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Signalling pathway activation by Photodynamic Therapy: NF-κB at the crossroad between oncology and immunology.

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Abstract.

The response of tumors to photodynamic therapy (PDT) largely varies upon the intensity of the stress created in the cancer cells but also in the local environment. Singlet oxygen has been demonstrated, in many instances, as being the primary reactive oxygen species generated by PDT and responsible for most of the cellular effects. Cancer cells have developed various sensors which activate signalling pathways in response to PDT and the nature of the activated pathway varies with the PDT stress intensity. At low dose PDT, signalling pathways allow cancer cells to both proliferate and to switch on pro-survival responses such as autophagy. Above a certain level of PDT stress intensity, cancer cells cannot cope with the numerous damage and signalling pathways leading to cell death are activated. Two types of regulated cell death have been shown to be induced by PDT: apoptosis and necrosis. Signalling pathways activating NF-κB transcription factors have the peculiarity to be activated both at low and high doses of PDT. These pathways coordinate the cross-talk between the immune system via the release of cytokines and chemokines and an anti-cell death response via the control of apoptosis and necrosis. Therefore, NF-κB induced by PDT appears to play a positive role in educating the immune system to fight tumors but also a negative role in helping cancer cells to survive to the stress generated by singlet oxygen. This is why NF-κB cannot easily be considered as a pharmacological target whose inhibition will favor tumor cells eradication by PDT.

1. Introduction.

Photodynamic therapy (PDT) is a rather non-invasive treatment of cancer and several non malignant pathologies.[1] Its mechanism of action is based on three essential components: a light absorbing photosensitizer, the presence of molecular oxygen in the tumor and visible light. This combination is known to generate reactive oxygen species (ROS), mainly singlet oxygen that has been demonstrated to be the cytotoxic species at the origin of the therapeutic effect.[2] The anti-tumor effect of PDT stems from three origins: (i) a direct cytotoxic effect caused by singlet oxygen leading to cell death occurring either by apoptosis, necrosis or autophagic cell death, (ii) damage to tumor vasculature and (iii) the activation of innate and adaptative immune responses. The contribution of these three mechanisms to tumor eradication by PDT largely depend on the physico-chemical properties of the photosensitizer which used, its intra-cellular localization, but also on the light dose delivered to the tumor and the time between the photosensitizer delivery and the light exposure.[3] Obviously, it turns out that an efficient tumor eradication by PDT could only be achieved when combining an efficient cancer cell death with a long-lasting anti-tumor immunity to control tumor recurrences by the immune system.[4] In that respect, apoptosis is considered in vivo as immunologically silent, tolerogenic or immunosuppressive and therefore unable to initiate an efficient immune response.[5] On the other hand, necrosis which has been considered for years as a sort of accidental cell death is in fact a highly regulated phenomena under the control of several signalling pathways.[6] By opposition to apoptosis, necrosis is highly inflammatory due to the sudden release of various intracellular molecules called “damage-associated molecular patterns” (DAMPS) that sensitize and attract phagocytes to the tumor, creating a pro-

inflammatory environment.[7] Therefore has recently emerged the concept of "immunological cell death" where treatment modalities eliminate cancer cells by an efficient cell death together with an increase in tumor immunogenicity.[8] PDT using photosensitizers such as hypericin has been remarkable in that sense. Indeed, hypericin is able to provoke a burst of singlet oxygen in the Endoplasmic Reticulum (ER) crucial for membrane exposure and release of DAMPS but also for causing an efficient cell death together with the ability to trigger anti-tumor immunity.[9]

