REVIEW ARTICLE

IL-37: a new anti-inflammatory cytokine of the IL-1 family

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ABSTRACT. The IL-1 family of cytokines encompasses eleven proteins that each share a similar β-barrel structure and bind to Ig-like receptors. Some of the IL-1-like cytokines have been well characterised, and play key roles in the development and regulation of inflammation. Indeed, IL-1α (IL-1F1), IL-1β (IL-1F2), and IL-18 (IL-1F4) are well-known inflammatory cytokines active in the initiation of the inflammatory reaction and in driving Th1 and Th17 inflammatory responses. In contrast, IL-1 receptor antagonist (IL-1Ra, IL-1F3) and the receptor antagonist binding to IL-1Rrp2 (IL-36Ra, IL-1F5) reduce inflammation by blocking the binding of the agonist receptor ligands. In the case of IL-37 (IL-1F7), of which five different splice variants have been described, less is known of its function, and identification of the components of a heterodimeric receptor complex remains unclear. Some studies suggest that IL-37 binds to the α chain of the IL-18 receptor in a non-competitive fashion, and this may explain some of the disparate biological effects that have been reported for mice deficient in the IL-18R. The biological properties of IL-37 are mainly those of down-regulating inflammation, as assessed in models where human IL-37 is expressed in mice. In this review, an overview of the role of IL-37 in the regulation of inflammation is presented. The finding that IL-37 also locates to the nucleus, as do IL-1α and IL-33, for receptor-independent organ/tissue-specific regulation of inflammation is also reviewed.

Key words: IL-37, IL-1F7, IL-1 cytokines, inflammation, IL-18, IL-18BP

BACKGROUND AND INTRODUCTION

The IL-1 family of cytokines and receptors

The family of interleukin 1 (IL-1) cytokines *(table 1)* is a family of protein molecules that play a key role in mediating the activation of innate immunity, the first line of defence against pathogenic micro-organisms and physical damage/stress. Stimulation of the innate immune system through Toll-like receptors (TLR) induces the production of inflammatory cytokines, thereby activating the more specific and effective adaptive response. Cytokines produced, such as IL-1 and IL-18, activate target cells through a group of receptors of the same family as TLR, the IL-1R-like family of receptors, thus amplifying the immune response. The existence of the large Toll/IL-1 receptor/Resistance (TIR) family (encompassing both TLR and IL-1R) suggests the importance of its signalling mechanism during evolution. The TIR-mediated effects need to be tightly controlled, unless they cause severe autoimmune and inflammatory diseases such as rheumatoid arthritis, systemic lupus erythematosus, and inflammatory bowel diseases. On the other hand, several proteins of the IL-1 cytokine family and of the IL-1R family are apparently involved in homeostatic cell-cell communication and in maintaining normal steady-state conditions, as has been described for IL-1 [1] and for the receptor TIR8/SIGIRR at the mucosal level [2, 3]. In addition, some of the cytokines of the IL-1 family are not inflammatory, but rather inhibit inflammation or trigger anti-inflammatory reactions. IL-1Ra (IL-1F3), the receptor antagonist of IL-1α and IL-1β, binds to IL-1RI and does not allow recruitment of the accessory chain IL-1RAcP, thus acting as an inhibitor of IL-1-dependent inflammation [1, 4]. IL-33 (IL-1F11) binds to T1/ST2 and recruits IL-1RAcP to deliver a signal for anti-inflammatory cytokine activation [5, 6]. Finally, IL-1F5 has been reported to bind IL-1Rrp2 and to inhibit NFκB activation in cells stimulated with IL-F6, IL-1F8 and IL-1F9, which all bind to IL-1Rrp2 and use IL-1RAcP as accessory chain, and to down-regulate LPS and IL-1β-induced local inflammation in the brain through involvement of TIR8/SIGIRR [7]. For this reason, it has been proposed that the three agonist cytokines IL-1F6, IL-1F8 and IL-1F9 be re-dubbed IL-36α, IL-36β, and IL-36γ, while IL-1F5 is re-designated IL-36Ra [8].

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Table 1
The IL-1 family of cytokines.

Name	Synonym	Expression	Receptor binding & activities
IL-1F1	IL-1α	Ubiquitous; mostly monocytes/macrophages	1. Binds to IL-1RI and forms complexes with IL-1RAcP; binds little to IL-1RII; does not bind to other IL-1R-like receptors 2. Cell-associated form most common (mature form cleaved by caspase-1-independent mechanisms) 3. Immunostimulating and inflammatory activities
IL-1F2	IL-1β	Ubiquitous; mostly monocytes/macrophages	1. Binds to IL-1RI and forms complexes with IL-1RAcP; binds well to IL-1RII; does not bind to other IL-1R-like receptors 2. Extracellular cytokine matured upon cleavage by caspase-1 3. Immunostimulating and inflammatory activities
IL-1F3	IL-1Ra	Ubiquitous; mostly monocytes/macrophages	1. Binds to IL-1RI but does not form complexes with IL-1RAcP; binds little to IL-1RII; does not bind to other IL-1R-like receptors 2. Secretory form and two intracellular forms 3. Antagonises IL-1α and IL-1β receptor-dependent activities
IL-1F4	IL-18, IGIF, IL-1γ	Monocytes, tissue macrophages, DC, Kupffer cells, osteoclasts, keratinocytes, epithelial cells, tumour cell lines	1. Binds to IL-18Rα and forms complexes with IL-18Rβ; binds to IL-18BP; does not bind to other IL-1R-like receptors 2. Mature cytokine cleaved by caspase-1 3. Induction IFN-γ in Th1 and NK cells; induces TNF-α, GM-CSF, IL-8, IL-6, IL-1β, FasL expression in different cell types
IL-1F5	IL-1Hy1, FILδ, IL-1H3, IL-1RP3, IL-1L1, IL-1δ, IL-36Ra	Placenta, uterus, skin (also psoriatic), brain, heart, kidney, keratinocytes, monocytes, B cells, DC	1. Does not bind IL-1RI, IL-1RAcP, IL-18R, AcPL, T1/ST2, TIGIRR, IL-1RAPL 2. Antagonises IL-1F9 in activating NFκB through IL-1Rrp2 and IL-1RAcP 3. Induction of IL-4-dependent anti-inflammation in the brain and glial cells, through involvement of TIR8
IL-1F6	FIL1ε, IL-36α	Spleen, lymph node, tonsil, leukocytes, bone marrow, foetal brain, monocytes, B cells, T cells	1. Does not bind to IL-1RI, IL-1RAcP, IL-18R, AcPL, T1/ST2, TIGIRR, IL-1RAPL 2. Activates NFκB and MAPKs through IL-1Rrp2 and IL-1RAcP
IL-1F7	IL-37, FIL1ξ, IL-1H4, IL-1RP1, IL-1H	Forms d and e only in testis and bone marrow; forms a, b, and c in lymph node, thymus, bone marrow, lung, testis, placenta, uterus, skin, colon, NK, monocytes, stimulated B cells, keratinocytes; brain only form a, kidney only form b, heart only form c. Absent in the mouse	1. Form b binds to IL-18Rα but does not complex IL-18Rβ; no agonist nor antagonist activity; does not bind IL-18Rβ, IL-1RAcP, T1/ST2 2. Form b binds to IL-18BP and enhances its IL-18 inhibitory capacity 3. Form b does not induce IFN-γ or IL-12 in cultured PBMC 4. Putatively active forms are a, b, and d, whereas forms c and e do not encompass the complete IL-1-like sequence 5. Mature IL-1-like cytokine upon cleavage by caspase-1 and/or other unidentified proteases
IL-1F8	FILη, IL-1H2, IL-36β	Bone marrow, tonsil, heart, placenta, lung, testis, colon, monocytes, B cells	1. Does not bind to IL-1RI, IL-18R, AcPL, T1/ST2, TIGIRR, IL-1RAPL 2. Activates NFκB and MAPKs through IL-1Rrp2 and IL-1RacP
IL-1F9	IL-1H1, IL-1RP2, IL-1ε, IL-36γ	Placenta, stimulated keratinocytes, epithelial cells, squamous cell-epithelia of oesophagus, psoriatic skin	1. Does not bind IL-1RI, IL-18R, T1/ST2 2. Activates NFκB and MAPKs through IL-1Rrp2 and IL-1RAcP 3. Expression up-regulated during chronic contact hypersensitivity and HSV infection 4. NFκB activation in IL-1Rrp2-transfected Jurkat cells down-regulated by IL-1F5
IL-1F10	IL-1Hy2, FKSG75	Basal epithelia of skin, proliferating B cells in the tonsil	1. Binds to soluble IL-1RI
IL-1F11	IL-33, NF-HEV	Constitutive in human lung epithelium and SMC, HEV endothelial cells, induced (by TNF-α and IL-1β) in fibroblasts, keratinocytes and at low level in macrophages and DC, absent in resting/activated macrophages, DC, T and B cells, NK, PBMC; in mouse highly expressed in stomach, lung, spinal cord, brain, skin, at lower levels in lymphoid tissue (LN, PP, spleen), pancreas, kidney, heart, activated BM macrophages and DC, absent in resting macrophages and DC, T and B cells, thymus	1. Mature form (not cleaved by caspase-1) binds to T1/ST2 and forms complexes with IL-1RAcP 2. Signalling through NFκB and MAP kinases 3. Induction IL-4, IL-5 and IL-13 in responsive Th2 cells and *in vivo*; activation of mast cells; *in vivo* increase of serum levels of IgG, IgA and IgE, induction of eosinophilia and splenomegaly; *in vivo* induction of mucosal lung and intestinal damage (eosinophil & mononuclear infiltrates, increased mucus production, epithelial hyperplasia & hypertrophy). 4. proIL-33 (NF-HEV) present in endothelial cells acts as a transcriptional repressor at the nuclear level by associating with heterochromatin and mitotic chromosomes

IL-1-like cytokines typically function by binding to a ligand-binding receptor, which then recruits a non-binding accessory chain [9]. The receptors of the IL-1 family are characterised by the presence of an intracellular TIR domain, which initiates the signalling cascade. Signalling is initiated by the approximation of the intracellular TIR domains of the two chains that form the active receptor complex. In the extracellular compartment, IL-1R chains typically encompass three immunoglobulin (Ig)-like domains [10]. The IL-1R family includes ten molecules *(table 2)*, four of them being signalling ligand-binding chains (IL-1RI, IL-18Rα, IL-1Rrp2, T1/ST2), two having been identified as accessory receptors (IL-1RAcP, IL-18Rβ), two orphan receptors (TIGIRR-1 and TIGIRR-2) and two which are unconventional receptors (IL-1RII and TIR8/SIGIRR). IL-1RII is a non-signalling ligand-binding receptor, devoid of an intracellular TIR domain, that can capture IL-1 and sequester IL-1RAcP into an inactive complex [21]. TIR8/SIGIRR is a single Ig-domain receptor unable to bind IL-1 ligands but involved in inhibition of inflammation and/or activation of anti-inflammatory processes [22, 23].

It is therefore of critical importance to understand the mechanisms by which these cytokines and receptors are activated, mediate signalling, and are down-regulated, with the long-term objective of understanding the pathogenic mechanisms of chronic inflammatory and autoimmune diseases.

IL-18 and IL-18BP

IL-18 is the major inducer of IFN-γ (it was originally defined as IGIF, IFN-γ-inducing factor), and plays an important role in promoting inflammatory Th1 and natural killer (NK) cell activation [24]. As is the case for IL-1β, there is a wealth of reports of disease association and correlations of IL-18 with chronic inflammatory, autoinflammatory, and autoimmune diseases [25-27].

IL-18 and IL-1β share a similar three-dimensional structure, are translated into inactive precursors without signal peptides, and remain in the cytoplasm until processed by caspase-1. However, there are differences between the two cytokines. For example, the IL-18 precursor protein is constitutively present in blood monocytes and tissue macrophages, in epithelial cells and keratinocytes in healthy subjects, and in tissues of healthy mice and rats, whereas IL-1β is not present in these same cells and tissues unless specifically stimulated by TLR agonists or endogenous cytokines. IL-18 binds to its receptor IL-18Rα and recruits the accessory protein IL-18Rβ to initiate cell activation, similarly to IL-1β binding to IL-1RI and recruiting IL-1RAcP. IL-18 binds to its receptor with low affinity (between 2 and 18 nM), as compared to IL-1 binding to IL-1RI (about 10 pM), and the accessory chain IL-18Rβ is required to stabilise the interaction and form a functional, high affinity (0.4 nM) receptor complex [28-30]. As is the case for IL-1β, soluble IL-18 receptors bind active IL-18 and reduce its activity. However, it was reported that either the soluble IL-18Rα chain, or the two α and β chains together are very weak in neutralising IL-18 compared to the soluble IL-1 receptors, and especially compared to the IL-18-binding protein (IL-18BP) [31].

IL-18BP is a soluble, IL-18-binding protein with high affinity for IL-18 [32], which acts as a potent inhibitor of IL-18 activity by preventing its binding to the IL-18R chains on target cells. The tight binding of IL-18 to the IL-18BP is an important part of the biology and the clinical significance of IL-18. The ligand-passing of IL-18 to cell bound receptor (as it occurs for monomeric soluble TNF p75 receptor) does not occur for IL-18BP due to its unusually high affinity [29, 32, 33]. IL-18BP (or a chimeric construct IL-18BP:Fc), has been tested in human whole blood in culture and clinical trials in plaque psoriasis and rheumatoid arthritis [34, 35]. The results show that potent effects in reducing cell activation and disease severity are achieved by intermediate dosages, while these activities are less evident at higher doses of IL-18BP. With the high binding affinity for IL-18, it was anticipated that IL-18BP would follow a strict, dose-response effect, whereby increasing doses of IL-18BP would proportionally decrease disease severity. However, this turned out not to be the case.

