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A phylogenetic view of the leukocyte ectonucleotidases

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ABSTRACT

The leukocyte ectonucleotidases are a recently defined family included in the last Human Leukocyte Differentiation Antigens Workshop, giving prominence to these membrane proteins whose catalytic activity is expressed outside the cell. Among the most important substrates of the leukocyte ectonucleotidases are extracellular ATP and NAD^+ whose transient increases are not immunologically silent but rather perceived as danger signals by the host. Among the host responses to the release of ATP, NAD^+ and related small molecules is their breakdown on behalf of a panel of leukocyte ectonucleotidases - CD38, CD39, CD73, CD157, CD203a and CD203c -, whose activities are concatenated to form two nucleotide-catabolizing channels defined as the canonical and non-canonical adenosinergic pathways. Here, after briefly reviewing the structure and function of the proteins involved in these pathwys, we focus on the genes encoding the ectoenzymes of these adenosinergic pathways. The chromosomal localizations of the enzyme-encoding genes yield a first level of information concerning their origins by duplication and modes of regulation. Further information was obtained from phylogenetic analyses that show ectoenzyme orthologs are conserved in major tetrapod species whereas examination of synteny conservation revealed that the chromosomal regions harboring the ADP-ribosyl cyclases on human chromosome 4 and the ENTPDase CD39 on chromosome 10 show striking similarities in gene content consistent with their being paralogous chromosomal regions derived from a vertebrate whole genome duplication. Thus the connections between some of the leukocyte ectoenzymes run deeper than previously imagined.

1. The leukocyte ectoenzyme family

Little was known of the surface molecules expressed by human leukocytes until the introduction of murine monoclonal antibodies as analytical tools. To establish order, international projects like the Human Leukocyte Differentiation Antigen (HLDA) workshops were set up, bringing together antibody producers, experts in human cell biology and clinicians. The idea was simple: to map the surface of human cells by means of antibodies that recognized target molecules. The results of these international efforts have been summarized in the CD (Cluster of Differentiation) Workshops. To date, over 400 CD molecules have been defined and grouped by structure and function into gene families [1].

A new addition made to the last update of the CD Nomenclature, published in 2015 [1], is the Ectoenzyme Family, an umbrella term that brings together leukocyte antigens endowed with catalytic activity [2] (Table 1). Although all nine enzymes listed in Table 1 are leukocyte ectoenzymes involved in signalling pathways that regulate cells of the immune system, here we will focus on six of the family members, namely CD38, CD39, CD73, CD157, CD203a and CD203c, as they are conjoined, or potentially so, in pathways that stem from the hydrolysis of the major extracellular nucleotides adenosine-5′-triphosphate (ATP) and nicotinamide adenine dinucleotide (β-NAD^+, or simply NAD^+ from here on) and lead to the production of extracellular adenosine, an extremely potent and pleiotropic small molecule which can undergo different fates and affect numerous physiological and pathological processes [3–5].

2. The substrates of the leukocyte ectoenzymes: extracellular ATP and NAD^+

ATP and NAD^+ are universal nucleotides that have played essential roles in the evolution of life, from forming the building blocks of the genetic code to supplying chemical energy to living cells via intermediary metabolism [6]. ATP and NAD^+ are involved in a staggering number of biochemical reactions which take place strictly inside the cell, allowing for the continuous replenishment of both nucleotides to maintain homeostasis. While the intracellular concentration of ATP reportedly spans from 1 to 10 mM, extracellular ATP lies in the nanomolar range, generating a very high gradient between intra- and extracellular ATP [6]. Likewise, the intracellular NAD^+ concentration is around 0.3 mM, decreasing about a thousand-fold to 0.1-0.5 μM in biological fluids [7].

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Table 1
Summary of the The Leukocyte Ectonucleotidase family[a].

CD#	Enzyme	Gene name	EC number
CD26	Dipeptidyl peptidase	*DPP4*	EC 3.4.14.5
CD38	NADase/ADP-ribosyl cyclase	*CD38*	EC 3.2.2.5/EC 2.4.99.20
CD39	Ectonucleoside triphosphate diphosphohydrolase	*NTPD1*	EC 3.6.1.5
CD73	5'- Ectonucleotidase	*NT5E*	EC 3.1.3.5
CD157	NADase/ADP-ribosyl cyclase	*BST1*	EC 3.2.2.5
CD203a	Ectonucleotide pyrophosphatase/ phosphodiesterase	*ENPP1*	EC 3.6.1.9/EC 3.1.4.1
CD203c	Ectonucleotide pyrophosphatase/ phosphodiesterase	*ENPP3*	EC 3.6.1.9/EC 3.1.4.1
CD296	ADP-ribosyltransferase	*ART1*	EC 2.4.2.31
CD297	ADP-ribosyltransferase	*ART4*	EC 2.4.2.31

[a] Only CD antigens in bold are the focus of this review.