2. Low Dose vs High Dose PDT

The cellular response to PDT strongly varies with the intensity of the treatment; i.e. low *vs* high dose PDT (Fig.1). When tumor cells are subjected to a low intensity PDT stress, they react by activating various signalling pathways allowing the cells to adapt to the modified redox cell homeostasis and to proliferate.[10] Several kinases involved in redox-controlled signalling pathways have been shown to be at least transiently activated such as MAP kinases p38 and c-Jun, PI-3K/AKT, p53, CDKs,...(Fig.1).[11] Activation of these signalling pathways ultimately lead cells to better survive to these new conditions and allow them to proliferate. Under these non-lethal PDT stress conditions, kinases important to initiate autophagy were shown to be activated.[12] Autophagy is primarily seen as a complex pathways ultimately leading to the sequestration of portions of cytoplasm in double-membrane vesicles which will fuse with lysosome to form autophagolysosomes in which the autophagic cargo will be degraded. Autophagy is then generally accepted as being a response protecting cell from injuries.[13] However, when autophagosomes are accumulating under higher stress level or when several

essential autophagy genes are deleted, autophagy can mediate cell death as an effector mechanism known as autophagic cell death.[14]

When stress exceed a critical duration or overcome an intensity threshold, cells start to die by apoptosis or necrosis (Fig.1).[15] This threshold can be considered as the level of oxidative damage a tumor cell can support before triggering cell death mechanisms. It is determined by many factors such as for example the level of anti-oxidative defense. In this sequence of events, autophagy and apoptosis often occur in a sequence in which autophagy precedes apoptosis.[13] In a similar way, autophagy also precedes necrosis. In L929 fibrosarcoma cells, it has been shown that autophagy can degrade catalase, an enzyme that detoxify hydrogen peroxide, thereby promoting necrosis[16]

3. **Activation of intracellular signalling pathways by PDT**

When tumor cells are subjected to low doses of PDT, the intracellular generation of singlet oxygen leads to a low cytotoxic effect because oxidation products generated in nucleic acids (guanine base oxidation, alkali-labile sites,..), lipids (lipoperoxides,..) and proteins (protein oxidation, protein cross-links,..) are either repaired or if left unrepaired are without drastic physiological consequences that could threaten cell survival. In this situation, tumor cells are known to adapt to the modified redox-controlled cellular context and one important adaptation is by turning on or off several redox-sensitive transduction pathways leading to the activation or repression of transcription factors and thereof to the activation or repression of gene transcription and mRNA translation.[17] Tumor cells have therefore intrinsic sensors to detect changes of the redox balance caused by singlet oxygen production. Among the sensors important for cell adaptation are

kinases and, particularly, redox-sensitive kinases such as protein tyrosine kinases (PTKs)[18] and kinases with redox-sensitive cysteine residues.[19] Receptor tyrosine kinases such as insulin receptor, Epidermal Growth Factor Receptor (EGFR) and Platelet-Derived Growth Factor Receptor (PDGFR) have been shown to undergo direct oxidation.[20] In the case of EGFR the direct oxidation of cys797 in the kinase active site enhances the activity of the receptor and of the dependent signalling pathway.[21]

Among the signalling pathways that are not dependent on PTKs, the NF-κB[22] and the p53[23] pathways turn out to be among the most important and, interestingly, were shown to be active over a rather large range of PDT stress intensity. Indeed, cell culture where PDT is leading to more than 50% cytotoxicity, NF-κB can still be seen translocated in the nucleus exerting its anti-apoptotic effects.[24]

The transcription factors NF-κB belong to the Rel family and is composed of five members: RelA (p65), p50, p52, RelB and c-Rel.[25] They work as hetero- or homo-dimers. In a resting cell, these dimers are sequestered in the cytoplasm by inhibitors of the IκB family such as IκBα, IκBβ, IκBε, p100 and p105. Following the so-called canonical pathway, ligand activated receptors belonging to the TNF receptor family, to IL-1 receptor family including Toll-like receptors lead to the phosphorylation of the inhibitors (mainly IκBα) on serine residues, to its K48 ubiquitination and degradation by the proteasome freeing NF-κB which is then translocated to the nucleus where it can activate gene transcription by RNA polymerase II (Fig.2). IκBα phosphorylation has been shown to be done by the IKKβ subunit of the IKK complex in the canonical pathway (Fig.2). For the IKK complex to be active, the NEMO subunit should undergo a linear polyubiquitination by LUBAC.[26] The detailed mechanisms by which the IKK complex and particularly

the IKKβ subunit is activated is still the subject of controversies and intense research since it varies upon the nature of the receptor which is situated upstream in the signalling pathway.