A second ligand binding to IL-18BP and IL-18Rα: IL-37

These findings in humans are consistent with data in animals, showing that in mice treated with recombinant mouse IL-18BP, higher doses of IL-18BP increased disease severity in collagen-induced arthritis [36]. The production of IFN-γ, TNF-α and IL-1β in cultured spleen cells was reduced by *in vivo* treatment with low doses, but not a high dose, of IL-18BP. The data support the concept that at high concentrations IL-18BP, while binding all available IL-18, is still available to bind another ligand. Such a ligand is likely to be one of the "orphan" cytokines of the IL-1 family that will bind to the IL-18Rα and trigger opposite (anti-inflammatory) effects or suppress IL-18-dependent inflammation (acting as a receptor antagonist). That the IL-18Rα may bind more than IL-18, and effect a totally different activity comes from a series of experimental data. Pancreatic islets and cells from mice deficient in IL-18Rα (IL-18Rα KO) have enhanced inflammatory activation in reseponse to various stimuli, although the pancreatic damage in mice deficient in IL-18 (IL-18 KO) is significantly decreased [37]. EAE in IL-18Rα KO mice is exacerbated as compared to its inhibition in IL-18 KO mice [38]. Inhibition of IL-18Rα (with antibodies or siRNA, or in cells from IL-18Rα KO mice) greatly enhances the inflammatory response of cells to IL-1β, as compared to wild type cells, while IL-18 KO cells display a decreased response [39]. Furthermore, this putative orphan ligand may also bind to the IL-18BP thus depriving the host of its ability to counter the inflammatory response due to IL-18 itself.

The orphan IL-1-like cytokine IL-1F7 was found to be able to bind to the IL-18Rα chain [30, 40, 41] and also to the IL-18BP [41]. It is remarkable that IL-1F7 (in particular the major splice variant IL-1F7b) is the one member of the IL-1 cytokine family that until very recently had no known functions, yet the protein is induced by TLR agonists in monocytes and is expressed in tissues from patients with autoimmune diseases. Another splice variant, IL-1F7a, is the only IL-1F7 isoform present in the brain. The role of IL-18 in the brain and the possibility that IL-1F7 could affect local IL-18 activity are issues of particular interest for the understanding of energy metabolism and metabolic disfunctions [37]. The unexpected inflammatory hyper-responsiveness of mice and cells in which

Table 2
Receptors of the IL-1R family[1].

Name	Synonym	Ligand	Expression
IL-1R1	IL-1RI	Initiates and amplifies the immune and inflammatory response upon binding the agonist ligands IL-1α and IL-1β; inhibited upon binding the antagonist ligand IL-1Ra; the co-receptor is IL-1R3; the soluble form also binds IL-1F10.	Expressed by all cells responsive to IL-1, predominant type of IL-1R on T cells, fibroblasts, epithelial and endothelial cells.
IL-1R2	IL-1RII	Binds IL-1β and, less efficiently, IL-1α and IL-1Ra; decoy receptor, unable to initiate signal transduction. Both membrane and soluble form have inhibitory activity.	Expressed by many cell types, particularly abundant on B cells, mononuclear phagocytes, polymorphonuclear leukocytes and bone marrow.
IL-1R3	IL-1RAcP	Co-receptor for IL-1R1 responsible for signalling after binding IL-1α or IL-1β; can form inactive complexes with IL-1R2 bound to IL-1; co-receptor for IL-1R6 activation by IL-36α, IL-36β, and IL-36γ; co-receptor for IL-1R4 activation by IL-33.	Ubiquitous; expressed by all cells responsive to IL-1.
IL-1R4	T1, ST2, ST2L, DER4, Fit-1	Binds IL-33 using IL-1R3 as co-receptor; negative regulator of TLR/IL-1R signalling.	Th2 cells, mast cells, fibroblasts.
IL-1R5	IL-18Rα IL-1Rrp1	Binds IL-18 using IL-1R7 as co-receptor; also binds IL-37b but without recruiting IL-1R7.	Mononuclear phagocytes, neutrophils, Th1 cells, NK cells, endothelial cells, smooth muscle cells.
IL-1R6	IL-1Rrp2	Binds IL-36α, IL-36β, and IL-36γ, using IL-1R3 as co-receptor; activation of NFκB and MAPKs.	Lung, epididymis, lower levels in testis and cerebral cortex (non-neuronal).
IL-1R7	IL-18Rβ AcPL	Co-receptor for IL-1R5 responsible for signalling after binding of IL-18.	Mononuclear phagocytes, neutrophils, Th1 cells, NK cells, endothelial cells, smooth muscle cells.
IL-1R8	TIR8, SIGIRR	Orphan receptor [11]; negative regulator of TLR4/IL-1R signalling [2]; responsible for anti-inflammatory homoeostatic signalling at the mucosal level [3]; involved in the IL-4-dependent anti-inflammatory signalling evoked by IL-1F5 in the brain and glial cells [7].	Ubiquitous (Northern blot [11]); abundantly expressed in mucosal epithelial cells and DC (Northern blot [3]).
IL-1R9	TIGIRR TIGIRR-1 IL-1RAPL2	Orphan receptor. Does not activate JNK, ERK, p38 [12], NFκB [13]. The ic domain (complete or only TIR) does not signal with IL-1R3 nor with IL-1R7 for NFκB activation (chimeras assay [13]).	Human: tested in brain (commercial Northern blot [14]); in adult brain frontal and temporal lobe, cerebellum (commercial Northern blot [14]), foetal brain (commercial Northern blot confirmed by real-time-PCR [15]); skin and weaker in liver, placenta and foetal brain (by PCR analysis on human cDNA commercial panel [13]). Not expressed in heart, liver, pancreas, skeletal muscle, testis, spleen, thymus, prostate, ovary, small intestine, colon, PBL, brain, lung (commercial Northern blot [13]). Mouse: diencephalon, spinal cord (E12.5), rostral cortex, cerebellum (E16.5), all brain and especially cortex layers III-VI, olfactory bulb, Purkinje cells, X cerebellar lobule, hippocampal CA1 region (P12.5; in situ hybridisation [14]).
IL-1R10	IL-1RAPL TIGIRR-2 IL-1R8	Orphan receptor; mutations/deletions involved in X-linked mental retardation [16]; does not activate NFκB [12, 13], ERK and p38; activates JNK [12] and mediates IL-1-dependent neuronal JNK activation [17]; the intracellular domain (complete or only TIR) does not signal with IL-1R3 nor with IL-1R7 for NFκB activation (chimeras assay [13]); inhibition of N-type voltage-gated calcium channel activity and Ca^{++}-dependent exocytosis through NCS-1 [18, 19].	Human: brain, heart, skeletal muscle (commercial Northern blot [13]); heart, brain, ovary, skin, and weaker expression in tonsil, foetal liver, prostate, testis, small intestine, placenta and colon (by PCR analysis on human cDNA commercial panel [13]), adult and foetal brain (commercial Northern blot [20]); no expression in spleen, lymph node, thymus, bone marrow, leukocytes, lung, liver, skeletal muscle, kidney and pancreas (by PCR analysis on human cDNA commercial panel [13]). Mouse: brain E10.5 upregulated at E12.5 then stable even in adult, primary neuronal and astroglial cells derived from foetal and newborn brain, primary neuronal cells from cerebellum and cortex, and other brain structure including hippocampus (RT-PCR [20]); primary olfactory cortex, entorhinal cortex, hyppocampus, mammillary bodies and supramammillary nucleus (in situ hybridisation [20]).

[1] In man, most of the IL-1R genes are clustered on chromosome 2 (IL-1R1, IL-1R2, IL-1R4, IL-1R5, IL-1R6, IL-1R7). Other receptors are on different chromosomes: IL-1R3 on chromosome 3, IL-1R8 on chromosome 11, while IL-1R9 and IL-1R10 are on chromosome 10.

IL-18Rα is inhibited, as compared to mice and cells deficient in IL-18 itself, has thrust IL-1F7 into the arena of investigation as the possible additional ligand for the IL-18 receptor, imparting a unique mechanism of action.

IL-1F7 is now being re-dubbed IL-37 [8]. This acronym will be used in this review except when mentioning genetic data, for which the original name, IL-1F7, will be used. The current knowledge about IL-37 will be reviewed and discussed hereafter, in light of the possible dual role of IL-1-like cytokines in regulating inflammation, and by considering the features of local *versus* systemic regulation.

GENE ORGANISATION AND EXPRESSION

The identification of IL-37 was reported by several independent groups in the year 2000. An EST apparently encoding an IL-1-like molecule (accession number AI014548) was found in the GeneBank™ [42]. This corresponded to an IMAGE clone with a stop codon upstream to the ORF, and no initial methionine. Upon screening of two additional cDNA libraries, another clone was isolated from the pancreatic tumour cell line HPT-4, which had a methionine and the full ORF that extended for 192 amino acids. The clone was dubbed FIL1ζ and corresponds to the IL-37a isoform. After three months, another group reported the identification of an IL-1-like molecule by screening public and commercial EST databases with the full length sequences of other IL-1F members (IL-1F5 and IL-1F9) [43]. The entire coding region of a new IL-1-like molecule was obtained from cDNA clones of the IMAGE consortium (accession numbers AI014548 and AI343258), which was dubbed IL-1H4. The predicted sequence of IL-1H4 is that of the IL-37b isoform. Two months later, a third study reported the identification of new IL-1-like sequences by searching for analogues within the IL-1 locus on chromosome 2 in parallel with searching the EST databases for corresponding sequences [44]. One of these sequences, IL-1RP1, was found by sequencing IL-1β-containing human BAC clones, and the corresponding cDNA clone was isolated from a library of human bronchial epithelial cells stimulated with TNF-α + PMA + cycloheximide. The IL-1RP1 sequence corresponds to that of the IL-37c isoform. Few months later, an additional study identified, through an EST search for sequences similar to IL-1Ra, a clone with an ORF encoding a protein of 218 amino acids that was called IL-1H or IL-1HLa [40]. PCR amplification in human cDNA libraries allowed identification of two clones, one identical to the EST clone (except for two base pair changes likely due to polymorphism) that was called IL-1HL, and a second clone containing a 120 bp in-frame deletion resulting in a 40 aa-shorter protein called IL-1HS. The IL-1HL and La forms correspond to IL-37b, whereas the IL-1HS form is IL-37c.

The locus containing the genes for most of the IL-1 cytokines is located on human chromosome 2. Nine out of the eleven IL-1F genes including *IL1F7* map to 2q13 [42, 43, 45-47]. Two genes are found on separate chromosomes: *IL18* maps to 11q22.2–q22.3 [48] and *IL33* is found on 9p24.1 [5].

The human *IL1F7* gene is located between the *IL1B* and *IL1F9* genes and the direction of transcription is oriented towards the telomere. The gene size is 3.617 kb (measured as the genomic distance beginning at the initiating methionine codon and ending at the stop codon). *IL1F7* is 78.932 kb away from *IL1B*, which is closer to the centromere, and 59.808 kb away from *IL1F9*, which is closer to the telomere [45, 49] *(figure 1)*.

In the mouse, the IL-1F gene cluster is also located on chromosome 2 [45]. Mouse *Il18* is found on chromosome 9, and mouse *Il33* is found on chromosome 19.

Figure 1 shows the genomic organisation of the human and mouse IL-1F loci. In general, both loci are conserved with respect to the order and orientation of the IL-1F genes. The only exception is *IL1F7*. This gene seems to be absent in mice, since no mouse genomic sequence or cDNA corresponding to human *IL1F7* has been found to date. Taylor *et al.* [45] suggest that *Il1f8* could be the missing orthologue to human *IL1F7*, and the orthologue to human *IL1F8* could have become a pseudogene in mouse. However, amino acid sequence similarities between human *IL1F8* and the alternative *Il1f8* locus in mice shown by the same group tend to disprove this theory. These results suggest that the *Il1f7* locus in mice is found elsewhere or that it has been lost as the result of an evolutionary event that took place in the IL-1 locus. It is interesting that the *IL1F7* gene is located in a region of human chromosome 2 that is conserved in gorilla but deleted in chimpanzee and bonobo. Consequently, *IL1F7* transcripts can be found in the peripheral blood of gorilla, but not chimpanzee or bonobo [50]. In the cow, the IL-1F locus is located in chromosome 11, and it includes the *IL1F7* gene in the same position as in the human cluster. From the RNA sequence, a predicted protein of 205 amino acids is closely related to the human IL-1F7b isoform [51].

The human *IL1F7* gene undergoes alternative splicing. This results in the expression of five different isoforms that are illustrated in *figure 2*. Besides isoforms a, b, and c that have been already described, two additional splice products have been identified (isoforms d and e) by PCR amplification from testis cDNA [45].

The *IL1F7a* (isoform 5, IL-37a) uses a unique start codon in exon 3 (prodomain), which is then spliced in exon 4 to 6 (forming the putative 12 β-strand-containing protein structure).

IL1F7b (isoform 1, IL-37b) encodes the longest transcript variant, consisting of exons 1 and 2 (prodomain) and exons 4 to 6.

The *IL1F7c* (isoform 4, IL-37c) is a transcript variant encompassing exons 1 and 2 (prodomain) followed by exons 5 and 6.

The *IL1F7d* (isoform 2, IL-37d) has a prodomain limited to exon 1 (exon 2 is missing) followed by the complete IL-1-like sequence encoded by exons 4 to 6.

The *IL1F7e* (isoform 3, IL-37e) is only composed of exon 1, 5, and 6.

