Regulation of Phospholipid Hydrolysis and Signaling by $cPLA_2$ and $sPLA_2$ in Activated Macrophages

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February 10, 2015

Transcription of the lecture presented on Tuesday, February 10, 2015 at the 6th International Conference on Phospholipase A_2 and Lipid Mediators, Tokyo, Japan (Slides 1 & 2).

Human blood monocyte-derived macrophages are very large cells and thus contain a high amount of lipids. These cells are relatively "easy" to transfect, which provides us with a useful model of a primary human cell to study long-term activation responses (Slide 3 – Human Blood Monocyte-Derived Macrophages) [1]. Depending on the stimulus, long-term activation of macrophages may result in two different kinds of responses (Slide 4 – Macrophage Polarization). On one hand there is the classical response or M1, which leads the macrophages to acquire a proinflammatory phenotype and is triggered by stimuli like LPS or IFNγ and leads to the secretion of proinflammatory cytokines such as those indicated in the figure; and on the other hand there is the M2 or alternative response, anti-inflammatory, pro-resolving, IL-4 is the typical stimulus and ultimately leads to the up-regulation of anti-inflammatory genes such as IL-10, TGFβ or ARG-1 among others. Well, human macrophages express a number of PLA_2s, $cPLA_2$, several $iPLA_2$s, $sPLA_2$-V, but not –IIA or –X (Slide 5 – PLA_2 Expression in Human Macrophages) and our interest was to analyze these genes under conditions of macrophage activation to M1 or M2. So, we stimulated the cells with LPS+IFNγ to obtain M1 macrophages, and Il-4 to obtain M2 macrophages (Slide 6 – Expression of Genes During Human Macrophage Polarization). The first thing we did was to make sure that the cells worked as expected, which was the case; M1 macrophages up-regulated pro-inflammatory genes, and M2 up-regulated anti-inflammatory genes. So, what about the PLA_2s? (Slide 7 – Expression of PLA_2s During Human Macrophage Polarization). What happens with the PLA_2s? Well we exposed them to LPS + IFNγ or IL-4 as before, same color code, and what we found was unexpected; LPS + IFNγ induced small or no increases in the expression levels of all these PLA_2s; however IL-4 induced the very strong expression of one gene, that of $sPLA_2$-V (Slide 7 – Expression of PLA_2s During Human Macrophage Polarization). This was unexpected since our previous experience with mouse cell lines had indicated that $sPLA_2$-V is upregulated by LPS; well, not in human macrophages [2-5]. This is a time-course of $sPLA_2$-V protein production by immunoblot, showing a 5-6-fold increase in protein after 24 h treatment with IL-4 (Slide 8 – Induction of $sPLA_2$-V Protein During Macrophage Activation by IL-4). Well, we wanted to determine whether this $sPLA_2$-V increase was a peculiarity of the IL-4 treatment or rather was a general feature of the M2 phenotype. To study this, we used other stimuli that induce M2 activation of macrophages, i.e. C-MSF and IL-10 (Slide 9 – Induction of $sPLA_2$-V Protein by Other M2 Stimuli). IN both cases $sPLA_2$-V was clearly augmented which tells us that $sPLA_2$-V can be regarded as a *bona fide* marker for the M2 activation state.

To study the role of $sPLA_2$-V in IL-4-activated cells we took advantage of siRNA inhibition and overexpression of the enzyme. (Slide 10 – $sPLA_2$ Activity of Human Macrophage Homogenates). When the cells were treated with IL-4 there was an increase in the $sPLA_2$ activity of the homogenates, which was almost completely blocked by our siRNA strategy. Overexpressing $sPLA_2$ led to increases in activity that were comparable to those found with IL-4. It is well established that IL-4 treatment leads to increased phagocytic capacity. These are macrophages exposed to zymosan, a classical phagocytosis stimulus [6-9] and you can see that the IL-4-treated cells showed a higher phagocytic response (Slide 11 – $sPLA_2$-V Depletion by siRNA Inhibits IL-4 Stimulated Zymosan Phagocytosis). What happens if we use cells depleted of $sPLA_2$-V by siRNA? Well, phagocytosis is clearly inhibited. This indicated that $sPLA_2$-V is important for the cells to show enhanced phagocytosis in response to IL-4. Next we used cells overexpressing $sPLA_2$-V and you can see that simply overexpressing the enzyme, leads to increased phagocytosis. Treating the overexpressing cells with IL-4 does not further increase the response, suggesting that we were already at the maximum with IL-4 or $sPLA_2$-V alone (Slide 12 – $sPLA_2$-V Overexpression Increases Zymosan Phagocytosis).