The intracellular/extracellular gradients of ATP and NAD^+ are perturbed by release of these nucleotides which, in humans, mainly occurs in disease conditions, such as infection, hypoxia, inflammation or cytotoxic treatment, all conditions that lead to cell disruption via physical stress, apoptosis or necrosis [7,8]. In addition, ATP and NAD^+ may also be released from intact cells in a more regulated manner, such as by exocytosis or by diffusion across selective membrane channels and transporters [9]. However, the release of ATP and NAD^+ into the immunological milieu is not merely a discharge of damage metabolites nor is it immunologically silent, as both these nucleotides are autocrine/paracrine signalling molecules and perceived by the host as danger signals [8]. Extracellular ATP can interact and signal through P2 purinergic plasma membrane receptors, promoting a response that is typically proinflammatory in nature, with inflammasome activation and production of proinflammatory cytokines [10]. Likewise, the release of NAD^+ into the extracellular space does not go unnoticed, and also NAD^+ can play an immunomodulatory role by binding purinergic receptors [7,10].

By binding and catabolizing extracellular nucleotides in the immunological milieu, the leukocyte ectoenzymes can terminate ATP- and NAD^+-driven signalling by converting the nucleotides to adenosine [7,8]. Again, these reactions are not immunologically silent as, unlike ATP, adenosine is powerfully immunosuppressant [11,12]. Thus the leukocyte ectonucleotidases can be viewed as 'immunological switches' capable of converting an ATP-rich, proinflammatory extracellular environment into an adenosine-rich, anti-inflammatory niche. Adenosine carries out its many immunosuppressive effects, such as blocking proinflammatory and inducing anti-inflammatory cytokines, and inhibiting leukocyte migration, by interacting with widely expressed P1 adenosine receptors [10,13]. Extracellular adenosine may undergo other fates, such as internalization via nucleoside transporters or be converted to inosine by adenosine deaminases (ADAs), essential enzymes of purine metabolism [4,11]. Another member of the leukocyte ectoenzyme family, CD26 or dipeptidyl peptidase (DPP4) binds ADA, thus transforming this normally cytosolic enzyme into a membrane-bound extracellular form that can convert adenosine to inosine [4].

3. The CD39/CD73 tandem and the extracellular pathway from ATP to adenosine

The network of enzymes responsible for the extracellular pathways that both consume and generate ATP were described by Yegutkin et al. in human leukocytes and non-leukocytic cells [14]. As more than 15 years have passed since its description, this pathway has become well consolidated and is often referred to as the canonical pathway or CD39/CD73 axis seeing as the principal actors are the ecto-nucleoside triphosphate diphosphohydrolase (NTPDase) CD39 (EC 3.6.1.5) and the 5′-nucleotidase (5′-NT) CD73 (EC 3.1.3.5). These enzymes work in series (Fig. 1): membrane-bound CD39 has high affinity for extracellular ATP and starts the pathway by hydrolyzing it completely to AMP, usually with little release of intermediary ADP [15]. The AMP generated by CD39 is then relayed to CD73 which hydrolyses it to adenosine (and inorganic phosphate).

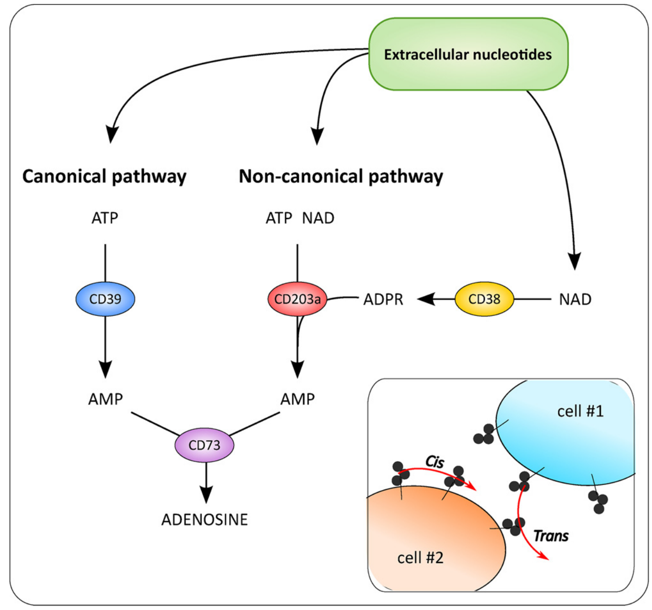

Fig. 1. Schematic representation of the canonical and non-canonical adenosinergic pathways. The extracellular pathways leading from ATP and NAD to adenosine are schematically represented. Inset: pathways proceed through ectoenzymes expressed in *cis* (same cell) and *trans* (on different cells) within a closed system such as the bone marrow niche.

