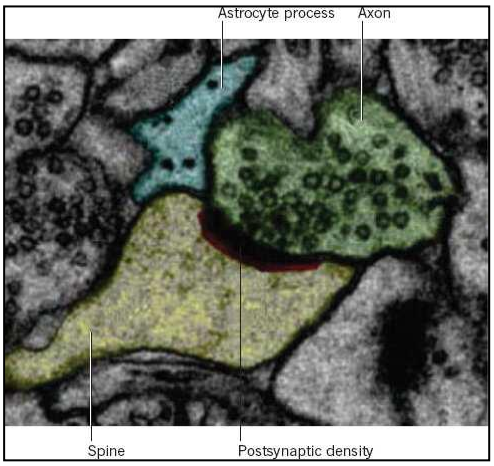


Figure 6: **The electron microscopy (EM) of the tripartite synapse.** The intimate association of astrocytes and synapses is both structural and functional. Here is shown a tripartite synapse in the hippocampus. The astrocyte process (blue) unsheathes the perisynaptic area. The axon of the neuron is shown in green, with the dendritic spine in yellow and the postsynaptic density in red and black.

2.1.2 Astrocytes display both detrimental and protective roles

During neuroinflammation, astrocytes seem to have both detrimental and protective activities. They can produce various pro- and anti-inflammatory cytokines, including IL-1, IL-6, IL-10, IL-12, IL-15, IL-23, IL-27, IL-33, IFN-a, IFN-b, TGF-b, TNF and several chemokines, including CCL2 (MCP-1), CCL3, CCL4, CCL5, CCL20, CCL5 (RANTES), CXCL8 (IL-8), CXCL10 (IP-10) and CXCL12 (SDF-1) [39]. The ability of astrocytes to release *in vitro* a wide variety of proinflammatory chemokines and cytokines, suggests that they may act as proinflammatory mediators [39] [69]. Nevertheless, equally strong *in vitro* evidences suggest that astrocytes may serve to mitigate or restrict inflammation by producing anti-inflammatory cytokines and ROS scavengers [39] [70] [71].

During CNS injury or inflammation, astrocytes upregulate GFAP protein in a process termed “reactive astrocytosis”, eventually leading to the formation of the so called “astroglial scar” [72]. The astrogliotic scar can prevent neural stem cell migration into the lesion, thus impairing remyelination and axonal regeneration. However, reactive astrocytosis may be also beneficial in neuroinflammatory diseases such as MS. Notably, as shown in figure 7, experimental autoimmune encephalomyelitis (EAE), the widely characterized model of human MS [73], is more severe in GFAP-deficient mice, with strongly enhanced immune cell infiltration [74, 75]. Consistent with these data, a recent study showed that inhibition of reactive astrocytosis after initiation of EAE leads to increased macrophages infiltration and enhanced severity of EAE [76], further supporting a role of astrocytes in limiting EAE lesions. As mentioned before, astrocytes, which are intimately associated with blood vessels [67] and constitute the glia limitans apposed to the BBB, regulate leukocyte trafficking into the CNS. At the BBB, astrocytic endfeet release, upon activation, inflammatory cytokines, including IL-6, TNF-alfa and IL-1beta: these factors can inhibit tight junctions in adjacent endothelial cells [77], thus increasing BBB permeability and favour influx of immune cells into the brain. On the other hand, reactive astrocytes can also promote tight junctions formation in endothelial cells and BBB integrity via Sonic hedgehog pathway, to counteract brain inflammation [78].

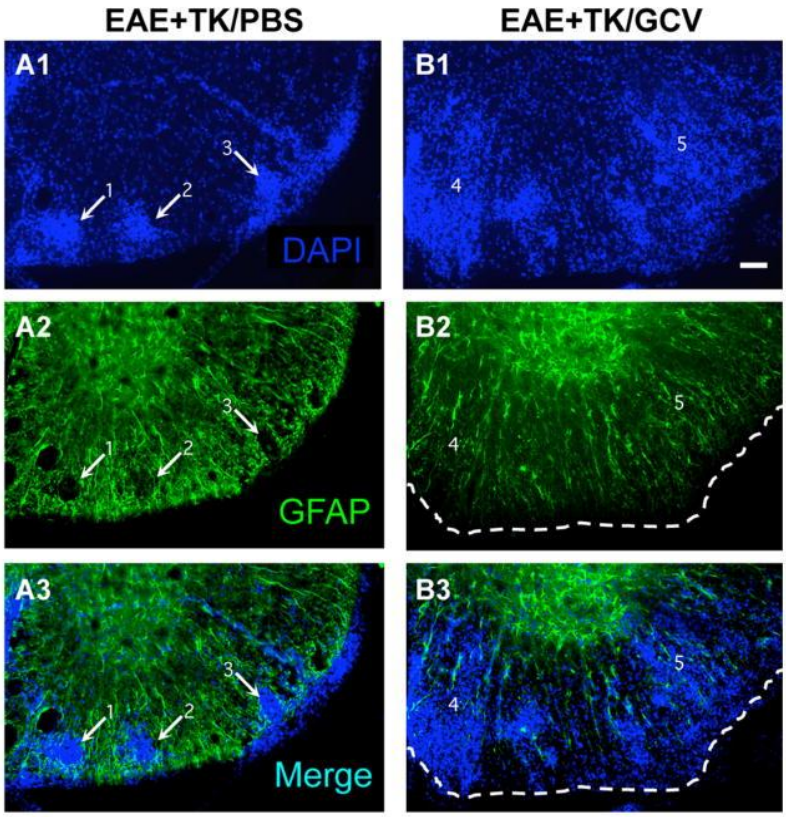


Figure 7: **Increased spread of infiltrating inflammatory cells during EAE with ablation of proliferating reactive astrocytes.** ***A1–A3***, ***B1–B3***, Single-channel and merged two-color fluorescence survey images of spinal cord sections stained for GFAP (green) and the nuclear counterstain, DAPI (blue), in GFAP-TK transgenic mice that were induced with EAE and given either PBS (EAE + TK/PBS) as a vehicle control (***A1–A3***), or GCV (EAE + TK/GCV) to ablate transgene expressing astrocytes (***B1–B3***). Note that in EAE + TK/PBS, DAPI-stained infiltrating inflammatory cells (***A1***) are found primarily in dense perivascular clusters (e.g., 1–3) surrounded by intensely stained GFAP-positive astrocytes (***A2***, ***A3***). In EAE + TK/GCV (***B1–B3***), inflammation is markedly increased and there is little perivascular clustering in comparison with EAE + TK/PBS; instead, DAPI-stained infiltrating inflammatory cells (***B1***) spread widely in the parenchyma of the white matter. GFAP staining shows that the white matter is substantially depleted of GFAP-positive astrocytes in EAE + TK/GCV (***B2***), and double staining reveals that the spread of DAPI-positive inflammatory cells is heaviest in areas depleted of astrocytes (***B3***). Scale bar, 50 μm.[75]

2.1.3 Astrocytes exhibit calcium-mediated intercellular communication

Astrocytes display very stable membrane potential and unlike neurons are not capable of generating action potentials [79]. Thus, astrocytes have classically been regarded as electrically silent, nonexcitable, cells. However, astrocytes display their excitability through variations in intracellular calcium [Ca2+]i [80-82]. Astroglial cells display spontaneous [Ca2+]i oscillations *in vitro* [83, 84] and it has been shown that even in situ astrocytes from brain slices show spontaneous [Ca2+]i oscillations [85, 86]. Many neurotransmitters stimulate [Ca2+]i elevations in astrocytes by activating corresponding receptors expressed on their surface. By the use of fluorescent Ca2+ indicators, it has been demonstrated that intracellular Ca2+ oscillations could be both spontaneous as well as evoked by neuronal activity. Spontaneous Ca2+ signals are mostly restricted to single astrocytes and occur independently of the activity of neighboring cells [87], while activity-mediated Ca2+ oscillations occur in groups of different astrocytes that respond to the same neuron-derived stimulus [88]. Both spontaneous and neuronal activity-evoked Ca2+ oscillations trigger the release of active transmitters from astrocytes, in a process named “gliotransmission”. Gliotransmitters comprise glutamate [56, 89], D-serine [90], tumor necrosis factor alfa (TNFa) [91] and ATP [92, 93] and many other active compounds. Remarkably [Ca2+]i oscillations can spread from one astrocyte to adjacent cells to form synchronous Ca2+ oscillations or Ca2+ waves. Different studies proved that calcium wave propagation among astrocytes is dependent on both an intercellular gap-junction pathway, involving calcium and inositol 1,4,5-trisphosphate (IP3) [84, 94-96], and an extracellular pathway, involving release of ATP and/or glutamate and activation of metabotropic receptors on neighbouring cells [97]. In the presence of the gap-junction blocker anandamide (100 μM), ATP release was found to represent the main factor controlling propagation of calcium wave in hippocampal cultures [93]. Consistently, subsequent findings revealed that both spontaneous and evoked Ca2+ propagation can be reduced or even abolished by purinergic antagonists or the ATP-degrading enzyme apyrase (figure 8A) [92, 97-99] indicating that extracellular ATP and activation of P2 receptors are largely responsible for spontaneous and evoked Ca2+ events. These findings identify extracellular ATP as the primary molecule involved in calcium-mediated cross-talk among astrocytes and between astrocytes and other cell types in the central nervous system.

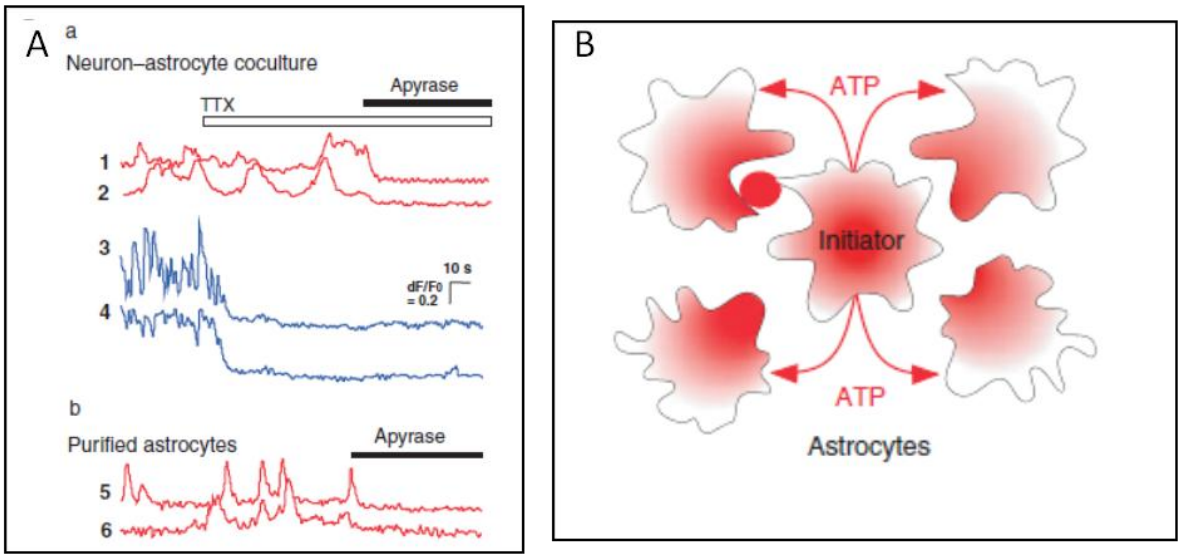


Figure 8: **Neuronal activity-independent Ca2+ transients in astrocytes.** (A) (a) Neuronal Ca2+ oscillations seen in the hippocampal neurons (blue traces shown as 3 and 4) are highly synchronous and are inhibited by tetrodotoxin (TTX) . Adjacent astrocytes (red traces shown as 1 and 2) also show slower and less synchronous Ca2+ oscillations. However, the synchronous Ca2+ oscillations in astrocytes are unaffected even when neuronal activities are inhibited by TTX, suggesting that astrocytes have mechanism(s) by which they form neuronal activity-independent Ca2+ transients. (b) Astrocytes reveal synchronous Ca2+ transients (red traces 5 and 6) when neurons are not present (purified astrocytes). Astrocytic Ca2+ oscillations seen in the presence of TTX or in purified astrocytes were abolished by the ATP-degrading enzyme apyrase. (B) Schematic cartoon of neuronal activity-independent astrocytic Ca2+ oscillations. One or some initiator astrocyte(s) release ATP, and this is followed by ATP-dependent Ca2+ transients that propagate into adjacent astrocytes.[98]

The source of astrocyte Ca2+ elevations appears to be almost exclusively inositol1,4,5-triphosphate receptor (IP3R) sensitive intracellular store, activated following G-protein coupled receptors GPCRs stimulation and involving the canonical phospholipase C (PLC) /IP3 pathway. Upon Gq GPCR activation, PLC hydrolyzes the membrane lipid phosphatidylinositol 4,5-bisphosphate (PIP2) to generate diacylglycerol (DAG) and IP3, leading to IP3R activation and Ca2+ release from the endoplasmic reticulum (ER) (figure 9). In fact, incubation of astrocytes with thapsigargin (2 µmol/L), an irreversible inhibitor of the sarco/endoplasmic reticulum (ER) Ca2+-ATPase, to deplete ER Ca2+ stores, completely prevents spontaneous Ca2+ oscillations [86, 100]. Moreover, spontaneous and evoked astrocyte Ca2+ elevations are almost completely abolished

by removal of the astrocyte-specific IP3R type2 (IP3R2) receptors [101-103]. Taken together the above evidences indicate that both evoked and spontaneous Ca2+ elevations are driven almost exclusively by Gq GPCR-coupled release from intracellular stores.

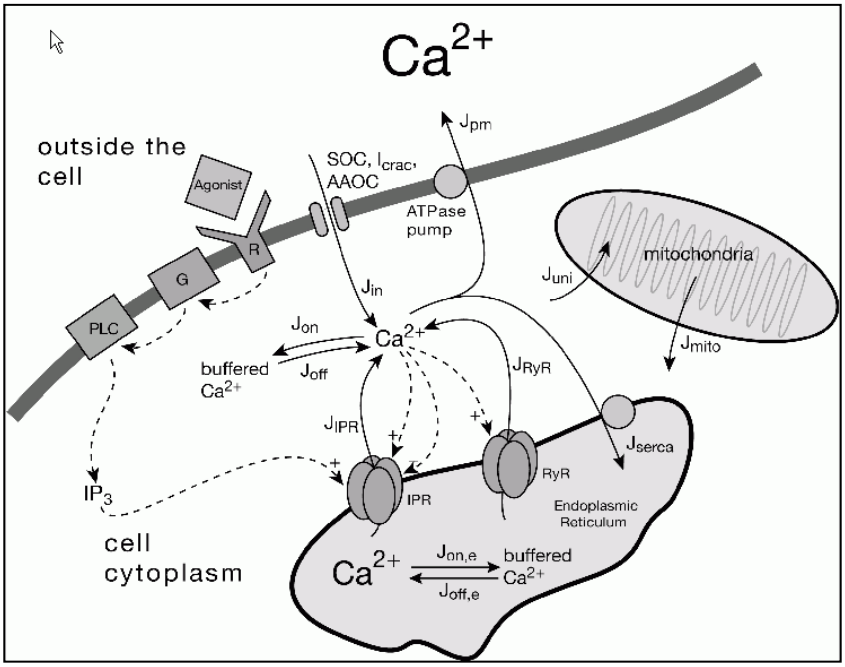


Figure 9: **Diagram of the major fluxes involved in the control of calcium oscillations in non-excitable cells.** Abbreviations: R (agonist receptor), G (G-protein), PLC (phospholipase C), SOC (store-operated channels), AAOC (arachadonic acid-operated channels), RyR (ryanodine receptor), Icrac (calcium release-activated channel).

2.1.4 Gliotransmission. Focus on ATP

Gliotransmission has been an exciting area of neuroscience research since its initial discovery in 1994 [56]. Gliotransmitters include a large number of neuroactive molecules, such as (a) excitatory, inhibitory and modulatory aminoacids (D-serine, glutamate, aspartate, GABA, glycine, homocysteine and taurine) [104, 105], (b) ATP and related nucleotides and nucleosides (purine, nucleotides), (c) eicosanoids and other lipid mediators (prostaglandins) [106] (d) neuropeptides (proenkephalin, angiotensinogen, endothelins, atrial natriuretic peptide (ANP), neuropeptide Y (NPY), (e) neurotrophins (nerve growth factor (NGF), neurotrophin-3, brain-derived

neurotrophic factor (BDNF) [107], (f) cytokines (interleukins, interferons, TNF-α) [91], (g) structurally associated chemokines and (h) growth factors. As I mentioned in the previous paragraph, a link between gliotransmission and Ca2+ evoked regulated secretion has been described only for some of the molecules released by astrocytes: in fact, while Ca2+ dependent release has been proven for glutamate [56, 89], ATP [92, 93] and D-serine [90], for many other active agents the Ca2+-dependency of their secretion has not been proven or tested yet. Moreover, alternative routes of release, independent from Ca2+ mobilization [108, 109] also exist for gliotransmitters, such as glutamate and ATP, which are secreted through vesicular mechanims [110]. For example ATP can be released from astrocytes by (1) Ca2+-dependent exocytosis [111-113] (2) by a regulated form of exocytosis ,(3) through hemichannels formed by connexins and pannexins, and finally (4) through plasmalemmal voltage-dependent anion channels and purinergic receptor (P2XRs) (figure 10).[109, 114]

Two different types of secretory vescicles mediate regulated ATP exocytosis: large dense core vesicles, which store secretogranin II (Sg II) [115-117], peptides (ANP) and a fraction of cellular ATP [93] [60, 118] and secretory lysosomes [119-121] which contain ATP but not glutamate. Secretory lysosomes have been recently suggested to represent the major vesicular compartment undergoing calcium regulated exocytosis in astrocytes [119-122]. To fuse with the plasma membrane, secretory lysosomes use the vesicular SNARE protein TI-VAMP, also called VAMP7, which is typical markers for these secretory organelles (figure 11)[123, 124].

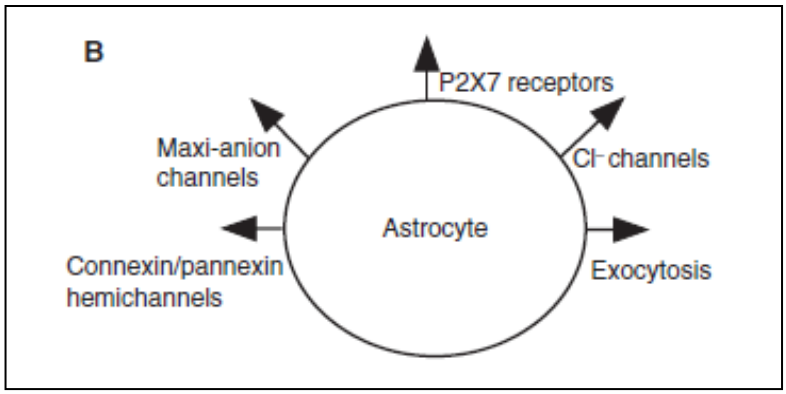


Figure 10: **Multiple pathways for the release of ATP.** Hemichannels of connexin or pannexin, maxi-anion channels, P2X7 receptors and Cl channels are pathways through which ATP can flow. In addition, the existence of exocytotic ATP is also suggested.

The existence of vesicular mechanisms of ATP secretion *in vivo* was supported by studies of Philip Haydon's laboratory, which were performed [60] in transgenic mice expressing, selectively in astrocytes, a dominant-negative SNARE domain, that blocks regulated exocytosis, Indeed, the authors found that secretion of ATP and its subsequent conversion to adenosine is strongly inhibited in the brain of these transgenic mice [60] [125]. Very recently it has been also identified the vesicular nucleotide transporter which mediates ATP storage within astrocytic vesicles: SLC17A9, a novel member of the anion transporter family.

