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THE MODULATION OF MACROPHAGE ACTIVATION BY TYROSINE PHOSPHORYLATION

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1. ABSTRACT

Activated macrophages are a critical component of our antimicrobial armamentarium. Unfortunately, the lipid mediators and free radicals that these cells produce are not only toxic to potential pathogens, but also to the host. Thus the modulation of these activities can mitigate an overzealous immune response and thereby prevent host cell injury. Two families of receptor tyrosine kinases (RTK) in macrophages, the RON/STK and the Tyro3 families of protein kinases, will be examined in this review with an emphasis on their roles in modulating the effector functions of activated macrophages. Both families of receptors are capable of down-regulating the inflammatory response of macrophages to lipopolysaccharide, and both families of RTK's are structurally related. An analysis of the intracellular domains of RON/STK and Tyro3 reveal a common multi-substrate binding site, which can recruit common signaling molecules such as growth factor receptor bound 2 (Grb2) and phosphatidylinositol 3-kinase (PI3-K). The observations relating to a modulation of macrophage effector mechanisms by these receptors open unexplored avenues for the development of pharmacological immunomodulators with the potential to exploit elements of this common pathway.

2. INTRODUCTION- MACROPHAGE ACTIVATION

Tyrosine phosphorylation events play a central role in macrophage activation. The fundamental importance

of protein tyrosine kinases in macrophage activation and inflammation was definitively shown in the mid 1990's when tyrosine kinase inhibitors were reported to inhibit LPS-induced lethality, as well as diminish the production of tumor necrosis factor-alpha (TNF-alpha) and nitric oxide (NO) by LPS-stimulated macrophages (1). Macrophage activation is a process, which requires the coordinate action of two signals, a priming signal followed by a stimulatory signal. The priming signal is invariably IFN-gamma and the stimulatory signal can be any number of microbially derived molecules interacting with one of the macrophage Toll-like receptors (TLR) (2). Studies to determine the pathway of macrophage priming by IFN-gamma, and the role of tyrosine phosphorylation in this process have been well-documented and summarized previously (3;4). Briefly, these studies have definitively identified the Janus family of tyrosine kinases to be an integral participant in IFN-gamma signaling. This is now a well-established signaling pathway, in which JAK kinases activate STAT transcription factors (5), which in turn activate IFN-gamma-inducible genes (6).

The second stimulatory signal is transduced to macrophages via the TLRs. This pathway of signal transduction from TLRs is the subject of much on-going investigation (2). Serine/threonine kinases have figured far more prominently in TLR-dependent cellular stimulation than do tyrosine kinases. In this pathway, TLR activation results in a cascade of serine/threonine kinase events culminating in the activation of NF-kappaB (6). Thus, macrophage activation involves the coordinate activation of both serine/threonine kinases from the TLRs and tyrosine kinases from the IFN-gamma receptor. Recent work suggests that TLR-4, the receptor for LPS, may activate additional signaling pathways, which results in STAT-1 activation via a pathway involving tyrosine phosphorylation. This second pathway of STAT-1 activation via TLR4 may explain the increased toxicity of LPS relative to agonists of other TLRs.

The role of Src and Syk non-receptor tyrosine kinases in macrophage activation responses remains somewhat controversial. Although several studies have indicated that inhibitors of tyrosine phosphorylation can diminish cellular responses to LPS (7-9), macrophages from mice lacking hck, fgr, and lyn, the three src family kinases commonly expressed in macrophages, were fully capable of becoming activated (10). Similarly, macrophages from syk-deficient mice became activated and responded to LPS by producing relatively normal levels of NO (11;12).

Two new families of receptor tyrosine kinases have recently been associated with macrophage activation. These are the RON/STK and the Tyro3 families of the receptor tyrosine kinases. Paradoxically these tyrosine kinases appear to inhibit macrophage activation events and function as physiological safeguards against hyperactivation of macrophages. This review will focus on the RON/STK and Tyro3 families of receptor tyrosine kinases as potential inhibitors of macrophage activation.