In many cancer cell types, NF-κB is fully inducible by ligand-receptor activation such as for example in HeLa cell (cervix cancer), T24 (bladder cancer), HCT116 (colon cancer), HEK293 (kidney cancer), MCF-7 (breast cancer),… meaning that in the resting cells NF-κB is sequestered in the cytoplasm and translocating to the nucleus only after receptor ligation. In other cancer cell types however, NF-κB can be present in the nucleus without the need of any receptor ligation due to the constitutive IKK activity or mutations in receptor associated proteins. This is the case of some cancer cells of neuronal origin[27] or several hematological cancer cells.[28] Therefore, NF-κB activation by PDT has only been seen in cell lines where it remains absent from the nucleus of non-activated cells and where the signalling is tunable. Similarly, the lack of NF-κB activation by PDT can be caused by the nature of the cancer cells studied or by the nature and/or the cellular localization of the photosensitizer which is used.

4. NF-κB signalling pathway activation by PDT

The first demonstration that NF-kB activation can be achieved by photosensitization has been done in 1993 by Ryter et al who showed that a 10-fold increased of NF-κB activity can be detected in the nucleus of mouse leukemia L1210 cells after photofrin mediated photosensitization.[29] This work was rapidly followed by the works of Legrand et al[30] and by Piret et al.[31] In these two studies, NF-κB was shown to be fully actived in a T lymphoblastic cell line (ACH-2) by a DNA binding photosensitizer (proflavine) and by lysosomotropic photosensitizer

(Methylene Blue) and by a cytoplasmic photosensitizer (Rose Bengal). In these papers, NF-κB activation was demonstrated by the presence of the heterodimer RelA/p50 in the cell nucleus after irradiation using a band shift assay method and by following IκBα degradation by Western Blot. Using HeLa cells and Photofrin as photosensitizer, Kick et al demonstrated that interleukin-6 (IL-6) can be released in cell supernatant after gene transcription directed by AP-1 but not by NF-κB.[32] This result may be seen as unexpected since the promoter of the IL-6 encoding gene is under the sole control of NF-κB; AP-1 playing only a very minor role in the control of the transcription of this gene.[33]

Following these initial works, Matroule et al have investigated the mechanism by which NF-κB is activated in colon cancer cells (HCT116 cells) by two pheophorbide derivatives (PPME, pyropheophorbide methylester and APP, aminopheophorbide) localized in the cell membranes.[34,35] These works uncovered a rather interesting mechanism with two NF-κB activation waves: (i) a rapid and transient phase taking place up to 1h after irradiation and (ii) a slow and long lasting NF-κB activation maintaining NF-κB up 24h in the nucleus (Fig.3). Analysis of the IκB inhibitory proteins by Western Blot demonstrated that IκBα was the only inhibitory protein involved; IκBβ, p100 and p105 remaining unaltered by the PDT treatment. The analysis of the signalling pathways led to unexpected results since the rapid phase was due to sequestration of the IL-1 signalling machinery by the pheophorbide derivatives activating the TRAF6 protein (likely by K63 ubiquitination) and then the IKK complex. This rapid phase of NF-κB activation was increased by isotopic substitution demonstrating that singlet oxygen was important in triggering the rapid phase. The sequestration of the IL-1 signalling pathways by the pheophorbide derivatives mediated photosensitization was