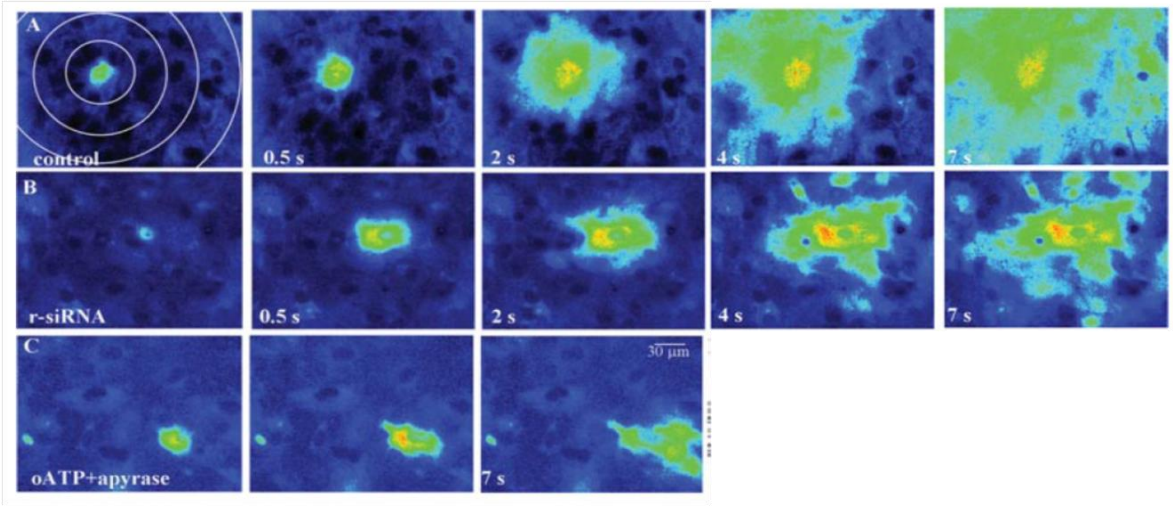


Figure 11: **TI-VAMP silencing reduces the amplitude and the speed of calcium wave propagation.** (A and B) Pseudo-colour images of the changes in the calcium concentration (F340/380 signal) in control (A) and TI-VAMP silenced (B) astrocytes taken 0.5, 2, 4, and 7 s after mechanical stimulation in the presence of 100 µM anandamide. (C) Pseudocolour images of calcium wave propagation at 0.5 and 7 s in astrocytes pre-incubated with the ATP degrading enzyme apyrase (30 µ/mL) and the purinergic receptor antagonist oATP (300 µM).[122]

2.2.1 Role of extracellular adenosine-5'-triphosphate (ATP) in the adaptive immune response

In healthy tissues, ATP is almost exclusively localized intracellularly (in the 5-10 mM range), whereas its extracellular concentration is very low (in the 10–100 nM range). After injury or in response to stimulation, cells may undergo formation of transient breaches in the plasma membrane from which ATP may easily efflux, driven by the large chemical gradients (10^6-fold gradient for ATP efflux; millimolar versus nanomolar). Extracellular ATP (eATP) concentration is maintained low by the activities of extracellular ecto-apyrases and ectoadenosine triphosphatases (ecto-ATPases), which metabolize ATP into adenosine 5'- diphosphate (ADP), adenosine 5'-monophosphate (AMP), and adenosine. Thanks to the function of these enzymes, leakage of a very small fraction of intracellular ATP is sufficient to elevate extracellular levels at concentrations active on purinoreceptors (P2Rs). Therefore ATP is generally considered an ubiquitous 'danger signal' or DAMP (Damage Associated Molecular Pattern), especially in the immune system. Extracellular ATP concentration can also increase in the absence of cell damage, as virtually all cells, possess secretory pathways for non-lytic ATP secretion (i.e. ABC transporters, non-selective large conductance channels such as connexins and pannexins, constitutive or stimulated exocytosis). [126, 127]. Among the purinoreceptors, P1Rs (now known as A1, A2, and A3 receptors) respond to adenosine but not to ATP, whereas all P2Rs respond to ATP, with some also responding to adenosine diphosphate (ADP), uridine 5'-triphosphate (UTP), or uridine 5'-diphosphate (UDP). Two main classes of purinergic P2 receptors have been identified: P2X receptors (P2X1- P2X7), which are Ca2+-permeable, nonselective ligand-gated ion channels, and G protein protein–coupled receptors (P2Y1, P2Y2, P2Y4, P2Y6, and P2Y11 to P2Y14 receptors), which bind also ADP, UDP, UTP, or uridine diphosphate glucose (UDP-glucose). Activation of P2 receptors regulates a myriad of cellular functions ranging from survival and proliferation to apoptosis. Therefore the final effect of extracellular ATP on a given cell depends on the composition of P2 receptors expressed on its surface .P2X1, P2X4, and P2X7 and several functional P2Yrs are found on T cells [128] as are. Most P2Rs are sensitive to micromolar concentrations of eATP. The P2X7 receptor is an unusual non-selective cation channel, whose activation requires higher ATP concentrations (>100 μM) compared to the other members of the P2X receptor family. ATP binding to P2X7

receptor induces a depolarizing ionic current within milliseconds. Persistent activation of the receptor leads to the formation of a pore permeable to larger molecules such as ethidium bromide. For a long time the molecular composition of the P2X7 pore has been a matter of a great discussion. However, it has been recently described that P2X7 receptor interacts with the pannexin-1 hemichannels which may constitute the path for uptake of large molecules [129].

2.2.2 Extracellular ATP shapes CD4+T cell functional responses

The importance of extracellular ATP for cell-to-cell communication in the nervous and vascular systems has been thoroughly studied for years, but its role in the immune system is less known. Several studies have shown that ATP regulates T-cell activation and function [42, 130]. However a coherent picture of responses evoked by ATP in T cells is not currently, available, because of the contrasting results obtained in different studies [131-133]. Two recent studies demonstrated that eATP is important for cell-to-cell communication in the immune system. In the first study, Grassi and collaborators showed that T cell activation induces ATP synthesis and release, thus activating purinergic receptors on T cells in an autocrine fashion [42]. Moreover, they demonstrated that the activation of P2X7 by ATP inhibits the suppressive potential and stability of T regulatory cells (Treg). In fact, increased ATP synthesis, induces the *in vivo* conversion of T regs to IL-17–secreting T helper 17 (Th17) effector cells [134]. In another study, the team of Takeda proposed that ATP released by commensal bacteria drives the differentiation of intestinal T helper 17 (TH17) cells [135]. More in details, Takeda and colleagues showed that i) commensal bacteria release large amounts of ATP, although not enough to deliver a “danger” inflammatory signal to the intestinal mucosa ii) the number of Th17 cells increases in bacteria-free mice upon treatmentg with adenosine 5'-O-(3-thiotriphosphate), a nonhydrolyzable form of ATP, while decreases upon treatment with apyrase, which degrades ATP. Finally the percentage of Th17 cells measured in a co-culture of T cells and DCs is strongly increased by the addition of the bacterial supernatant, and this effect is apyrase sensitive.

All these findings highlight a major role of ATP in the intercellular communication in the immune system.

3.1.1 *Modulators of extracellular ATP levels: The ectonucleotidases CD39 and CD73.*

The pool of extracellular nucleotide (NTPs) is controlled by ectonucleotidases (E-NTPDases), which remove NTPs by degrading them to NMP (nucleoside monophosphate) [136, 137]. Members of this enzyme family are expressed on the surface of various cells, including astrocytes, microglia and T cells in the brain. Of this family, E-NTPDase1 (also called CD39) is the dominant ectoenzyme in the immune system [138]. As shown in figure 12, CD39 dephosphorylates ATP to AMP, removing one phosphate at a time with a modest appearance of ADP [139]. Monophosphonucleosides (for example, AMP) are further hydrolysed to nucleosides (adenosine) by ecto-5'-nucleotidase (CD73), a glycosyl phosphatidylinositol-anchored enzyme located at the cell surface [140]. In addition to CD39 and CD73, which are the major nucleotide metabolizing enzymes that regulate immunity and inflammation, there are other less well characterized cell surface-associated enzymes, involved in the catabolism of extracellular nucleotides, which include alkaline phosphatases, pyrophosphatases, and phosphodiesterases, as well as the counteracting ATP-regenerating ectoenzymes adenylate kinase and nucleoside- diphosphate kinase [141]. Although the impact of extracellular ATP on the immune system is well established [142], the importance of CD39 in immune regulation is not yet clear. Besides removing a proinflammatory stimulus, CD39 may also act in concert with CD73, to produce adenosine. This nucleoside exhibits mostly inhibitory and antiproliferative effects [142]. Hence, the overall effect of CD39 activity should be mainly immune suppressive.

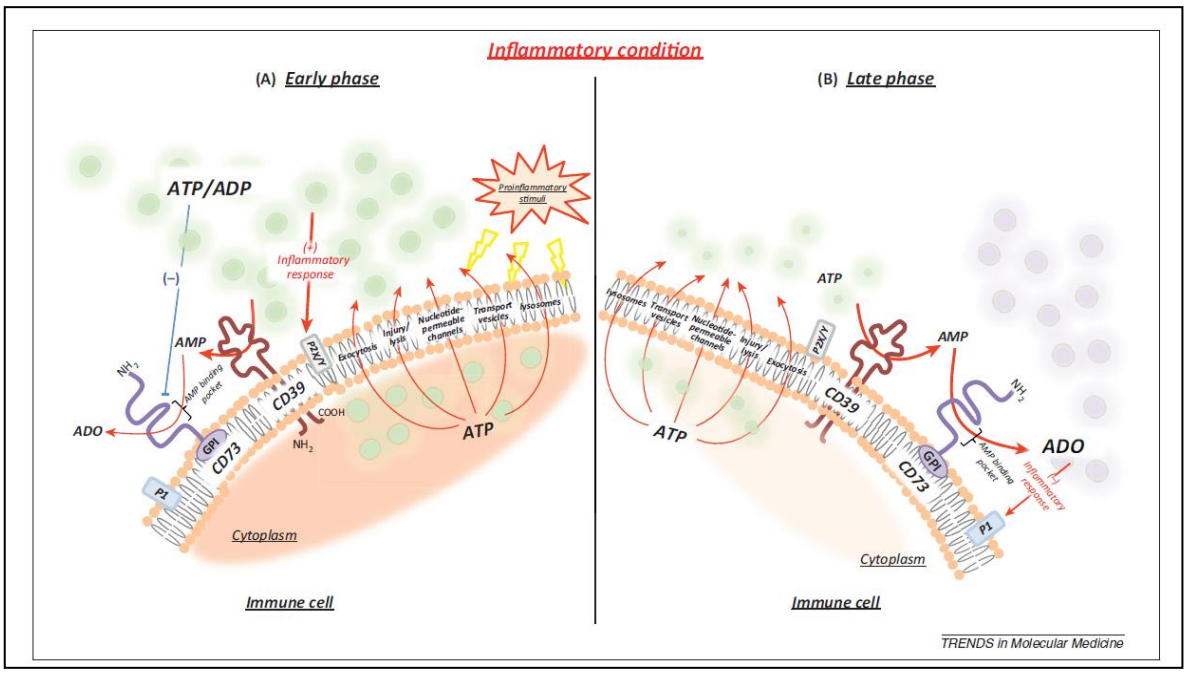


Figure 12: **CD39/CD73 in inflammatory event.** The occurrence of pathological events, such as inflammation, promotes a massive accumulation of ATP, which serves as a key 'danger' signal, triggering a series of proinflammatory responses (A). However, negative feedback also takes part in this context because increased ATP secretion, as observed in the early phase of inflammation, is followed by its sequential degradation to AMP by CD39, and to adenosine by CD73. Adenosine promotes a depressive action on the immune cell activity and exerts a potent anti-inflammatory effect.[143]

3.1.2 T regulatory cells express CD39/CD73 ectonucleotidases

Several regulatory mechanisms are used to maintain immune homeostasis, preventing autoimmunity and moderating the inflammation induced by pathogens and environmental insults. In this regard, Tregs are widely considered the primary mediators of peripheral tolerance, playing a pivotal role in preventing autoimmune diseases, as well as in limiting chronic inflammatory disorders. There are a number of mechanisms by which Tregs suppress proliferation and cytokine production by other cells, including depletion of IL-2, direct toxicity through granzyme or perforin, or via cytokines, such as IL-10, TGFb, or IL-35 [144]. A specific feature of Foxp3+ Tregs is the surface expression of CD39 and CD73, which are increasingly used as markers of this

lymphocyte population [145, 146]. The catabolic activity of the CD39/CD73 axis is synchronized with the activation status of the cells. Indeed, murine Tregs display increased CD39 activity only upon activation of their T cell receptor (TCR), while the enzyme is inactive in non-stimulated cells [147]. The increase in the ATP metabolizing activity appears to be critical for the immunosuppressive activity of Tregs [148]. It was speculated that the enhanced CD39 activity allows the entrance of Tregs into inflamed regions, where it reduces the extracellular ATP levels, thereby decreasing P2 receptor-mediated Treg cell death [148]. Deaglio et al. [149], using CD39-deficient mice, showed that CD39, in concert with CD73, facilitates the pericellular generation of adenosine, which mediates many of the immune suppressive and anti-inflammatory activities of Tregs.

3.1.3 Th17 Cell immunosuppressive activity via ectonucleotidase expression

Th17 cells are a subtype of CD4+ T lymphocytes, characterized by high expression of IL-17A (also called IL-17), a proinflammatory cytokine involved in the pathogenesis of various autoimmune diseases [12, 150]. Th17 cells are important for host defense against extracellular microorganisms and are involved in the pathogenesis of diverse immune-mediated diseases, including multiple sclerosis [151]. In addition to IL-17A, Th17 cells are characterized by secretion of IL-17F, IL-21, and IL-22, and expression of the IL-23 receptor (IL-23R) [152]. IL-17A, IL-17F, IL-22, and IL-21 contribute to inflammation through several mechanisms [153], by inducing the recruitment and expansion of myeloid cells including neutrophils and macrophages [154]. Th17 are also characterized by the expression of the transcription factor retinoid orphan nuclear receptor (encoded by RORC), which specifies the Th17 cell lineage [155]. Characterization of the phenotypic and functional properties of Th17 cells has been carried out in vitro after differentiation from naı¨ve CD4+ T lymphocytes. In mice, Th17 cells are commonly obtained by culturing naı¨ve CD4+ T cells isolated from spleen and lymph nodes in the presence of antibodies to CD3 and CD28 as well as the polarizing cytokines transforming growth factor-b (TGF-b) and IL-6 [156, 157]. IL-23, an IL-12

family member, does not initiate Th17 differentiation alone but favors Th17 proliferation and maintains the Th17 cell transcriptional program. However, it was recently shown that TGF-b is dispensable for the induction of Th17 cells. Indeed, Th17 cells can be obtained from naı¨ve CD4+ precursors upon culturing with IL-1b, IL-6, and IL-23 without TGF-b [158]. In addition to their capacity to produce IL-17A and express the transcription factor RORgt, Th17 cells produce IFN-Ɣ and express T-bet (Th1 lineage). Taken together, these data suggest that Th17 polarization of murine naı¨ve CD4+ T cells during T cell receptor (TCR)-mediated activation in vitro can be obtained through at least two pathways, one requiring TGF-b with IL-6, and the other involving the proinflammatory cytokine IL-1b with IL-6 and IL-23 without TGF-b. Studies regarding the role of Th17 cells in cancer and tumor progression, showed that despite the ability of Th17 cells to promote tissue inflammation and autoimmunity, Th17 cells can also act as immunosuppressor cells with the capacity to suppress the immune response against tumors and consequently enhance cancer growth. At least two separate mechanisms have been identified that may sustain Th17 immunosuppressive effect: (i) Th17 cells can progressively convert to Tregs because of their plasticity, and (ii) they can produce immunosuppressive adenosine upon TGFb-dependent ectonucleotidase expression. Th17 cells differentiated with TGF-b and IL-6, but not those induced with IL-1b, IL-6, and IL-23, express CD39 and CD73 ectonucleotidases on their surface [159]. The ectonucleotidase expression actually determines the effectors immunoregulatory function of Th17 cells. The concomitant expression of these two enzymes transforms ATP or ADP liberated by damaged cells in the tumor microenvironment into adenosine which play a major role in immunosuppression [149]. Ectonucleotidase expression on Th17 cells is determined by TGFb and IL-6 signaling through the transcription factors Gfi1 (growth factor independent protein 1) and Stat3 (signal transducer and activator of transcription 3): TGF-b downregulates Gfi1, a repressor of ectonucleotidase expression, whereas IL-6 activates Stat3, a promoter of ectonucleotidase expression (figure 13). The combined effect results in the transactivation of the CD39 and CD73 promoters [159]. These results suggest that ectonucleotidase expression actually determines the effector immunoregulatory function of Th17 cells.

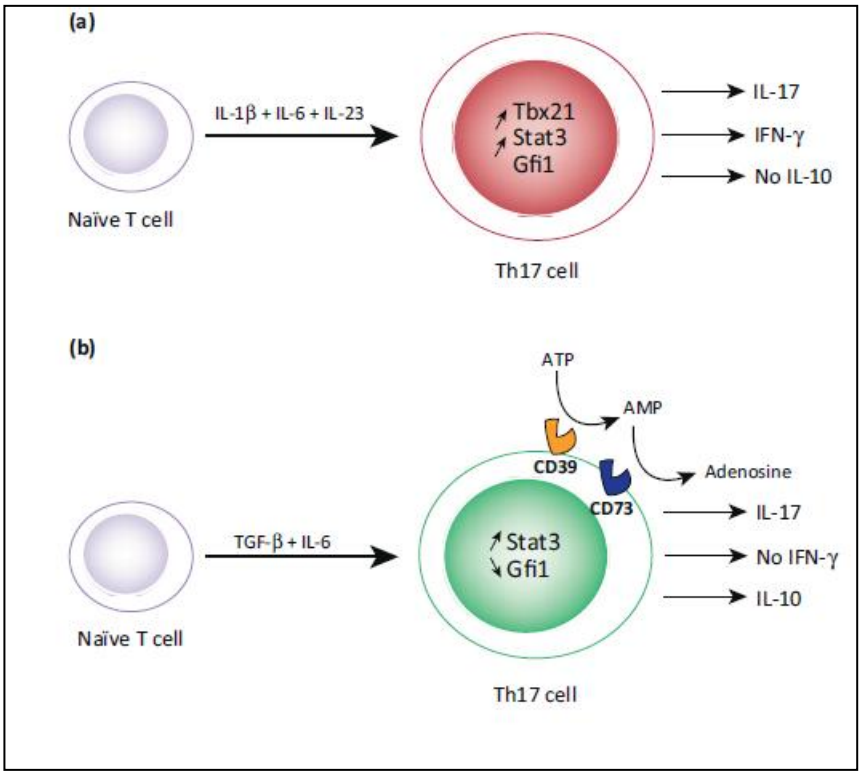


Figure 13: **Contrasting effects of T helper 17 (Th17) cells on cancer progression.** Mouse Th17 cells secreting interleukin (IL)-17A and expressing the Th17 transcription factors signal transducer and activator of transcription 3 (Stat3) and RORgt (retinoid orphan nuclear receptor gt) can be induced from naı¨ve CD4 T cells using IL-1b, IL-6, and IL-23 (a) or transforming growth factor-b (TGF-b) and IL-6 (b). (a) Th17 cells differentiated without TGF-b express the Th1 cell transcription factor Tbx21 and secrete interferon-g (IFNg) but not the immunosuppressive cytokine IL-10. They also express the transcription factor growth factor independent protein 1 (Gfi1) . Upon adoptive transfer, these cells promote activation of CD8 effector T cells and tumor regression in an IL-17- and IFN-g-dependent manner. (b) Th17 cells generated in the presence of TGF-b fail to produce IFN-g but secrete IL-10. They also feature reduced expression of Gfi1, resulting in the expression of the CD39 and CD73 ectonucleotidases, which can convert ATP to adenosine, thereby contributing to the inhibition of anticancer immunity.[160]

4. Multiple sclerosis and EAE. The possible scenario of astrocyte- T cell interaction

Multiple sclerosis is a chronic, progressive inflammatory disorder which principally affects the brain and spinal cord. Pathological hallmarks of MS are the inflammatory plaques and the presence of inflammatory cells and their products in the brain lesions.

CD4 T cells, autoreactive against myelin, play a major role in the pathogenesis of MS. They pass into the parenchyma in response to chemotactic signals through the BBB [161]. Once entered the CNS, T cells are re-activated by microglia, which bear MHC class II and secrete pro-inflammatory cytokines (figure 14) [162]. Autoreactive T cells mediate damage against neurons, their axons and myelin sheaths. Indeed the key morphological feature of MS is demyelination of nerve axons, which leads to block of signal conduction or conduction slowing at the site of demyelination. The most common form of MS, termed relapsing– remitting MS (RRMS), is associated with acute inflammatory episodes resulting in neurological deficits. Patients may experience some recovery between relapses, but 80% of patients with RRMS evolve to a more progressive form, termed secondary progressive MS (SPMS). This form is associated with a gradual loss of neurological function and ascending paralysis and is thought to be independent of inflammation. The best characterized and studied animal model of MS is the experimental autoimmune encephalomyelitis (EAE). EAE is induced in susceptible animals by immunization with myelin antigens, emulsified in complete Freund's adjuvant (CFA) [163] or by adoptive transfer of activated myelin-specific CD4+ T cells from mice with EAE into naive recipient mice (figure 14) [164]. The EAE model has provided a useful tool for studying the inflammatory processes throughout the disease course. However, there are significant differences between MS and EAE as indicated by the fact that certain therapies had opposite outcomes in the murine and human patologies. EAE is a multi-step inflammatory process initiated by Th1 and Th17 cells. During actively induced EAE, myelin-specific Th1 or Th17 cells are activated and expand in the peripheral lymphoid tissues in response to myelin peptide and complete Freund's adjuvant (CFA) immunization. Activated Th1 or Th17 cells cross the blood brain barrier (BBB) thanks to the interaction of very late activation antigen 4 (VLA-4) expressed on their surface with vascular cell adhesion molecule (VCAM) expressed on endothelial cells [32]. Figure 15 shows that infiltrating T cells are then re-activated by brain-resident antigen presenting cells (including microglia, macrophages and myeloid dendritic cells) [165]. Interaction with APCs triggers the release of inflammatory cytokines from Th1 or Th17 cells thereby causing local inflammation and demyelination of white matter tracts, which reduces the ability of axons to conduct electrical signals. Astrocytes are involved in nearly all processes within the brain, but their contribution to EAE pathogenesis is not certain. *In vitro* studies suggest that they also produce cytokines that modulate T cells function as well as chemokines that may attract additional peripheral inflammatory

leukocytes. A number of studies have highlighted the central role of Th17 cells in the development and pathogenesis of EAE. Firstly, mice lacking RORgt, IL-17 or IL-23 as well as mice treated with IL-17-blocking antibodies are less susceptible to EAE than wild-type or untreated mice. Secondly, EAE can be induced by transfer of either Th-17 or Th-1 cells [166, 167] and the Th-17/Th-1 ratio of infiltrating cells determines where inflammation occurs in the CNS [168].