3. RON/STK

3.1. Identification of the receptor and its ligand

In 1978 Leonard and Skeel isolated macrophage stimulating protein (MSP) from human serum (13). When added to resident macrophages, this protein enhanced the phagocytosis of complement-opsonized erythrocytes and induced cell chemotaxis. This early observation suggested that macrophages express a receptor for MSP that could influence effector functions of monocytic cells. The receptor was later identified by screening of cDNA libraries of human keratinocytes with a hallmark motif for protein tyrosine kinases and referred to as *recepteur d'origine nantais* (RON) (14). One year later an independent group performed RT-PCR analysis of hematopoietic stem cells and identified a stem cell-derived tyrosine kinase (STK). This proved to be the murine homologue to RON (15). Thus far, the only ligand identified for these two receptors is MSP-1 (sometimes referred to as HGFL), and consequently for the purpose of this review, RON/STK will be considered as one.

RON/STK is expressed to variable degrees in macrophages and macrophage-like cells. The degree of expression depends on the cell type and the differentiative state of the cell. The receptor was detected in peritoneal and dermal macrophages as well as osteoclasts, however alveolar macrophages, spleen macrophages and blood monocytes express little or no RON/STK (16-18). Receptor expression appears to be dependent on the differention stage of the monocytic cell (16), and maturity of peritoneal macrophages correlates with an up-regulation of RON. In the adult mouse RON/STK is expressed in many organs (16;19-21). During embryogenesis, the receptor is involved in the development of epithelial, bone and neuro-endocrine tissues (22) and deletion of RON/STK has proven to be lethal for murine embryos (23).

3.2. The structure of RON/STK

Structurally, RON/STK exhibits features that are common to other RTK's (Figure 1). Several specific features place RON/STK in the MET proto-oncogene super-family (24) of receptor tyrosine kinases. (i) RON is translated as a single precursor, which forms the mature disulfide-linked 185 kDa heterodimer after proteolytic cleavage (19;25). Both of the extracellular subunits combine to form the ligand-binding domain. (ii) Tyrosine residues 1238 and 1239 of the beta chain constitute the typical neighboring tyrosine residues in the catalytic domain, responsible for the regulation of the enzymatic activity after autophosphorylation (26). The 35-kDa alpha chain has no intrinsic kinase activity (19). (iii) The tyrosine residues at 1353 and 1360 of the beta chain provide a functional bidentate docking site in the C-terminal tail for high affinity interactions with src homology 2 (SH2) modules of other signaling proteins (27), such as growth factor receptor bound 2 (Grb2) or phosphatidylinositol 3-kinase (PI3-K). Although RON/STK shares these common features with the MET proto-oncogene super-family, the unique C-terminal tail of this molecule is divergent enough to characterize RON as a new member of a separate (sub)family, RON/STK (14;15;28-30).

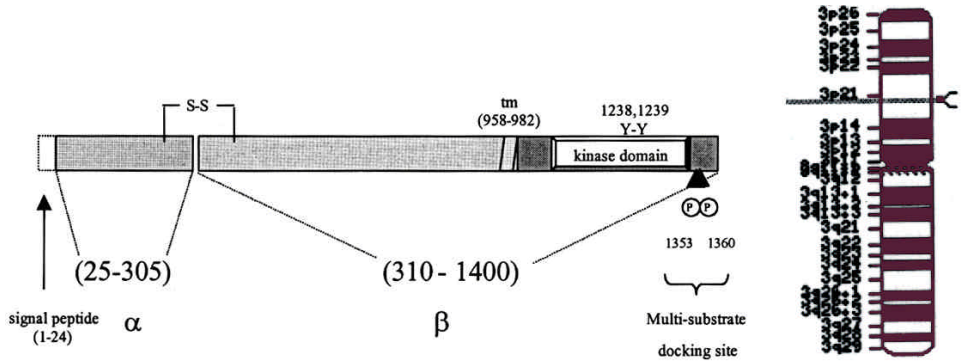

Figure 1. Diagrammatic illustration of structural elements of the human RON receptor tyrosine kinase. The single precursor protein with its signal peptide is processed to the bipartite RON receptor with its extracellular alpha chain covalently linked by disulfide bonds (S-S) to the signal transducing beta chain. The beta chain is divided into an extracellular, transmembrane (tm) and an intracellular domain. RON contains two juxtaposed tyrosine residues at position 1238 and 1239 respectively (Y-Y) in the kinase domain of the beta chain. A carboxy-terminal docking site for multiple substrates with *src* homology 2 (SH2) domains is composed of two phosphorylation sites for tyrosine at positions 1353 and 1360. Signaling proteins with SH2 moieties, such as the adaptor molecule growth factor receptor-bound 2 (Grb2), were found to be recruited to the multiple substrate-docking site (triangle). The inset depicts the chromosomal localization (3p21) of the human RON gene provided by courtesy of Online Mendelian Inheritance in Man (http://www.ncbi.nlm.nih.gov/omim/). References (19), (25), (27), (81), and (82).