specific since there was no effect on other pathways such as the Tumor Necrosis Factor (TNF) signalling shown by the lack of effect of a TRAF2 dominant negative mutant. When the sequestration of the IL-1 signalling machinery is deactivated (i.e. between 1 and 2 hours after irradiation), a second slower and long-lasting phase was starting. This phase involved lysosomes and the cytoplasmic release of ceramide (Fig.3). Ceramide release was likely due to the stimulation by PDT of an acidic sphingomyelinase activity present in the lysosomal membrane. Interestingly, none of these two NF-κB activation phases could be inhibited by water-soluble or lipid-soluble antioxidants demonstrating a close interaction between the pheophorbide derivatives and the IL-1 receptor machinery during the initiation of the first rapid wave of NF-κB activation. Therefore, these experiments tend to rule out the role of ROS in the IKK activation. This was further reinforced by the expression of IκBα mutated proteins. Indeed, pheophorbide mediated NF-κB activation was totally inhibited in a cell line expressing the IκBα protein where the two serines 32 and 36 were mutated in alanine. On the hand, expression of the IκBα protein where the tyrosine residue 42 was mutated does not affect NF-κB activation; this tyrosine residue being phosphorylated under oxidative stress conditions by c-Syk.[36,37]

The mechanism of NF-κB activation in non-transformed endothelial cells has been investigated after low dose PDT with the same pheophorbide derivatives.[38] This was shown to promote a very efficient NF-κB activation in endothelial cells. Interestingly and by opposition to what was seen in colon cancer cells, the activation is occurring in a single wave leading to a rather sustained nuclear translocation of NF-κB (up to 24h). In this cell type, NF-κB activation is ROS-

dependent and IKK-independent underscoring again the influence of the cell type in the nature of the signalling pathway that is involved in NF-κB activation.

NF-κB activation in a promyelocytic cell line (HL-60 cells) was also studied after PDT mediated by a benzoporphyrin derivatives (BPD, Verteporfin) both at high or low doses.[39] At high dose of PDT, HL-60 cells undergo apoptosis and the analysis of the IκB proteins by Western Blot showed that the level of IκBβ remained unaffected while IκBα was degraded in a caspase-independent pathway. At low dose PDT, a very rapid and important NF-κB activation was observed by band shift assay. Using luciferase reporter gene assay, it was shown that the RelA/p50 heterodimer was transcriptionally active capable of replenishing the level of IκBα protein levels in the treated cells.

Unexpectedly, an ER-localized photosensitizer like hypericin was shown to be unable to efficiently activate NF-κB in two susceptible cell lines such as T24 and HeLa cells.[40] The reason for this lack of NF-κB activation by hypericin is still unclear despite the fact that other signalling pathways such as p38 MAPK and JNK were heavily activated in similar conditions. Very interestingly, hypericin mediated PDT in these two cell lines was shown to strongly activate the expression of the cyclo-oxygenase 2 (COX-2) expression whose gene transcription is controlled by NF-κB. This apparent discrepancy is in fact due to a post-transcriptional mRNA stabilisation under the p38 MAPK control leading to an increased translation of the COX-2 protein and not by a transcriptional activation of the COX-2 gene by NF-κB.[40]

Recently, it was shown that low-dose PDT treatment of melanoma B78-H1 cells with pheophorbide leads to NF-κB activation which can block apoptosis and allow cells to recover from the injuries caused by the treatment.[41] This has been

correlated with a low level of NO production, the expression of the anti-apoptotic Snail protein and the reduction of the expression of pro-apoptotic RKIP. The role played by NF-kB in the modulation of Snail and RKIP was shown to be central in the cellular rescue occurring in cells receiving a low-dose PDT. Rather similarly, breast cancer cells treatment with ALA-PDT at a dose causing about 20 % of cell apoptosis allowed to visualize NF-kB nuclear translocation by Western Blot or confocal fluorescence microscopy.[42] Based on the use of several pharmacological inhibitors, it was suggested that the signaling pathway leading to NF-kB activation was involving the axis PI-3K, AKT and the IKK complex. Importantly, these authors also demonstrated that the NF-kB controlled up-regulation of the inducible nitric oxide was at the origin of a NO release by these cells which effectively counteract the pro-apoptotic effect of ALA-PDT. [42]