4. The CD38/CD203a/CD73 triad and the extracellular pathway from NAD^+ to adenosine

Another ectoenzymatic pathway was proposed over twenty years ago, whose function in human T lymphocytes was to recycle NAD^+-derived metabolites [5]. As evidence in favour of the signalling power of adenosine accumulated, this route was re-examined by Horenstein et al. who demonstrated the existance of an alternative pathway leading to extracellular adenosine production, stemming from the breakdown of NAD^+. This pathway has since become established as the non-canonical adenosinergic pathway and is mediated by the CD38/CD203a/CD73 axis [16] (Fig. 1). The principal actors are: (i) the NAD nucleosidase CD38 (EC 3.2.2.5), a member of the ADP ribosyl cyclase family that mostly converts NAD^+ to ADP ribose (ADPR) directly or through formation and degradation of cyclic ADP ribose to ADPR [17]. The conversion of NAD^+ to ADPR could in theory also be catalysed by the NAD nucleosidase CD157 (alias BST1), also a member of the ADP ribosyl cyclases and a paralog of CD38 [18] ; (ii) the ecto-nucleotide pyrophosphatase/phosphodiesterase (NPP) CD203a (alias PC-1) (EC 3.6.1.9). This enzyme has a broad substrate specificity and can use as substrate either NAD^+ (cleaving it to AMP and nicotinamide mononucleotide), ATP (producing AMP and inorganic phosphate) or the ADPR generated by CD38 from NAD^+ (forming AMP) [19]; The conversion of nucleotides performed by CD203a could in theory also be catalysed by its paralogue CD203c which, however, proceeds through production and breakdown of ADP; and finally there is (iii) CD73 which catalyzes the conversion of AMP to adenosine [19], representing the point of convergence between canonical and non-canonical pathways.

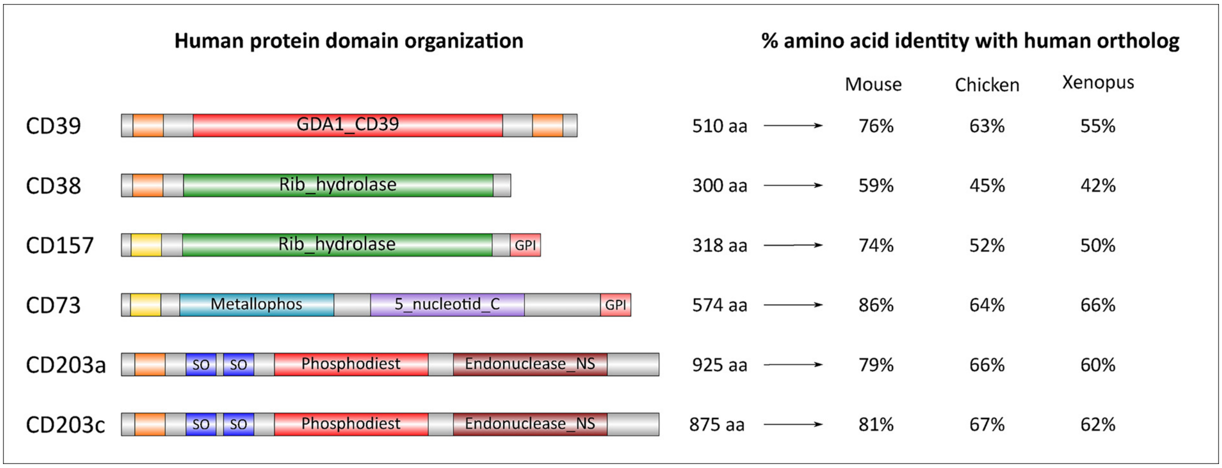

Fig. 2. Domain organization of the leukocyte ectonucleotidases. Constitutent Pfam domains of each protein are indicated. N-term orange squares represent transmembrane domains; yellow squares are signal peptides. On right panel, amino acid conservation between individual human ectoenzymes and their respective orthologs in mouse, chicken and frog.

5. Topology and domain organization of the leukocyte ectonucleotidases

By definition, the ectoenzymes that compose the canonical and non-canonical adenosinergic pathways are located on the plasma membrane with their catalytically active domains facing the extracellular environment. There are other common features shared by CD38, CD39, CD73, CD157, CD203a and CD203c: for example, they are all glycosylated proteins with conserved disulphide-forming cysteines, and they all undergo oligomerization on the cell surface [15,20,21]. However, the membrane topology of the ectonucleotidases is not uniform: CD38, CD203a and CD203c are type II transmembrane proteins whereas CD73 and CD157 are GPI-linked (Fig. 2). CD39 has a more unusual structure with respect to its family members: this glycoprotein is anchored to the cell membrane by two transmembrane regions, one at the N-term, the other at the C-term, separated by a large extracellular loop containing the catalytic domain.