3.3. The biological activity of RON/STK

As the name of its ligand, MSP-1, would imply, RON/STK was originally identified as a positive regulator of macrophage responsiveness. Indeed, the original studies of MSP demonstrated that it could not only improve C5a-mediated chemotaxis of monocytic cells, but also act as chemoattractant itself (31). MSP was also shown to stimulate the phagocytosis of C3bi-opsonized erythrocytes (32;33), and induce the expression of macrophage scavenger receptors, resulting in an increase in the endocytosis of LDL (34). More recent work, however has demonstrated that RON/STK can modulate macrophage activation, and inhibit some of the activities typically associated with activated macrophages. The best evidence for this is that mice heterozygous for RON (35) or mice expressing a tyrosine kinase domain defective form of RON (36) were more sensitive to letal endotoxemia than were wild-type mice. These mice also experienced enhanced irritation to phenols and enhanced tissue damage during cell-mediated inflammatory responses (36). Consistent with these *in vivo* observations is the *in vitro* demonstration that the ligation of STK/RON inhibited the secretion of TNF-alpha by macrophages, and down-regulated the expression of iNOS, decreasing the production of NO (36-40). RON/STK protects murine macrophages against LPS-induced apoptosis by decreasing endogenous nitric oxide (NO) production and preventing the nuclear accumulation of p53 (41).

Interesting recent data have suggested that the stimulation of macrophages by MSP can induce a population of macrophages with properties that are similar to those of an alternatively activated macrophage (42). MSP treatment of macrophages not only inhibited the production of (proinflammatory) TNF- alpha, IL-12 and NO, but it also up-regulated several genes that are typically associated with alternative activation, including arginase, the scavenger receptor type A (SR-A), and the IL-1R antagonist (IL-Ra) (42;43). These intriguing observations suggest that this receptor and the signals emanating from it upon ligation lead to the development of a macrophage phenotype involved in wound healing rather than in killing microbes and mediating inflammation.

4. TYRO3 FAMILY OF RTK

4.1. Identification of the receptor and its ligand

A related family of RTK called the Tyro3 family is also expressed on macrophages. These receptors were discovered around the time that MSP was identified, their signaling appears to be analogous to the RON/STK receptor signaling pathway, and they modulate macrophage activation in ways that are similar to RON/STK. The first member of this family was initially isolated from cells of leukemia patients (44) in 1991. It was called Axl, but it has also been referred to as Ufo (45), Ark (adhesion-related kinase) (46) and Tyro 7 (47). Two additional members of the family have been identified, and designated Tyro3 and Mer (or Nyk). Tyro3 was discovered during a search for protein tyrosine kinases expressed in the central nervous system (47;48). Other names for Tyro3 include Brt (49), Etk (50), Rse (51), Tif (52), Dtk, Rek (53) and Sky (54). Mer is the third member of this family. It was originally described in the murine system and designated as Nyk (55). The extracellular domain of Mer exhibited sequence similarity to the c-MET receptor tyrosine kinase superfamily.

The ligand for all three Tyro3 family members is Gas6, a term derived from the growth arrest-specific gene 6 (56-58). Gas6 is expressed in a variety of tissues including lung, intestine, endothelium, bone marrow, spleen, central nervous system and other tissues (59-61). Gas6 is comprised of five domains: the gamma-carboxyglutamaic acid (Gla) residues, a loop region, a stretch with four epidermal growth factor (EGF)-like repeats, and two globular (G) domains at the carboxy terminus. The Gla domain of Gas6 binds phosphatidyl serine on apoptotic