5. PDT pro-inflammatory effects are under the control of NF-κB.

In human cells, there are about 500 genes whose transcription is activated by NF-κB.[43] Among these genes those coding for proteins having immune functions are rather numerous (Fig.4).[44] Indeed, many pro-inflammatory cytokines such as IL-1, TNFα, IL-6, chemokines such as IL-8, MCP-1 and MIP-1α and adhesion molecules such as ICAM-1, VCAM and selectins have been shown to be under the control of NF-κB.[43] Pro-inflammatory cytokines have been shown to be produced by tumor cells after PDT both in vitro and in vivo and in several settings, i.e. various tumor types and photosensitizers.[45] The extracellular release of pro-inflammatory cytokines and chemokines after PDT is responsible for the inflammatory response associated to the treatment and the influx of neutrophils and other immune cells to the tumor but also important for the cross-talk between

the tumor and its vasculature.[46] Indeed, PDT has been shown to be capable of leading to the expression of several adhesion molecules in endothelial cells essential for the chemo-attraction of immune cells from the peripheral circulation into the tumor.[47]

In conditions of low dose PDT mediated with PPME, endothelial cells released many chemokines such as IL-8, MCP-1, I-309 and L32 and NF-κB is instrumental in these effects.[48] Both transcriptional and post-transcriptional effects are needed for an efficient release of these molecules. Indeed, cytokine- and chemokine-encoding mRNA are rather unstable and oxidative stress conditions such as those obtained during PDT allow mRNA stabilization through the binding of factors in the 3'-UTR regions.[49]

In vivo studies with the mammary EMT6 tumor model, PDT with HPPH induced an up-regulation of the adhesion molecule E-selectin in the tumor-associated microvasculature.[50] On the other hand, PPME when used to photosensitize endothelial cells promoted the transcription of genes encoding for several adhesion molecules but the translocation of these proteins from the ER to the plasma membrane can sometimes be altered by the oxidative stress caused in the ER which redirect adhesion proteins to degradative compartments such as lysosomes.[48] Therefore such pheophorbide derivatives could impair immune cell attraction to the tumor by down-regulating adhesion molecules expression in endothelial cells; a relevant event in the search of PDT conditions that can augment the dialog between the injured tumor and the immune system.

6. NF-κB activated by PDT is anti-apoptotic.

In addition to genes coding for pro-inflammatory cytokines and chemokines, there is another set of genes controlled by NF-kB which is important for the tumor adaptation to the PDT stress (Fig.4). These are genes coding for proteins with anti-apoptotic functions such as for example Bcl-2, Bcl-XL, Mcl-1, and survivin.[51]

When tumor cells are treated with pheophorbide derivatives in conditions yielding up to 50 % cell apoptosis, NF-κB translocated into the nucleus playing pro-survival functions.[52] Indeed, inhibition of NF-κB activation in these conditions by over-expressing a dominant-negative version of IκBα, called super-repressor which does not allow NF-κB to be liberated from its inhibitory molecules, sensitized colon cancer cells to apoptosis. Bcl-2 protein phosphorylated on serine 70 seems to be important in the protection against apoptosis in these conditions.[52]

From these data, it is obvious that induction of NF-κB in tumor cells dying by apoptosis after PDT, should be considered as a brake to cell death. Pharmacological inhibition of NF-κB in this situation could be a way to sensitize tumor cells to apoptosis.

In glioblastoma cell lines treated by high dose ALA-PDT (less than 50 % cell survival), a rather weak NF-κB activation can be observed.[53] This is likely because these cells already have a constitutive IKK activity in their cytoplasm. However, despite this rather low activation, NF-κB inhibition by both genetic and pharmacological means strongly sensitize glioblastoma to cell death.[53] Analysis of several apoptosis read-outs indicated that glioblastoma cells did not die by apoptosis but rather by necrosis.[54] Indeed, a necrosome containing both RIPK-1 and RIPK-3 has been shown to be formed very quickly after irradiation. Very interestingly, a transient autophagy flux has been also identified in these conditions.[54] Therefore inhibition of both autophagy and IKK complex activity

strongly sensitized glioblatoma to necrosis. Knowing the difficulty to eliminate glioblastoma cells and to avoid their high recurrence, ALA-PDT performed in the presence of NF-κB and autophagy inhibitors could provide patients with treatment modalities improving their survival to this deadly cancer.