$sPLA_2$-V is an enzyme, so it seems logical to wonder whether these effects on phagocytosis are related to changes in phospholipid content (Slide 13 – Lipidomic Analyses). To answer this question we carried out lipidomic analyses by LC/MS [10-14]. The following slide shows the major PC species in human macrophages (Slide 14 – Phosphatidylcholine (PC) Species in IL-4-Treated Human Macrophages). When we treated the cells with IL-4 we saw no differences compared with the unstimulated cells. When $sPLA_2$-V-depleted cells were used, again we saw no changes in IL-4-treated cells versus untreated cells. I said before that these cells are very large and have lots of lipids, so we speculate that if the IL-4 response is small any effect would be obscured by the high amount of lipid present. This slide shows the major PE species and again no differences (Slide 15 – Phosphatidylethanolamine (PE) Species in IL-4-Treated Human Macrophages); PI species, again no differences (Slide 16 – Phosphatidylinositol (PI) Species in IL-4-Treated Human Macrophages). Next we went on to examine lysophospholipids, which are obviously the primary products of phospholipase A_2s. These are the major lysolipid species (Slide 17 – Lysophospholipid Species in IL-4-Treated Human Macrophages). As before, no differences between IL-4-treated versus control cells; however, when we examined the $sPLA_2$-V-depleted cells a very striking change was observed, highlighted by the yellow arrows. The levels of all lysoPE species decreased significantly. Note that the drop in lysoPE levels is observed in the IL-4-treated cells, but not in the otherwise untreated cells, indicating that the drop is related with the activation state of the cell. In other words, in the IL-4-treated cells there is a turnover of lysoPE whose levels are maintained by $sPLA_2$-V (Slide 18 – LysoPE levels are maintained by $sPLA_2$-V in IL-4-treated cells). So, what is the biological consequence of this finding? To study this we went back to our phagocytosis assay and what we wanted to determine is whether adding exogenous lysoPE has any effect on phagocytosis (Slide 19 – LPE Restores Phagocytosis in $sPLA_2$-V-Deficient Cells. Zymosan). As before, IL-4 increased phagocytosis of zymosan particles and the presence of lysoPE did not show any significant effect. But, what happens if we use $sPLA_2$-V-depleted cells? (Slide 20 – LPE Restores Phagocytosis in $sPLA_2$-V-Deficient Cells. Zymosan). The IL-4 response is inhibited and the addition of lysoPE almost completely restores the response, so lysoPE is substituting for $sPLA_2$-V under these conditions. This experiment was carried out using zymosan as the phagocytic stimulus. We repeated it using this time live bacteria, E. coli, as a phagocytic stimulus, and the results were the same, see, lysoPE restoring the IL-4 effect (Slide 21 – LPE Restores Phagocytosis in $sPLA_2$-V-Deficient Cells. Bacteria). We also assayed other lysolipids, lysoPC and LysoPI, and neither of these restored phagocytosis the way lysoPE did (Slide 22 – LPC and LPI Do Not Restore Phagocytosis in $sPLA_2$-V-Deficient Cells). As a conclusion of these experiments, we have seen that LysoPE is involved in IL-4-induced phagocytosis and that this lysoPE is produced by $sPLA_2$-V (Slide 23 – LysoPE Is Involved in IL-4-induced Phagocytosis). To study the mechanism for these $sPLA_2$-V effects, we thought it could be