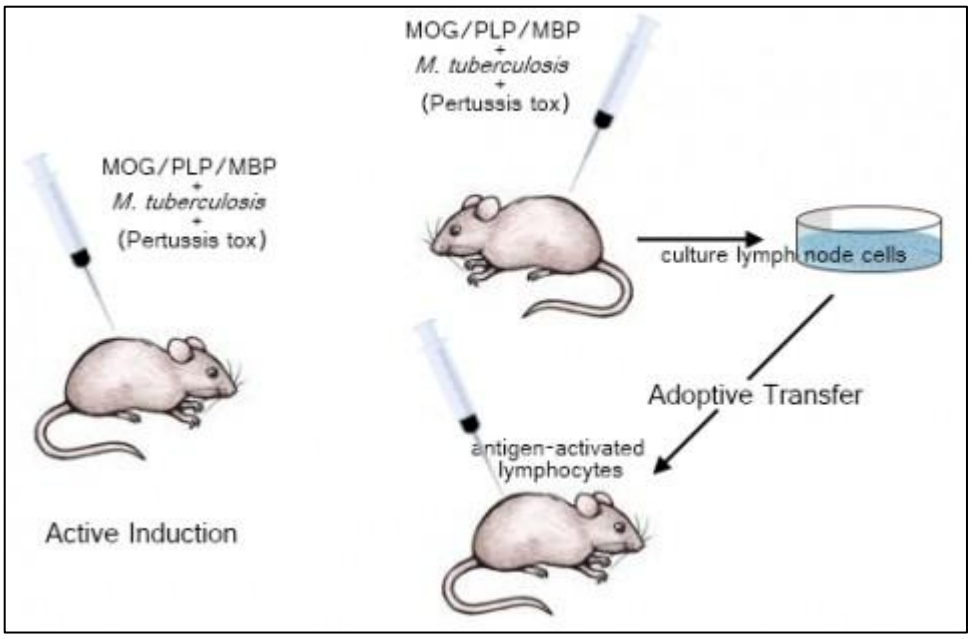


Figure 14: **EAE can be instigated in one of two ways.** Active induction involves injecting animals with antigens such as brain or spinal cord tissue or myelin proteins such as MOG, PLP, or MBP—along with *Mycobacterium tuberculosis* and often pertussis toxin, both of which act as immunostimulants. For adoptive transfer, researchers first immunize an animal with these antigens and immunostimulants, and after a week or two remove its lymph nodes, extract antigen-primed lymphocytes, and grow them in culture. These activated immune cells are then injected into another animal to induce the disease.

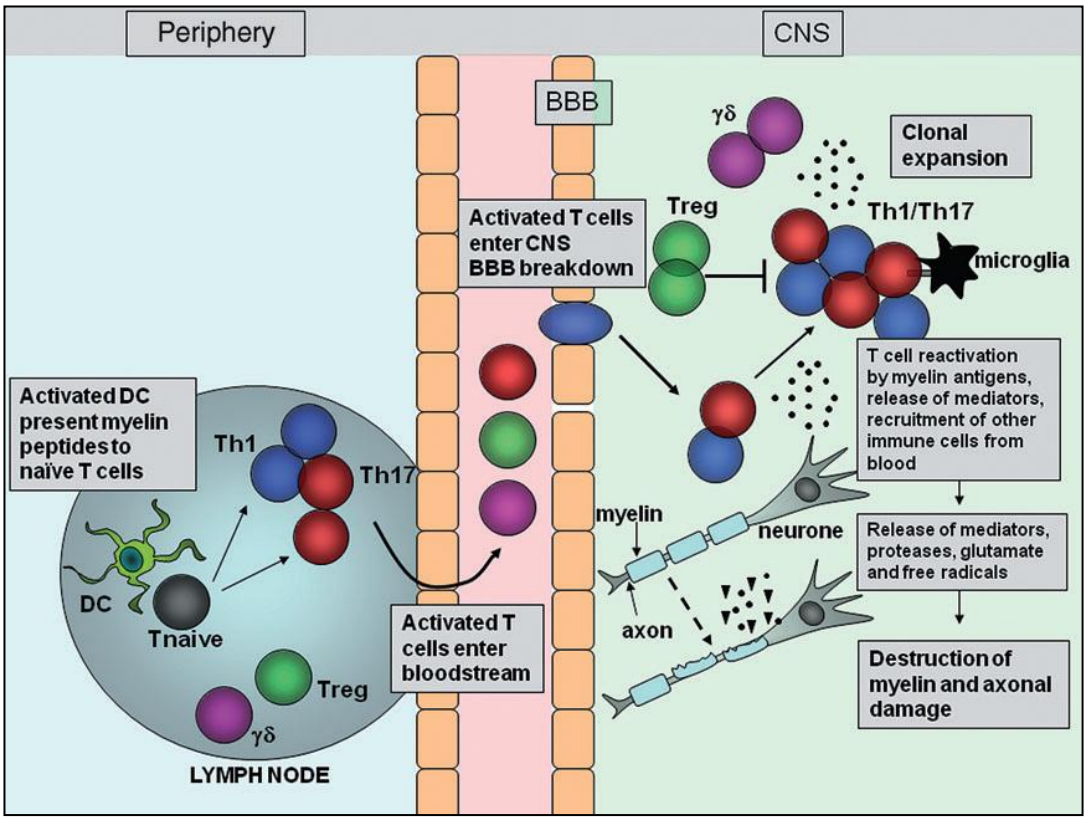


Figure 15: **Migration and effector function of T cells in the central nervous system (CNS) during experimental autoimmune encephalomyelitis (EAE).** After immunization with myelin antigens, complete Freund's adjuvant (CFA) and pertussis toxin, dendritic cells (DCs) are activated in the lymph nodes by Toll-like receptor (TLR) agonists within the mycobacterium tuberculosis component of CFA, and present myelin antigen to naive T cells. The activated myelin-specific T cells enter the bloodstream and traffic to and enter the CNS. Breakdown of the blood–brain barrier (BBB) occurs, allowing recruitment of other inflammatory cells into the CNS. T cells entering the CNS encounter their cognate myelin antigens and become reactivated by local APC. T cells expand and release inflammatory mediators which help recruit other immune cells to the site of inflammation. Activation of local microglial cells and infiltrating cells results in production of proteases, glutamate, reactive oxygen species and other cytotoxic agents which promote myelin breakdown. Damage to the myelin sheath surrounding axons is followed by axonal damage and neurological impairment. [16]

MATERIALS AND METHODS

Mice

The animal use procedures, performed according to the EC Directive 86/609/EEC, were approved by the Institutional Animal Care and Use Committee of the Humanitas Research Institute. Animals were sacrificed after gentle carbonarcosis (by slowly rising CO2 inside the cage) to minimize pain and discomfort.

Primary cell cultures

Astrocytes cultures: cortical and hippocampal astrocytes were obtained from 2 day-old C57BL/6 mice. Cortex and hippocampus were freshly dissected, cut into small sections and washed in Hank's Balanced Salt Solution supplemented with Hepes /Na pH 7.4 (10 mM), MgSO4 (12 mM), 50 U/ml Penicillin and 50 µg/ml Streptomycin. The tissue was then dissociated with 2.5 mg/ml trypsin type IX in presence of 1 mg/ml deoxyribonuclease (DNase, Calbiochem) for 10 min at 37 °C in two subsequent steps and the supernatants obtained were diluted 1:1 in medium containing 10% fetal bovine serum (FBS). The cells were plated in MEM (Life Technologies) supplemented with 10% FBS, 33 mM glucose, 100 mM Na2+/ Pyruvate (Lonza), 50 U/ml penicillin-G and 50 ug/ml streptomycin and maintained in 75 cm2 flasks (1 for pup) at 37 °C in a humidified 5% CO2 incubator. Pure cultures (> 99.5%) of astrocytes were obtained by shaking flasks at 220 rpm for 24 h at 37 °C at day 2 and 6 after plating. Shaking medium (5 ml/flask) was Minimum Essential Medium with Hank's salts, supplemented with 10% horse serum, 33 mM glucose, 200 mM Ultraglutamine (Lonza), 10 mM Hepes/ Na pH 7.4, 50 U/ml Penicillin and 50 µg/ml Streptomycin.

T-cell cultures: CD4+ T cells were isolated from peripheral lymph nodes and spleens of C57BL/6 female mice by positive selection with anti-CD4 immunomagnetic beads (MiltenyiBiotech). CD4+ T cells were stimulated with plate-bound anti-CD3 (10 ng/ml) and anti-CD28 (5 ng/ml) (Biolegend) mAbs for 40 h in RPMI-1640 medium supplemented with 5% FBS, 100 U/ml penicillin and 100 g/ml streptomycin.

Microglia and peritoneal macrophages cultures: Primary microglia cells were obtained from astrocytic layers by shaking the flasks for 45' at 230 rpm, 10–12 days after dissection. Detached cells (about 90% microglia with a 10% astrocytic contamination) were plated in multiwells (150,000 cells per well in 12 well plates) coated with poly-L-ornithyne hydrobromyde (100 µg/ml). Cultures usually contained 95% microglial (CD11b+) cells. To obtain peritoneal macrophages culture, mice were killed by asphyxiation with CO2 cellular material was aspirated from peritoneum and spun at 400 × g at 4 °C for 10 min. Cell culture were seeded in RPMI with 10 % FCS at $1x10^6$ cells/well in 12-well plates cultured at 37 °C in an atmosphere of 5 % CO2. After 1 h non-adherent cells were removed by washing three times with PBS. After 24h adherent macrophages/ microglial cells were co-cultured with CD4+ T cells at macrophage/ microglia: T cell ratio 1: 2.

Astrocyte/ T-cell co-cultures

Astrocytes were dissociated using trypsin-EDTA (Life Technologies-BRL), collected by centrifuging at 800 g for 10 min and then re-plated on multiwells (12 well plates; 300.000 cells/well) coated with poly-L-lysine (0,02 mg/ml). The same day of astrocytes plating, CD4+ isolated T cells were activated with CD3/CD28 mAbs for 40 h and then plated in 24-well plates alone or in 12 well plates in co-culture with astrocyte. Astrocyte:T-cell ratio was 1:2 and the co-culture was maintained in RPMI-1640 medium supplemented with 5% FBS, 100 U/ml penicillin and 100 g/ml streptomycin in the presence of 90 U/ml IL-2. In order to prevent cell-cell contact between astrocytes and T-cells, a transwell system was used (0.3 uM membrane pore; Transwell System, Corning).

Antibodies and Immunofluorescence analysis

The following antibodies were used: mouse anti-GFAP (1:500; Sigma), rabbit anti-GFAP (1:400; Synaptic System), rabbit anti-IBA1 (1:200; Wako), (PE)-conjugated rat anti-MHCII (1:100; eBioscience), FITC-conjugated Armenian Hamster anti-CD80 (1:100, eBioscience), PE-conjugated rat anti-CD86 (1:100, Biolegend), rabbit anti-CD3 (1:150, Dako), mouse anti-CD4 (1:300; Abcam), APC-conjugated rat anti-CD73 (1:150,

Biolegend), Pe-Cy7-conjugated rat anti-CD39 (1:200, eBioscience). Secondary antibodies used were conjugated with the following fluorophores: Alexa-488, Alexa-555 or Alexa-633 (Invitrogen, San Diego, CA).

Immunofluorescence analysis: cell cultures plated on poly-lysine pre-coated glasses were fixed in a solution containing 4% formaldehyde and 4% sucrose for 20' at room temperature, and the washed with PBS 1X. Briefly, cells were washed three times in a buffer solution containing low salt concentration (150 mM NaCl, 10 mM Phosphate Buffer 240 mM pH 7.4) followed by three washes with buffer solution containing high salt concentration (500 mM NaCl, 20 mM Phosphate Buffer 240 mM pH 7.4), and then permeabilized with GSDB 1X (15% Goat serum, 0.3% tryton, 450 mM NaCl, 20 mM Phosphate Buffer 240 mM pH 7.4) for 30 min at room temperature. The primary antibodies were diluted in GSDB 1X and incubated for 2 hours at room temperature. After three washes of 10 min with high salt solution, the secondary antibodies (diluted in GSDB 1X) were incubated for 1 hour at room temperature in the dark. Cells were then washed three times with high salt solution followed by three washes with low salt solution 5 min each, and incubated with DAPI solution. Coverslips were mounted onto glass using the mounting medium for fluorescence Vectashield (Vector Laboratories). Images were acquired using a Leica SPE confocal microscope equipped with an ACS APO 63X/1.30 Oil objective. Images were processed by using Photoshop (Adobe) and ImageJ Software.

Bioluminescence Assay to Measure ATP

ATP levels in the extracellular saline (KRH) of astrocyte monolayers alone, CD4+ T cell WT or *NTPDase1*$^{-/-}$ and astrocyte- T cell co-culture, in the presence of the ecto-ATPase inhibitor ARL (100 μM, Sigma), were measured using a luciferin/luciferase-based ATP determination kit (Molecular Probes, Leiden, NL) according to the manufacturer's instructions and a luminometer (Infinte F500, Tecan). Each sample was run in triplicate. Samples were assayed within 5 min of collection.

Calcium imaging

Astrocytes cultures used for calcium imaging experiments were plated onto glass coverlips of 25 mm diameter pre-coated with poli-L-lysine (0.02 mg/ml). Cells were loaded with Oregon Green-488 (Molecular Probes) for 1 h at 37°C, 5% CO2 in RPMI 5% FBS. Coverslips were mounted on a recording chamber (Warner Instruments, Hamden, CT) and placed on the stage of an IX-71 inverted microscope (Olympus, Hamburg, Germany) equipped with an EMCCD (electron-multiplying CCD) camera (quantem 512x512, photometrics). Illumination was obtained using a light-emitting diode LED (Cairn research, Optoled Lite), with an oil-immersion 40X objective (1.3 NA). Regions of interest (ROIs) of about 15-pixel area were drawn on the cell cytoplasm of virtually all the cells in the recorded field. The time lapse recording of spontaneous calcium dynamics was performed with an acquisition rate of 5 Hz for 200 seconds and off-line analyzed with MetaFluor software (Molecular Devices). Cultures were imaged in KRH (Krebs-Ringer-Hepes) extracellular solution containing (mM) 2 CaCl2, 125 NaCl, 1,2 MgSO4, 25 HEPES, 5 KCl, 6 glucose, 1,2 KH2PO4, pH 7.3.

Calcein exchange experiments

Astrocytes were incubated for 30 min at 37°C with 1 µM of the gap junction-permeant green fluorescent dye Calcein Acetoxymethyl ester (AM; Molecular Probes). As negative control we used 1 µM of gap junction-impermeant CellTracker CMFDA (Alexa Fluor 488) (5-Chloromethylfluorescein Diacetate). After three washes with PBS solution containing 2% FBS, astrocytes were co-cultured with activated T cells. Calcein transfer from dye-loaded astrocytes to CD4 T cells was evaluated by flow cytometry (FACSfortessa, Becton Dickinson, UK) and the percentage of green Calcein-positive CD4+ T cells were quantified.

Flow cytometric analysis

Floating CD4+ T cells were collected by three wash in PBS 2% FBS, while adherent T cells were detached from astrocyte monolayer by Accutase (Millipore). Then they were surface stained with the following conjugated antibody: FITC-conjugated anti-CD4 (Biolegend), V450-conjugated anti-CD45.2 (BD), PE-conjugated anti-Cd11b

(Biolegend), Pecy7- conjugated anti-CD39 (eBioscience), APC-conjugated anti-CD73 (Biolegend), FITC- conjugated anti-CD49d (integrin a4 chain, BD) and PE-conjugated anti-CD29 (integrin b1 chain, BD). The analysis was performed by using FlowJo Software.

Intracellular Cytokine Secretion Analysis

Cytokine producing T cells were detected by intracellular staining after three hours of incubation with PMA (100 nM) and ionomycin (1 uM). Brefeldin A (10 ug/ml) was added for the last 3 hours of incubation together with PMA and ionomycin thus preventing cytokine release into the medium. After cell surface staining, T cells were fixed and stained with the following mAbs: PE-conjugated anti-IL-17, APC-conjugated anti-IFN-g, APC-conjugated anti-IL-10, PercP-conjugated anti-TNFalfa and APC-conjugated anti-FoxP3 to score Tregs, according to the intracellular staining protocol for the Cytofix/Cytoperm kit (BD Bisciences). The FlowJo Software was used for the off-line analysis.

Pharmacological treatments

In order to modulate T-cells CD39/CD73 activity, 50U/mL Apyrase (Sigma) was directly added to the cell cultures. In other samples, T-cells were preincubated with 30 ug/ml CD29 (integrin beta1 chain, BD), or with 200 uM periodate-oxidized 2′,3′-dialdehyde ATP (oATP) (Sigma) and rocked gently for 1.30 h at room temperature. T cells were then centrifuged and added to astroglia. Astrocytes were incubated with 2 uM Thapsigargin for 20', washed three times in PBS and incubated with T cells. T-cells cultured alone were incubated with the following drugs: 1-100 uM ATPγS (Sigma), 100 uM Bz ATP (Sigma) or with 10 ng/ml IL-6 for 24h.

ELISA assay

Cell supernatants were assayed for IL-6 or TNF-alfa production by a specific sandwich-type enzyme-linked immunoabsorbent assay (ELISA) (DuoSet ELISA Development kit) according to the manufacturer's instructions (R&D System). Optical density (OD) values

(set to 540 nm or 570 nm) were plotted on a standard curve and expressed as pg/10^6 cells recovered.

Quantitative real-time RT-PCR (qPCR)

CD4+ T cells were stained with an antibody against CD4 and CD45.2 and sorted by FACSaria (Beckton Dickinson). Total RNA was extracted using TRI REAGENT (Sigma) according to the manufacturer's instructions, including DNase I genomic DNA degradation step. RNA concentration was determined by Nanodrop. 1-2 µg of total RNA were reverse transcribed to generate cDNA, using high capacity cDNA reverse transcription kit (Applied Biosystems). Q-PCR was performed using Real Time Taqman universal master mix (Applied Biosystems) according to a standard protocol, using 25 ng of template cDNA in a total volume of 10 ul. All primers were used at the final concentration of 900 nM. Q-PCR was performed using a Viia7 instrument (Applied Biosystem). The relative mRNA levels were calculated using the comparative Ct method, using GAPDH as a house keeping gene.

Statistical analysis

The statistical analysis between two groups of data was assessed using the two-tailed t-Test, whereas the analysis of two or more groups was performed by using the one-tail ANOVA test followed by Tukeys post-hoc test. In some experiments two-way ANOVA test followed by Bonferroni post-hoc test was used to compare different treatment groups. Data was shown as means ± S.E.M. $P<0.05$ was considered significantly different. ****=$p< 0.0001$; *** = $p<0.001$; **= $p<0.01$; *= $p<0.05$.

AIM OF THE PROJECT

Astrocytes are involved in many aspects of central nervous system (CNS) physiology and pathophysiology, including synapse formation and function, stimulation of neurite growth and branching, modulation of synaptic transmission and plasticity, regulation of blood–brain barrier (BBB) and myelination of axons. Whether and how astrocytes might be detrimental or protective during neuroinflammation has been intensely investigated with controversial results. The principal aim of my thesis was to dissect at the cellular and molecular levels the interaction of astrocytes with CD4 T helper cells, which play a pivotal role in shaping the adaptive immune response and can be responsible of immunopathological conditions, including multiple sclerosis and other neuroinflammatory processes. Adhesive contacts can be established between astrocyte and T cell, which may reciprocally influence the activity of the two cells. In addition, astrocytes and CD4+ T cells may communicate through secretion of signalling molecules during their cross-talk.

Main objectives of this project were:

1. To assess whether T cell activation modify adhesiveness to astrocyte and this in turn affect astrocyte function (e.g. Ca2+ signaling in astrocytes)
2. To investigate the role and possible reciprocal regulation of purinergic signaling in activated T cells and astrocytes upon their interaction
3. To dissect the dependence on cell-cell contact versus soluble mediators for phenomena occurring during astrocyte-T cell cross-talk
4. To investigate the possible role of gap junctions and integrins in astrocyte-T cell communication
5. To characterize transcriptional regulation and functional polarization of T cell upon interaction with astrocyte

RESULTS

TCR stimulation enables CD4 T cell adherence to astrocyte.