In addition to the five IL-37 splice products, it is worth mentioning that a chimeric transcript has been found, composed of exons 1, 4 and 5 of IL-37 (identical to the N-terminal part of IL-37d except for a small difference in the splice junction between exons 1 and 4), spliced immediately into the 5'UTR of the full length IL-36γ (IL-1F9) message [45]. The presence of this chimeric transcript was detected in testis and placenta, but its functional significance remains obscure.

mRNA expression of IL-37b is low in human monocytes and transfected RAW264.7 cells in the absence of stimu-

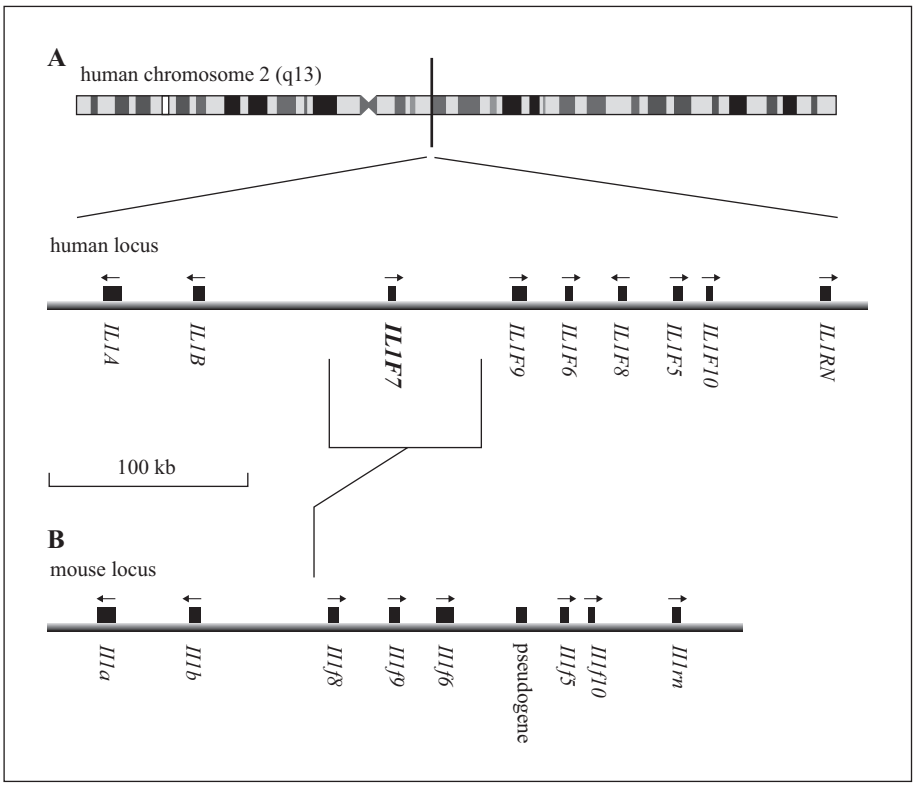

Figure 1

Genomic organisation of the human and mouse IL-1 loci.
Panel **A** shows the IL-1 family locus on human chromosome 2, while panel **B** illustrates the IL-1 locus on mouse chromosome 2. Arrows indicate the transcriptional direction. Conservation between human and mouse IL-1 loci is shown by the same order and orientation of all family genes except mouse *Il1f8*. In fact, *Il1f8* occurs at the expected *Il1f7* position in a reversed orientation as compared with its human orthologue. Furthermore, a pseudogene, which shows significant nucleotide sequence similarity with the functional human copy of the gene, is found in the expected position of mouse *Il1f8*, whereas no apparent orthologue for human *IL1F7* can be found in the mouse IL-1 locus.

lation. Indeed, IL-37b transcript instability was observed being the result of untranslated region-independent control elements [52]. Upon stimulation with LPS, up-regulation of transcription and increased mRNA stability and protein production was observed. IL-37 mRNA stability is apparently regulated by instability elements present in exon 5, as exon 5 deletion can significantly increase mRNA stability of both IL-37b and IL-37c. Since all IL-37 isoforms contain exon 5, it is expected that the same instability element is present in all, to ensure an increase in mRNA stability only in inflammatory conditions. A similar mRNA stabilisation upon LPS stimulation can be observed for IL-18 [52].

PROTEIN STRUCTURE AND CHARACTERISTICS

The alternative splicings giving rise to the five isoforms of IL-37 and their amino acid sequences are shown in *figures 2* and *3*.

IL-37a has a unique N-terminus encoded by exon 3, with a stop codon immediately upstream of the starting methionine. The sequence encoded by exon 3 does not resemble that of a typical signal peptide, and is thought to give rise to a prodomain that is processed in the mature form of the protein [45]. In all other isoforms, exon 3 is missing and translation starts from exon 1. The exons 4 to 6 encode 12 putative β-strands predicted to form the β-trefoil structure, which is characteristic of the IL-1 family [53]. This suggests that IL-37a might be a functional cytokine.

IL-37b is the best characterised IL-37 isoform, and the one with the longest sequence (218 amino acids). The N-terminal sequence encoded by the first two exons (exons 1 and 2) represents the prodomain that is described as being cleaved off upon cytokine maturation. Like isoform a, IL-37b also contains the segment encoded by exons 4 to 6 that bears the 12 β-strands required for the IL-1-like β-trefoil secondary structure. Thus, IL-37b is expected to be biologically functional. Isoform b was reported to form homodimers under experimental conditions [30], and this seems to occur also under physiological conditions in LPS-stimulated PBMC [54]. Experiments in which the protein was expressed beginning from the second methionine in exon 1 indicated that the sequence between M1 and M12 is necessary for optimal expression [40]. Pan *et al.* also reported a polymorphism in IL-37b protein, *i.e.*, two conservative amino acid exchanges (Gly31 → Val and Thr42 → Ala) in the variant IL-1HL [40].

IL-37c was first described by Busfield *et al.* [44]. It is identical to isoform b, except for an in-frame deletion due to splicing of exon 2 to exon 5. The lack of the sequence encoded by exon 4 (encompassing the first three β-strands) is predicted to cause misfolding of the protein, without formation of the β-trefoil structure. It is unlikely that isoform c can function as a cytokine.

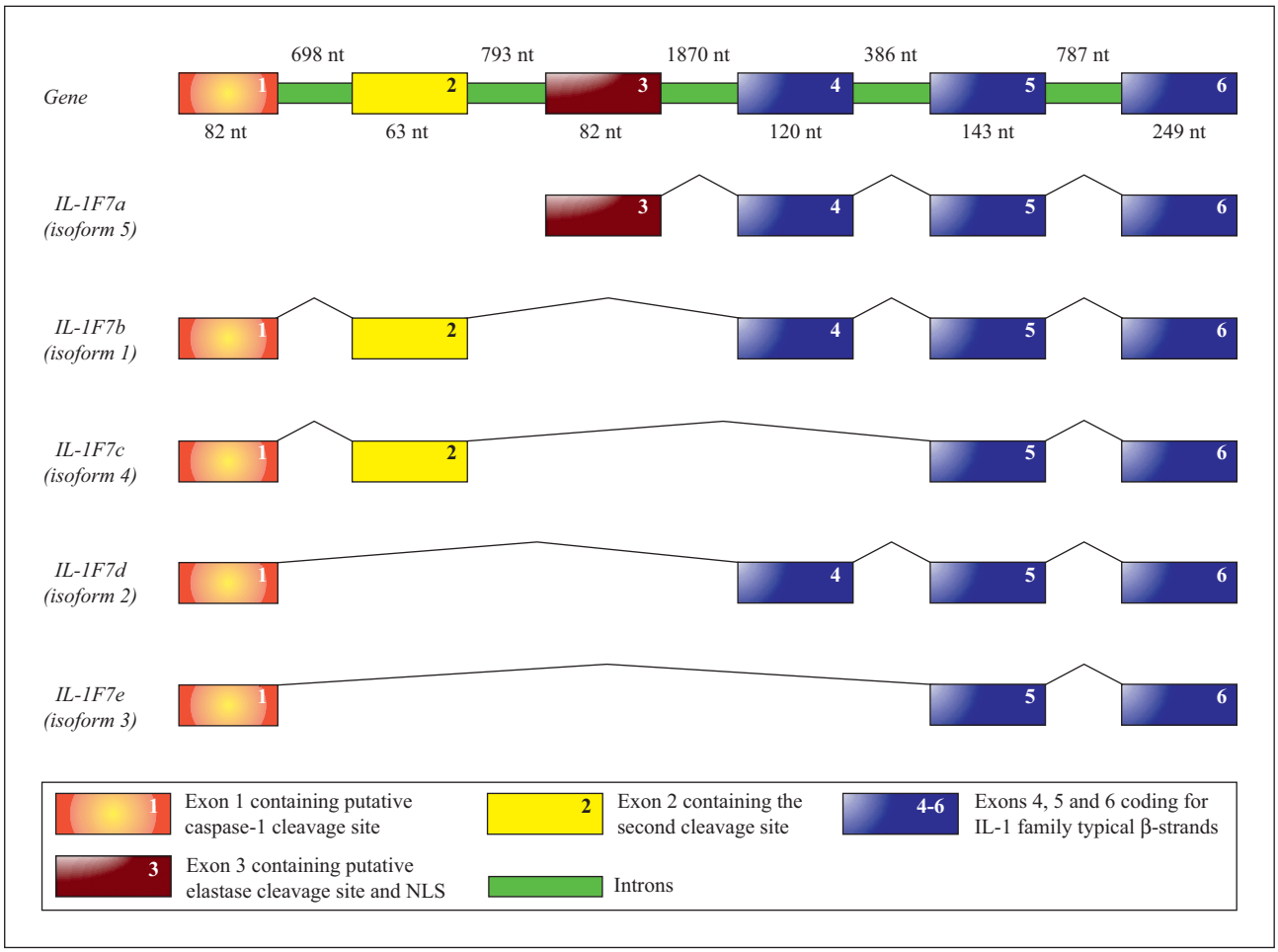

Figure 2

Exon-intron structure of the human *IL1F7* gene.
Exon usage in the five splice isoforms is depicted. The size of exons is indicated just below the exon boxes. The size of the intervening introns (green) is shown at the top.

IL-37d consists of exons 1, 4, 5 and 6; compared with IL-37b, only exon 2 is missing. Hence, all twelve required β-strands are present and can form the three dimensional β-trefoil structure. Thus, IL-37d could represent another functional form of the IL-37 cytokine.
IL-37e consists of exon 1, 5 and 6. Due to the lack of exon 4 (coding for the first three β-strands), it is unlikely that it can fold into the conserved IL-1 family 3D structure and bind to the IL-18 receptor.

Protein processing

Cytokines of the IL-1 family are characteristically synthesized as precursor molecules containing a pro-peptide domain which lacks a classical hydrophobic leader sequence. Caspase-1 is considered to be the major cleaving enzyme responsible for maturation of IL-1 precursors and the extracellular export of active cytokines, in particular IL-1β and IL-18 [55]. From mRNA sequences, IL-37 isoforms are also predicted to be expressed as pro-proteins that need to be processed to produce the mature, IL-1-like cytokine. All the available protein studies have been performed on the most abundant isoform, IL-37b.
IL-37b is synthesized as a pro-protein which, after LPS stimulation, is processed to its mature form [30, 56]. A caspase-1 cleavage site has been predicted in the sequence encoded by exon 1 between residues D20 and E21. A study of *in vitro* IL-37b maturation by caspases 1 to 10 and Granzyme B showed that caspase-1 cleavage was the most efficient, with much lower maturation rates attained by caspase-4, and no activity with other enzymes [30]. It is possible that *in vivo* IL-37b is cleaved by other proteinases, or sequentially by caspase-1 and other enzymes. Indeed, overexpression of the entire precursor form of IL-37b in HEK 293 or CHO cells yielded a soluble cytokine starting at amino acid V46, suggesting a second cleavage site downstream of the putative caspase-1 site in the sequence encoded by exon 2 [40]. This hypothesis is supported by the observation that the intracellular processing of pro-IL-37b in RAW264.7 cells, stably transfected with IL-37b, can be inhibited only partially by caspase-1 or pan-caspase inhibitors [56]. IL-37b overexpressed in HEK 293, both in the form of precursor and mature cytokine, tend to homodimerise with an association constant of 4 μM and 5 nM, respectively [30]. The biological significance of such homodimerisation, which is not shared by other IL-1 family members, remains unknown.