7. Conclusions

Despite its use as a first line treatment for actinic keratosis,[55] PDT is still not enough considered as an important modality for the treatment of many solid tumours.[1] Among the numerous factors at the origin of this lack of recognition by many oncologists, the large heterogeneity in the cellular responses to PDT is one of these factors. Experimental evidences discussed in this perspective about the molecular signalling leading to the activation of NF-κB showed that the tumor cells respond to PDT in large variety of manner. Based on the photosensitizer which is used, its intracellular localization and its physico-chemical properties, the inducibility of NF-kB will largely depend on the nature of the tumor cell type and on its interaction with the vasculature.

Analysis of the molecular pathways leading to NF-κB activation by PDT revealed that this transcription factor is situated at the crossroad between the tumor biology and the immune system. Unfortunately, it is a double-edge sword. NF-κB is certainly important for the immune cells attraction in the tumor and therefore in establishing an immune response that should avoid recurrence. On the other hand, it also protects tumor from death both by apoptosis and necrosis. Pharmacological inhibition of NF-κB during PDT should therefore be performed with caution and after showing that tumor cell eradication is improved without interfering with an immune response.

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Figure captions.

Figure 1:

Cellular responses to PDT vary with the intensity of the treatment. At non-lethal PDT stress levels (Low dose PDT), cells react by activating several signalling pathways leading to proliferation or and autophagy. Above a certain threshold, PDT causes cell death (High dose PDT) and start to switch on distinct signalling pathways leading to apoptosis or necrosis.

Figure 2:

NF-κB canonical activation pathway. Upon ligation of IL-1 on its cognate receptor, TRAF6 is K63-polyubiquitinated (in pink) allowing the IKK complex to be activated by phosphorylation of the IKKβ subunit and linear polyubiquitination of NEMO (in yellow) by the LUBAC complex. This leads to the phosphorylation and the K48 polyubiquitination of IκBα (in blue) which is then degraded by the proteasome allowing the NF-κB transcription factor (RelA and p50) to translocate to the nucleus and activate transcription of target genes by RNA polymerase II.

Figure 3:

PDT treatment of colon cancer cells by PPME or APP is inducing an intense and rapid activation of NF-κB. The rapid phase is triggered by the pheophorbide derivative at the level of the IL-1 receptor causing activation of the IKK complex, phosphorylation of IκBα and its K48 polyubiquitination and degradation by the proteasome. The late phase is taking place after the activation of an acidic sphingomyelinase in the lysosome membrane and the release of ceramide which

is also able to activate the IKK complex. These two pathways allow NF-κB to translocate into the nucleus and to activate gene transcription.

Figure 4:

Cellular responses caused by the PDT-mediated NF-κB activation. At low dose PDT, NF-κB causes cell proliferation and the release of pro-inflammatory cytokines, chemokines and prostaglandins. At high dose of PDT, cell apoptosis and necrosis are down-regulated by NF-κB.