In order to characterize astrocyte/CD4+ T-cell interaction, we have co-cultured mixed mouse glial cells, containing both astrocytes and microglia, or purified astrocytes with either naïve or polyclonally activated CD4 T cells (previously stimulated with CD3 and CD28 antibodies for 40 h). CD4 T cells were plated on a glial monolayer at an astrocyte to T cell ratio of 1:2. Differential interference contrast (DIC) microscopy at 48 to 96 h after initiation of co-culture revealed a substantial fraction of activated but not naïve CD4+ T cells adhering to microglial cells or astrocytes. Figure 1 shows a representative picture of activated T cells co-cultured with mixed glial cells, in which microglia are stained with the myeloid marker isolectin IB4-FITC (left panel). As expected, activated T cells preferentially interacted with IB4 positive microglia, which are known to be professional antigen presenting cells (APCs). Nevertheless, several flatten CD4 T cells were found to adhere to the astrocyte monolayer, both when co-cultured together with glial cells and purified astrocytes (middle panel in figure 1). Adherent T cells represented approximately 50% of total T cells in culture and maintained a stable interaction with astrocytes, which resisted several washing passages (data not shown). Conversely, unstimulated CD4 T cells did not establish direct physical contact with astrocytes and were confined in the floating cell fraction (right panel).

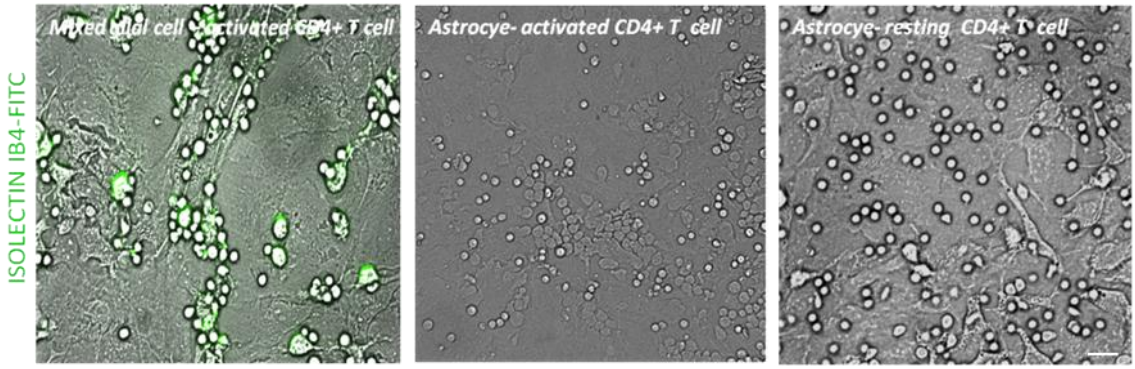


Figure 1**: Mixed glia or purified astrocyte-T cell co-culture.** DIC image of mixed glial cell monolayer (left) or astrocyte purified monolayer (middle) co-cultured with activated T cell or purified astrocytes in the

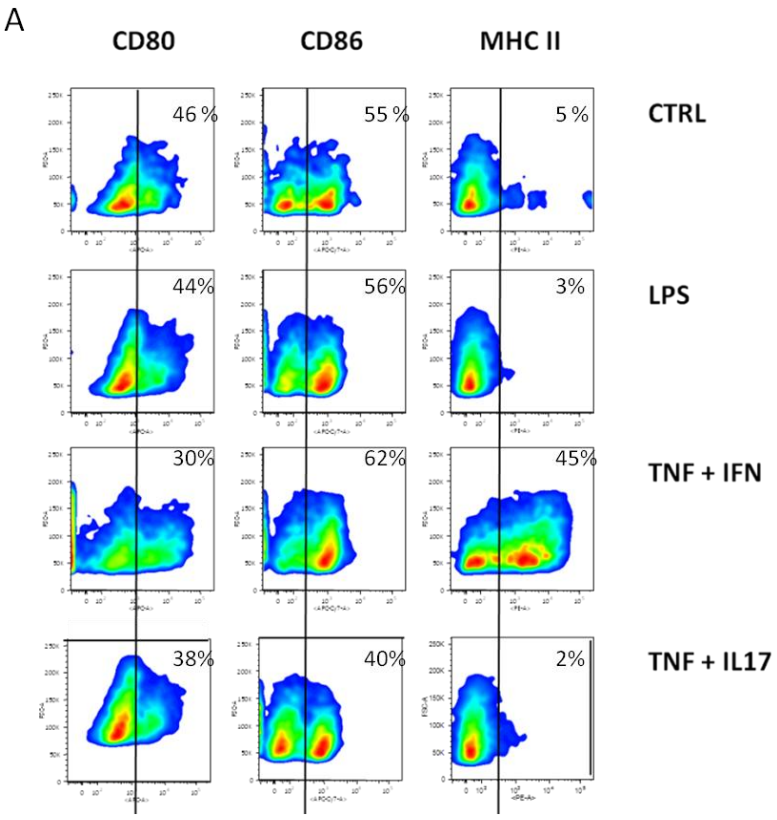
presence of resting (unstimulated) T cell (right). Isolectin IB4 conjugated to FITC was used to label microglial cells. Scale bar 20 um.

Astrocytes functionally interact with T cells although they display a low potential to act as professional APCs.

Conflicting results have been published relative to the presence of MHCII and co-stimulatory molecules in astrocytes [39, 169]. Indeed, while a large body of evidence indicates that activated microglia cells upregulate MHC-II on their surface both *in vitro* and *in vivo* [170, 171] , MHC-II expression has been reported in astrocytes *in vivo* only upon prolonged inflammation and *in vitro* after interferon-gamma (IFN-γ) and Tumor Necrosis factor- alfa (TNF-α) treatment [36, 172]. Several papers reported that while having no influence alone on class II MHC expression, TNF-α enhances IFN- γ induced class II MHC expression on astrocytes [173] [174]. Accordingly, we observed that IFN-γ and TNF-α strongly up-regulated MHC-II, CD80 and CD86 expression *in vitro*, as indicated by flow cytometry analysis (figure 2A), whereas exposure to IL17 and TNF-α or to LPS was ineffective. Altogether these findings confirmed and extended previous evidences supporting the crucial role of INF-γ in promoting the antigen presenting capability of astrocytes by inducing upregulation not only of MHC-II but also of co-stimulatory molecules.

We next investigated the effect of CD4+ cells on MHC-II and CD80/86 expression by astrocytes. Immunofluorescence analysis showed that while astrocytes cultured alone do not express MHC-II, when cultured in the presence of activated CD4 T cells, they clearly expressed MHC-II on their surface (red staining, figure 2B). Flow cytometry analysis (figure 2C) confirmed MHC-II induction on astrocytes co-cultured with activated CD4+ T cells respect to astrocytes cultured alone or activated with LPS. Notably, unstimulated (resting) CD4+ T cells did not induce MHC-II expression on purified astrocyte (figure 2B, second panel). As shown in figure 2D, immunofluorescence analysis revealed that T cell were not as efficient as IFN-γ added in culture in promoting CD80/86 expression, while flow cytometry (figure 2E) detected only a slight increase in CD86 expression on astrocyte after exposure to activated T cell. As a positive control of professional antigen presenting cells (APCs), T cells were co-cultured with peritoneal macrophages: the orange trace in the histogram shows that activated T cells significantly induced CD86 and to a lesser extent CD80 on macrophage surface.

We also investigated by immunohistochemistry whether MHCII expression could be detected on astrocytes *in vivo*, by analyzing the spinal cords of mice affected by Experimental Autoimmune Encephalomyelitis (EAE), the mouse model of human multiple sclerosis. Whereas strong MHC-II immunoreactivity was present in the vast majority of IB4+ microglial cells, we could detect only rare MHC-II positive astrocytes and we could rarely detect MHC-II in astrocyte plasma membrane (figure 2F). Overall our *in vitro* and *in vivo* data indicate that although astrocytes have the potential to act as APCs, they do not efficiently upregulate costimulatory molecules upon exposure to activated CD4+ T cells. Thus astrocytes are likely able to deliver a signal to T cells albeit with reduced strength than “professional” APCs, which are characterized by higher density of MHC-II and CD80/86 on their surface.



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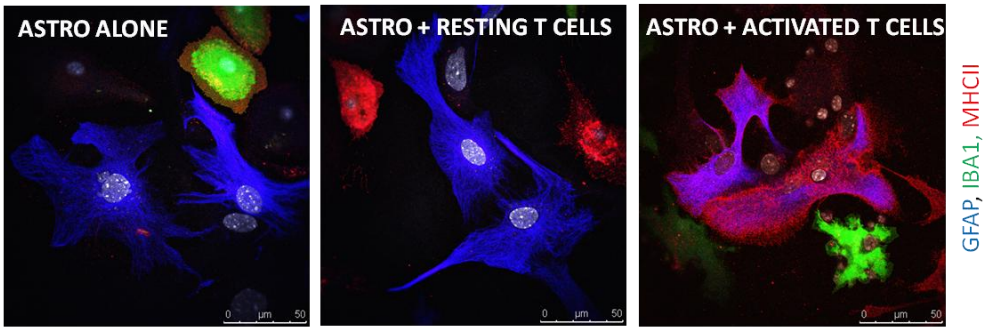
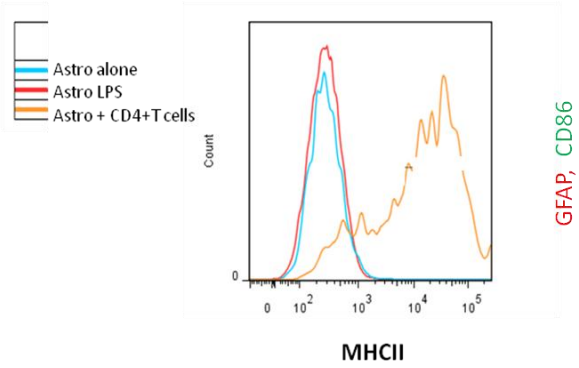

ASTRO ALONE
ASTRO + RESTING T CELLS
ASTRO + ACTIVATED T CELLS
GFAP, IBA1, MHCII

C


Astro alone
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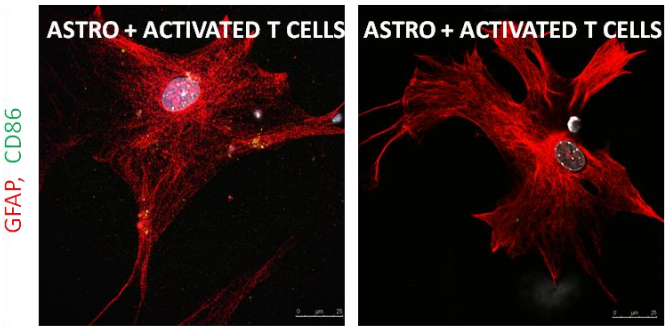
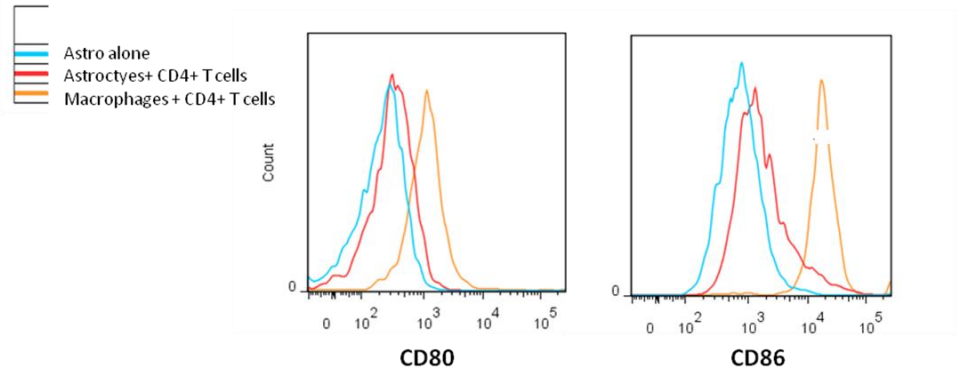

ASTRO + ACTIVATED T CELLS
ASTRO + ACTIVATED T CELLS
GFAP, CD86

E


Astro alone
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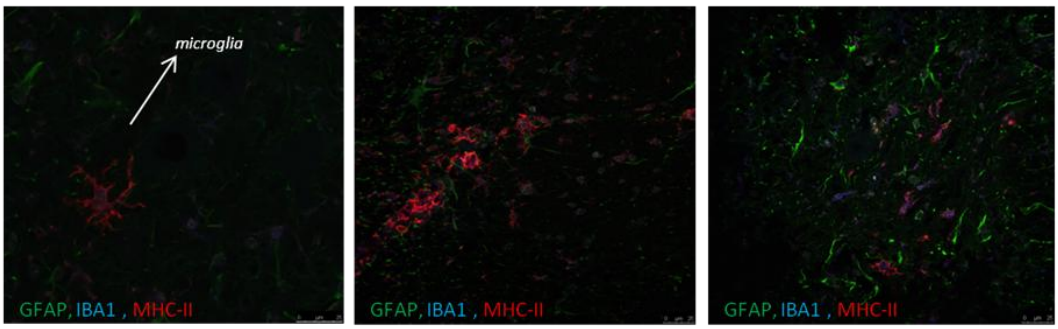
Count
CD80
CD86

F


microglia
GFAP, IBA1 , MHC-II
GFAP, IBA1 , MHC-II
GFAP, IBA1 , MHC-II

:

Figure 2 : **Analysis of cell surface protein expression by primary murine astrocytes after addition of activated CD4+ T cell.** (A) MHC class II and costimulatory molecules (CD80 and CD86) on astrocytes were analyzed by flow cytometry in the absence (CTRL) and 72 h after the addition of LPS (400 ng/ml) or IFN-γ and TNF-alfa (25 ng/ml and 20 ng/ml, respectively) or IL17 and TNF-alfa (100 ng/ml and 20 ng/ml respectively). Representative histograms are shown. (B-C) Activated T cells induce MHCII-expression on astrocyte. Immunofluorescence and flow cytometry of MHC-II expression on astrocyte alone (left), co-cultured with resting (middle) or activated T cells (right). Confocal images of astrocytes culture stained for the astrocyte marker GFAP (blue), IBA1 (green) to detect microglial cells and MHC-II (red), DAPI for the nuclei (gray). Scale bar 50 um. (D) Confocal images of astrocytes GFAP positive (red) double stained for CD80 or CD86 (green). Scale bar 25 um. (E). Histograms showing CD80 and CD86 expression by astrocyte cultured alone (blue line), astrocytes co-cultured with activated T cell (red line) or CD80/CD86 expression by peritoneal macrophages upon T cell culture as positive control (orange line). (F) Immunostaining of spinal cord slices from EAE mice. Stage 2 of the disease. Astrocytes are positive for GFAP (green), microglial cells IBA1 positive (blue) and MHC-II molecules are shown in red. Scale bar 25 um.

Activated but not resting T-cells reduce spontaneous calcium oscillations in astrocytes.

It is well known that cortical astrocytes display spontaneous $[Ca^{2+}]_I$ oscillations both in culture [83, 84] and in acute brain slices [85, 86]. Moreover, such spontaneous increases in intracellular calcium concentration can occur in the absence of neuronal activities [175] [84] [87, 98] and are mainly caused by both extracellular Ca2+ influx and intracellular Ca2+ release from the endoplasmic reticulum (ER)[176]. Whether immune cells like CD4+ T cells can influence spontaneous calcium oscillations in astrocytes is unexplored to date. In order to address this point, astrocytes cultured alone or co-cultured with activated T cells for 96 hours were loaded with the calcium sensitive dye Oregon green (figure 3A) and the percentage of cells showing spontaneous calcium transients were measured. We observed that while in astrocytes sample almost 40% of the total recorded cells exhibited spontaneous calcium transients, in astrocytes co-cultured with activated T cells this percentage was significantly reduced (figure 3A-B). Strikingly, co-culture with non-stimulated, resting naïve T cells did not affect the percentage of astrocytes displaying calcium oscillation (figure 3C).

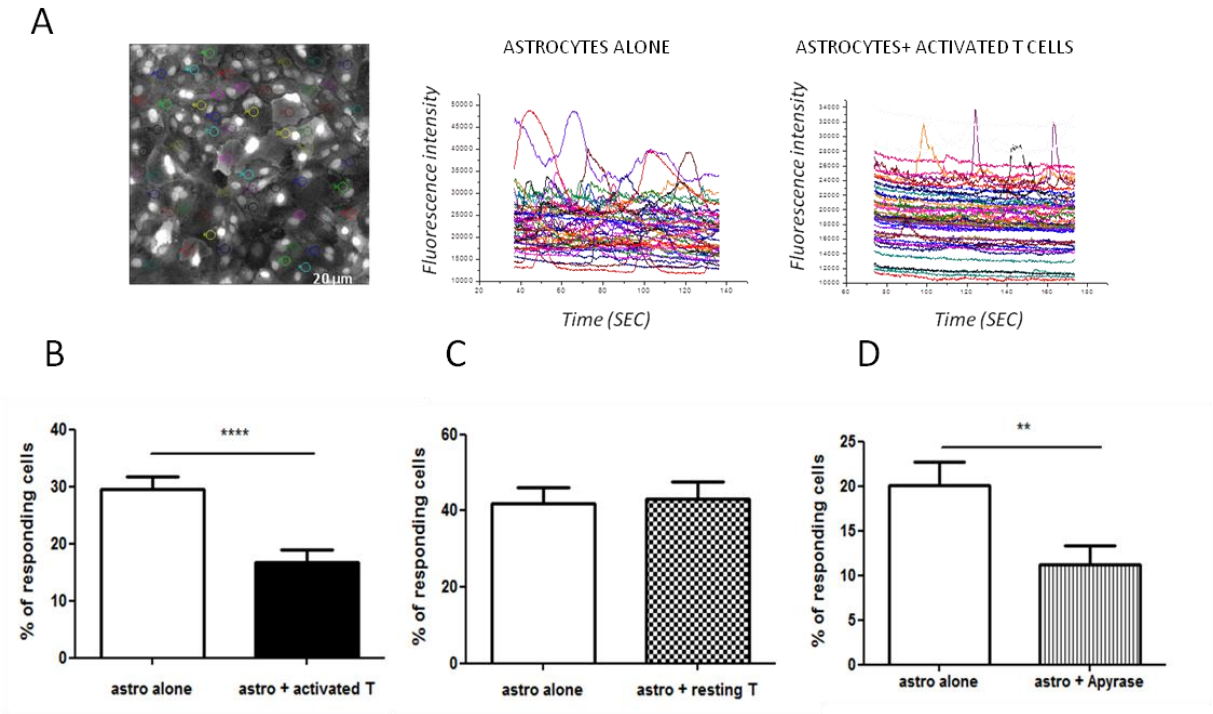


Figure 3: **Spontaneous intracellular calcium oscillation in astrocyte are downregulated by the presence of activated T-cells.** (A, left panel) Representative fluorescence image of cultured astrocytes loaded with the calcium sensitive dye Oregon Green (Fluorescence 505 nm). Regions of interest (ROIs) were drawn on the cytoplasmatic portion of all the cells present in the recording field (scale bar 20 um). Representative traces of spontaneous Ca2+ transients measured in astrocytes alone (middle panel) and in astrocytes cultured with activated CD4+ T cells (right panel). (B-D) Quantitative analysis of the percentage of astrocytes displaying calcium oscillations under different conditions. (B) The percentage of cells exhibiting spontaneous calcium transients was significantly reduced in the presence of activated T cell (n exp= 4 ; n=64 fields astrocyte alone, n=62 fields astrocyte with T cells), but not in the presence of resting T cells (C), (n exp= 2 ; n= 33 fields astrocyte alone, n=32 fields astrocytes with resting T cells). (Mean ± SD). (D) Astrocytes treated with the ATP –degrading enzyme, Apyrase (30 U/ml) for 1 h displayed a significantly reduction of cells showing spontaneous calcium oscillation (n exp= 3 ; n= 27 fields astrocyte alone, n=38 fields astrocyte Apyrase treated) (Mean ± SEM). Statistical significance was evaluated with Student's t-test, ****$p < 0.0001$, **$p < 0.01$).

Among soluble factors involved in the regulation of astrocyte calcium signalling, ATP is known to be one of the most important (Cortina et al. 2000, P.B. Guthrie 1999 J Neurosci). Moreover Koizumi S. and collaborators showed that application of apyrase, an ATP hydrolyzing enzyme able to convert ATP to ADP and AMP, abolished spontaneous Ca2+ oscillations in cultured hippocampal astrocyte, thus suggesting that spontaneous

Ca2 oscillations in astrocytes are tightly dependent on extracellular ATP levels [98]. Thus, we confirmed these observations by incubating cultures of cortical astrocytes for 1 hour with 30 U/ml apyrase. Also in our experimental setting apyrase significantly reduced the percentage of astrocytes showing calcium oscillations (figure 3D). Altogether these results suggest that extracellular ATP plays an important role in the generation of spontaneous calcium transients in astrocytes and that activated CD4+ T cells might modulate spontaneous cytosolic Ca2+ elevations in astrocytes by affecting extracellular ATP levels.

Reduction of extracellular ATP levels upon astrocyte-CD4 T cell contact.