The caspase-1 site (between amino acids D20 and E21, encoded by exon 1) is present in isoforms b, c, d, and e. The experimentally identified maturation site between residues F45 and V46 (encoded by exon 2) is only present in isoforms b and c. The IL-37a isoform does not use exons 1 and 2, containing these two cleavage sites, but possesses a unique sequence encoded by exon 3. Therefore, it is hypothesized that an alternative cleavage site is present

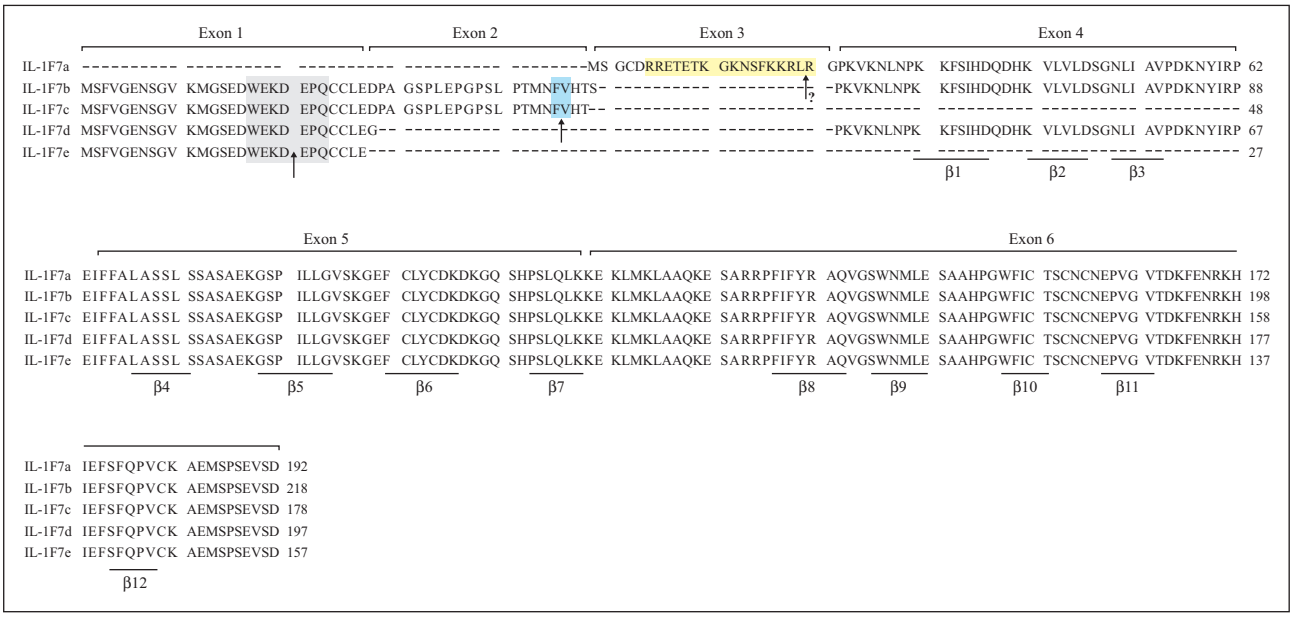

Figure 3

Alignment of the predicted amino acids sequences of the five IL-37 isoforms.
The sequences were aligned using the free-ware programme 'ClustalW' (www.ebi.ac.uk/clustalw/). The exon position is shown according to the arrangement in the gene. Positions of predicted β-strands are underlined (prediction by PSIPRED; http://bioinf.cs.ucl.ac.uk/psipred/). Propeptide cleavage sites are marked by black arrows. The predicted caspase-1 cleavage site is located between D20 and E21 (grey shaded area; present in isoforms b-e). Another experimentally-detected cleavage site is located between F45 and V46 (blue shaded area; present in isoforms b and c), and a predicted, unique elastase cleavage site in exon 3 of IL-1F7a is positioned between L21 and R22 (arrow with question mark). Exon 3 also contains a putative, bi-partite nuclear localisation signal (NLS) found with ProSite (http://www.expasy.ch/prosite/), spanning residues R5 to R22 (highlighted in yellow).

in this sequence. By bioinformatic analysis (http://db.systemsbiology.net:8080/proteomicsToolkit/proteinDigest.html), a putative elastase cleavage site can be identified within the N-terminal sequence of IL-37a, upstream of the β-trefoil structure, between L21 and R22 *(figure 3)*. Thus, while the mature IL-37b isoform, starting at residue V46, is predicted to be a 173 amino acid-long protein, the mature IL-37d isoform (cleavable only at the caspase-1 site) is expected to be a 177 amino acid-long molecule, and the mature IL-37a (cleavable by elastase) should be a protein of 172 amino acids. The sequence differences in the three mature isoforms would therefore reside exclusively in the first few N-terminal amino acids:

Isoform a: RG PK...

Isoform b: VHTS PK...

Isoform d: EPQCCLEG PK...

It is worth noting that the two isoforms that which are not predicted to yield complete IL-1-like proteins, IL-37c and IL-37e, maintain the same pro-domain sequence of IL-37b and IL-37d, respectively. This could suggest that the incomplete isoforms may act as regulators of complete IL-37 isoforms, either by competing with the correct splicing of active isoforms, or by inhibiting maturation of active isoforms acting as alternative substrate for the cleaving enzymes.

Expression profile

IL-37 is expressed in a variety of normal tissues and tumours [40, 43, 44, 52]. *Table 3* summarises the expression profiles of the different IL-37 isoforms. It is notable that there are some isoforms of IL-37 that seem to be expressed in a tissue-specific fashion. IL-37a is the only isoform expressed in the brain, IL-37b is the only one present in kidney, IL-37c is the heart-specific isoform, IL-37d and e are only expressed in bone marrow and testis [45]. All the isoform-specific expression studies of IL-37 are based on RT-PCR and Northern blotting. Use of primers/probes for common exons (*e.g.*, exon 6) or immunohistochemical staining with polyclonal antibodies raised against IL-37b did not allow discrimination between isoforms. In any case, immunocytochemical staining of peripheral blood mononuclear cells (PBMC) revealed that the IL-37 protein is mainly present in the cytoplasm of monocytes, while in solid tissues it is often associated with plasma cells. The IL-37 staining shows a granular pattern in close proximity to the Golgi and ER and partly associated with the plasma membrane, a pattern that suggests translocation via secretory vesicles [30, 41].

The IL-37 protein is endogenously present at low levels in human PBMC and can be upregulated by inflammatory stimuli and cytokines (TLR agonists, IL-1β, IL-18, TNFα, IFN-γ, TGFβ), while other factors are inactive or inhibitory (IL-12, IL-32, GM-CSF+IL-4) [54]. Of particular interest is the finding that PBMC treatment with GM-CSF+IL-4 (the conditions that induce differentiation of monocytes to dendritic cells, DC) downregulates IL-37 expression, in agreement with the finding that IL-37 inhibits DC activation [54], thus suggesting that DC differentiation is consequence of inflammation and does not occur in conditions of inhibition of inflammation.

As a general finding, the levels of IL-37 mRNA expression in human tissues (liver, chronically inflamed bowel, etc.) appears to be lower than the production of the IL-37 protein (Bufler, unpublished). This might be due to a very tight regulation of IL-37 mRNA by the coding region instability elements, resulting in the rapid disappearance of mRNA, while the protein persists for longer time.

Table 3
Characteristics of IL-37 isoforms

Name synonym	Accession numbers (RefSeq)	Expression
IL-37 (all forms)	Exclusively expressed in human cells, not detected in mice	**YES** in: cytoplasm of plasma cells in epithelial crypts and germinal centres of tonsils, in lamina propria of normal colon, in stroma of colon carcinomas (with pAb anti-IL-37b) [30]; associated with ER/Golgi in plasma cells of tonsil germinal centres and tonsil epithelial cells (perinuclear), skin sweat glands (apical), skin sebaceous glands (perinuclear), normal colon epithelium (moderate, perinuclear and apical), normal breast , placental syncytial trophoblast, breast carcinomas (more intensely than normal breast), some colon carcinoma cells (less intensely than breast carcinomas), melanomas (moderate), lung carcinomas (moderate) (with pAb anti-IL-37b) [30]; nuclear localisation in outer skin epidermal cells (with pAb anti-IL-37b) [30]; blood monocytes (granular staining) (with pAb anti-IL-37b) [41]; fully differentiated keratinocytes in stratum granulosum of skin (in situ with probe in the coding region of IL-37c) [44]; PBMC, DC, A431, KG-1, THP-1 (in all upregulated by PMA), SK-LU-1 (with primers and probe for exon 6) [40]; most abundant in testis, thymus, uterus, present in muscle, brain, lung, spleen, prostate, low levels in heart, adrenal glands, stomach, liver, salivary glands, pancreas, kidney (with primers and probes for exon 6) [40]; CCL-247 (colon carcinoma), ductal mammary carcinoma, normal thalamus (with unspecified SAGE tags) [30]; RAJI , CCL-247, placenta (RT-PCR with unspecified probes for IL-37b) [30]. **NO** in: prostate carcinoma cells, majority of colon carcinoma cells (with pAb anti-IL-37b) [30]; blood lymphocytes (with pAb anti-IL-37b) [30].
IL-37a Isoform 5 FIL1z IL-1F7a	mRNA: NM_173205.1 Protein: NP_775297.1 Source seq: AF201832 Consensus CDS: CCDS2107.1 UniProt/Swiss-Prot: Q9NZH6-2	**YES** in: lymph nodes, thymus, bone marrow, placenta, colon, lung, testis, colon carcinoma (by RT-PCR with primers for exons 3-4) [42]; lymph nodes, placenta, colon, lung, testis, brain (by RT-PCR with exon-specific primers) [45]; THP-1, U937 (increased by LPS), HL60, IMTLH (bone marrow stromal cell line), HPT-4 (pancreas cell line) (by RT-PCR with primers for exons 3-4) [42]; blood NK+IL-12, blood monocytes +LPS, stimulated blood B (SAC+CD40L) (by RT-PCR with primers for exons 3-4) [42]; blood monocytes (upregulated by LPS and LPS+IFN-γ) (by real-time PCR with isoform-specific primers) (Boraschi, unpublished). **NO** in: spleen, tonsil, foetal liver, leukocytes, heart, liver, skeletal muscle, kidney, pancreas, prostate, ovary, small intestine, colon (by RT-PCR with primes for exons 3-4) [42]; kidney, heart (by RT-PCR with exon-specific primers) [45]; T lymphocytes, B lymphocytes (by RT-PCR with primers for exons 3-4) [42].
IL-37b Isoform 1 IL-1H4 IL-1HL IL-1Hla IL-1F7b	ESTs, BACs: AI014548 AI343258 AF200496 BC020637 mRNA: NM_014439.3 Protein: NP_055254.2 Source seq: AF251118 Consensus CDS: CCDS2103.1 UniProt/Swiss-Prot: Q9NZH6-1, QR7U00	**YES** in: blood monocytes (upregulated by LPS and LPS+IFN-γ) (by real-time PCR with isoform-specific primers) (Boraschi, unpublished); lymph nodes, placenta, colon, lung, testis, kidney (by RT-PCR with exon-specific primers) [45]. **NO** in: brain, heart (by RT-PCR with exon-specific primers) [45].
IL-37c Isoform 4 IL-1HS IL-1RP1 IL-1F7c	mRNA: NM_173204.1 Protein: NP_775296.1 Source seq: AF251120 Consensus CDS: CCDS2106.1 UniProt/Swiss-Prot: Q9NZH6-3	**YES** in: lymph nodes, placenta, colon, lung, testis, heart (by RT-PCR with exon-specific primers) [45]; blood monocytes (upregulated by LPS and LPS+IFN-γ) (by real-time PCR with isoform-specific primers) (Boraschi, unpublished). **NO** in: brain, kidney (by RT-PCR with exon-specific primers) [45].
IL-37d Isoform 2 IL-1F7d	mRNA: NM_173202.1 Protein: NP_775294.1 Source seq: AY071840 Consensus CDS: CCDS2104.1 UniProt/Swiss-Prot: Q9NZH6-4	**YES** in: testis, bone marrow (by RT-PCR with exon-specific primers) [45]. **NO** in: lymph nodes, placenta, colon, lung ,brain, kidney, heart (by RT-PCR with exon-specific primers) [45]; unstimulated and LPS-stimulated blood monocytes (by real-time PCR with isoform-specific primers) (Boraschi, unpublished).
IL-37e Isoform 3 IL-1F7e	mRNA: NM_173203.1 Protein: NP_775295.1 Source seq.: AY071841 Consensus CDS: CCDS2105.1 UniProt/Swiss-Prot: Q9NZH6-5	**YES** in: testis, bone marrow (by RT-PCR with exon-specific primers) [45]. **NO** in: lymph nodes, placenta, colon, lung ,brain, kidney, heart (by RT-PCR with exon-specific primers) [45]; unstimulated and LPS-stimulated blood monocytes (by real-time PCR with isoform-specific primers) (Boraschi, unpublished).

RECEPTOR BINDING AND MECHANISMS OF ACTION OF THE EXTRACELLULAR IL-37

Two different ligands binding to the same receptor: a concept consistent with the IL-1 family of ligands and receptors

What is peculiar about the IL-1 receptor family is its promiscuity. The intracellular domains of nearly all its receptors are highly homologous, not only within the IL-1R family, but also with the intracellular domains of the TLR. The concept that the same receptor (IL-1RI) binds either IL-1α or IL-1β was established many years ago, although IL-1α and IL-1β share minimal homology and only partial structural similarities. Upon binding either ligand, the IL-1 receptor accessory protein (IL-1RAcP) is recruited forming a complex that transduces a proinflammatory signal resulting in the expression of many inflammatory genes. A third ligand, the IL-1 receptor antagonist (IL-1Ra), also binds to the IL-1RI but does not recruit the accessory chain and is therefore unable to signal. Recently, the ligand for T1/ST2, for 13 years an orphan member of the IL-1 receptor family, was reported and given the name IL-33 (or IL-1F11). After IL-33 binding to T1/ST2, the same IL-1RAcP chain is recruited [5, 6, 57]. The complex of T1/ST2 plus IL-33 plus the IL-1RAcP triggers a signal that results in Th2 and allergic responses, thus quite different from those triggered by IL-1 with the same IL-1RAcP chain. The structure of IL-33 is most similar to that of IL-18, yet IL-33 recruits the IL-1RAcP, not the IL-18Rβ chain. Other examples exist. Three members of the IL-1 family (IL-36α, β, and γ, *i.e.*, IL-1F6, 8, and 9) bind to the IL-1Rrp2 and use IL-1RAcP as accessory chain leading to activation of NFκB and MAPK [58].