With a view to identifying orthologs of the ectonucleotidases in other vertebrate species, we examined their domain composition using the Pfam database. Although CD38 and CD157 are topologically different (type II and GPI, respectively) and in opposite orientation with respect to one another (CD38: C-term$_{exo}$; CD157: N-term$_{exo}$), their domain organization is similar and formed by a single *ribosyl-hydrolase* domain (Pfam 02267). These proteins are the smallest of the ectonucleotidase group (~ 300 aa). Human CD39, a larger protein consisting of 510 aa, has an extracellular region formed by a *GDA1_CD39* protein domain (Pfam 01150) which contains evolutionarily conserved motifs known as ‘apyrase-conserved regions’ [21]. The CD73 polypeptide contains 574 aa, and the CD73 ectodomain contains two different components: an N-term *Metallophos* domain (Pfam 00149) that binds Zn^{2+} and Ca^{2+} ions required for catalysis, and a *5_nucleotidase_C-terminal* domain (Pfam 02872).

Human CD203a and CD203c are the largest proteins of the group, formed by 875 and 925 aa, respectively, with a large extracellular catalytic domain containing an N-term *Phosphodiesterase* domain (Pfam 01663) and a C-term *Endonuclease_NS* (non specific) domain (Pfam 01223). As expected, human paralog protein pairs show amino acid sequence conservation: this is relatively low for the ADP-ribosyl cyclases that 37% aa identity between CD38 and CD157, and higher for CD203a and CD203c, which share 54% aa sequence.

6. Expression pattern of the leukocyte ectonucleotidases

Each one of the leukocyte ectonucleotidases has a distinctive pattern of expression within cells of the immune system and the endothelial cells that line the vasculature and lymphatic systems. The function of the canonical and non-canonical pathways does not require that the ectoenzymes forming a pathway be expressed on the same cell but by cells in sufficient proximity that the product of one enzyme can become the substrate of the next in the series [16]. This concept is similar to that of receptors that interact with ligands on another cell, *i.e.,* a *trans* interaction, or with a ligand on the same cell (*cis* interaction) [22] (Fig. 1, inset). In the CD39/CD73 axis, the first-step catalyst CD39 was originally described as a B lymphocyte activation marker but is broadly expressed in other cells of the lymphoid compartment (activated T cell subsets, NK cells) and the myeloid compartment (neutrophils and monocytes) [23]. CD39 is also expressed in vascular endothelium, where it plays a crucial role in hemostasis and thromboregulation. Human CD73 is widely expressed in T, B and myeloid cells, overlapping with CD39 expression [24].

The first component of the non-canonical pathway, the NAD glycohydrolase represented by CD38, is expressed in distinct steps of T and B lymphocyte differentiation and activation, and also in monocytes and NK cells [25]. The NAD glycohydrolase represented by CD157 is instead characterized by a prevalent myeloid pattern of expression, being a marker of neutrophils and monocytes, but is also expressed in bone marrow stromal cell populations [26]. CD203a is expressed in T and B lymphocytes, and plasma cells [27,28], wheras CD203c is a marker of basophils and is also expressed in mast cells [29].

7. The leukocyte ectonucleotidases as moonlighting proteins

One aspect of the leukocyte ectoenzymes that should not be neglected is the fact that most appear to lead double lives, having been firstly selected by evolution to perform as enzymes but subsequently recruited to perform a novel, or moonlighting, function unrelated to catalysis [30,31]. A common thread in this respect is the capacity of the leukocyte ectoenzymes to influence cell adhesion and migration.

Proceeding with the ectoenzymes in numerical order: for a long time CD38 has been known to be involved in orchestrating migration of cells of the immune system, such as human mature dendritic cells [32], and T lymphocyte-endothelium adhesion [33], while *Cd38*-deficient mice revealed a role in neutrophil chemotaxis [34]. The NTPDase CD39 plays a role in homotypic adhesion in activated B cells [23] and its role in monocyte chemotaxis and angiogenesis was a major finding in *Cd39*-deficient mice [35]. CD73 is also involved in leukocyte trafficking and regulates lymphocyte-to-vascular endothelium interactions, and between B cells and dendritic cells [15]. CD157 influences adhesion and migration of human neutrophils and monocytes [26], and associates with β1/β2 integrins [36]. CD157 also binds to fibronectin (with high affinity) in the extracellular matrix (ECM) [37]. CD203a/NPP1 also interacts with ECM components, notably heparin and other glycosaminoglycans [15]. CD203c/NPP3 contains the tripeptide Arg-Gly-Asp RGD motif which may mediate its association with integrins [15]. In addition, transfection of CD203c into NIH/3T3 cells modified their migration capacity [38]. Therefore, while the catalytic performance remains the principal functional role of the leukocyte ectoenzymes, they have all adapted to perform secondary functions that influence migration and adhesion of cells of the immune system.