Among bioactive molecules secreted by astrocytes, ATP is a key messenger, which may signal to CD4+ T cell [42, 130]. ATP is released from astrocytes through gap junction hemichannels or through exocytosis of ATP-storing vesicles. Once released, ATP can activate purinergic ionotropic receptors present on neighbouring T cells [126, 177], thus facilitating Ca2+ entry and consequently regulating T cells activation [178]. To explore the possible role of ATP signalling in astrocyte-T cell interaction, we evaluated whether ATP levels in the pericellular space of astrocyte were modulated by activated T cells. We performed the luciferin/lucyferase assay to determine ATP concentration in the supernatants of astrocytes cultures, T cells, and astrocytes together with activated T cells. Our data revealed a significant reduction of extracellular ATP level in astrocyte-T cell co-cultures (figure 4A). This result suggests that activated CD4 T cells either inhibit ATP release or hydrolyze extracellular ATP.

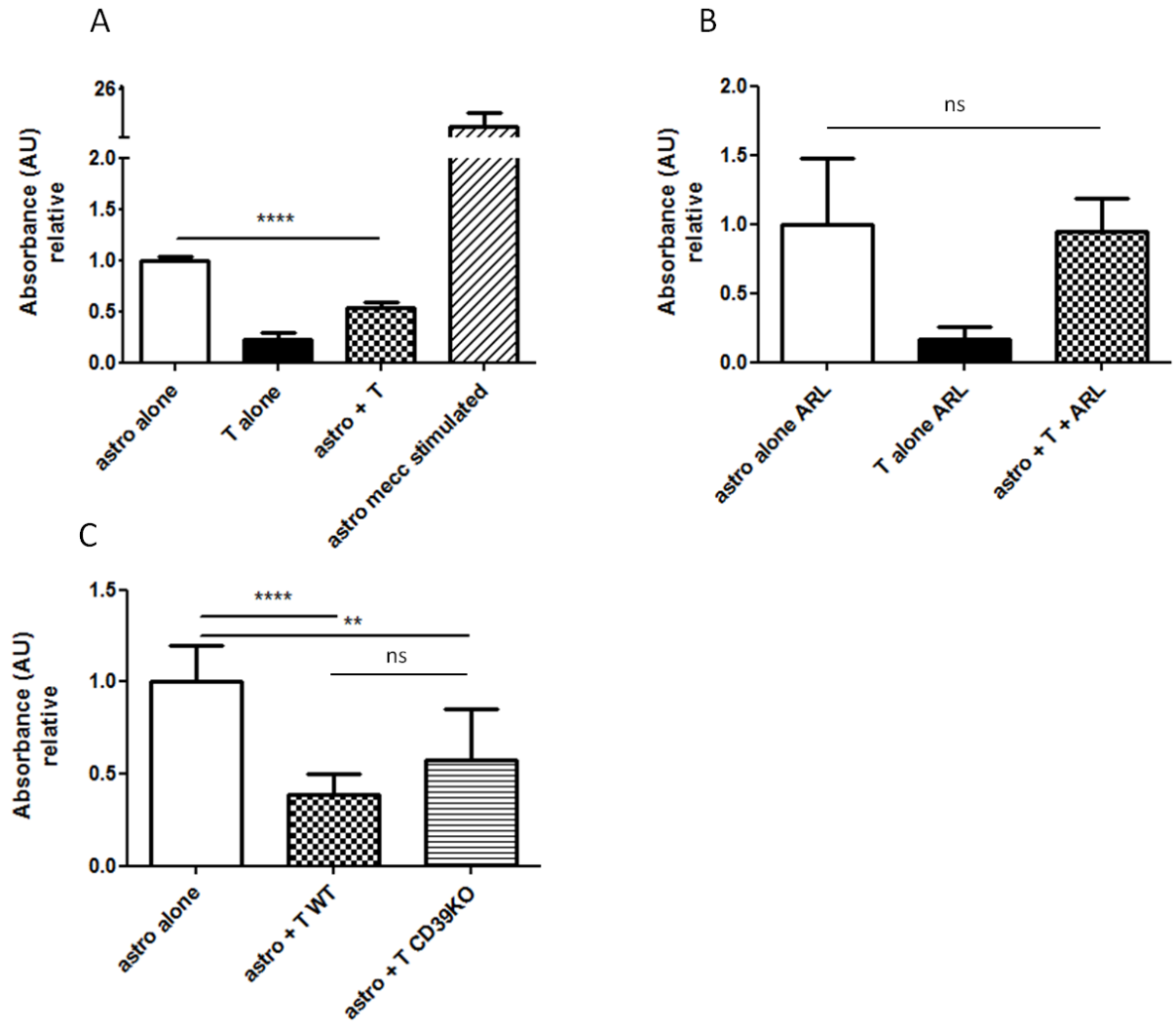


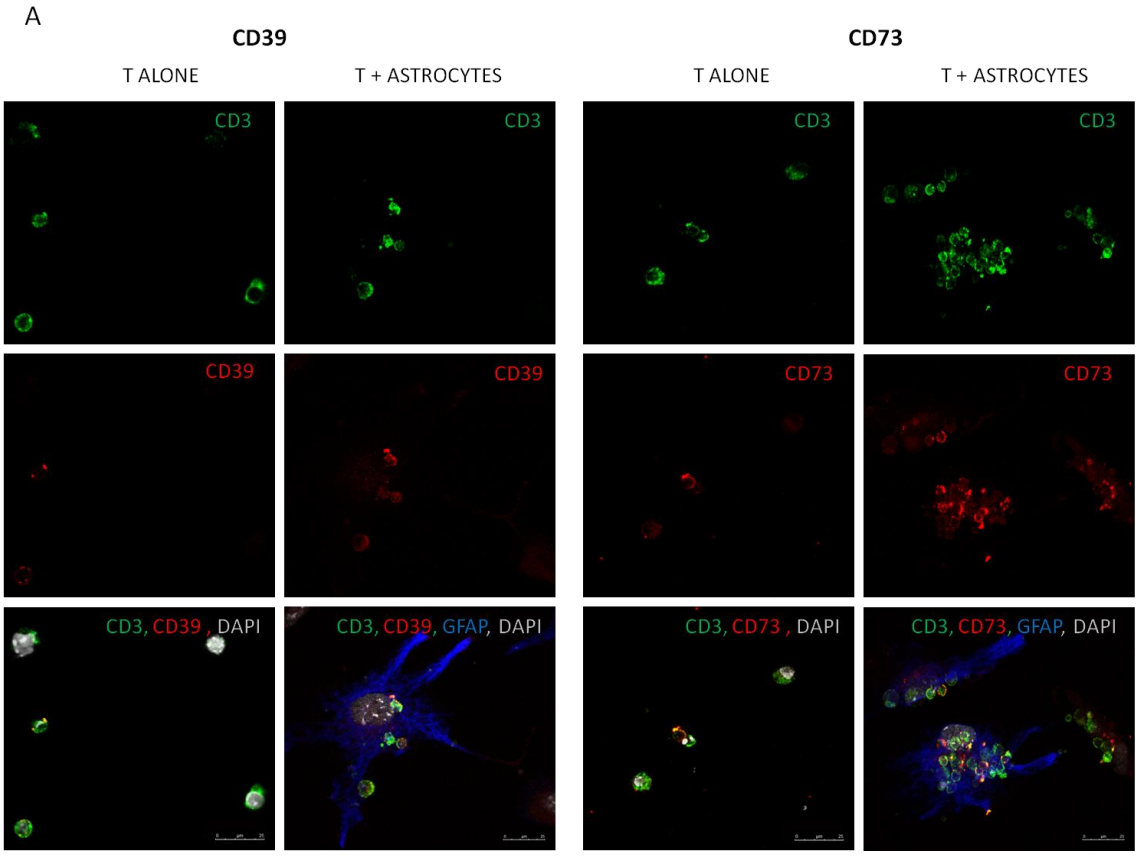
Figure 4: **Detection of significantly reduced ATP level in astrocyte-T cell co-culture.** (A) ATP content in the supernatants of astrocytes culture, mechanically stimulated astrocytes, T cells and astrocytes/T cells co-culture detected by luciferase assay. Measurements were performed after 72 h of co-culture. Each sample was made in triplicate and measures were performed in KRH. N exp= 5; (Mean ± SEM). (B) ATP content in the supernatants of astrocytes , T cells and astrocytes/T cells co-culture in the presence of 100 uM ARL, an inhibitor of NTPDase1/CD39, for 72h. n exp= 2; (Mean ± SD). (C) Extracellular ATP in the supernatants of astrocytes co-cultured with T cells form WT or *NTPDase1*$^{-/-}$ (CD39KO) mice; n exp= 2; (Mean ± SD). Statistical significance was evaluated with Student's two tailed t-test, p**< 0.01 ****p < 0.0001.

A large body of literature reports that CD4+ T cells express on their surface ectoenzymes involved in the regulation of extracellular ATP levels: CD39, the dominant ectoenzyme in the immune system, converts ATP to ADP and AMP, whereas CD73 converts AMP to adenosine [138] [139]. We thus investigated whether extracellular ATP reduction in astrocyte-T cell co-culture could be attributed to the activity of ectonucleotidases expressed by activated T cell. To address this point, we firstly measured extracellular ATP by the luciferin/luciferase assay in astrocytes incubated in the presence of ARL (100 uM), which partially but significantly inhibits NTPDase1/CD39 activity. Histograms in figure 4B shows that pharmacological inhibition of CD39 prevented the reduction of extracellular ATP observed in astrocyte-T cell co-culture. Moreover, we used the same assay to measure ATP in the supernatant of astrocytes co-cultured with CD4+ T cells isolated from *NTPDase1*$^{-/-}$ mice, in which the gene encoding CD39 is deleted (figure 4C). T cells lacking CD39 protein significantly reduced extracellular ATP levels, although to a lesser extent than wild type T cells. The lack of effect of *NTPDase1*$^{-/-}$ CD4 cells is likely due to residual ectonucleotidase activity provided by other enzyme isoforms expressed in T cells.

Overexpression of ectonucleotidases in CD4 T cells upon astrocyte co-culture.

To substantiate the hypothesis that CD39 and CD73 were induced in astrocytes/T cells co-cultures and they modulated extracellular ATP levels, we analyzed surface expression of CD39 and CD73 on CD4+ T cells in different culture conditions by confocal microscopy and flow cytometry. Immunofluorescence staining (figure 5A, right panel) revealed that T cells, identified by CD3 immunoreactivity (green), adhering to GFAP positive astrocytes (blue) clearly expressed CD39 and CD73 on their surface (red). Positive surface staining for both ectonucleotidases was also detected in a percentage of T cells cultured alone (figure 5A, left panel). Thus, flow cytometry allowed us to quantify the percentage of CD39 and CD73 positive CD4+ T cells and to evaluate possible changes in the expression level of these proteins on T cell surface upon astrocyte co-culture. 48 and 96 hours after astrocyte exposure (astrocyte: T cell ratio 1:2 as previously described), CD4+ T cells floating on astrocytes and adhering to them were surface stained for CD4, CD45, CD39 and CD73 and then analyzed by FACS as described in materials and methods. Figure 5B shows a representative density plot of

CD39 /CD73 positive cells at different times of culture. We found that T cells adherent to astrocytes displayed surprisingly higher CD39 and CD73 surface levels compared to control T cells cultured alone. In detail, after 96h of co-culture, the percentage of CD39 and CD73 positive cells increased from about 12% (among T cells cultured alone) to 47 % (among T cells adhering to astrocytes) for CD39 and from 49% to 86% for CD73. Moreover, T cells adhering to astrocytes displayed a progressive increase of CD73 positive cells over time also, both among suspended or adherent T cells. In T cells adhering to astrocytes, number of CD73 positive cells increased from 75% CD73 positive detected at 48h to 86% at 96h of co-culture (right panel, figure 5B). The histogram in figure 5C well represents the effect mediated by astrocyte on CD39 and CD73 induction on suspended (orange line) or adherent (red line) T cells. These results indicate that contact with astrocyte results in strong upregulation of both ectnucleotidases in T cells.



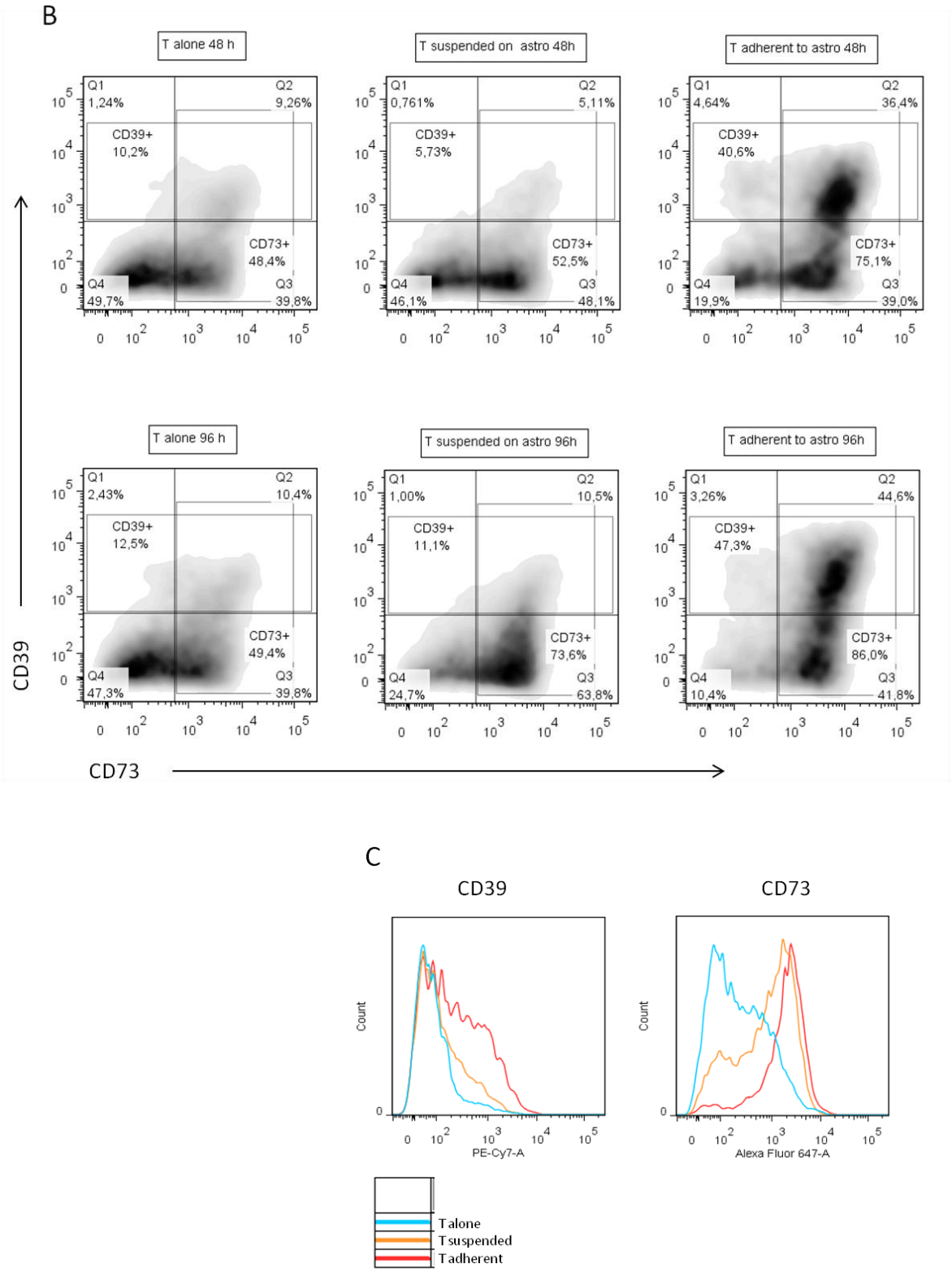
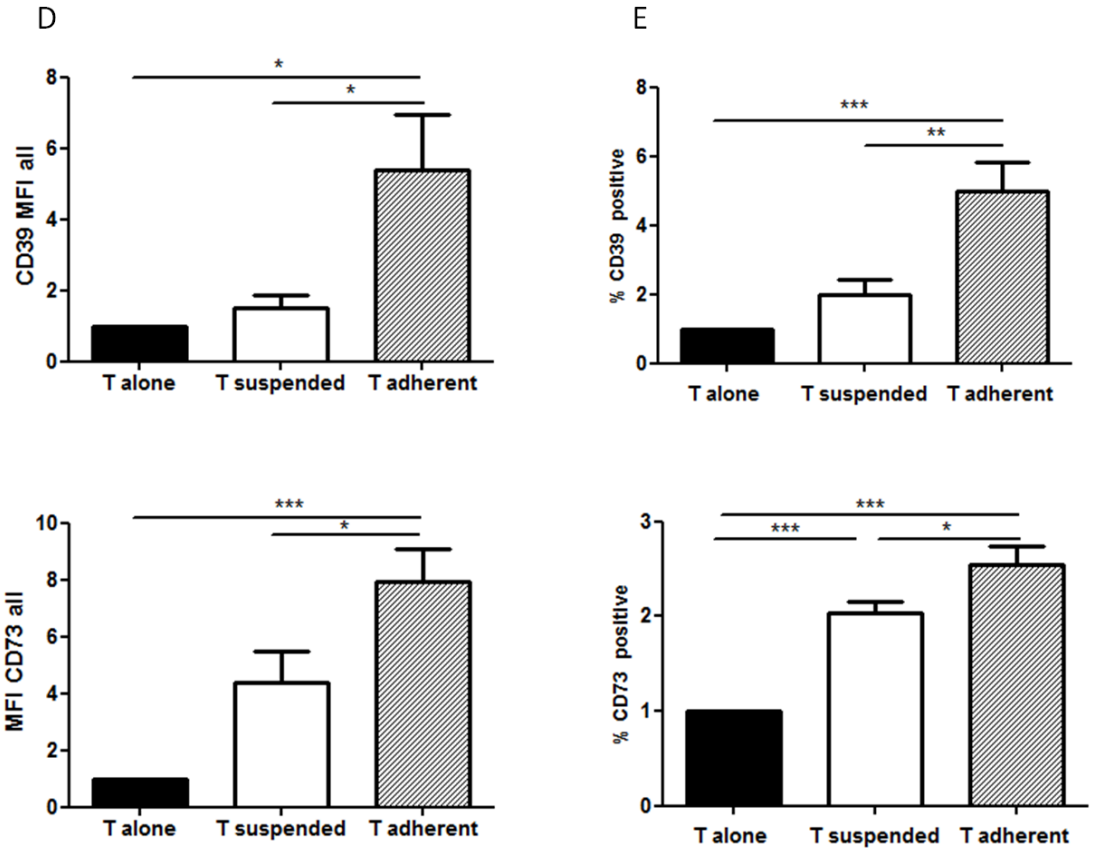


Figure 5: **CD39 and CD73 were significantly upregulated in T cells adhering to astrocyte.** (A) Immunofluorescence staining of T cells alone (left) or T cells co-cultured with astrocyte (right) labelled with CD3 (green) to stain T cells, GFAP to visualize astrocytes (blue) and with CD39/CD73 (red). DAPI for the nuclei (gray). Scale bar 25 um (B) Representative density plot of the percentage of CD39 /CD73 positive cells after 48 h (higher panel) or 96 h (lower panel) of co-culture. Numbers reported in dot plots

indicate the percentage of CD39/CD73 positive cells within the quadrant. (C) Histograms representative of CD39/CD73 induction on T cells floating or adhering to astrocyte respect to T cells alone.

Mean fluorescence intensity (MFI) of T cells either floating or adherent to astrocytes was used as reference parameter to reveal possible changes in ectonucleotidase expression under different culture conditions. MFI values, normalized on T cells cultured alone and measured on the CD39/CD73 total population, revealed that CD39 expression increased by 5 fold in T cells physically associated to astrocytes, while CD73 increased more than 6 fold in adherent T cells respect to T cells alone (figure 5D). Moreover, no significant increase in CD39 expression was detected in suspended T cells, while CD73 expression increased by 4 fold in floating T cells respect to the control. Accordingly, the percentage of CD39 and CD73 positive cells was significantly higher in T cells adhering to astrocytes as compared to floating T cells (figure 5E). We *also evaluated ectonucleotidases expression on astrocyte surface (figure 5F). In line* with previous observations [179, 180] astrocytes were positive for both CD39 and CD73. However, we did not observe significant changes of CD39 and CD73 expression in astrocytes alone respect to astrocytes exposed to activated CD4 T cells.



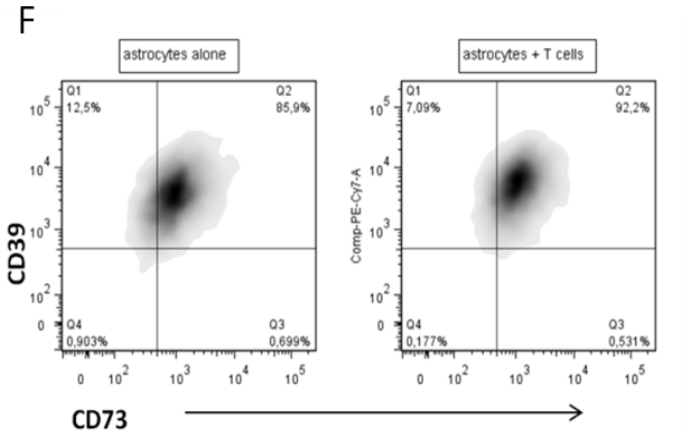


Figure 5: **CD39 and CD73 were significantly upregulated in T cells adhering to astrocyte.** (D-E) Analysis of CD39/CD73 relative MFI (measured on the total population) (left) and the percentage of positive cells (right) measured by FACS after 96h of co-culture with astrocytes. N exp = 5. Data are expressed as the MFI ± SEM and compared by Anova one way test with Tukey's multiple comparison post test. Values are normalized on T cells alone.p*< 0.05, p**< 0.01, p***< 0.001 . (F) Analysis of CD39 and CD73 expression by astrocytes in flow cytometry.