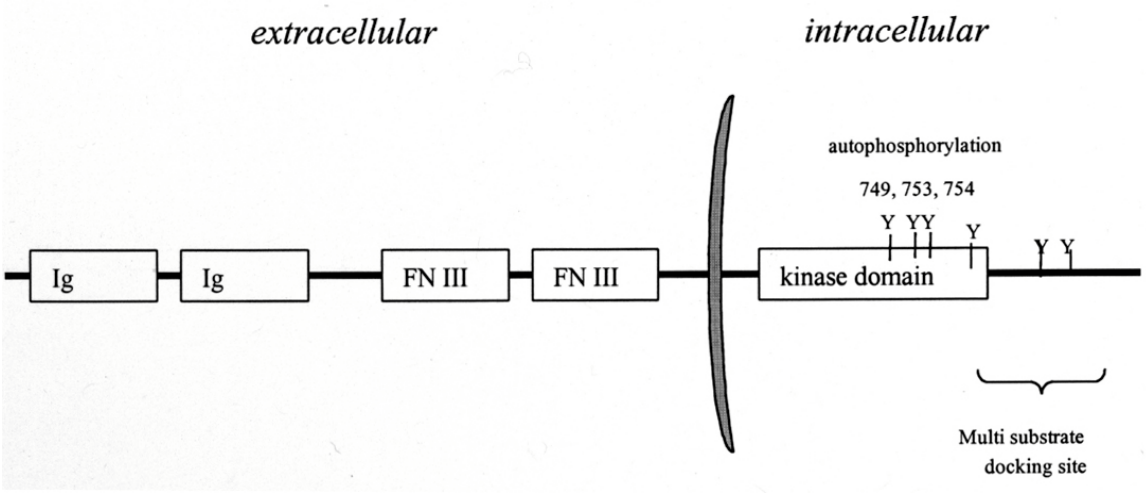

Figure 2. Characteristic structure of Tyro3 receptor tyrosine kinases. Depicted is the prototypic structure of Mer (Nyk). The extracellular ligand-binding domain region is composed of two immunoglobulin-like motifs (Ig) followed by two fibronectin type III motifs (FN III). The intracellular kinase domain contains triplicate tyrosine residues Y749, Y753, Y754 that are autophosphorylatyed after ligand binding. The carboxy-terminal multi-substrate binding comprises additional tyrosine residues that transduce the signal through SH2 domains of Grb2 and PI3-K. References (53;70;71).

cells and opsonizes them for uptake by macrophages via Tyro3 family members (62). The G domains of Gas6 are sufficient for binding to Tyro3 kinases. These domains display some homology to the steroid hormone-binding globulin protein (SHBG) (63).

Members of the Tyro3 family are expressed in a variety of tissues and cell types. Tyro3 kinases could be detected in many organs including kidney, liver, brain, spleen, bone marrow (64), testis (Tif (52)), ovaries, retina (Rek (53)) and the central nervous system (48;49). Mer is not only produced in adult tissue, but also during the morula, blastocyst, yolk sac and fetal liver stages. At least two and possibly all three of the Tyro3 members are expressed on monocytes, and treatment of these cells with phorbol ester or IFN-gamma invariably resulted in the up-regulation of their expression (65-67).

4.2. Structure of Tyro3 kinases

The Tyro3 family displays structural elements that are unique to the Tyro3 family, as well as common structural elements that indicate their relationship to the MET proto-oncogene superfamily (Figure 2). The unique features of the Tyro3 RTK family are: (i) the extracellular moiety, which includes two immunoglobulin like domains adjacent to two fibronectin type III-like domains (66;68;69); (ii) the KWIAIE(S) motif of the tyrosine kinase domain; (iii) the three functional autophosphorylation sites (Tyr-749, Tyr-753, Tyr754) of the kinase domain (70). At least two other shared characteristics of the Tyro3 members suggest a relationship to the MET proto-oncogene superfamily. The extracellular domain of Nyk (Mer) was reported to be similar to the MET superfamily (55). The multi-substrate binding site for PLC-gamma, PI3-K, Grb2, Src and Lck (71;72) constitutes the second characteristic feature which can also be found in the MET proto-oncogene family.

4.3. The biological activity of Tyro 3 in macrophages

There are several reports indicating an involvement of Tyro/Axl/Mer kinases in the effector functions of macrophages (62;73-75). Knockout mice lacking any one or, for that matter, all three of the Tyro3 family members are still viable. Using knockout mice it was recently demonstrated that Mer contributes to the down-regulation of the inflammatory response of macrophages to LPS. A recent comparison of wild-type with Mer-deficient cells showed a dramatic increase of the production of TNF-alpha and IL-1 after LPS challenge of peritoneal macrophages deficient in Mer (73). Furthermore, the elevation of TNF protein and mRNA correlated with an increased binding of NF-kappaB p50 and p65 to the κ3 region of the TNF-alpha promoter. Peritoneal macrophages from triple mutant mice deficient in Tyro3, Axl and Mer displayed an elevated constitutive production of IL-12 as well as an inducible overproduction of IL-12 relative to wild-type cells (74). These findings indicate that Tyro3 members down-regulate the production of TNF-alpha, IL-1 and IL-12, three macrophage proteins that are typically secreted by classically activated macrophages.