8. What the chromosomal localizations of the leukocyte ectonucleotidases tell us

While the physiopathology of the adenosinergic pathways and their constituent proteins have already been the subject of detailed analyses, we now focus on novel aspects of the ectonucleotidases that emerge when we examine the chromosomal localization and genomic conservation in major vertebrate models of the genes that encode these proteins. An immunological audience might not be aware that almost one third of human proteins are related in origin [39]. This is because the birth of genes most frequently occurs by a process of gene duplication [40], although new genes may also be recruited *de novo* from noncoding sequences [41]. The generation of duplicated DNA sequences may occur on a small-scale, such as the tandem duplication of a single gene, or may involve an entire genome, as in whole genome duplication (WGD) [42]. WGDs have occurred in major eukaryotic lineages [43,44]; in the vertebrate lineage, two rounds of whole genome duplication have occurred, as first hypothesized by Susumu Ohno (the 2R-hypothesis) [45] in whose honour WGD duplicated paralogous genes are now known as 'ohnologs'. Because of the vertebrate WGDs, 20–30% of human protein-coding genes are believed to be related [39].

Tandem duplication is the mechanism of origin of *CD38* and *BST1* (encoding CD157), the vertebrate ADP-ribosyl cyclase/NAD glycohydrolases, located in tandem array on human chromosome 4, at 4p15.1 (Fig. 3). The *BST1* and *CD38* gene duplicates show strong conservation of intron/exon structure, and they are located in tandem throughout the tetrapod lineage of vertebrates suggesting that the duplication occurred in early vertebrate evolution [46]. The genes encoding CD203a (*ENPP1*) and CD203c (*ENPP3*), located in human at 6q23.2, are also in tandem array throughout tetrapod evolution and examination of their structure and gene tree indicates their origin by duplication in early vertebrate evolution (see *ENPP1* and *ENPP3* at www.ensembl.org). Thus, thanks to their origin by tandem duplication, some ectonucleotidases are not randomly distributed throughout the genome, and may be clustered for functional reasons, such as co-ordinated mRNA expression [47]

Other potentially important regulatory features emerged from a simple analysis of the chromosomal localization of the leukocyte ectonucleotidase genes. For example, the NTPDase CD39, encoded by *ENTPD1,* is located on human chromosome 10, at 10q24.1. At the same chromosomal address, but on the opposite DNA strand, we find *ENTPD1-AS1*, a long noncoding antisense RNA (lncRNA). In general, these types of RNA genes generate transcripts which are implicated in gene transcription regulation [48]. The function of *ENTPD1-AS1* remains to be characterized but it has recently emerged as one of a set of six immune-related lncRNAs that have been proposed as prognostic markers in glioblastoma [49].

The gene encoding CD73, designated *NT5E*, is located on human chromosome 6, at q14.3. According to recently published work, expression of CD73 is also regulated by a *MIR30A*, a small noncoding RNA located at human chromosome 6q13 that is implicated in both tumor promotion and tumor suppression in a variety of human cancers [50].

Another important aspect of ectonucleotidase gene regulation that is currently evolving is the role of alternative splicing (AS). We have recently described the crucial role of AS in generating canonical, catalytically active human CD157 [51] whereas Maloney et al. have described a single nucleotide polymorphism in the human *CD39/ENTPD1* promoter that increases protein expression and catalytic activity, leading to increased risk of thrombosis [52]. Human *CD73/NT5E* is negatively regulated by AS which leads to production of a shorter, catalytically inactive form, so far described in liver disease [53]. *CD203a/ENPP1* variants with phenotypic effects in various tissues have been described [54].

9. Conserved synteny and the evolutionary histories of the leukocyte ectonucleotidases

We next examined the presence of orthologous genes (*i.e.,* same gene, different species) encoding the leukocyte ectonucleotidases in four major tetrapod models: human (*Homo sapiens,* Hsa*)*, mouse (*Mus musculus*, Mmu), chicken (*Gallus gallus*, Gga) and frog (*Xenopus tropicalis*, Xtr). We also examined the local gene order to evaluate conservation of synteny which, if maintained over major evolutionary timescales, may indicate sharing of regulatory elements [55].

Orthologs of the human genes encoding proteins that form the CD39/CD73 axis are conserved from human to xenopus (Fig. 3). As for the local gene order, the genes surrounding the *CD39/ENTPD1* orthologs show relatively low levels of conservation of synteny (Fig. 3), being better conserved between the two mammalian orthologs and between the two non-mammalian orthologs. This dichotomy of conservation is also demonstrated by the presence of the paralog *ENTPD7* close to *CD39/ENTPD1* in chicken and xenopus, but not in human and mouse.

The genomic neighborhood surrounding *CD73/NT5E* is well conserved from human to xenopus most notably in the upstream genes (Fig. 3). The syntenic block incorporating *CD203a/ENPP1* is also well conserved in tetrapods, as is the presence in tandem of its paralog *CD203c/ENPP3*. The syntenic block surrounding *BST1* and *CD38* on human chromosome 4 is highly conserved, as shown by the similarities in gene order in mouse chromsome 5, chicken chromosome 4 and xenopus chromosome 1 (Fig. 3).