Selective CD73 induction in activated T cell by contact with astrocyte

To investigate whether CD39 and CD73 induction on T cells was a specific feature of astrocytes, we co-cultured CD4+ cells with peritoneal macrophages or with microglial cells, at the same cell to cell ratio (APCs :T cells ratio of 1:2). A similar upregulation of CD39 (by about 5 fold) to that one detected after astrocyte exposure, was detected on T cells adhering to macrophages or microglia (figure 6A, upper panel), with 50% of total T cells becoming CD39 positive 96h after microglia or macrophages co-culture (not shown). On the contrary, the percentage of CD73 positive T cells contacting microglia or macrophages was not significantly different from T cells cultured alone (figure 6B, lower panel). In fact, exposure to macrophages or microglia resulted in significantly lower percentage of CD73 positive T cells with respect to astrocyte co-culture (figure 6B lower panel). Moreover, CD73 MFI of T cells co-cultured with macrophages or microglia was similar to the MFI of T cells cultured alone (figure 6A lower). Altogether these data indicate that CD73 is selectively upregulated in previously activated CD4 cells upon contact with astrocytes but not professional APCs.

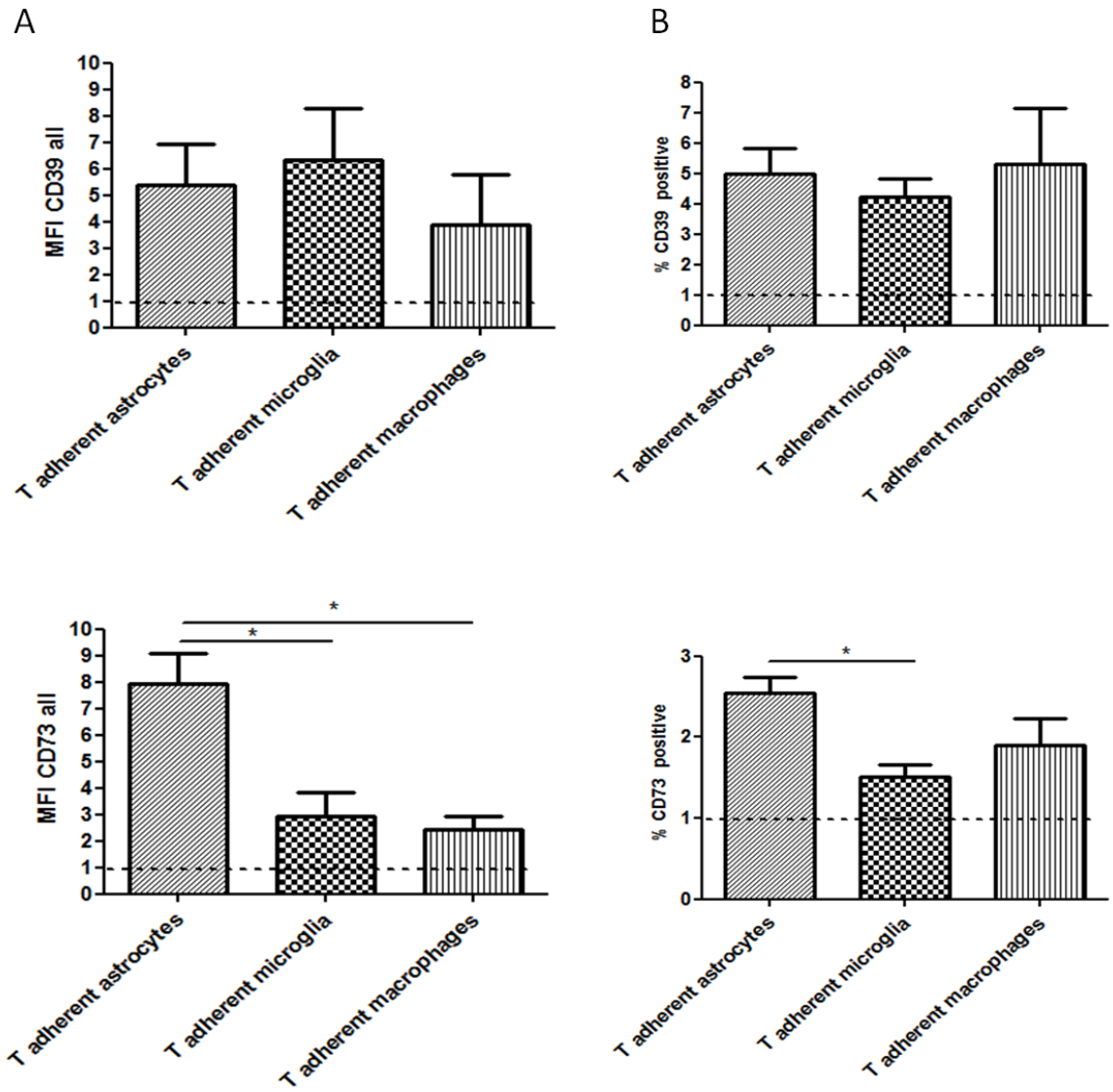


Figure 6: **Astrocyte-specific CD73 induction on T cells.** (A-B) Histograms represent the percentage or MFIs values calculated on CD39/CD73 positive or total T cell population respectively, cultured with astrocytes/ macrophages/ microglia. Values are normalized on T cells alone (not show). n exp= 5 (astrocytes); n= 3 (microglia); n= 2 (macrophages MFI ± SD) . Data are expressed as the MFI ± SEM and compared by Anova one way test with Tukey's multiple comparison post test. p*< 0.05

Astrocyte contact-dependent downregulation of CD4.

Astrocytes promote downregulation of TCR expression and suppress upregulation of CD25 upon T-cell activation [181]. We observed a similar downregulation of the CD4 co-receptor in activated T cells upon contact with astrocytes, macrophages or microglia. The histograms and dot plot in figure 7A (lower panel) clearly shows that T cell cultured alone or suspended on astrocytes monolayer expressed high levels of the CD4 receptor on their surface. Conversely, T cells contacting astrocytes completely downregulated the co-receptor. To investigate whether CD4 molecules were internalized after contact, we performed intracellular staining of T cell. Figure 7B shows that CD4 was actually internalized in T cells adhering to the astrocyte monolayer. Nevertheless, this effect was not astrocyte-specific and in line with the literature we observed the same CD4 downregulation also in T cells contacting peritoneal macrophages and microglial cells.

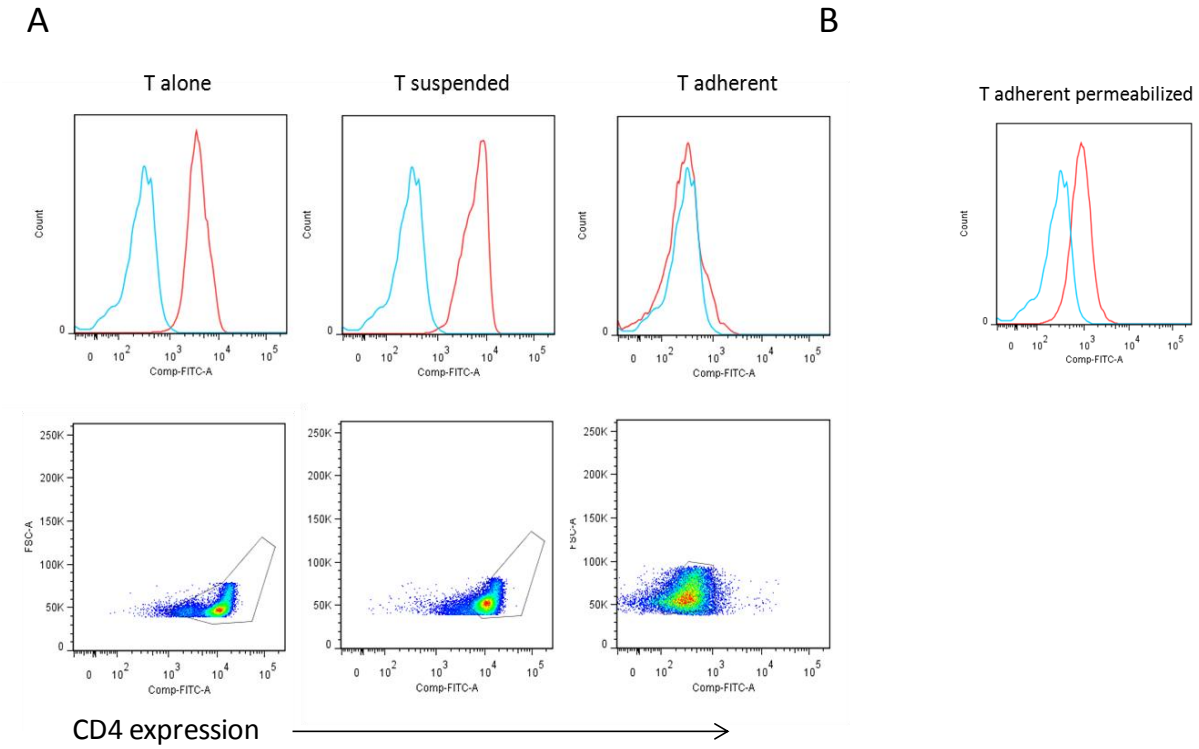
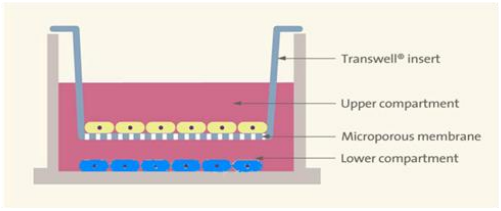


Figure 7: **CD4 receptor is downregulated after astrocyte- contact.** (A) Histograms (upper panel) and dot plot (lower panel) show different CD4 co-receptor levels in T cells alone, suspended or adherent to astrocyte (red line). The blue line represents Isotype control. (B) T cells adhering to astrocyte were permeabilized and stained for CD4 to detect intracellular CD4. Blue line represents Isotype control.

Physical cell contact mediates CD39/CD73 upregulation on CD4+ T cell

Stronger induction of ectonucleotidases in T cells adherent to astrocytes as compared to floating T cells suggested that physical cell contacts may play a relevant role in the process. To definitely assess the role of cell-to-cell contact in CD39 and CD73 induction in astrocytes-T cells co-cultures, CD4+ lymphocytes were separated form astrocytes in the co-culture by a 0.3 μM membrane of transwell system. Analysis in flow cytometry both at 48h and 96h (not shown) revealed that CD39 and CD73 upregulation was completely abrogated on T cells separated from astrocytes by transwell (figure 8). This result strongly support the notion that cell-to-cell contact could be crucial in regulating ectonucleotidase expression in T cells by astrocytes. Nevertheless, the transwell system beside avoiding physical contact of T cells with astrocytes may also prevent T cell activation by labile soluble factors such as nucleotides triphospate produced by astrocytes, which might influence expression of the ectonucletidases. This could be especially true for CD73 expression, which is induced by exposure to astrocytes but not to APCs.

A



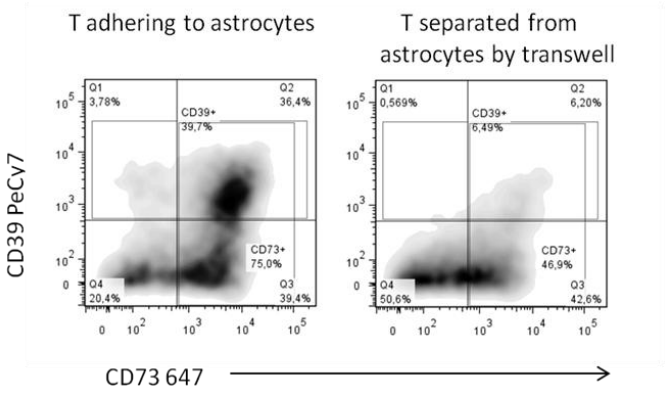


Figure 8: **Astrocyte/T cell contact and Il-2 cytokine are critically involved in CD39/CD73 induction.** (A) FACS analysis of CD39 /CD73 expression in T cells separated from astrocytes by transwell (0.3 uM membrane pore) for 48h.

Lack of evidence for gap junctions mediated interaction between astrocytes and CD4+ T cells.

To elucidate the nature of astrocyte-T cell interaction we focused on the possible molecules responsible for their reciprocal contact. Both T cells and astrocytes express proteins of the connexin and pannexin families (Cx32, Cx43, Cx30, pannexin1), which form membrane hemichannels that mediate release of signalling molecules into the pericellular space [41-43]. Through formation of intercellular channels, called gap junction, connexins also allow the direct transfer of signaling factors between adjacent cells including cAMP, Ca2+, adenosine-5-triphosphate (ATP), inositol 1,4,5-trisphosphate and morphogens [45]. Notably, recent studies showed that T cells may form heterotypic gap junction with adjacent dendritic cells [182]. To investigate possible formation of gap junction between astrocytes and T cells, astrocytes were loaded with Calcein AM 1 uM, a gap junction permeant green fluorescent dye, and then co-cultured with CD4+ activated T cells. Calcein AM is readily retained in the cytosol after the acetoxymethyl ester is cleaved by intracellular esterases. The resulting compound has a molecular weight of 520 Da, and thus is able to pass through gap junctions. After astrocyte loading, we analyzed possible dye transfer from astrocytes to T cells by flow cytometry, a technique which allows a precise evaluation of the amount of transferred dye in recipient cells through the analysis of MFI. After 24 and 48 hours no dye transfer from astrocyte to T cell in the co-culture was detectable (figure 10), suggesting that gap junctions (GJs) were not formed between the two cell types. As positive control, CD4+ T cells were co-cultured with peritoneal macrophages, which, according to literature, form GJs with T cells [182]. Already after 24h of co-culture with peritoneal macrophages, CD4+ T cells took up significant amount of the fluorescent dye. Histograms in figure 10 show a significant increase in MFI of T cells co-cultured with calcein AM loaded macrophages, while MFI of T cell co-cultured with calcein AM loaded astrocyte did not change with respect to T cells cultured on astrocytes labeled by CMFDA as control.

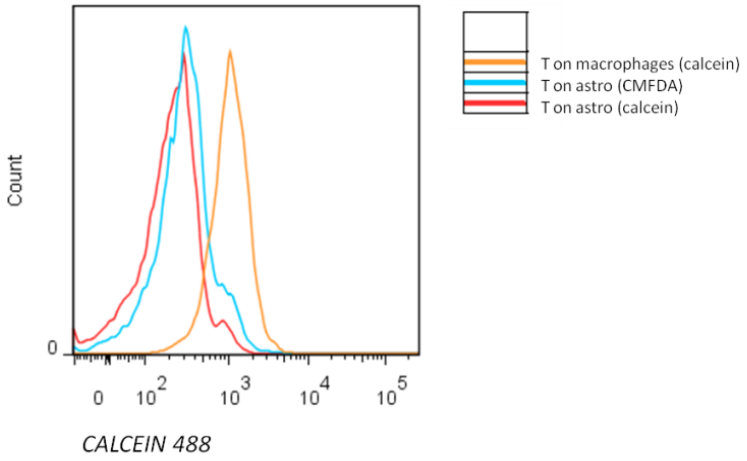
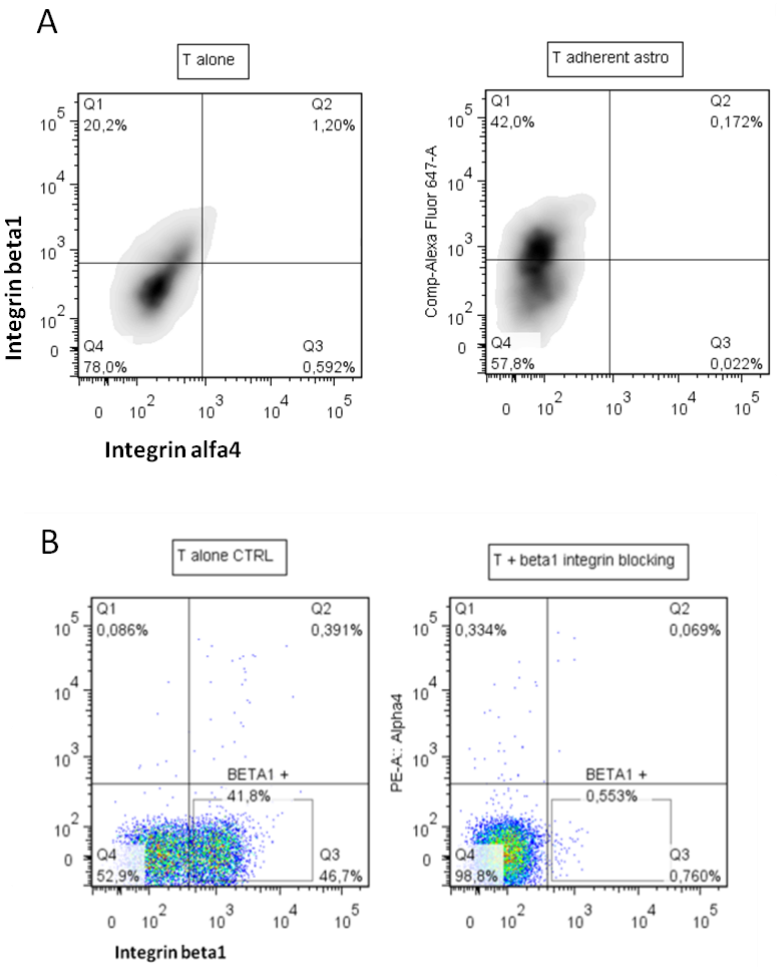


Figure 10: **Lack of gap junctions detection between astrocyte and T cell.** Direct intercellular communication through gap junction channels was assessed using flow cytometry by measuring the amount of calcein AM (1 uM) transferred from astrocytes or peritoneal macrophages (as positive control), to lymphocytes after 48 h of co-culture.

B1 integrin participates to astrocyte-T cell contact

Searching for adhesion receptors that could mediate T cells adhesion to astrocytes and consequently contribute to CD39 and CD73 upregulation in T cells, we focused on integrins. Indeed, previous studies demonstrated that beta1 integrin plays an important role in the epithelial/T-cell and astrocyte/ T cell interaction [46-49]. The group of Colombatti M. discovered that alfa3/beta1 integrins are active components of the molecular complex mediating astrocyte / T cell binding and another group showed that the subunits of VLA-4 integrin, alfa4/beta1, are necessary for T cell contact-induced generation of proinflammatory molecules in astroglia. Thus, we investigated the role of beta1 integrin in astrocyte/T-cell adhesion by assessing whether a beta1 blocking mAb affected ectonucleotidases upregulation on T cells. First, FACS analysis revealed that activated CD4+ T cells, upon astrocytes contact, expressed on their surface beta1 but not alfa4 integrin subunit (figure 11A). Incubation of T cells with the beta1 blocking mAb (figure 11B) resulted in reduced CD39 expression, albeit non-significantly, in T cells that adhered to astrocyte (figure 11C). We did not detect changes in CD39 expression on T

cells suspended on astrocytes, or in CD73 expression on T cells either suspended or adherent to astrocytes. These results indicate that CD39 upregulation depends at least in part on integrin-mediated physical contact between astrocyte and T cell.