IL-37 binds to IL-18Rα and IL-18BP

As for IL-1α and IL-1β that bind to the same receptor IL-1RI, recombinant IL-37b was shown to bind to the IL-18Rα chain, thus being the second ligand for this receptor after IL-18 [30, 40, 41]. On the other hand, IL-37b is unable to interact with the IL-1 receptor IL-1RI, the accessory protein IL-1RAcP, the T1/ST2 receptor, or the IL-18Rβ [30, 40]. Both proIL-37b and mature IL-37b bind to an IL-18Rα-Fc fusion protein, with binding of the mature form approximately 5 to 10 times stronger compared to the immature form [30]. The results from BIAcore assays indicate that the affinity of IL-37b for IL-18Rα (130 nM) is at least 50 times lower compared to the affinity of IL-18 for IL-18Rα (2.3 nM). Binding features of mature IL-37b to the IL-18Rα do not fit into a simple 1:1 interaction model, and association/dissociation can only be measured by a bivalent model (that implies two subsequent stages of association and dissociation). It should be mentioned that the mature IL-37b used in this study was a recombinant product purified from *E.coli* and containing a His_6 tag, and that most of it was found in dimeric or aggregated form [30]. Whether dimerisation is a naturally occurring event for IL-37b remains to be established.

The possibility of IL-37b being an agonist or antagonist of IL-18 was examined using human cell lines (the human acute myelogenous leukemia KG-1, and the NK cell line NKO) [30, 41]. In contrast with IL-18, which induced a dose-dependent increase in IFN-γ production, neither immature nor mature IL-37b could achieve the same effect, even at high concentrations. Moreover, the presence of excess IL-37b did not have a significant impact on either IL-18-dependent or independent IFN-γ production. Thus, IL-37b binds to the IL-18Rα with low affinity and this binding is non-competitive for IL-18. In addition, binding of IL-37b to IL-18Rα failed to recruit the IL-18Rβ chain (as shown by cross-linking experiments), implying that signalling through the active, ternary IL-18 receptor complex cannot be initiated by IL-37b binding to the IL-18Rα [41].

The same investigators also showed, by chemical cross-linking, that both immature and mature IL-37b can bind to the recombinant third extracellular Ig-like domain of the IL-18Rα [44]. That IL-37b would bind to this domain was hypothesized after the prediction that IL-18 would do so. Indeed, the third IL-18Rα domain is essential for IL-18 binding and recruitment of the accessory protein [59]. IL-18 binding to both IL-18Rα and to IL-18BP has been reported to involve the same key amino acid residues on the IL-18 surface (E42 and K89) [60]. Furthermore, sequence alignment of IL-18BPa against the PDB identified the third domain of IL-1RI (PDB entry 1itb) as most similar to the Ig fold of the IL-18BP [33]. The conclusion was that IL-18 binds to the third domain of the IL-18Rα (highly homologous to IL-1RI) and to the similarly structured IL-18BP, through engagement of residues E42 and K89. Bufler *et al.* have hypothesized that, upon alignment of the IL-37b sequence with that of IL-18, the two key residues are conserved in IL-37b (E35 and K124), thus implying similar binding features to the IL-18BP and to the third domain of the IL-18Rα [41]. However, from NMR solution structure and mutagenesis of IL-18, and molecular modelling of interaction with IL-18Rα, it seems that IL-18 interacts with its receptor in a fashion very similar to that of IL-1β binding to IL-1RI, *i.e.*, by contacting two sites on the receptor, located in domains 1-2 and in domain 3, respectively [61]. Homology molecular modelling of IL-37b binding to IL-18Rα ([40] and *figure 4*) suggests that IL-37b principally interacts with the first and second receptor domain, as is the case for IL-1Ra, whereas interaction with domain 3 is probably very low, especially when compared to the binding of IL-1 to IL-1RI. Therefore, the interaction of IL-37 with the third receptor domain is speculative and needs further proof. Indeed, upon alignment of the IL-37b and IL-18 sequences, taking into consideration both the sequence and the structure with the predicted position of the twelve IL-1-like β strands, there is no evidence for conservation of the two key residues within the IL-37b structure *(figure 4)*. In addition, the predicted E35 residue of IL-37b may be absent in the mature cytokine, which appears to be naturally cleaved at residue 45 [40]. Further studies on the characterisation of IL-18 binding to IL-18Rα and IL-18BP indeed demonstrated that the IL-18 residue E42 is not involved in binding, and that the residue K89 has little importance for binding to IL-18Rα, but it is involved in IL-18BP binding, as also suggested by the fact that mutation of this residue (resulting in defective engagement of the inhibitor) significantly increases the biological activity of IL-18 [29, 61]. In any case, it is important to mention that information provided by homology models (as in the case of IL-37b and IL-18Rα) needs

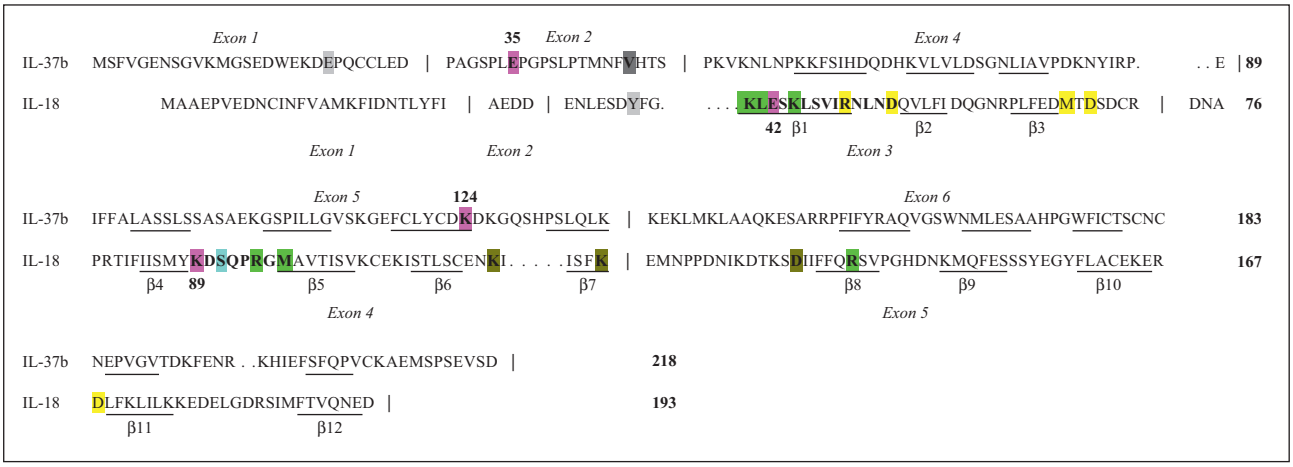

Figure 4

Alignment of IL-37b and IL-18 protein sequences.
The secondary structure of IL-37b has been predicted with PSIPRED (http://bioinf.cs.ucl.ac.uk/psipred/). The secondary structure of IL-18 is that derived from the NMR analysis [61] with small adjustments. Alignment has been performed for the mature sequences (from V46 of IL-37b and from Y37 of IL-18) with the programme YAP ("Yet Another Alignment Program" developed at the Biocomputing Group of the University of Bologna, http://gpcr.biocomp.unibo.it/) that takes into consideration both the sequence and the secondary structure. Coding exons are indicated above the IL-37b sequence and below the IL-18 sequence, and defined within the sequences by vertical bars. β-strands are underlined (numbering β1-β12 is indicated below the aligned sequences). Light gray: N-terminus of mature protein (putative for IL-37b, as predicted from caspase-1 cleavage site [30]) (ProteinDigest: http://db.systemsbiology.net:8080/proteomicsToolkit/proteinDigest.html and PeptideCutter: http://expasy.org/tools/peptidecutter/peptidecutter_references.html). Dark gray: experimentally-detected N-terminus [40]. Magenta: key amino acids E42 and K89 of IL-18, and E35 and K124 in IL-37b [29, 33, 41, 60]. Yellow: IL-18 amino acids of the "site I", interacting with domains 1 and 2 of the IL-18Rα [61]. Green: IL-18 amino acids of the "site II", interacting with domain 3 of the IL-18Rα [29, 61]. Olive green: IL-18 amino acids of the "site III", possibly interacting with the IL-18Rβ [61]. Blue: IL-18 amino acid partially important for binding to IL-18Rα [29].

to be validated experimentally after resolution of the crystal structure (which is presently not available). Models in the *figure 5* are preliminary homology models, with IL-37b modelled on the crystal structure of IL-1Ra, and IL-18Rα modelled on the crystal structure of IL-1RI (Boraschi & Lucchesi, unpublished). Indeed, two crystal structures of IL-1RI are available, that of IL-1RI binding to the agonist ligand IL-1β, and that of IL-1RI binding to the antagonist ligand IL-1Ra. Thus, two homology models of IL-18Rα have been calculated, modelled after either IL-1RI structures (model 1, calculated on the IL-1RI/IL-1β structure; model 2, calculated on the IL-1RI/IL-1Ra structure). Interaction of IL-18 with the receptor was calculated using the receptor model 1, while interaction of IL-37b with the receptor was calculated using the receptor model 2. Attempts to model the IL-37b/IL-18Rα interaction using the receptor model 1 gave unsatisfactory results. Thus, it appears that IL-37b binds to the IL-18Rα in a fashion that resembles that of an antagonist.

Two independent studies have shown that both immature and mature IL-37b are unable to bind to immobilised IL-18BP-Fc fusion proteins when using a BIAcore assay [30, 41]. However, cross-linking of IL-37b with IL-18BP using BS^3 suggested that interaction can occur between IL-18BP and both proIL-37b and the mature cytokine [41]. The inability to observe binding in more physiological situations suggests that binding of IL-37b to IL-18BP is very weak. In an *in vitro* assay of IL-18-dependent IFN-γ production by NKO cells or PBMC, the inhibitory effect of exogenous IL-18BP was apparently increased in the presence of mature IL-37b [41]. Although statistically significant, this effect was very limited (maximum 21% additional decrease as compared to IL-18BP alone), and it was much less pronounced (and not significant) for proIL-37b. It is interesting to note that the additional inhibiting effect of a fixed dose of mature IL-37b was only observed when low concentrations of IL-18BP were used (3.12 to 6.25 ng/ml). The hypothesis to explain this behaviour proposes that the IL-37b/IL-18BP complex is able to recruit the IL-18Rβ accessory chain into an inactive complex, thus decreasing its availability to form active receptor complexes with IL-18/IL-18Rα [41], similarly to the "co-receptor competition" mechanism described for inhibitory IL-1RII capturing IL-1β and sequestering IL-1RAcP into a non-signalling complex [21]. In this case, IL-18BP may have a dual effect on the inhibition of IL-18-mediated inflammatory responses, by either capturing the agonist IL-18 or by sequestering the accessory chain through IL-37b engagement. The observation that two of the three IL-18 residues allegedly involved in engagement of IL-18Rβ are conserved in the IL-37b sequence/structure (K126 and K136; *figure 4*) supports this hypothesis. Along this line, it is noteworthy that the IL-37b/IL-18BP complex cannot bind the soluble form of IL-18Rβ (as shown in cross-linking experiments). Indeed, engagement of soluble accessory protein would dampen the inhibitory potential of IL-37b/IL-18BP by competing with its binding to the membrane-associated signalling accessory chain [41]. In any case, the formal proof of the formation of the trimeric complex IL-37b/IL-18BP/membrane IL-18Rβ is still missing.

IL-37 receptor complex

Overall, data on the interaction between IL-37 and potential binding partners remain limited. Several independent groups have shown that this molecule binds to the IL-18Rα [30, 40, 41]. However the effects of this interaction are not clear since direct agonist or antagonist effects on the function of IL-18 have not been observed thus far. It

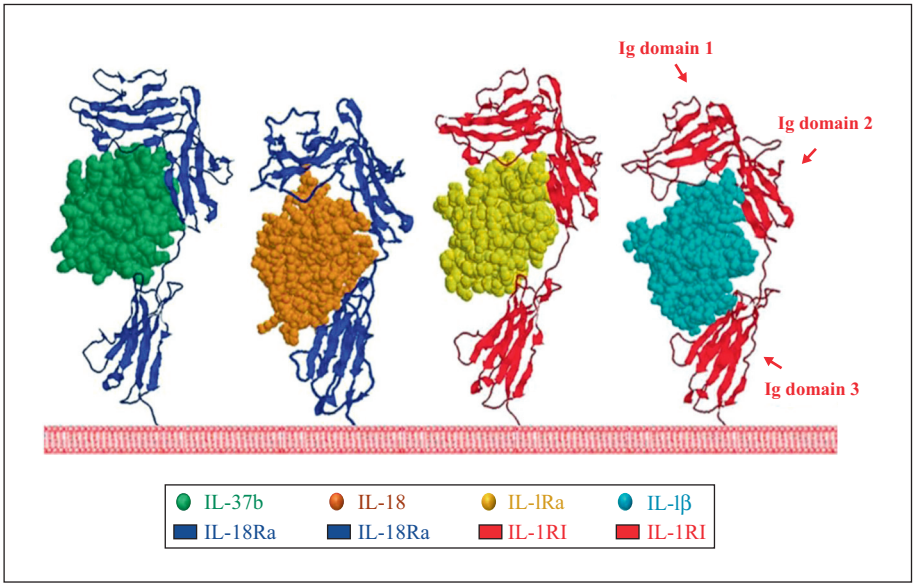

Figure 5

Homology model of IL-37 interaction with IL-18Rα.
The model of IL-37/IL-18Rα binding (far left) is compared to other ligand/receptor interaction models in the IL-1 family. Far right: IL-1β/IL-1RI crystal structure (PDBid: 1ITB, IL-1β represented in cyan and IL-1RI in red); centre-right: IL-1Ra/IL-1RI crystal structure (PDBid 1IRA, IL-1Ra represented in yellow and IL-1RI in red); centre-left: model of IL-18/IL-18Rα interaction (IL-18 represented in orange and IL-18Rα in blue). On the far left is the model of IL-1F7b/IL-18Rα interaction. The IL-1F7b homology model (light green) was built on the IL-18 NMR structure (PDBid: 1J0S) and docked to the IL-18Rα model (blue) that was built on the IL-1RI crystal structure (PDBid: 1IRA). This interaction model shows more similarity with IL-1Ra/IL-1RI structure as compared with the IL-1β/IL-1RI structure, especially at the third Ig-like domain level, where contact with receptor is looser for IL-37 and IL-1Ra and tighter for IL-1β and IL-18. The position of the three Ig domains of the receptor is shown in the far-right model and applies to all other models. A tight cytokine/receptor complex is expected to be required for recruiting the accessory protein, thus the inability of IL-37 in recruiting IL-18Rβ could be due to its incomplete binding to IL-18Rα. Homology models were built using Modeller 9v2 (freely available for academic use at http://salilab.org/modeller/); dockings were obtained using AutoDock 4.0 suite ([62]; freely available for academic use at http://autodock.scripps.edu/) and refined with short runs (maximum 5 ns) of molecular dynamic using Gromacs 3.3.3 (freely available as binaries or source at http://www.gromacs.org/).