Fig.1

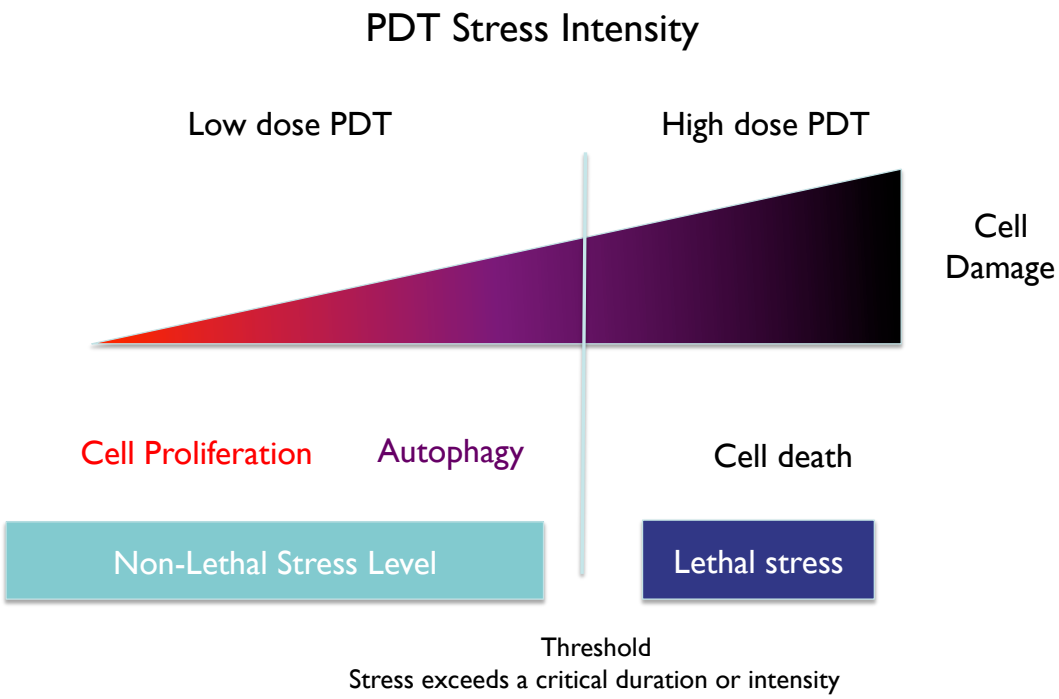
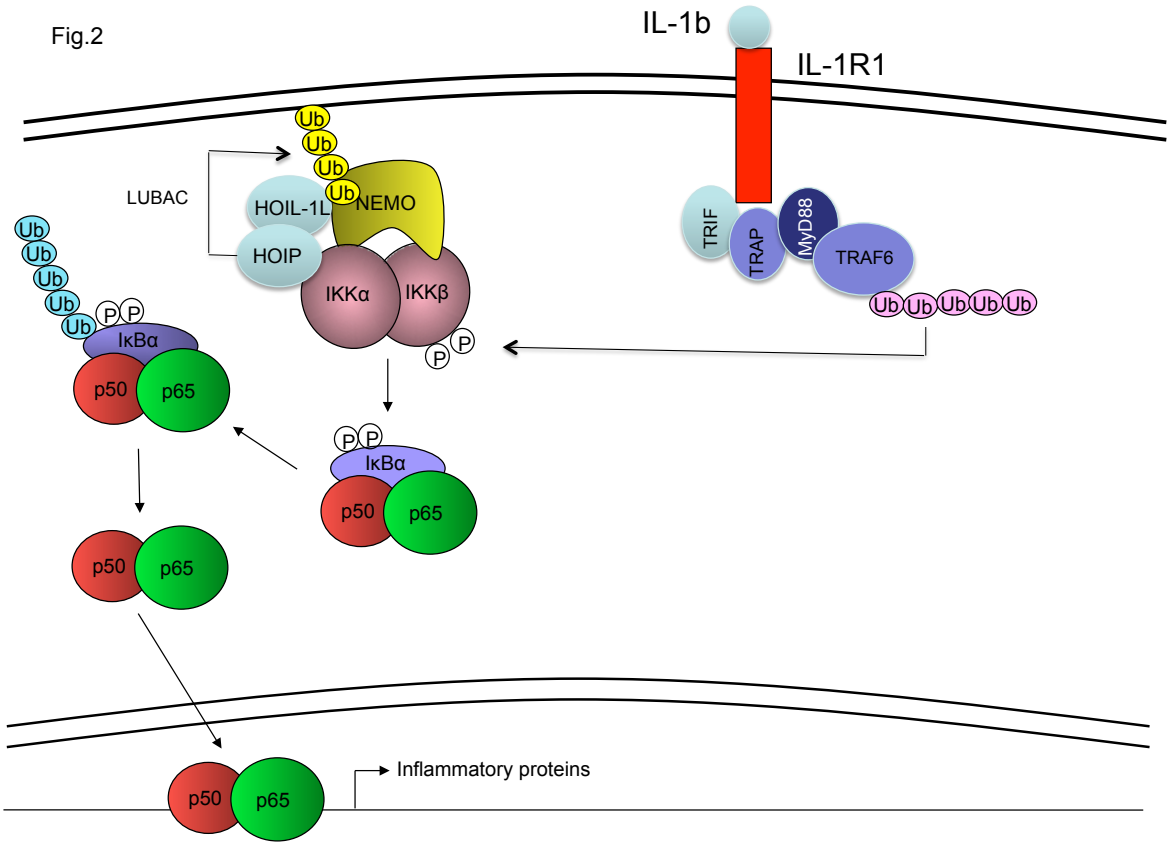