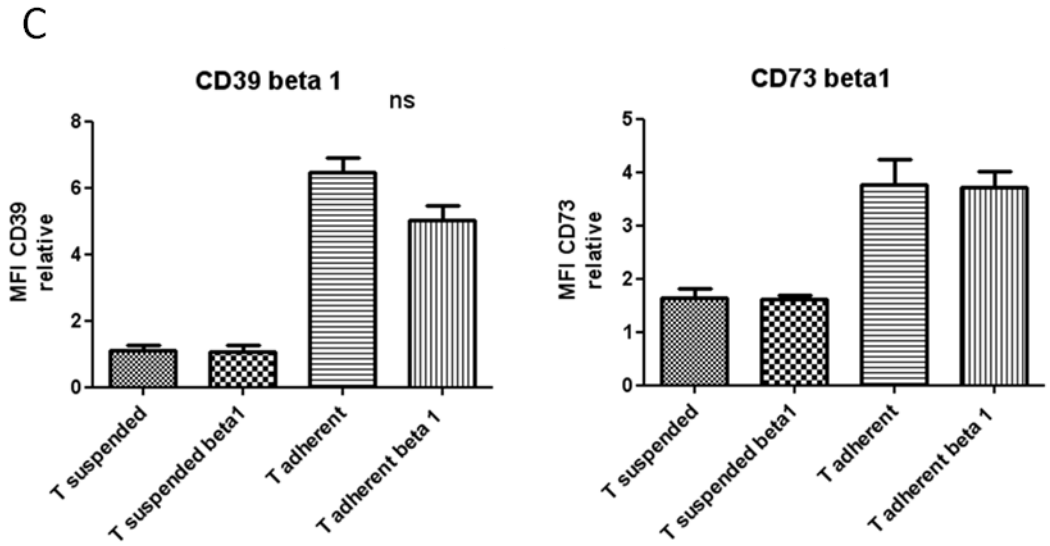
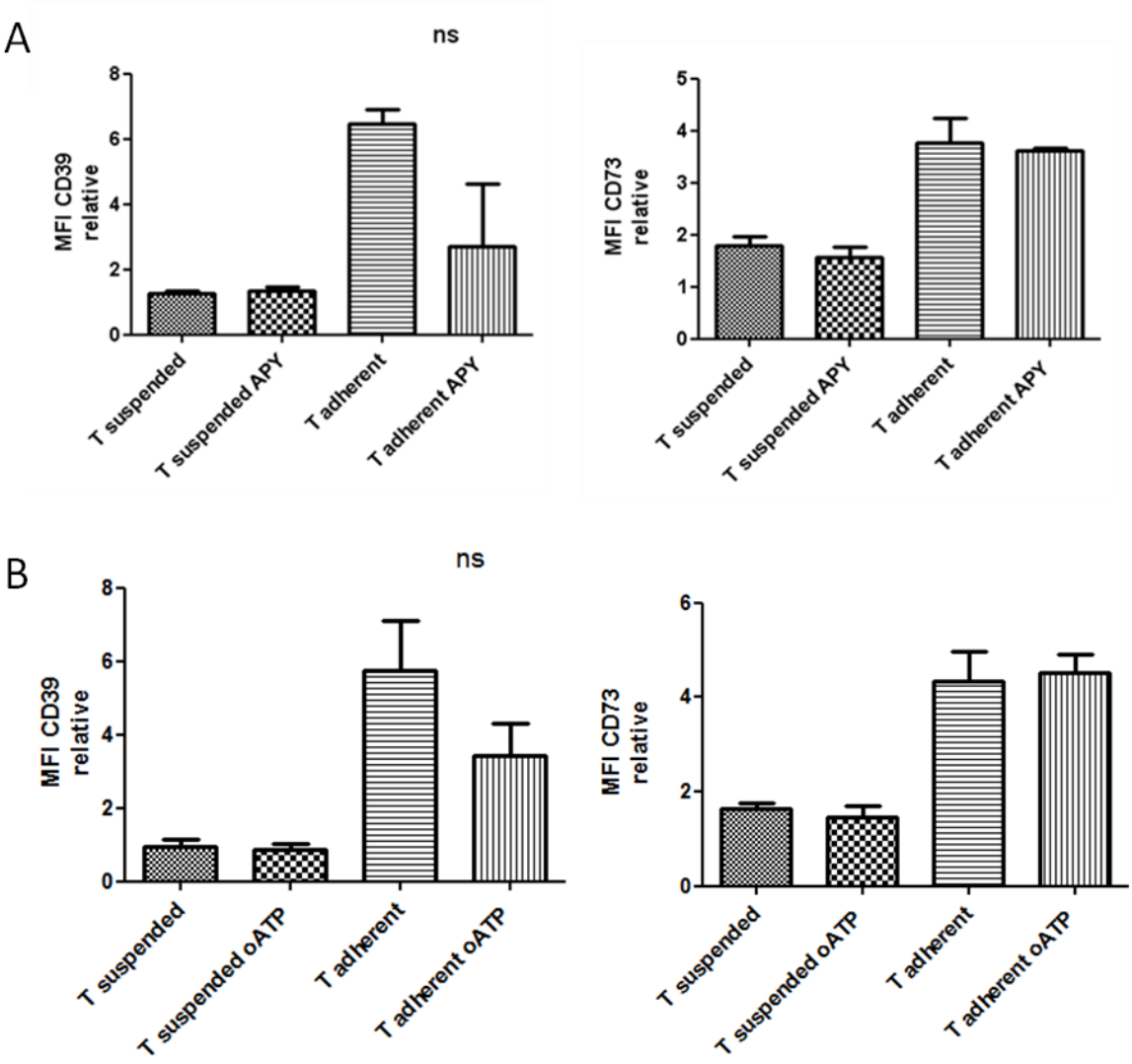


Figure 11: **The role of beta1 integrin in CD39/CD73 expression in T cells co-cultured with astrocytes.** (A) Density plot of beta1 and alfa4 integrins expression on activated T cells alone or contacting astrocytes. (B) Incubating T cells for 1.30 h with anti-beta1 integrin mAb (30 ug/ml) blocks beta1 integrin binding sites (right). Site inhibition was evaluated by FACS staining with PE coniugated anti-beta 1 integrin mAb (C) Incubation with anti-beta1 integrin (30 ug/ml) mAb partially prevented CD39 induction on T cells adhering to astrocytes (expressed as MFIs calculated on total T cell population). No observable changes in CD73 expression. Values are normalized on T cells alone (not shown). N exp = 3. Data are expressed as the MFI ± SEM and statistical significance was evaluated with Student's two tailed t-test.

ATP plays an important role in CD39 upregulation on T cell adhering to astrocyte.

Overall data obtained until now suggested that the interplay between astrocyte and T cell is crucial in CD39/CD73 upregulation. Our results excluded that this interaction could be mediated by gap junctions and, in line with previous findings, we observed that integrin beta1 is involved in astrocyte-T cell adhesion. Moreover data obtained by transwell experiment revealed that physical contact with astrocytes is of absolute importance for ectoenzymes induction on T cell surface. However, these experiments did not exclude that soluble factors and short-range acting molecules released by astrocyte may contribute in ectonucleotidase induction. Among soluble molecules, extracellular nucleotides and in particular adenosine triphosphate (ATP) could be significantly involved in this mechanism. In order to verify this hypothesis, we incubated astrocyte/T cell co-culture with the ATP degrading enzyme Apyrase, which converts ATP to ADP and AMP. After 48h we analyzed ectoenzymes levels on T cells surface in flow cytometry. In the presence of Apyrase at 50 U/ml, we observed a reduced increase in CD39 on T cells which adhered to astrocytes, while CD73 MFI was unchanged (figure 12A). This result suggested an important role of extracellular ATP in CD39 induction on T cells. To further support this conclusion, we pre-incubated T cells with the P2X antagonist periodate-oxidized 2′,3′-dialdehyde ATP (oATP) to pharmacologically prevent extracellular ATP sensing by the T cell. Histograms in figure 12B show that oATP reduced CD39 increase on T cells contacting astrocytes, while had no effect on T cells floating on astrocyte or on CD73 expression. To directly evaluate the contribution of

ATP on ectoenzyme upregulation we incubated CD4+ cells for 24 (figure 12C) and 96h (not shown) with ATP-γS, a non-hydrolyzable phosphorothioate ATP analog that cannot be hydrolyzed by ATPase, or with the prototypic P2X7 agonist 2′(3′)-O-(4-benzoylbenzoyl) ATP (BzATP). Although ATP seems to be crucially involved in CD39 upregulation in T cells/astrocytes co-culture, T cells incubation with either agonist per se did not result in significant CD39 or CD73 induction on T cells (figure 12C), thereby suggesting that other factors during T cell/astrocyte interaction enables the purinergic signaling, which contributes to CD39 upregulation.



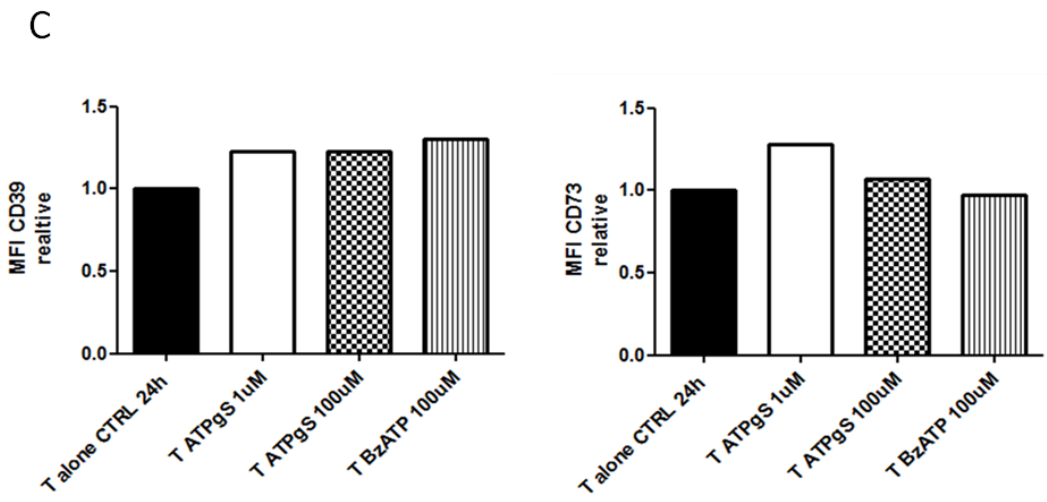


Figure 12: **CD39/CD73 induction is partially dependent on extracellular ATP levels.** (A) Histograms show flow cytometric analysis of CD39/CD73 MFIs calculated on total T cells population in the presence of Apyrase (50 U/ml) in the co-culture, n exp= 2 (Mean ± SD) or (B) preincubating T cells for 1.30 h with oATP (200 uM) n exp = 4 (Mean ± SEM). (C) CD4+ T cells cultured for 24 h in the presence of ATP-γS 1 / 100 uM or with BzATP 100 uM. MFIs are measured on total T cells. Statistical significance was evaluated by Student's two tailed t-test (A-B) and by Anova one way test with Tukey's multiple comparison post test (C).

Inhibition of astrocytic spontaneous calcium waves by tapsigargin completely prevent CD73 induction.

As already mentioned in the introduction, astrocytes are able to communicate one with each other thorough spontaneous intracellular calcium [Ca2+]i waves [80, 81]. It is also known that astrocytic Ca2+ oscillations can trigger the release of several soluble factors called gliotransmitters, in a process named "gliotransmission". The gliotransmitters released by astrocytes comprise glutamate [56, 89], D-serine [90], tumor necrosis factor alfa (TNFa) [91] and ATP [92, 93]. We therefore investigated whether spontaneous calcium waves in astrocytes are involved in CD39/CD73 upregulation on T-cells. In order to address such point, we took advantage of an irreversible inhibitor of

sarco/endoplasmic reticulum Ca2-ATPase pump (SERCA), thapsigargin, to deplete the intracellular store of calcium in the endoplasmic reticulum of astrocytes. Thapsigargin treatment can inhibit spontaneous Ca2+ oscillations for over 2 days even after the drug was washed away (figure 13A).

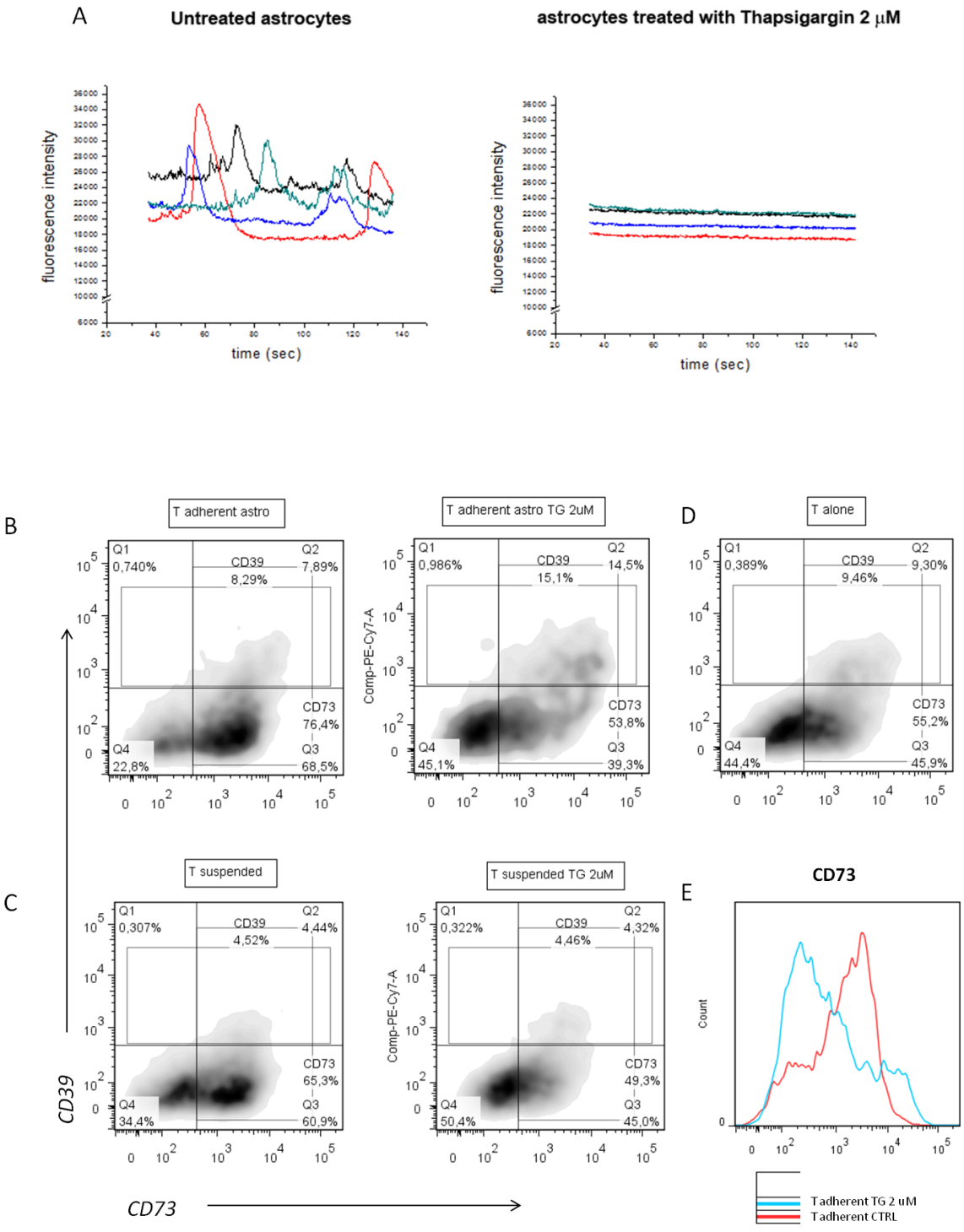


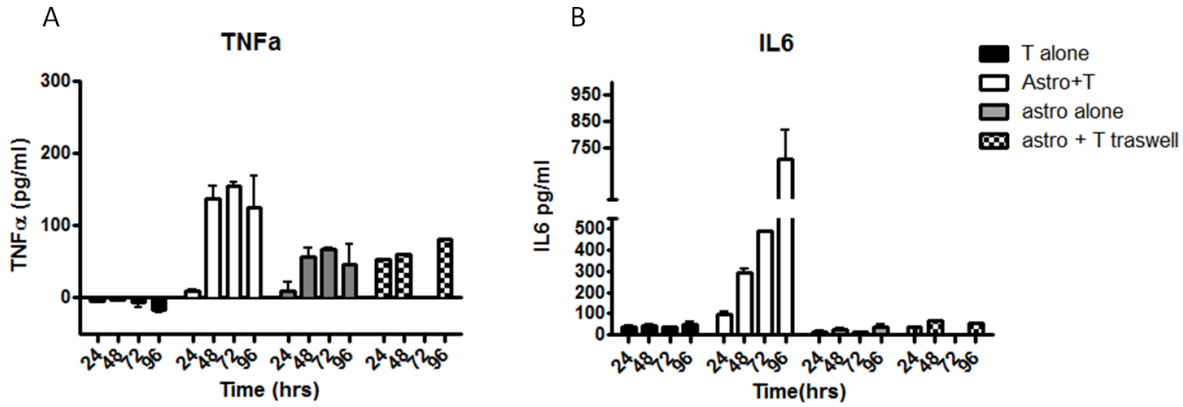
Figure 13: **T cells contacting astrocytes in which Ca2+ signals were pharmacologically inhibited show basal CD73 expression level.** Astrocyte monolayers were treated with thapsigargin (2 uM) for 20 min and then washed three times with PBS. The thapsigargin-treated astrocytes remained Ca 2+ signal-deficient for at least 48 h after the withdrawal of the drug (A). Representative density plot of CD39/CD73 induction at 48h on T cells adhering (B) or suspended (C) on thapsigargin-treated astrocytes. (D) T cells cultured alone. (E) Histogram shows CD73 expression on adherent T cells. N exp=2

Thus, activated T cells were co-cultured with thapsigargin-pretreated astrocytes and after 48h ectonucleotidases expression in T cells was evaluated by FACS analysis. We found that CD73 expression level on both adherent (figure 13B) and suspendend (figure 13C) T cells cocultured with thapsigargin-pretreated astrocytes was comparable to CD73 expression level measured in T cells cultured alone (figure 13D), indicating that the inhibition of spontaneous calcium oscillation in astrocytes completely prevented the upregulation of CD73. This result strongly suggested that a putative soluble factors released by astrocytes through a calcium-mediated mechanism might be critical for the upregulation of ectonucleotidases observed in T-cells.

Increase in TNFalfa and IL6 secretion in astrocyte-T cell co-culture.

The list of cytokines produced by astrocytes *in vitro* and/or *in vivo* comprises interleukin (IL)-1; IL-6; IL-10; interferon (IFN)-a; IFN-b, tumor necrosis factor (TNF)-a; transforming growth factor (TGF)-b; colony-stimulating factors GM-CSF, M-CSF, and G-CSF; and chemokines, including RANTES, IL-8, monocyte chemoattractant protein-1 (MCP-1), and IFN-g-inducible protein-10 (IP-10) [39]. Among other cytokines, IL-6 can be critical in the maintenance or limitation of focal inflammatory processes owing to its ability to regulate the survival/expansion of lymphocytes and the development/differentiation of astrocytes, which invariably develop reactive gliosis during infective/degenerative diseases of the CNS [183]. IL-6 gene expression is regulated in different tissues by a variety of effectors molecules, including those involved in adhesive interactions (i.e.,integrins) [184]. Myelin basic protein (MBP)-specific clonal T cells are able to

induce IL-6 gene expression in U251 astrocytes both by release of soluble factors and adhesion [48]: through astrocyte induction, T cells may indirectly regulate the availability of a cytokine crucial in modulating fate and behavior of cell populations involved in the pathogenesis of MS inflammatory lesions. We therefore measured IL-6 and TNF alfa levels in T cells, astrocytes and in astrocyte-T cell co-culture. In line with previous finding, we detected significantly higher TNFalfa (14A) and IL-6 (14B) levels in astrocyte-T cell co-culture respect to astrocyte or T cell alone. A peak of IL6 production was observed after 96 hours of co-culture, time at which we detected the greater CD73 induction on T cell. To understand whether a link between cell contact, IL6 production and ectoenzymes upregulation on T cells could exist, we stimulated activated T cell with IL6 (10 ng/ml), and subsequently analyzed CD39/CD73 expression on T cells. After 24h (figure 14C) and 96h (not shown) of IL-6 stimulation we did not observe any change in CD39 and CD73 expression on T cell, thus excluding a major role for this cytokine in ectoenzymes induction on T cells.



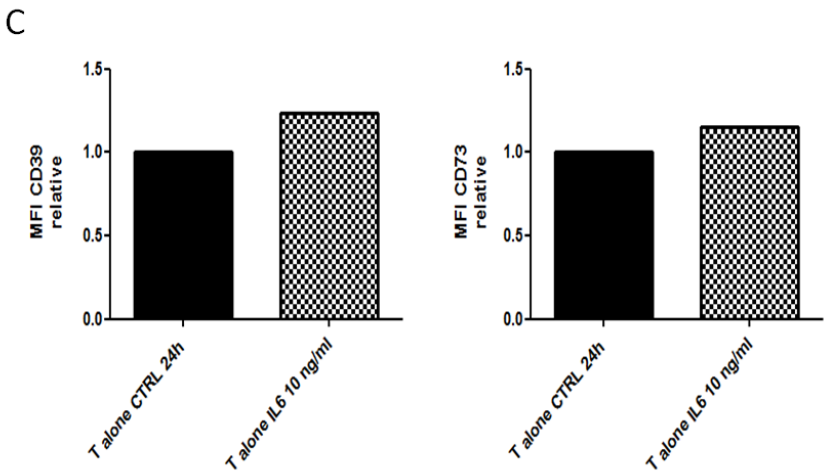
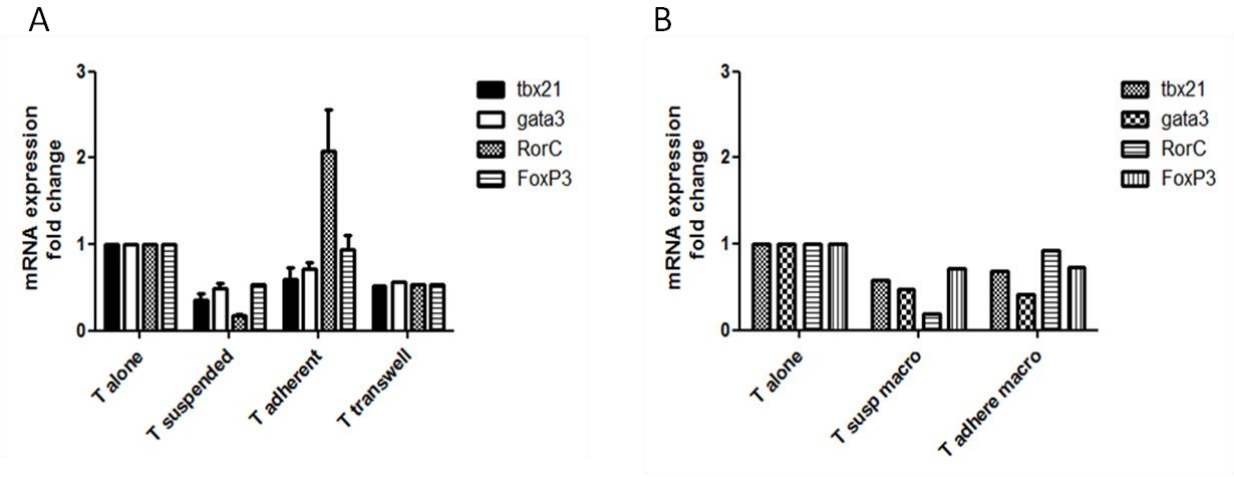


Figure 14: **IL-6 and TNFalfa production in astrocyte/T cell co-culture.** (A-B) Quantification of TNFalfa and IL-6 by ELISA in culture supernatants of astrocytes, T cells and astrocytes/T cells co-culture at different times (24, 48, 72, 96 h). Histograms represent the average of duplicates obtained in two independent experiments (Mean ±SD). (B) Relative CD39/CD73 MFI obtained in flow cytometry on CD4 T cells incubated with IL-6 (10 ng/ml) for 24h. Values are normalized on T cell alone.