should be stressed that, except for the first study [40], evaluation of interaction with receptors was performed with recombinant IL-37b generated in *E.coli* systems, which could result in lack of post-translational modifications, insoluble protein expression in inclusion bodies, need for denaturation/renaturation procedures, risk of inappropriate folding and/or aggregation, with consequent loss of activity. In addition, the *E.coli*-expressed mature IL-37b used in the BIAcore, cross-linking, and biological assays still possessed the engineered His_6 tag used for purification [30, 41], and this might also affect the protein conformation. Whether the IL-37b/IL-18Rα complex, once formed, does subsequently interact with an accessory protein is again a matter of speculation. Indeed, cross-linking experiments failed to demonstrate the formation of ternary complexes of the extracellular domain of the IL-18Rβ accessory protein with the IL-37b/IL-18Rα complex [41]. It is possible that the very low affinity of the interaction between IL-37b and IL-18Rα and the consequent instability of the complex may prevent recruitment of IL-18Rβ in a sufficiently stable and measurable ternary complex even if this occurs. The availability of mammalian-derived, correctly cleaved and folded IL-37b will tell us whether IL-37b does indeed induce the formation of a ternary complex with IL-18Rα and IL-18Rβ or not.

Negative signals from IL-1 receptors

In general, nearly all investigations of the IL-1 family of receptors have focussed on binding with an IL-1-like ligand and documenting a positive signal thereof. This is the case for IL-1α, IL-1β, IL-18, IL-36α, IL-36β, IL-36γ, and IL-33. Some IL-1 family members are receptor antagonists or anti-inflammatory cytokines. The IL-1 receptor antagonist (IL-1Ra) is well established as a treatment for humans with various inflammatory diseases. IL-36Ra has been described as being able to inhibit signalling (NFκB and MAPK activation) induced by IL-36α, IL-36β, and IL-36γ [63], all ligands that are active through IL-1Rrp2 and IL-1RAcP [58].

It remains an attractive hypothesis that IL-37b binds to the IL-18Rα, blocking the ability of IL-18 to bind to the same receptor, thereby acting as a naturally occurring receptor antagonist for IL-18, as it is the case of IL-1Ra for IL-1β. However, several studies have failed to demonstrate the ability of recombinant IL-37b to act as an antagonist for IL-18 (*in vitro* stimulation of IFN-γ production in IL-18-responsive cells) [30, 41]. Also in this case, the extremely low affinity of *E.coli*-derived recombinant IL-37b for the IL-18Rα makes it unlikely that it could compete with IL-18. Again, availability of mammalian-derived IL-37b may help in solving the issue of whether IL-37b does indeed act as a receptor antagonist or not.

An alternative possibility is that the IL-18Rα may deliver a negative signal depending on the ligand and the accessory chain. Thus, by binding IL-37b, the IL-18Rα may recruit an accessory chain other than IL-18Rβ and initiate an activation pathway leading to anti-inflammation. The best candidates for such "negative" accessory receptor chain are the orphan receptors of the IL-1 family, the single domain receptor TIR8/SIGIRR (IL-1R8), TIGIRR-1 (IL-1R9), and TIGIRR-2 (IL-1R10). In mice deficient in TIR8/SIGIRR,

there is greater susceptibility to lethal LPS challenge [2] and inflammation in experimental colitis and lung infection [64, 65]. Recently, IL-36Ra (highly homologous to the antagonist IL-1Ra, but unable to bind to IL-1RI [13]) was found to be able to inhibit the inflammatory effects of LPS and IL-1β in the brain *in vivo* and in brain tissue/cells *in vitro* by a mechanism requiring TIR8/SIGIRR [7]. The anti-inflammatory effect of IL-36Ra is apparently confined to brain cells (possibly to astrocytes), as no effect could be seen in mouse LPS-stimulated DC, macrophages or spleen cells [7], nor in human articular chondrocytes and synovial cells stimulated by IL-36β [66]. The TIR8/SIGIRR-dependent effect of IL-36Ra goes through induction of the anti-inflammatory cytokine IL-4 [7]. Therefore, this anti-inflammatory effect is apparently due to the activation of an anti-inflammatory pathway rather than inhibition of an inflammatory pathway. Even if no direct interaction of IL-36Ra with TIR8/SIGIRR has been demonstrated, it is noteworthy that this IL-36Ra-dependent TIR8/SIGIRR-mediated effect occurs locally, being involved in the fine tissue-specific anti-inflammatory activation, in contrast to the generalised inhibition of inflammatory signalling by the closely related IL-1Ra, which blocks IL-1 effects on every IL-1RI-bearing cell/tissue. It is therefore tempting to speculate that TIR8/SIGIRR may act as accessory chain for anti-inflammatory signalling also in the case of IL-37 binding to IL-18Rα complex. Alternatively, the orphan receptors TIGIRR-1 and TIGIRR-2 may have this function. It is of interest that TIGIRR-1 and TIGIRR-2 have very different organ distribution, and thus may be involved in organ-specific regulation *(table 2)*. The existence of five different splice variants of IL-37, with different exon usage for the N-terminal propeptide, different maturation sites, and different tissue distribution provides further support to the hypothesis of a tissue-specific regulation by IL-37 through an organ-specific accessory receptor chain. Indeed, it should be noted that in some tissues only one of the five isoforms is expressed: IL-37a in the brain, IL-37b in the kidney, IL-37c in the heart (Table 3). Since TIGIRR-1 and TIGIRR-2 are abundantly expressed in brain, we would like to propose that one of the TIGIRR receptors may be the accessory chain for mature IL-37a (the brain IL-37 isoform).

In *figure 6*, the hypothetical mechanisms of IL-37 interaction with receptor chains are depicted.

BIOLOGICAL FUNCTIONS

The distinct intracellular role of IL-1 family proteins

The observation that the precursor form of each member of the IL-1 family, with the exception of the IL-1 receptor antagonist IL-1Ra, lacks a signal peptide suggests persistence of an evolutionarily conserved role of these proteins as intracellular factors. Indeed, there is growing evidence for intracellular roles for cytokines and growth factors of the IL-1/FGF family. For instance, IL-1α, which is rarely found in the extracellular compartment and is primarily a cell-associated cytokine [67], has been proposed to regulate cell migration, proliferation, senescence, and differentiation through intracrine mechanisms and intracellular pathways independent of its cell-surface membrane receptors [68]. Nuclear translocation of the IL-1α precursor, mediated by a consensus nuclear localisation sequence (NLS) within its N-terminal portion [69], has been shown to be critical for the intracellular functions of IL-1α [70]. The acidic N-terminal pro-piece of the IL-1α precursor, but not the C-terminal mature form, specifically interacts with several nuclear targets (the histone acetyltransferases p300, PCAF and Gcn5, and the growth suppressor necdin) [71, 72]. Another report claims an important role for the IL-1α precursor as an intracrine proinflammatory activator of transcription [68]. Together, these observations indicate that IL-1α is a dual-function protein that acts as both a nuclear factor and a pro-inflammatory cytokine. A similar duality of function has been shown for high-mobility group box 1 (HMGB1) protein, an abundant, chromatin-associated protein involved in transcriptional regulation that is released by necrotic cells and secreted by activated macrophages during inflammation and functions extracellularly as a potent proinflammatory cytokine [73-75].

The most recently described cytokine of the IL-1 family IL-33, a cytokine that signals via the T1/ST2 receptor and induces Th2-associated cytokines, is identical to NF-HEV (nuclear factor of high endothelial venules), a nuclear factor associated with heterochromatin *in vivo* and mitotic chromosomes in living cells, that possesses potent transcriptional repressor properties [5, 73, 74]. IL-33, similarly to IL-1α, may function both as a proinflammatory cytokine and as an intracellular nuclear factor involved in transcriptional regulation. In IL-33, nuclear localisation, heterochromatin-association, and targeting to mitotic chromosomes were all found to be mediated by an evolutionarily-conserved, homeodomain-like helix-turn-helix (HTH) motif within the N-terminal domain [76, 77]. The transcriptional repressor properties of IL-33 are associated to this HTH motif. This domain is predicted to exhibit structural homology with the homeodomain and other HTH DNA-binding domains, but has no similarity with the N-terminal propeptide of other IL-1 family cytokines. Cleavage of IL-33 and export of the C-terminal IL-1-like cytokine moiety have been proposed to occur by a caspase-1-mediated mechanism [5]. However, the observation that the predicted cleavage site for caspase-1 is not conserved in the canine, bovine, and porcine IL-33 orthologues, and the lack of evidence for IL-33 processing *in vivo* either for endogenous IL-33 in HEV endothelial cells or for ectopic IL-33 in HEK-293T epithelial cells, have cast some doubts about the physiological relevance of caspase-1 maturation of IL-33 [77]. Indeed, it appears that IL-33 does not need maturation for binding to its receptor to exert its cytokine activity [78]. Caspase-1 has no effect on full length IL-33, which, in turn, can be cleaved by the action of caspase-3 into two fragments unable to bind the T1/ST2 receptor and inactive as cytokine, while still able to translocate to the nucleus [78]. It would be interesting to explore the possibility that extracellular IL-33, similarly to HMGB1, may be released by activated or dying macrophages during inflammation after hyperacetylation of lysine residues [79], or that it may function, similarly to IL-1α, as a membrane-associated cytokine [67, 80, 81].

The precursor form of IL-37 is also found abundantly in the cytoplasm of producing cells by immunocytochemical staining with a polyclonal antibody to IL-37b (although no identification of the different isoforms is possible in

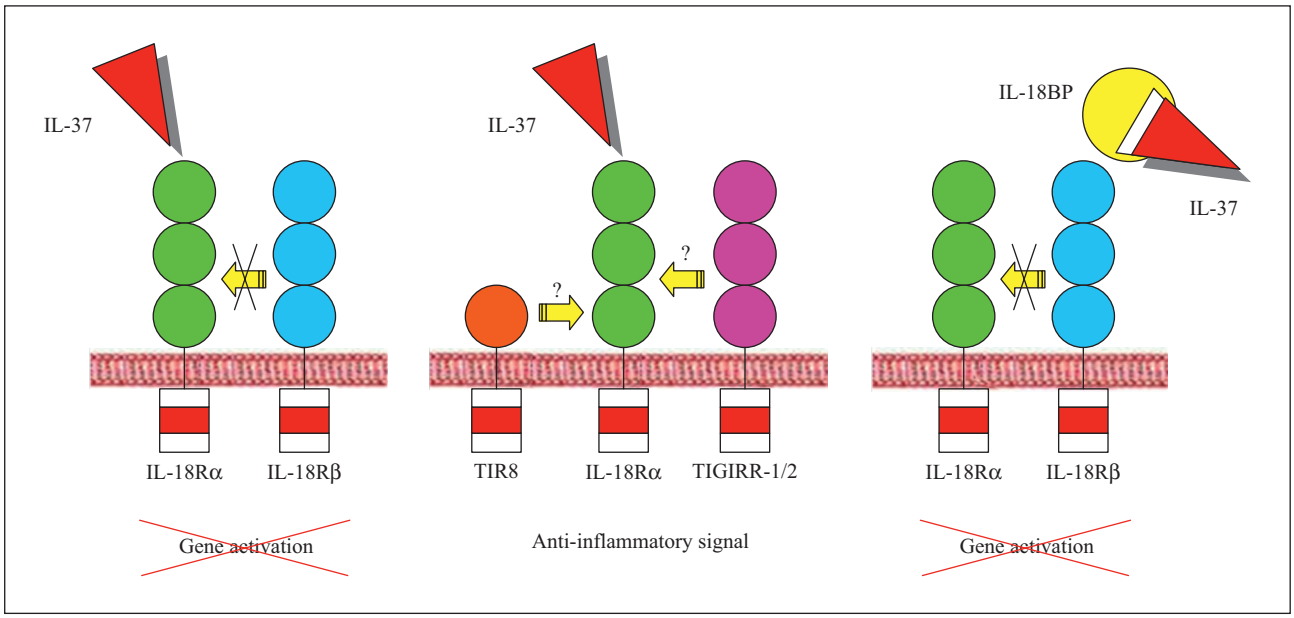

Figure 6

Proposed mechanisms of the biological functions of extracellular IL-37.
IL-37 interacts with the IL-18Rα, but does not induce the recruitment of the IL-18Rβ chain, thus failing to activate an inflammatory response. On the other hand, binding of IL-37 to the IL-18Rα might result in the recruitment of the TIR8/SIGIRR or TIGIRR-1/2 orphan receptors, which could act as "negative" accessory chains leading to activation of an anti-inflammatory response. Finally, the interaction between the complex of IL-37 and IL-18BP with the membrane-associated IL-18Rβ could prevent the recruitment of this accessory chain to the IL-18/IL-18Rα complex and the subsequent inflammatory gene transcription.

such studies) [30, 41]. It was shown that pro-IL-37 is present intracellularly in monocytes and PBMC, and is up-regulated by LPS and other TLR ligands but not by IL-4 [52, 54]. It should be noted that in human PBMC the IL-37 protein appears in non-reducing SDS-PAGE as a 45 kDa band (probably due to homodimerization) [54]. The hypothesis that IL-37 may have an intracellular role was investigated using different IL-37b fusion proteins in transfected murine macrophage-like RAW264.7 cells [54, 56]. These constructs encompass the full sequence of the pro-IL-37b fused to different fluorescent proteins (CFP at the N-terminus, and YFP at the C-terminus). In unstimulated cells, low levels of expressed fusion proteins could be detected both in the cytoplasm and in the nucleus. LPS stimulation upregulated expression of the transfected IL-37b constructs by increasing mRNA stability. Likewise, the pro-IL-37b proteins were significantly increased in LPS-stimulated cells, but only the IL-37b-YFP protein accumulated in the nucleus, at variance with CFP-IL-37b that was equally distributed throughout the cytoplasm and nucleus. This suggests a preferential translocation to the nucleus of the matured IL-37b (*i.e.*, that maintaining the C-terminal fluorescent tail), as compared to the unprocessed pro-protein (*i.e.*, that with the N-terminal fluorescent protein). Indeed, in cells transfected with the mature IL-37b-YFP translocation to the nucleus was evident upon LPS stimulation [56]. In transfected RAW264.7 cells, the IL-37 protein has an apparent MW of 25 kDa in non-reducing conditions [54]. LPS stimulation induced partial cleavage of the CFP-IL-37b pro-protein (about 27%), a maturation that could be inhibited by a specific caspase-1 inhibitor (by about 40%), and more efficiently, but not completely, by a pan-caspase inhibitor (about 75% inhibition) [56].