Fig.2



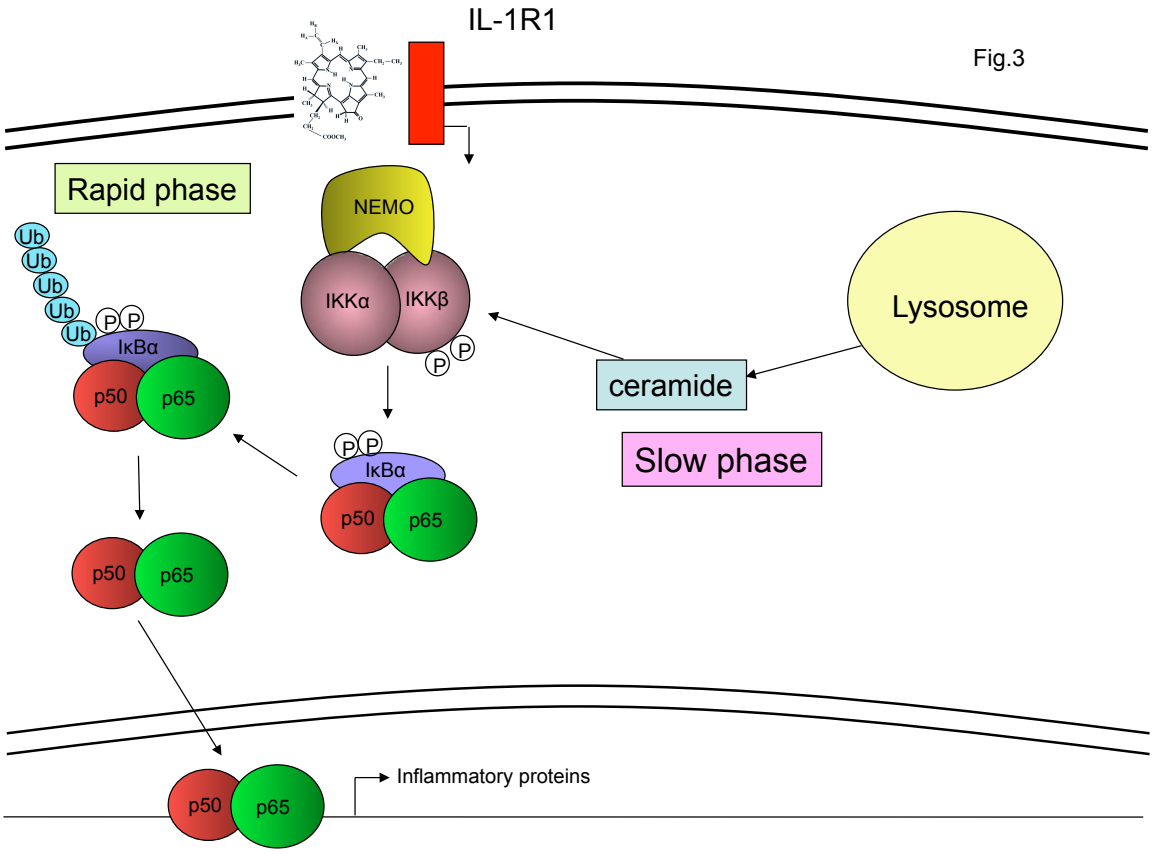


Fig.3

Fig.4