Being familiar with the gene content of the chromosomal region containing *BST1* and *CD38* on human chromosome 4, we were surprised to find similar genes surrounding *CD39/ENTPD1* on chromosome 10. As is shown in Fig. 4, in close proximity to the *CD38/CD157* cluster lies *CC2D2 A* (encoding Coiled-Coil And C2 Domain Containing 2 A protein) which is important for cilia formation. *CC2D2 A* has an important paralog, *CC2D2B*, which is located on chromosome 10, in close proximity to *CD39/ENTPD1*. Telomeric of *CC2D2 A* lie: (i) *CLNK* (Cytokine Dependent Hematopoietic Cell Linker), a member of the SLP76 family of immunoreceptor adaptors and (ii) *ZNF518a* (Zinc finger protein 518a), a regulator of nuclear gene transcription. Both *CLNK* and *ZNF518b* have human paralogues: *BLNK* (B cell linker) and *ZNF518b* (Zinc finger protein 518b), respectively. These too are located on chromosome 10 close to *CD39/ENTPD1*. If we proceed centromerically from the ADP-ribosyl cyclases, we find the genes *LCORL* (Ligand-Dependent Nuclear Receptor Corepressor-Like Protein) and *SLIT2* (Slit Guidance Ligand 2). Likewise, downstream of *CD39/ENTPD1* we find their paralogs *LCOR* (Ligand-Dependent Nuclear Receptor Corepressor protein) and *SLIT1* (Slit Guidance Ligand 1).

The presence of paralogs in the chromosomal regions harbouring the ADP-ribosyl cyclases and *CD39/ENTPD1* suggest they are ohnologs. As mentioned in section 8, these conserved syntenic blocks are remnants of the early vertebrate tetraploidizations due to WGD. Paralogous chromosomal regions comprising parts of human chromosomes 4 and 10 have been previously described [56], although the connection between *BST1/CD38* and *CD39* genes seems novel.

Although the notion of co-ordinated regulation between ectonucleotidases is highly speculative, support for the notion of gene dosage balance and interlocus collaboration between ohnologs has emerged