The finding that it is the mature form of IL-37b that translocates to the nucleus is in agreement with the fact that, from bioinformatical analysis, IL-37b does not present any distinctive nuclear localisation sequence in its propeptide sequence. Indeed, the same holds true for isoforms c and d, which share with IL-37b all or part of the propeptide (Lucchesi & Boraschi, unpublished). However, it is noteworthy that IL-37a, the isoform that uses a unique propeptide (encoded by exon 3), encompass a very typical NLS just upstream of the putative elastase cleavage site (*figure 6*) [82]. This observation stresses the hypothesis that different IL-37 isoforms can play distinct roles in different anatomical areas (IL-37a is exclusively located in brain tissues), and that these cytokines may have a dual role, both as nuclear gene regulators and as inflammatory/anti-inflammatory cytokines upon cleavage in situations of stress.

Biological effects of intracellular IL-37

The biological effect of over-expression of IL-37b in the murine macrophage-like RAW264.7 cells was assessed on LPS-induced production of inflammatory and anti-inflammatory cytokines [56]. As compared to cells transfected with the empty vector, IL-37b-overexpressing cells responded to LPS with a less pronounced production of soluble TNFα, IL-6, MIP-2, and of cell-associated IL-1α. A decrease in IL-10 production was also observed but this was not statistically significant. No difference in the production of MIP-1α was detected, showing that the decrease is selective for some cytokines. More recent data have implemented these findings. In IL-37b-overexpressing RAW264.7 cells, cytokine induction by a wide array of inflammatory stimuli (other TLR ligands besides LPS, IL-1β, TNFα) was found to be inhibited as compared to mock-transfected cells [54]. An array of cytokines was assessed in IL-37b-overexpressing cells stimulated with LPS, and significant decreases were

shown for a series of inflammatory molecules (IL-1α, IL-1β, TNFα, IL-6) and for GM- and M-CSF, while the Th2-related cytokine IL-13 was increased. However, IL-17 and MCP-1 production was enhanced in IL-37b-overexpressing cells, and IL-1Ra was inhibited, suggesting a more complex regulatory role for IL-37b rather than a purely anti-inflammatory activity. These findings were confirmed in other cell lines, *i.e.* in human macrophagic THP-1 cells, and in human alveolar epithelial A549 cells, which, upon IL-37b overexpression, were hyporeactive to LPS or IL-1β in terms of inflammatory cytokine production (IL-1β, TNFα, and IL-8 for THP-1; IL-1α and IL-6 for A549). To confirm these data in normal cells, the production of the IL-37 protein in human PBMC was assessed after stimulation with a variety of agents, and was found to be upregulated, in terms of intracellular protein production (please note that the antibody used for Western blotting, raised against recombinant IL-37b, most likely does not discriminate between IL-37 isoforms). RNA interference blocked IL-37 appearance and, concomitantly, enhanced production of IL-1α, IL-1β, TNFα, IL-6, GM-CSF, M-CSF, G-CSF and other factors. No effect was seen on production of IL-1Ra or IL-10, while IL-5 production was decreased.

These data appear to indicate that increased levels of IL-37 (whether intracellular or extracellular is not known) correlate with decreased response to inflammatory stimulation both in macrophagic and epithelial cells. It would be interesting to validate these results, to assess the effect of IL-37 overexpression *versus* knock-down in M2 alternatively activated and deactivated macrophages (with IL-4, IL-10, TGF-β, dexamethasone, etc.), *i.e.* in a situation in which macrophages do not exert inflammatory effects but rather anti-inflammatory, tissue-remodelling activities.

While investigating the signaling events leading to decreased inflammation, it was noted that IL-37 can interact with Smad3, as assessed in a proteomic-based search for Smad3 interactors [83]. In IL-37b-overexpressing A549 cells, less reactive to activating stimuli, association of IL-37 with phosphorylated Smad3 was shown [54]. On the other hand, inhibition of Smad3 (with the specific inhibitor SIS3 or with a specific siRNA) could increase the constitutive and stimulated production of inflammatory cytokines (IL-1α, IL-1β, IL-6, IL-8, TNFα) in IL-37b-overexpressing RAW264.7 and THP-1 cells, suggesting that Smad3 is key in IL-37-mediated inhibition of inflammatory cytokine production. In THP-1 cells stimulated with LPS plus IFN-γ, overexpression of IL-37b inhibited phosphorylation of several kinases involved in various pathways of cell activation. Possibly as a consequence of such modulation, IL-37b-overexpressing RAW264.7 cells apparently grew at a slower pace and showed fewer morphological changes upon LPS stimulation when compared to mock-transfected cells, suggesting impaired functional reactivity.

The *in vivo* effect of IL-37b has been examined in IL-37b transgenic (tg) mice, in which low constitutive expression levels of the cytokine expression were obtained (despite a constitutively active CMV promoter), which could be increased by LPS treatment [54]. The metabolic effects of a sublethal endotoxic shock were significantly decreased in IL-37b-tg mice as compared to normal littermates, in terms of hypothermia, metabolic acidosis, dehydration, rise in potassium concentration, and liver damage. In addition, in IL-37b-tg mice the LPS challenge was unable to induce significant circulating and organ levels of inflammatory cytokines (IL-6, IL-1β, IL-17, IFN-γ, *etc.*), while anti-inflammatory cytokines such as I-309, IL-13 and IL-10 were equally induced by LPS in wild type and IL-37b-tg mice, and IL-4 and IL-27 were actually increased in IL-37b-tg animals. LPS-induced *in vivo* activation of DC and macrophages was also reduced in tg mice, and their blood cells responded to LPS *ex vivo* with a severely impaired production of inflammatory cytokines. Also *in vivo*, the effects of IL-37b are apparently mediated by Smad3, since LPS-induced lung inflammation, down-regulated in IL-37b-tg mice, is re-established upon delivery of anti-Smad3 siRNA [54]. In an experimental model of intestinal bowel disease (dextran sodium sulfate[DSS]-induced colitis), the severity of the intestinal inflammation was significantly lower in IL-37b-tg mice as compared to wild type controls [84]. Is it noticeable that despite the presence of the constitutive CVM promoter, IL-37b expression was absent in the uninflamed colon, but was significantly induced (6-7x) following epithelial damage by DSS. Concomitantly, the clinical and histological scores for colitis were decreased. These include colonic infiltration by all types of leukocytes (in particular macrophages, neutrophils, eosinophils and DC), and colonic production of inflammatory cytokines (IL-1β, TNFα, IL-17). Other colonic cytokines induced by experimental colitis were not different between IL-37b-tg and wild type mice (IL-6, CXCL1), whereas the anti-inflammatory cytokine IL-10 was significantly increased in the transgenic animals. While amplifying the anti-inflammatory circuits (IL-10 induction), IL-37b did not appear to exert its effects through them, since inhibition of IL-10 *in vivo* by anti-IL-10R antibodies did not affect the mildness of colitis in IL-37b-tg mice. Transfer of IL-37b-tg bone marrow to irradiated wild type recipients could fully trasfer the protection from colitis, indicating that myeloid cell-derived IL-37b was indeed responsible for protection [84].

All these findings tend to indicate that IL-37 is a down-regulator of the inflammatory responses, by selctive inhibition of inflammatory cytokine production through a Smad3-dependent mechanism. However, much remains to be investigated, in particular since no kinetic evaluation of cytokine production has yet been performed, and the different contributions of the IL-37 isoforms have not yet been considered. In addition, it would be important to identify which of the observed effects of IL-37 are the consequence of a putative transcriptional regulation upon translocation to the nucleus, and which depend on the receptor-mediated activation by the soluble cytokine.

A significant activity has been demonstrated for IL-37b *in vivo* in a murine fibrosarcoma model [85]. A full-length IL-37b-expressing adenovirus could induce intracellular production of both precursor and processed IL-37b in A549 lung carcinoma cells. The extracellular proteins were consistent with the processed form, with and without post-translational modifications [85]. Intratumoral inoculation of the construct in established mouse fibrosarcomas caused a significant delay and regression of the tumour (in particular after multiple injections). This effect was due to a Fas-dependent mechanism (as for IL-18) and needed the presence of T and B lymphocytes, IL-12 and IFN-γ, but not NKT cells, thus suggesting a role for IL-37 in the passage to an adaptive type of immunity [85].

Convergence of positive and negative signals for the IL-18Rα

As mentioned above (see 1.3), unexpected differences can be observed between mice deficient in IL-18 (IL-18 KO) and mice deficient in the IL-18Rα (IL-18Rα KO). A study of the role of IL-18 in the development of experimental allergic encephalomyelitis (EAE) reported marked differences between IL-18 KO and IL-18Rα KO mice, with IL-18 KO animals fully susceptible to EAE induction, and IL-18Rα KO completely resistant [38]. In the same study, IL-18-independent engagement of IL-18Rα was found to be essential for the capacity of macrophages and DC to support Th17 polarisation [38]. The authors came to the conclusion that an additional ligand for the IL-18Rα exists that has effects distinct from those of IL-18. Other studies have confirmed the hypothesis of a second ligand for IL-18Rα. For example, IL-18 KO mice exhibit a significant delay in rejecting pancreatic islet allographs, whereas in IL-18Rα KO mice rejection is accelerated compared to wild type (WT) controls [37]. Consistently, IL-18 KO mice have markedly less inflammation and produce reduced levels of cytokines compared to WT controls. In contrast, IL-18Rα KO mice are hyper-responsive and produce greater amounts of pro-inflammatory cytokines compared to WT controls. Both KO strains were extensively backcrossed onto the C57BL/6 background. A third study employed KO mice carefully backcrossed into the lupus-prone MLR *lpr/lpr* strain in which all background strain 129 had been eliminated [86]. IL-18Rα KO mice developed full-blown lupus, which was indistinguishable from WT mice, a quite unexpected finding when considering the important role of IL-18 in the autoimmune pathogenesis of lupus. Indeed, IL-18 is a well-established agonist in lupus, and mice in which endogenous IL-18 is inhibited have little disease and better survival than controls [25]. On the other hand, the administration of IL-18 to lupus-prone mice worsens the disease manifestations and reduces survival [87]. Lupus-prone *lpr/lpr* mice lacking the IL-18Rα exhibit no protection from developing the disease, consistent with other observations in IL-18Rα KO *versus* IL-18 KO mice. The data are also in agreement with the findings in animals showing that with higher IL-18BP doses the anti-inflammatory benefit is lost and disease worsens. The hypothesis is that at higher doses IL-18BP is taking the unknown ligand from the IL-18Rα.

Thus, the obvious conclusion of all these findings is that another ligand also binds to the IL-18Rα and delivers a negative signal that regulates cytokine production and inflammation in a fashion distinct/opposite from that of IL-18. IL-37 could be this unknown ligand. However, the equivalent of IL-37 has not been yet identified in mice. Thus, the IL-18-independent, IL-18Rα-dependent effects observed in KO mice cannot be readily attributed to *bona fide* IL-37. A functional orthologue of IL-37 may be present in mice, as in the case of the murine IL-8 homologue KC. On the other hand, a much wider search for IL-37-like transcripts using more sensitive techniques needs to be performed before excluding the existence of mouse IL-37.

From all these data, it is tempting to speculate that a complex network of ligand-receptor-accessory chain interaction may regulate the different functional outcomes. Thus, IL-18Rα could ligate both IL-18 and another ligand (IL-37 in man, an unknown molecule in the mouse) and use different accessory chains (IL-18Rβ for IL-18 binding, unknown for IL-37) to exert different activities. In additon, experimental data seem to suggest that IL-18 itself may bind to a receptor different from IL-18Rα (IL-18 induces lupus, but IL-18Rα is not necessary). Since IL-18 can exert both inflammatory Th1-related activities [88] and anti-inflammatory Th2-related effects [89, 90], it might be hypothesized that, on different cellular targets, the cytokine may bind with low affinity to different binding chains (IL-18Rα *versus* another chain), and make use of IL-18Rβ for stabilising binding, or (and possibly in parallel) use the same IL-18Rα binding chain with a co-receptor other than IL-18Rβ. IL-37 may have similar features, as suggested by the different patterns of activity that have been reported (inflammatory and anti-inflammatory) in different systems.