Transcriptional Th17 signature in T cell adhering to astrocyte.

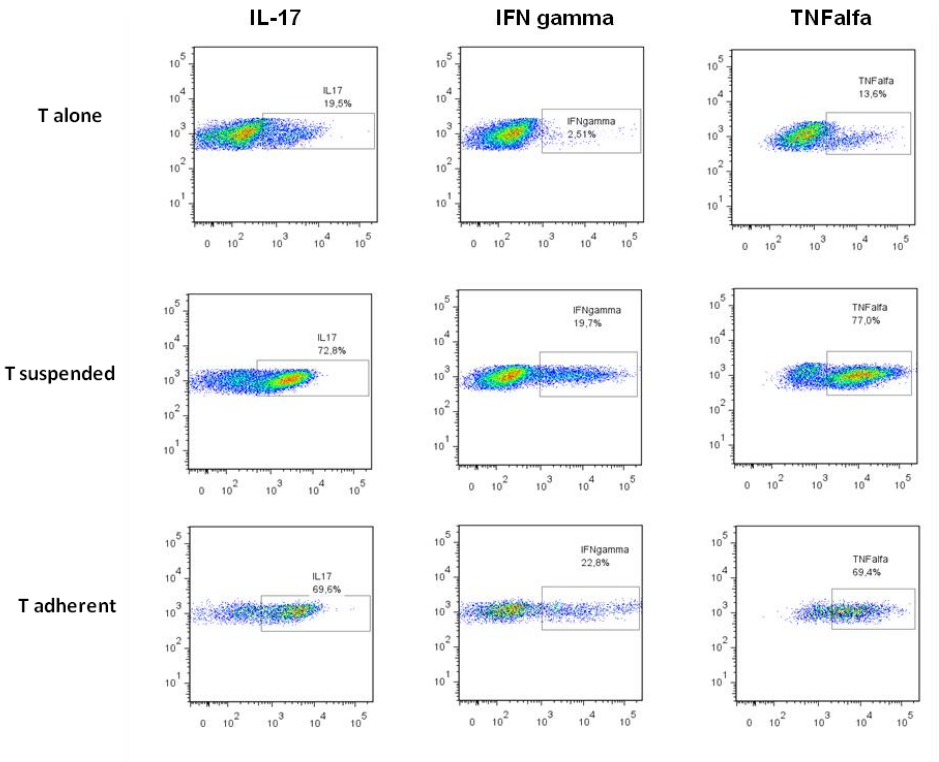
Interleukin-17 producing T cells (Th-17 cells) represent a distinct lineage of CD4+ T cells that can differentiate from uncommitted naive T cell precursors under the aegis of the transcription factors RORgt and RORa and the polarizing cytokines transforming growth factor-b, IL-6 and IL-23 [155, 185]. Ghiringhelli's group has recently described Th17 cells generated with the polarizing cytokines IL-6 and TGF-b, which express CD39 and CD73 ectonucleotidases and suppress effector T cells likely through adenosine generation [159]. Transcritptional regulation of ectonucleotidases was dependent on IL-6-driven Stat3 activation and TGF-b-mediated downregulation of zinc finger protein growth factor independent-1 (Gfi-1). In order to define whether activated T cells co-cultured with astrocytes and in particular adherent T cells, may acquire an effective regulatory or inflammatory phenotype, we performed real time PCR (qPCR) for markers of distinct T cell phenotypes on both floating and astrocytes-adherent CD4+ T cells at

48 h of co-culture. Transcriptional regulation of master switches for T cell functional skewing, including Tbet for T helper Th1 cells, GATA3 for Th2, RORC for Th17 cells and Foxp3 for Tregs was investigated on sorted CD4 cells. We found that Rorc mRNA was specifically induced in T cell adhering to astrocyte, as compared to T cell alone, but not in floating cells (figure 15A). Moreover Rorc transcription was completely prevented by separating astrocyte and T cell by culture in transwell. RorC was specifically induced in T cells adherent to astrocytes since we did not observe significant changes in RorC transcription rate in T cells co-cultured with peritoneal macrophages (figure 15B).



Besides transcription factors, intracellular staining with relevant cytokine-specific antibodies was performed to assess the effector potential of T cells (figure 15C). After 48h of co-culture accordingly with RorC upregulation, astrocytes-adherent T cells displayed a significant increase in intracellular IL17 production respect to T cells alone. Furthermore, adherent CD4+ T cells showed increased intracellular IFNgamma respect to T cells alone. This result is highly coherent with previous data which have demonstrated that astrocytes promote the generation of IFN-g and IL-17 citokines in concanavalin A- and myelin basic protein-stimulated lymph node cells from rats [186]. We also observed that TNFalfa levels increased more than half both in suspended and adherent T cells. We did not observe detectable changes in IL10 production, and in FoxP3 trasncription.

C


IL-17
IFN gamma
TNFalfa
T alone
T suspended
T adherent
IL17 19,5%
IFNgamma 2,61%
TNFalfa 13,6%
IL17 72,8%
IFNgamma 19,7%
TNFalfa 77,0%
IL17 69,6%
IFNgamma 22,8%
TNFalfa 69,4%

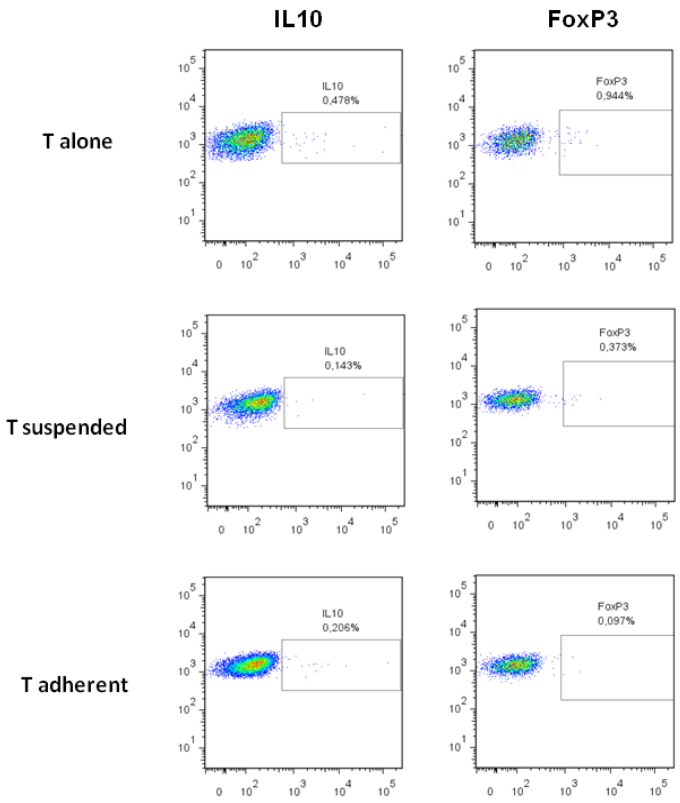

IL10
FoxP3
T alone
T suspended
T adherent
IL10 0,478%
FoxP3 0,944%
IL10 0,143%
FoxP3 0,373%
IL10 0,206%
FoxP3 0,097%

Figure 15: **Increase in Rorc transcripts (Th17 program) in T cells adhering to astrocytes.** (A) qRT-PCR analysis indicated no significant changes in mRNA levels of the transcription factors Tbx2 coding for Tbet (Th1 program), GATA3 (Th2 program) and FoxP3 (T regulatory phenotype) in activated T cells both floating and adhering to astrocytes respect to activated T cells alone after 48h of co-culture. mRNA expression was quantified by RT-PCR in 2 independent experiments. mRNA levels of the different genes were first normalized to the GAPDH housekeeping gene, and then expressed relatively to the levels found in CD3/CD28 activated control T cells. Statistical significance was analysed by two-way ANOVA test with Bonferroni post test (mean ±SD). (B) Analysis of transcription factors expressed by T cells co-cultured with peritoneal macrophages (n= 1). (C) Flow cytometry analysis of the intracellular cytokines: IL17, IFNgamma, TNFalfa, IL10 and the transcription factor FoxP3 in CD4+ cells in the indicated culture conditions. Rapresentative dot plot.

DISCUSSION

Astrocytes, the most abundant cells into the brain, play a crucial role in CNS function both in normal and pathological conditions [56, 57] [58, 59] [60] [61]. Together with endothelial cells, astrocytes are closely apposed to the blood brain barrier (BBB), and constitute the glia limitans, which regulate immune cells entry into the CNS [39]. Disturbed integrity of BBB and massive infiltration of leukocytes are important features in the pathogenesis of many neuroinflammatory diseases, such as multiple sclerosis (MS) and its animal model experimental autoimmune encephalomyelitis (EAE) [1] [2]. The role of leukocytes in promoting astrocyte capacity to present antigen to T cells and initiating production of pro- and anti-inflammatory mediators has been addressed in a number of studies with controversial results [39, 174, 187]. Astrocytes represent a source of various cytokines relevant for maintenance and/or regulation of the inflammatory response within the CNS [50]. However, whether and how astrocytes might play a protective or detrimental role during CNS inflammation has not been elucidated yet. The essential aim of my thesis was to contribute knowledge into the reciprocal interaction of astrocytes with activated CD4+ T helper cells, which play a pivotal role in the initiation of CNS infiltration by inflammatory leukocytes in neuroinflammation.

Astrocytes are non-excitable cells, nevertheless, one of their principal regulatory pathways is based on intracellular Ca2+ oscillations [80-82], which could be both spontaneous or evoked by neuronal activity. Our experiments revealed that astrocytes cultured in the presence of activated CD4+ T cells display significantly reduced spontaneous basal calcium oscillations. Previous T cell activation was crucial in endowing T cells with the potential to inhibit astrocytes Ca2+ oscillations since unstimulated CD4+ T cells did not showed the same effect. Adenosine triphosphate (ATP) is one of the main extracellular mediators involved in the regulation of calcium signaling in astrocyte [92, 93]. Koizumi and collaborators convincingly demonstrated that not only neuronal evoked, but also spontaneous Ca2+ oscillations are tightly dependent on extracellular ATP [98] [188]. In agreement with previous observations, we showed that incubation of astrocytes with Apyrase (30 U/ml), an ATP hydrolyzing enzyme able to convert ATP to ADP and AMP, significantly reduced the percentage of

astrocytes showing basal calcium oscillations. We hypothesized that the reduction in calcium transients detected in astrocyte/T cell co-cultures could be directly linked to the modulation of extracellular ATP levels performed by T cells. In fact, experiments with luciferin–luciferase based assay showed a decrease in extracellular ATP concentration in astrocytes culture medium when activated T cells were present. This result represents the first evidence that ATP is an extracellular mediator which play a key role in the interaction between astrocyte and activated CD4+ T cell. We therefore postulate that activated CD4+ T cells might respond to and hydrolyze ATP released by astrocytes through the expression of ectonucleotidases. Notably, the plasma membrane ectonucleotidase CD39 (E-NTPDase1) is the dominant ectoenzyme in the immune system [138] that dephosphorylates ATP to AMP by removing one phosphate at a time with modest residual ADP detectable [139]. In addition, T cells can express CD73, a glycosyl phosphatidylinositol-anchored enzyme [140] that hydrolyses AMP to nucleosides (adenosine) and Pi. These two ectonucleotidases are characteristically expressed by immunosuppressive Foxp3+ T regulatory (Treg) cells and their activities were hypothesized to be crucial for Treg cells mediated immune tolerance [147, 149]. A first indication for the involvement of ectonucleotidases activity in T cell mediated modulation of Ca2+ transient in astrocytes came from the observation that ARL 67156, which partially but significantly inhibits NTPDase1/CD39 activity, prevented the reduction of extracellular ATP detected in our astrocyte-T cell co-culture system. We therefore investigated the expression of ectonucleotidases in T cells cultured with or without astrocytes. FACS experiments revealed that activated CD4+ T cells co-cultured with purified astrocytes robustly upregulated CD39 and CD73 expression on their surface. CD4 T cells adhering to astrocytes displayed a surprisingly elevated increase in the percentage of CD39/CD73 double positive T cells as compared to the T cell population floating on astrocytes. We could not rule out whether suspended T cells previously or less intensely adhered to astrocytes, nevertheless this finding suggests that astrocyte contact might be required for ectonucleotidases upregulation on the surface of activated CD4+ T cells.

In order to assess the selective role of astrocyte in CD39/CD73 induction on T cells, we co-cultured T cells with different antigen presenting cells (APCs), namely microglial cells (CNS resident APCs) and peritoneal macrophages, and analyzed CD39 and CD73 expression in T cells by FACS. Whereas CD39 was induced on adherent T cells to a similar extent by all three cell types, CD73 was selectively upregulated upon contact

with astrocytes but not with other APCs. This result demonstrates the selective role of astrocytes in inducing CD73 ectonucleotidase on T cells. Furthermore it suggests the existence of an astrocyte-T cell contact dependent cross-talk based on the induction of ectonucleotidase activity in T cells and the reciprocal modulation of ATP signaling in astrocytes. In fact, impeding direct contact between astrocyte and T cell by a transwell culture system, CD39/CD73 upregulation in T cells was completely prevented. So far we postulate that physical contact with astrocytes is crucial for ectoenzymes induction on T cells.

We could eventually demonstrate that astrocyte/T cell communication was not mediated by gap junctions formation, therefore we addressed whether integrin-mediated adhesion was involved in astrocyte-dependent ectonucleotidases upregulation in T cells. Previous data showed that integrins are indeed implicated in astrocyte/T cell binding. In particular, alfa3/beta1 integrins were shown to be the active components of the molecular complex mediating astrocyte-T cell interaction [48], whilst the alfa4/beta1 dimer was necessary for T cell contact-induced generation of proinflammatory molecules in astroglia [49]. We investigated whether blocking beta 1 integrin would affect ectonucleotidases upregulation on T cells in a similar way as observed in the transwell culture. In spite of substantial effect of beta1 integrin blockade on CD39 expression, CD73 upregulation was not affected. Therefore, we concluded that direct cell–cell contact partially involving integrin beta 1 is required for CD39 induction on T cells adhering to astrocytes.

It is worth pointing out that the transwell system employed to address the cell-cell contact dependence for CD39/73 upregulation in T cells, beside avoiding physical contact between T cells and astrocytes, might possibly prevent T cell conditioning by labile soluble factors and/or short-range acting molecules released by astrocytes. We cannot therefore exclude that soluble molecules acting in the very pericellular space might contribute to ectonucleotidases induction on T cells. Astrocytes are indeed secretory cells able to release chemical transmitters in response to various stimuli in a process called “gliotransmission” [56, 89] [90] [91] [92, 93] and ATP could well conform to the kind of mediators involved in this activity. Astrocytes are considered the main source of physiologically released ATP in the CNS [93]. Astrocytes can release ATP (1) by a Ca2+-dependent exocytosis [111-113] (2) by a regulated form of exocytosis,(3) through hemichannels formed by connexins and pannexins, and finally (4) through

plasmalemmal voltage-dependent anion channels and purinergic receptor (P2XRs) [109, 114]. We addressed whether extracellular ATP contributed to ectonucleotidases upregulation in T cell upon contact with astrocyte. Addition of Apyrase to astrocyte-T cell co-culture or pre-treatment of T cells with the P2XRs antagonist oATP before culture with astrocytes (thus inhibiting ATP mediated signaling by P2XRs in T cells), both independently resulted in substantial inhibition of CD39 upregulation. Although extracellular ATP appeared involved in astrocyte mediated CD39 induction, the incubation of T cells with a non-hydrolyzable ATP analog, ATPγS, or with the prototypic P2X7 (the main P2X receptor subtype expressed in T cell) agonist BzATP did not result in significant increase of surface CD39. Altogether these data point to a role of pericellular ATP in regulating CD39 expression in adherent T cells in the context of astrocyte/T cell co-culture. Accordingly, the increase in surface CD39 was barely evident in non-adherent, i.e. floating, T cells. It will be interesting to investigate whether microglia and macrophages, which can both release ATP, induce CD39 upregulation upon contact with T cells by the same purinergic signaling pathway.

In contrast to CD39, CD73 expression was selectively induced by T cells exposure to astrocytes but not to microglia and peritoneal macrophages. As mentioned above, peculiar of astrocytes is the release of gliotransmitters triggered by spontaneous and neuronal activity-evoked Ca2+ oscillations. The actually known gliotransmitters include, further ATP[92, 93], glutamate [56, 89] and D-serine [90]. To address whether blockade of Ca2+ signaling in astrocytes affected CD73 modulation in T cells, we depleted the intracellular calcium store in astrocytes ER by thapsigargin, an irreversible inhibitor of sarco/endoplasmic reticulum Ca2+-ATPase pump, which abolish cytosolic Ca2+ elevations. This treatment completely prevented CD73 upregulation induced in both adherent and suspendend T cells, thus suggesting that a Ca2+ dependent, possibly soluble, factor was responsible for this effect. We are currently investigating which gliotransmitter could be critical in inducing CD73 upregulation in T cells.

An important aspect of astrocyte/T cell interaction for CNS pathophysiology emerging from our study stems from the role of CD73 in regulating BBB permeability. Indeed, extracellular adenosine generated by CD73 activity promotes T cell infiltration into the brain during EAE, while blockade of the A2A adenosine receptor inhibits BBB crossing by T cells [7]. Accordingly, mice lacking CD73, which are unable to hydrolyze extracellular AMP to adenosine, were protected from EAE [8]. The correlation between astrocyte contact and CD73 upregulation on activated T cells could be important to

assess a possible pathogenetic significance of astrocytes activity in leukocytes infiltration of the brain.

Moreover, we detected IL-6 and TNF-alfa levels in astrocyte-T cell co-culture, which were significantly higher with respect to astrocyte and T cell cultured alone. Astrocytes represent the major source of IL-6 in the CNS [189]; elevated IL-6 levels are detected in injury, infection, stroke, and a wide range of CNS disorders, including MS, where IL-6 could be detrimental as evidenced by the finding that IL-6 knockout mice are not permissive to EAE [190]. The coordinate regulation of IL-6 and CD73 might generate a pro-inflammatory environment permissive to leukocyte infiltration into the brain. Accordingly, analysis of T cell polarization in astrocyte/T cell co-culture revealed skewing of T cells toward IFN-γ and IL-17 secreting Th1 and Th17 phenotypes, which are both crucially involved in MS pathogenesis. Astrocytes selectively induced in adhering T cell high levels of Rorc transcript encoding the transcription factor RORγT, which specifies the Th17 lineage. This effect was not observed in T cells co-cultured with peritoneal macrophages, and was completely prevented by transwell culture. A recent study by Chalmin F et al. uncovered a new Th17 cells subtype characterized by the expression of both CD39 and CD73 which display immunosuppressive activity [159]. These immunoregulatory Th17 cells were differentiated *in vitro* in the presence of TGF-b and IL-6, thus reminiscent of the phenotype acquired by a significant fraction of T cells adhering to astrocytes in our co-cultures. Preliminary experiments not reported in the thesis addressing the immunosuppressive potential of Th17 cells generated in our astrocyte/T cell co-cultures showed pro-inflammatory but not immunoregulatory activity of this cell subset, thus suggesting that astrocyte might contribute to priming activated T cells for a peculiar brain-specific effector program.

Finally, the experiments presented in this thesis unravel a so far unknown reciprocal signaling between astrocyte and activated CD4+ T cell, which is dependent on cell-cell contact and soluble astrocyte released gliotransmitters. Understanding the significance of this cross-talk and identifying astrocytes-derived molecules regulating CD4 T cell function might unravel new therapeutic targets in MS and other neuroinflammatory conditions.

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