The function of macrophages as antigen-presenting cells may also by negatively influenced by the Tyro3 family. Peritoneal macrophages of triple mutant mice deficient in Tyro3, Axl and Mer constitutively express higher levels of MHC class II molecules than did wild-type macrophages. Macrophages from deficient mice were slightly more phagocytic than normal, and dendritic cells from deficient mice could be induced to overexpress B7.2, suggesting a role for these RTKs in dampening the initial phases of an adaptive immune response.

4.4. RON dependent signaling in macrophages

Signaling through both families of RTK's is similar. Consequently, we will illustrate the salient points of this cascade using RON, the system that has been most thoroughly

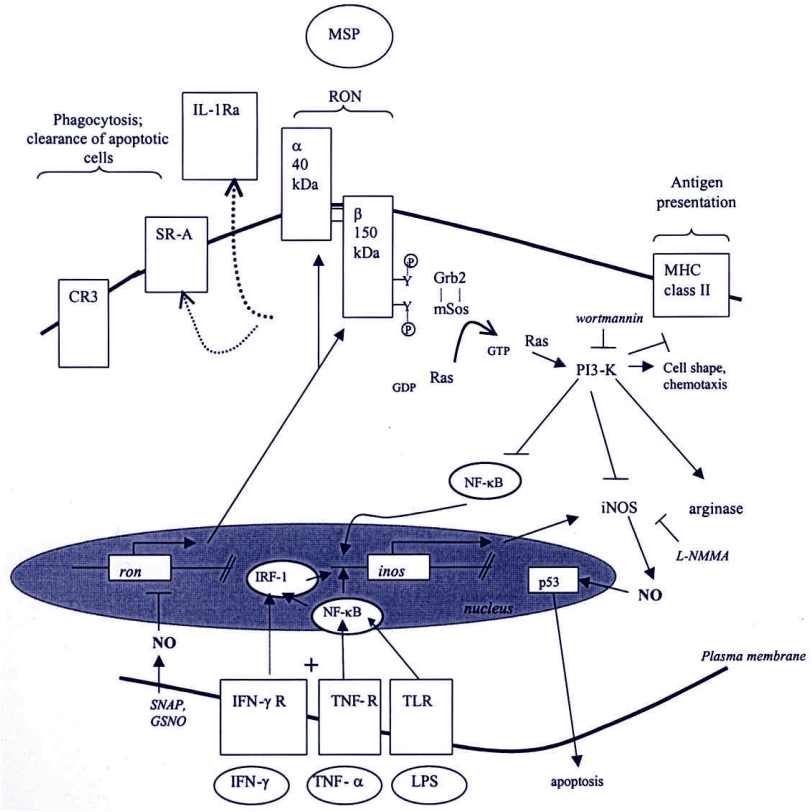

Figure 3. Model of RON/STK dependent signaling in macrophages. The binding of MSP to the heterodimeric receptor RON results in autophosphorylation of two tyrosine residues of the beta chain. As a consequence, cytoplasmic complexes of growth factor receptor bound 2 (Grb2) and Sos are recruited to the docking site of RON where they convert Ras-GDP into its active form. This leads to the activation of phosphatidylinositol 3-kinase (PI3-K) which affects phagocytosis, cell shape, and chemotaxis. The inhibition of the nuclear translocation of NF-kappaB prevents the transcription of the interferon regulatory factor 1 (IRF-1) as well as the inducible nitric oxide synthase (iNOS). Conversely, macrophage activation leads to the expression of iNOS and production of nitric oxide (NO) which prevents the transcription of the gene for RON (ron). Signaling via RON up-regulates scavenger receptor type A (SR-A) expression and provides a secondary signal required for the phagocytosis via complement receptors (CR3). Further details are discussed in the text.