IL-37 is present in human disease states

Expression of IL-37 is apparently linked to inflammation and inflammatory cells. In immunocytochemical staining with a polyclonal antibody raised against IL-1F7b, detectable staining was found in the cytoplasm and in granules of monocytes (within PBMC) from normal individuals in the absence of stimulation [41, 52, 54]. IL-37 could be significantly increased by 20 h stimulation of cells with LPS, other TLR ligands (Pam_3CKS_4, CpG), and cytokines such as IL-1β, IL-18, IFN-γ, TNFα, and TGFβ [54]. However, this cell-associated IL-37 protein yielded an unexpectedly high band in Western blot (about 45 kDa as compared to the 25 kDa proteins found in IL-1F7b-overexpressing RAW264.7 and A549 cells). Thus, natural IL-37 in PBMC is either dimeric, or undergoes post-translational modifications that increase its mass. Since only cell-associated IL-37 has been examined, it is unlikely that this could be due to glycosylation. Also, since the polyclonal antibody may cross-react with other IL-37 isoforms, it cannot be known if one or more IL-37 isoforms are present in the high MW band. The issue of the natural IL-37 form in human PBMC needs a deeper evaluation.

Association of IL-37 with disease can be inferred from immunohistochemistry with antibodies and by RT-PCR with specific primers. Indeed, initial studies were performed with tools (*e.g.*, primers recognising sequences in exons 5-6, polyclonal antibodies) that could not distinguish the different isoforms. Therefore, most of the information relates generically to IL-37, and only limited data are available for the specific isoforms.

The presence of the IL-37 mRNA and protein has been detected in human inflammatory and autoimmune disease states. Using both affinity purified polyclonal antibodies and a monoclonal antibody raised against IL-37b (which, however, may not distinguish between isoforms), the IL-37 protein was detected in synovial cells of patients with rheumatoid arthritis, in alveolar macrophages from patients with *Mycobacterium avium* infections, in the foam-like cells of atherosclerotic coronary and carotid artery plaques, in psoriatic plaques, and in the lamina propria macrophages of patients with Crohn's disease (Dinarello, unpublished). Using real-time PCR experiments, significant expression of IL-1F7b could be found in monocytes of lupus patients with severe disease (Boraschi, unpublished).

Expression of IL-37 (detected with primers in exons 5 and 6 that recognise all five isoforms) was detected in liver

and at higher levels in subcutanoeus and visceral fat of obese patients [91]. Liver IL-37 expression correlated positively with the body mass index (BMI) and negatively with γ-glutamyltransferase (GGT), and the subcutaneous fat IL-37 correlated negatively with BMI, serum insulin and homeostasis model assessment (HOMA) index. After weight loss, many inflammation-related and metabolic parameters were decreased (including BMI, GGT and HOMA index), and the IL-37 expression levels were significantly increased in subcutaneous fat but not in liver. Thus, it appears that liver IL-37 increases as consequence of inflammation, possibly as a mechanism attempting to re-establish homeostasis, while in the adipose tissue IL-37 appears to be directly involved in the decrease in inflammation.

The EST of IL-37a has been found in colon tumours [39]. IL-37 expression was detected in a colorectal carcinoma cell line (CCL-247), and ductal breast carcinoma both by SAGE tags and RT-PCR [30]. It should be noted that 35-40 cycles of PCR are necessary for detection, suggesting a low level of IL-37 expression. Immunohistochemical analysis of normal and diseased human tissues with anti-IL-37b revealed significant IL-37-related staining in plasma cells present in colon, breast, skin, tonsils, placenta, as well as in some tumours of the same tissues. Less intense staining was found in colon carcinoma stromal cells as compared to the breast carcinomas. Melanoma and lung carcinoma showed low levels of staining, whereas prostate carcinoma cells were negative. The strong IL-37 expression in plasma cells (both in normal and in pathological tissues), as well as in RAJI B lymphoma cells, suggests a potential role in immunoglobulin production and B cell activation in diseases such as multiple myeloma and B cell lymphomas, colon carcinoma, and inflammatory bowel disease [30].

In psoriatic keratinocytes, IL-37 expression was assessed only after 24 h of culture and was not different than expression in normal keratinocytes. Expression was not affected by stimulation with IL-17 [92]. It should be noted that in this study, primers for exons 1-2 were used that recognised both IL-37b and c isoforms, but not the other isoforms. Relative expression of the five IL-37 isoforms in the skin is, at present, unknown.

Several polymorphisms in the *IL1* gene cluster on chromosome 2 have been found to correlate with a range of inflammatory diseases, with significant variability among different populations. Polymorphisms that include some in the *IL1F7* gene have been indentified in psoriatic arthritis [93]. Association between a single nucleotide polymorphism (SNP) in the *IL1F7* gene and ankylosing spondilitis (AS) in the HLA-B27-positive Han Chinese population has been recently reported [94]. The study shows that only one of the two known *IL1F7* SNPs is present in the selected population (rs3811047, A to G in exon 2, leading to the replacement of threonine in position 42 by alanine), and that this SNP significantly correlates with disease in a cohort of HLA-B27-positive patients and controls. A significant association between the A/G polymorphism and drinking habits was also found in these patients [95]. It is noteworthy that the amino acid in position 42 is present only in the isoforms IL-37b and c, since the other isoforms do not use exon 2. The T42 is not maintained in the mature form cleaved between residues F45 and E46, while it would be present if the IL-37b and c precursors are cleaved by caspase-1 between D20 and E21, as well as in the intracellular uncleaved precursors. No hypothesis as to the putative functional role of IL-37 polymorphisms in contributing to disease is at present possible.

The IL-18Rα chain is expressed in most cells/tissues in the resting state, including epithelial, epidermal, mesenchymal and macrophagic cells. The IL-18Rβ chain (*i.e.*, the accessory protein of IL-18 receptor complex) is generally not expressed in resting cells, but only upon activation or in disease states. When both chains of the IL-18R are expressed on cells, IL-18 triggers a classic portfolio of inflammatory genes. A growing number of studies report the association between increased levels of IL-18 and disease severity in humans. Animal studies using IL-18 KO mice or *in vivo* inhibition of endogenous IL-18 activity (by cDNA vaccination, administration of neutralizing antibodies to IL-18, or IL-18BP) show marked decreases in inflammation, metastasis and autoimmune processes (reviewed in [26]).

Therefore, it becomes important to understand why there is more inflammation in mice in the absence of the IL-18Rα. We consider the likelihood that IL-37b binds to the IL-18Rα, but then recruits another accessory chain (not the IL-18Rβ), forming a complex, which then delivers an inhibitory signal. Thus, in the absence of the IL-18Rα, this inhibitory process is disabled and, as a result, there is more inflammation and greater production of inflammatory cytokines.

CONCLUSIONS AND PERSPECTIVES

Ten years after the first description of IL-37, it is becoming evident that this cytokine may set a new paradigm for regulation of inflammation. Up to very recently, the IL-1 family cytokines appeared to include inflammatory cytokines (such as IL-1 and IL-18) that activate target cells through a receptor-mediated mechanism shared with TLR receptors, and one anti-inflammatory cytokine antagonist (IL-1Ra) capable of blocking IL-1-dependent activation by mechanical occupation of the receptor. A series of studies however, have indicated that other "orphan" members of the family may have anti-inflammatory activity, by direct activation of alternative pathways. It has been shown that IL-36Ra is capable of inhibiting IL-36-dependent NFκB activation in Jurkat cells transfected with the IL-1Rrp2 receptor [63], although other groups have failed to confirm the result [58]. Recent data show that IL-36Ra can effectively inhibit IL-1β- or LPS-induced inflammation in the brain or brain cells by a mechanism requiring the IL-1R member TIR8/SIGIRR and which is dependent on induction of the anti-inflammatory cytokine IL-4 [7]. This study proposes two very important issues:

- the IL-1-like cytokines can directly activate anti-inflammatory pathways (not only inhibit inflammatory activation by antagonising receptor binding of inflammatory ligands, as in the case of IL-1Ra);
- the IL-1-like cytokines may exert their effects in a organ/tissue-specific fashion (IL-36Ra could not inhibit inflammatory activation of non-brain cells, despite the presence of TIR8/SIGIRR).

Another very important study in this context is that describing the anti-inflammatory activation brought about by IL-33, an IL-1-like cytokine that is identical to

transcriptional regulator NF-HEV, present mainly in high endothelial venules of organised secondary lymphoid organs (tonsils, Peyer's patches, lymph nodes) [76]. Upon release outside the producing cells by an unknown mechanism, IL-33 can bind to the IL-1R-like receptor T1/ST2 (mainly expressed by mast cells and Th2 lymphocytes) and, together with IL-1RAcP, activate these cells to produce IL-2, IL-4, IL-5 and IL-13 (in Th2 cells), or TNFα, IL-1β, IL-6 and IL-13 (in mast cells) [5, 6]. These studies set forth again some important issues:

- the IL-1-like cytokines can activate alternative pathways of inflammation (as in mast cells), as well as anti-inflammatory processes (as in Th2 cells), depending on the type of target cells bearing the specific receptor;

- the IL-1-like cytokines can be the end-product (in cases of acute stress) of an intracellular protein endowed with different functions and mechanisms of action.

Thus, the functional characteristics of IL-37 should be examined in view of these considerations. As is the case for many of the IL-1 family cytokines, IL-37 is synthesized as a long precursor protein that remains intracellular, and is cleaved and released only upon additional inflammatory stimulation. Indeed, experimental data suggest that in the case of IL-37b, it is the mature protein, rather than the precursor, that translocates to the nucleus. Although nothing is known about the other four isoforms, is it however expected that they could behave differently, and to be involved in their mutual regulation. IL-37c has the same N-terminal sequence as IL-37b, thus it can compete with proIL-37b as a target for the same cleaving enzyme, however its incomplete C-terminal sequence will give rise to an abortive, inactive cytokine. Thus, production of proIL-37c can function as a down-regulatory mechanism for limiting maturation of IL-37b. The same is true for IL-37e that has the same N-terminal sequence as IL-37d but is expected to give rise to an incomplete, non-functional cytokine upon cleavage. Moreover, IL-37e shares with IL-37b the caspase-1 cleaving site (in the sequence encoded by exon 1), so it should also be able to compete partially with the maturation of IL-37b. However, it cannot compete with IL-37b maturation at the second site in exon 2 (which is missing in the IL-37e sequence). Thus, IL-37b maturation should be efficiently down-regulated by IL-37c, but only partially by IL-37e. On the other hand, IL-37d maturation should be competed equally well by IL-37e and IL-37c, as all share the same caspase-1 cleavage site. The differential expression of all these isoforms in the various tissues and cell types will help clarify the complex network of mutual regulation. In this view, a case apart is that of IL-37a, which is the only IL-37 isoform expressed in the brain, thus it cannot be regulated by other IL-37 isoforms in this location. Indeed, IL-37a has a unique propeptide, not shared by other isoforms, with a characteristic elastase cleaving site. This would suggest that IL-37a maturation is promoted by inflammation (elastase is a major enzyme of PMN), and its down-regulation in the brain occurs through IL-37-independent mechanisms. However, in other locations, for instance in blood monocytes, IL-37a, b, and c are co-expressed and upregulated by inflammatory stimuli (LPS). In this situation, it is the activation of the different cleaving enzymes (elastase *versus* caspase-1 *versus* unknown IL-37b-cleaving enzyme) that will determine the maturation rate of the different isoforms. It should be noted that, independently of the isoform and cleaving enzyme, isoforms a, b, and d (*i.e.* those predicted to have the complete IL-1-like β-trefoil structure), all give rise to practically the same mature cytokine, and are therefore expected to bind to the same receptor and trigger the same range of biological effects. At variance with all other isoforms, IL-37a carries in its propeptide a classical bi-partite NLS, suggesting that the precursor protein, in addition to the mature polypeptide, can translocate to the nucleus. This makes IL-37a very different from IL-37b, for which it has been reported that translocation to the nucleus occurs only for the mature protein, in that maturation would not be needed for the putative transcriptional regulatory events.

The biological effects of IL-37 are mostly anti-inflammatory, with a significant and selective inhibition of the expression of a wide range of inflammatory factors consequent to over-expression of IL-37b. This finding seems to be related to intracellular expression of IL-37b, although a direct effect of transcriptional regulation has not been proven. It remains to be understood how and when IL-37 is released from producing cells, if and how it triggers IL-18Rα-dependent IL-18Rβ-independent effects, and which accessory chain(s) it can recruit.

In summary, the five IL-37 isoforms can be expressed differentially in different tissues, and be regulated and cleaved differently during inflammation. They can possibly regulate each other, either by competing for the maturing enzymes, or by other unknown mechanisms at the level of mRNA splicing. The fact that the mature forms of the three putatively active isoforms are practically identical further stresses the importance of the cleavage regulation for the production of the mature cytokine(s). The activity of mature IL-37 can be independent of receptor ligation, as the intracellular mature IL-37b can translocate to the nucleus and is therefore expected to have transcriptional regulatory functions. Once released from cells (*e.g.*, upon inflammatory cell death), the mature IL-37 can bind to IL-18Rα. The receptor-dependent effects of IL-37 are poorly understood, but it seems that a different accessory chain may be used by the IL-37/IL-18Rα complex. It is tempting to speculate that the orphan receptors TIGIRR-1 and TIGIRR-2 (mostly expressed in the brain) may serve as accessory chains at the brain level, while other chains (*e.g.*, TIR8/SIGIRR) could be involved in anti-inflammatory effects in other tissues (for instance, epithelial surfaces).

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