important to investigate the compartmentalization of lysoPE synthesis, i.e. where in the cells is this lysoPE produced or, in other words, to determine the subcellular localization of the enzyme that produces it. To this end we used cells transfect with sPLA$_2$-V tagged to EGFP [4,5] (Slide 24 – sPLA$_2$-V Does Not Translocate to the Phagosome in Human Macrophages). These are experiments we published a few years ago [15]. In the resting cell, you can see the cytoplasm is studded with green dots which most likely represent secretory granules. When we put these cells to phagocytize zymosan, the red balloons, the green sPLA$_2$-V dots in the cytoplasm disappear but we do not see any accumulation of green color around the particles, which suggests that the enzyme does not interact significantly with the phagosome. This is something that surprised us, since previous work by others had demonstrated that sPLA$_2$-V translocates to the phagosome in murine macrophages. Yet another striking difference between mouse and human cells. This behavior of sPLA$_2$-V also contrasts with that of cPLA$_2\alpha$, which does translocate to the phagosome (Slide 25 – cPLA$_2\alpha$ Translocates to the Phagosome in Human Macrophages). These are cells transfected with EGFP-cPLA$_2$ [16,17], exposed to zymosan and you can clearly see the translocation of the enzyme, shown even better in pseudocolor. We have also shown that phosphorylation of cPLA$_2\alpha$ is required for translocation (Slide 26 – S505A cPLA$_2\alpha$ Mutant Does Not Translocate to the Phagosome) [18]. On the top there is the same as that shown in the previous one, translocation of the enzyme. If, however, we transfect a mutant where Ser505 is replaced with Ala which prevents phosphorylation, the enzyme does not translocate at all. In human macrophages, phosphorylation of cPLA$_2\alpha$ is catalyzed by JNK, as shown in this classical experiment using MAPK inhibitors [18]. The inhibitors for ERK and p38 do nothing, whereas the JNK strongly inhibits phosphorylation (Slide 27 – JNK Phosphorylates cPLA$_2\alpha$ During Zymosan Phagocytosis). We we assayed these inhibitors in the phagocytosis assay, here the control with the translocated enzyme, the inhibitors of ERK and p38 do little or nothing and here in the golden frame, the JNK inhibitor strongly blocks translocation (Slide 28 – JNK Inhibitor Blocks cPLA$_2\alpha$ Translocation to the Phagosome) [18]. Now, what is cPLA$_2\alpha$ doing in the phagosome? The results we have in this regard are certainly striking (Slide 29 – cPLA$_2\alpha$ Inhibition Modifies the Pattern of Phagosome Internalization). On top this is a macrophage after 2 h exposure to zymosan, the phagocytosed particles appear to be concentrated deep inside the cell, around the nucleus. However if we use pyrrophenone, a cPLA$_2\alpha$ inhibitor [19,20], there are many particles around the nucleus, but there are also many dispersed around the cytoplasm. And if we use cells depleted of cPLA$_2\alpha$ by siRNA, we see the same, many cells dispersed around the cytoplasm. Here on the right there is the quantification. So with this we can show a scheme like this indicating that there are two PLA$_2$s implicated in phagocytosis in human macrophages. On one hand, sPLA$_2$-V which we do not exactly where is working. We speculate perhaps at the plasma membrane, near the phagosome, where it hydrolyzes PE to generate lysoPE, which is required to regulate the extent, amount, of particles ingested. On the other hand, we have the cPLA$_2\alpha$, which does interact with the phagosome and somehow regulates internalization of the particles (Slide 30 – Distinct Roles for sPLA$_2$-V and cPLA$_2\alpha$ in Regulating Phagocytosis). And a last question that I would like to address is whether these two enzymes interact, whether there is cross-talk between them. And the answer is: yes, there is cross-talk. If we take cells depleted of sPLA$_2$-V by siRNA, put them to phagocytize zymosan and examine the phosphorylation state of cPLA$_2\alpha$, we find that there is a significant decrease; here on the right you have the quantification. So this is interesting because if sPLA$_2$-V regulates cPLA$_2\alpha$ phosphorylation and cPLA$_2\alpha$ phosphorylation is important for translocation to the phagosome; hence sPLA$_2$-V regulates cPLA$_2\alpha$ translocation to the phagosome (Slide 31 – sPLA$_2$-V Depletion by siRNA Inhibits cPLA$_2\alpha$ Phosphorylation). Just to conclude, thanks to the people of my lab, in particular Julio Rubio, a postdoc who carried out most of the results that I have presented today, many in collaboration of María Balboa and her lab in our institute; thank as well our sponsors, and thank you very much for your attention.

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