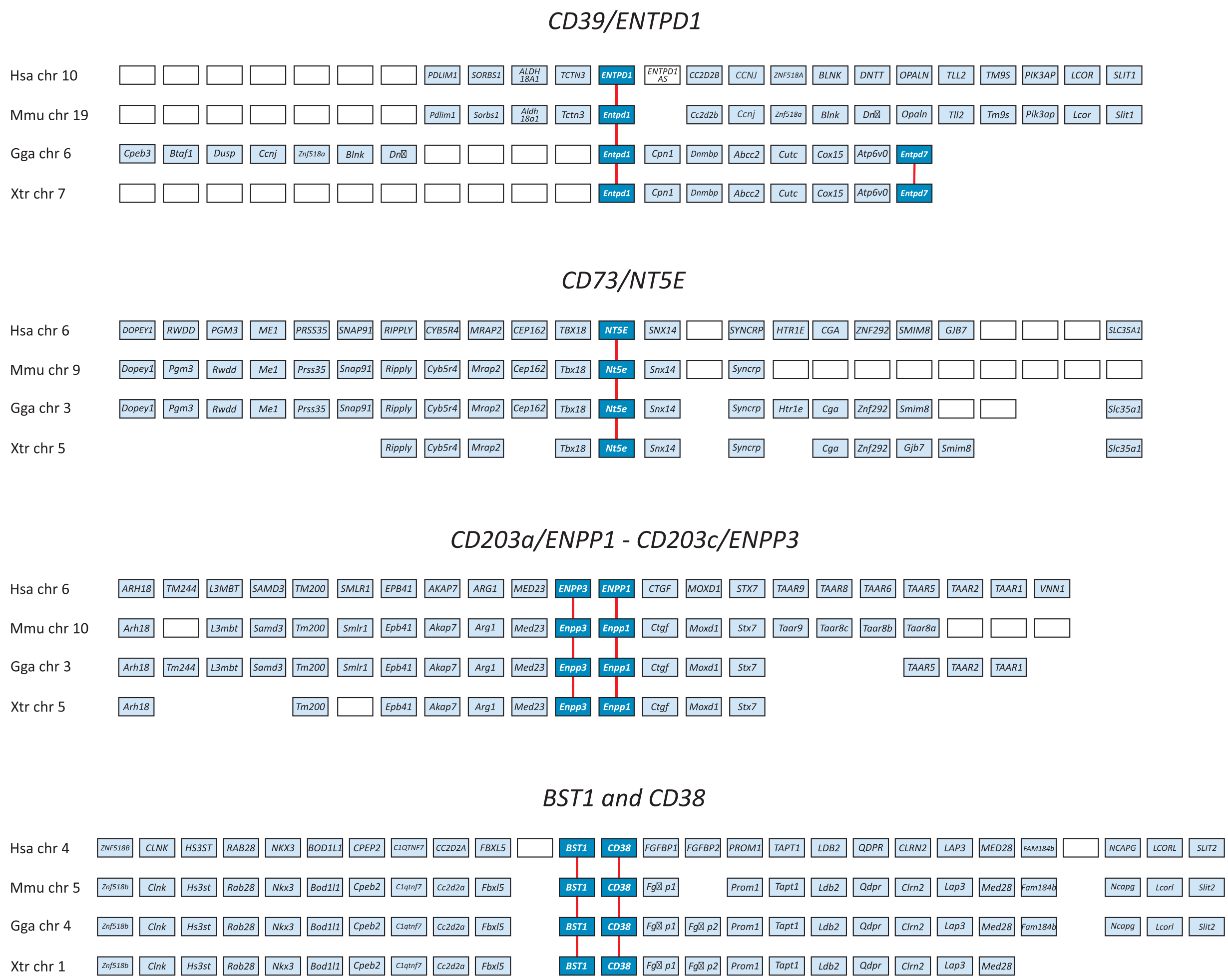

Fig. 3. Chromosomal neighborhood of the genes encoding the leukocyte ectonucleotidases. The genomic surroundings of the genes encoding CD39, CD73, CD203a/C and CD38/CD157 are shown in human, mouse, chicken and frog. Ectonucleotide genes, petrol blue boxes; orthologs are connected by the red line. Other conserved genes, light blue boxes. Unconserved genes, white boxes. No box, no gene.

[39]. An important example of paralog collaboration has recently emerged and it involves genes in linkage with *BST1/CD38* and *CD39*: coordinated expression of *LCOR* (Hsa chr 4) and *LCORL* (Hsa chr 10) paralogs is required to form two vertebrate-specific proteins (PALI1 and PALI2) that form part of the polycomb repressive complexes which repress transcription in target genes and regulate the maintenance of cell identity in cancer and development [57].

10. Mirror pathways in the parasite *Schistosoma mansoni*

The search for homologs of the ectonucleotidases beyond vertebrates proved particularly fruitful in one particular invertebrate. Just as human leukocytes present an array of nucleotide-catabolizing enzymes on their surface, so too does an important human parasite that resides in the human vascular system, the platyhelminth *Schistosoma mansoni*. The outer tegument of adult worms, which interacts with the host environment, is endowed with a panel of ectoenzymes that can perform the canonical and non-canonical pathways described here on the leukocyte surface. While degradation of ATP to adenosine has been described as being carried out with schistosome homologs of CD39 (SmaATPDase 1) and CD203a (SmPDE, phosphodiesterase) [58], the presence on the tegumental surface of a relative of CD38 and CD157, called SmNACE for NAD-catabolizing enzyme, implies that extracellular NAD can also be degraded [59]. Thus, the different roads to adenosine production, through destruction of either ATP or NAD^+, have been well travelled in evolution.

11. The leukocyte ectonucleotidases in translational medicine

By working our way backwards from CD antigen to gene to phylogeny, we find that the leukocyte ectonucleotidases have long and complex histories, and some have had curious beginnings. For example, CD39 started out as an apyrase isolated from the potato (*Solanum tuberosum*) [60] while the prototypic ADP-ribosyl cyclase was discovered in the sea slug *Aplysia californica* [61]. The ectoenzymes have come a long way since their initial identification, through the genetic, biochemical and immunological characterizations that have been reviewed here. The next important step in the cultural evolution of the ectonucleotidases is their relevance in human disease, where they are now being exploited clinically as therapeutic targets [62]. Striking examples are the successes obtained using anti-CD38 monoclonal antibodies in hematological diseases, most notably in multiple myeloma [63]; CD38's paralog CD157 is also a candidate for antibody treatment in acute myeloid leukemia [64]. CD73 has become a target in cancer therapy, with anti-CD73 antibodies being used in conjunction with conventional forms of treatment [65]. CD39 has been recently described as one of the 'master drivers of immune regulation' and is being viewed as a target for immune checkpoint-based therapy [66]. In addition, antibodies raised against schistosome NTPDase 1 cross-react with human CD39 and modulate macrophage function, opening new avenues of investigation and treatment for this important helminthic infection [67]. Finally, also CD203c is under investigation as a suppressant in ATP-dependent chronic allergy [68]. Thus, yet another common feature of