studied. The proximal signaling down-stream of RON was investigated with cells, which were transfected with RON (76). This approach revealed the involvement of Grb2, Sos and Ras (76). A ligand-dependent association between Grb2 and the beta-chain of RON was established. Furthermore, Grb2 was immunoprecipitated with Sos in MSP-1-stimulated cells. Finally, MSP initiated the guanine exchange activity of Sos, which converted Ras-GDP to active Ras-GTP measured by the release of 3H-labeled GDP. The two functional tyrosine residues at 1353 and 1360 of the bidentate docking site in the C-terminal tail of the beta chain allow the recruitment of signaling proteins with SH2 modules (27). Most of the experimental evidence indicates that PI3-K is down-stream of this signaling cascade. Co-immunoprecipitation experiments detected the binding of PLC-gamma, the p85 subunit of PI3-K, Shc and Grb2 at Y1353 and Y1360 (77). Furthermore, the inhibition of PI3-K by either wortmannin or a dominant-negative PI3-K blocked all down-stream effects of RON/STK activity. These treatments impaired the STK-mediated suppression of the NO production and iNOS expression (39), the chemotactic response to MSP-1 (78), the protection against apoptosis by decreasing nuclear accumulation of p53 (41), and the MSP-induced shape change in murine resident peritoneal macrophages (78). From these observations a model of signaling arises in which Sos–Gbr2 is recruited to the multiple substrate binding-site of the RON beta chain, followed by Ras and PI3-K activation (Figure 3). The activation of PI3-K following receptor ligation may explain the inhibition of apoptosis associated with RON/STK, as well as the enhancement of phagocytosis. Previous studies in different experimental systems have demonstrated that both of these processes are mediated by PI3-K.

In 2001 a noteworthy structural feature of the Tyro3 family was proposed (74) whose functional importance is yet to be elucidated. Tyro3 receptors may have an immunoreceptor tyrosine-based inhibitory motif (ITIM) in their cytoplasmic domain. The motif is not truly a consensus sequence but does have a charged amino acid at position 2 (79). The question remains unanswered whether this non-classical ITIM domain actually recruits phosphatases and contributes to the down-regulatory activities of Tyro3 RTKs.

An interesting feedback inhibition loop has been described between RON/STK and iNOS (Fig.3). On one hand, RON/STK activity decreases iNOS expression. On the other hand, the production of NO can down-regulate the expression of RON/STK. The suppression of iNOS by RON/STK appears to be associated with a reduced nuclear translocation of NF-kappaB (40) following receptor ligation. This is consistent with the observed decrease in the activity of an NF-kappaB reporter gene following receptor ligation. The down-regulation of RON expression following macrophage activation by IFN-gamma and TNF-alpha could be mimicked by treating cells with NO donors GSNO or SNAP. The decrease of RON/STK expression occurs at the level of transcription as demonstrated by RT-PCR mRNA analysis and reporter gene constructs driven by the RON promoter (80). In conclusion, iNOS expression by inflammatory stimuli suppresses RON synthesis via NO. These effects are counterbalanced by RON signaling which inhibits iNOS expression by a negative regulation of NF-kappaB.

4.5. Similarities between RON/STK and Tyro3 kinases in macrophages

Comparison of both RTK families indicates a number of similarities with regard to their biological effects in macrophages. These effects coincide with structural similarities shared between the two families and with parallels in signaling molecules induced by both families of receptors. The ligation of both receptors modulates the activation of macrophages by LPS, resulting in decreased inflammatory cytokine production. This modulation of macrophage activation may be the most important observation made pertaining to these receptors because it has the potential to lead to novel anti-inflammatory therapeutics based on these receptors and their signaling molecules. However, this modulation is not the only shared biological consequence of receptor ligation. Both families of receptors are able to enhance some aspects of macrophage phagocytosis, and both families contribute to monocyte chemotaxis. Finally, both families appear to mediate anti-apoptotic effects. The commonality of these biological effects may be traced to structural similarities in the docking site for multiple signaling molecules in the cytoplasmic part of these receptors. In conclusion, a comparison between the two families of receptor tyrosine kinases RON/STK and Tyro3 indicates similar biological activities due to a prototypic signaling pathway in macrophages involving the motif of a bidentate multi-substrate docking site of RTKs, recruitment of SH2 molecules like Grb2, activation of PI3-kinase and down-regulation of NF-kappaB activity.

5. ACKNOWLEDGEMENTS

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