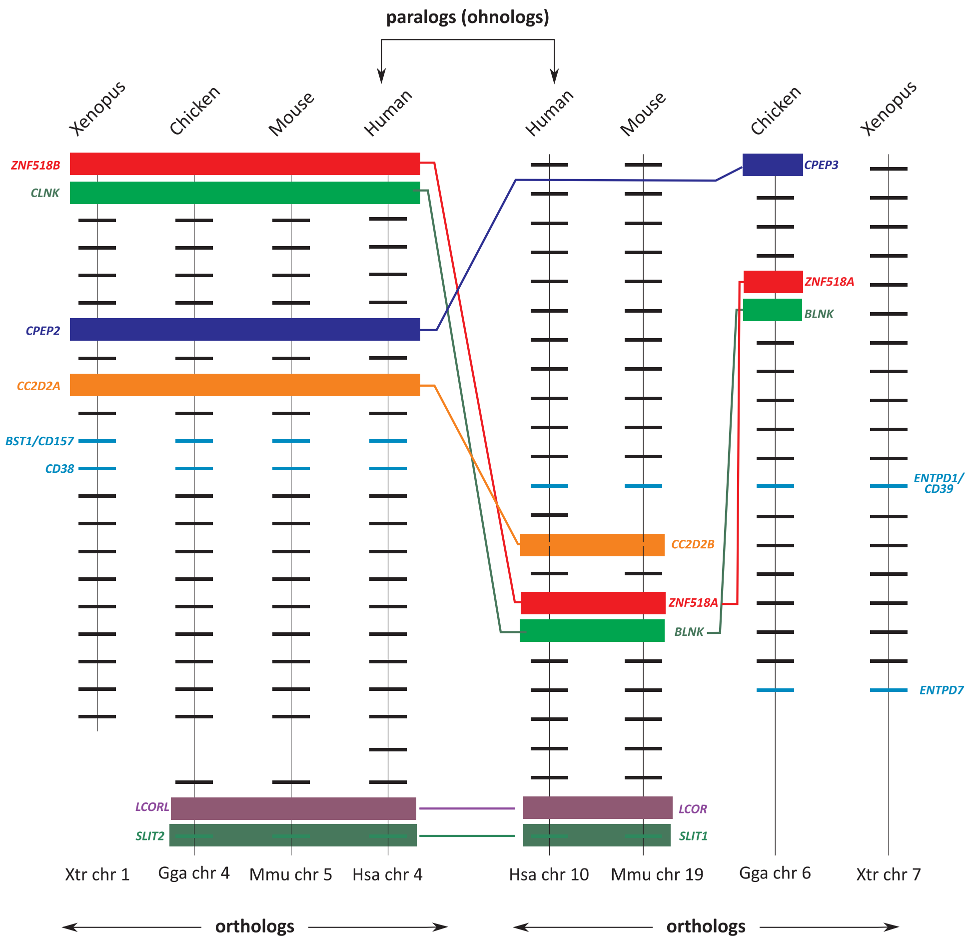

Fig. 4. Conserved synteny between chromosomal regions bearing the ADP-ribosyl cyclases and CD39. Left panel, conserved synteny among tetrapod species of the region bearing *CD38* and *CD157* orthologs. On right, conserved synteny among tetrapod species of the region bearing *CD39* orthologs. Paralogous pairs (*e.g., ZNF518B*, left panel, *ZNF518A*, right panel) are highlighted and connected with a gene-specific color.

the leukocyte ectonucleotidases is their emergence as potent treatment targets in inflammation, cancer and immunity, together with new therapeutic approaches focusing on adenosine-mediated immunosuppression [12].

12. Conclusions

The leukocyte ectonucleotidases form a newly-defined family of enzymes that collaborate through canonical and non-canonical pathways that lead to the production of adenosine, a pleiotropic small molecule whose powerful effects help regulate the function of the immune system. Comparative genome analyses of the genes encoding the ectonucleotidases reveal a previously unsuspected genomic connection between the ADP-ribosyl cyclases *BST1/CD38* and *CD39/ENTPD1,* suggesting the link between these enzymatic pathways may be deeper than previously thought.

Bioinformatics

Protein sequence retrieval

For protein analyses, the following reference sequences were used, obtained from NCBI (https://www.ncbi.nlm.nih.gov/) or Ensembl (https://www.ensembl.org/index.html). CD38: *Homo sapiens* P28907; *Mus musculus* P56528; *Gallus gallus* ADQ89191; *Xenopus laevis* BAL72804. CD39: *Homo sapiens* P49961; *Mus musculus* P55772; *Gallus gallus* NP_001292388; *Xenopus laevis* NP_001085737; CD73: *Homo sapiens* CD73 P21589; *Mus musculus* NP_035981; *Gallus gallus* ENSGALP00000025482; *Xenopus laevis* NP_001089463. CD157: *Homo sapiens* CD157 Q10588; *Mus musculus* NP_033893; *Gallus gallus* ADQ89192; *Xenopus laevis* XP_018108764. CD203a: *Homo sapiens* (Acc. no. P22413); *Mus musculus* NP_001295256; *Gallus gallus* XP_004940275; *Xenopus tropicalis* XP_012819124. CD203c: *Homo sapiens* NP_005012; *Mus musculus* Q6DYE8; *Gallus gallus* XP_003641087; *Xenopus tropicalis* XP_002936359.

Pairwise protein sequence alignment

To compare proteins sequences between non-human with the human counterpart, we used the protein BLAST program at NCBI using BLOSUM45 as scoring matrix.

Genome analysis

Gene structure, chromosomal localization, gene order and synteny blocks were analysed by Genomicus (version 91.01) (http://www.genomicus.biologie.ens.fr), by Gene Cards (http://www.genecards.org), by Entrez gene (https://www.ncbi.nlm.nih.gov/gene/) and (Ensembl https://www.ensembl.org/index.html).

Declarations of interest

None.

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