Plasmalogens, Polyunsaturated Fatty Acids, Ferroptosis, and Innate Immunity

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Polyunsaturated fatty acids such as arachidonic aid (20:4n-6) are indispensable components of innate immune signaling. Plasmalogens are phospholipids with a vinyl ether bond in the glycerol sn-1 position, instead of the more common sn-1 ester bond present in 'classical' glycerophospholipids. This kind of phospholipids is particularly rich in polyunsaturated fatty acids, particularly arachidonic acid. Beyond their involvement in the cellular metabolism of arachidonic acid, plasmalogens perform a varied number of functions. Membrane plasmalogen levels may determine parameters of the plasma membrane such as fluidity and the formation of microdomains that are necessary for efficient signal transduction leading to optimal phagocytosis by macrophages. Also, plasmalogens may be instrumental for the execution of ferroptosis. This is a non-apoptotic form of cell death that depends on iron.

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(**Slide 1** – Samuelsson's quote). It is important to keep in mind that not all membrane glycerophospholipids possess the same chemical structure (**Slide 2** – Not All Glycerophospholipids…). 80% of the total membrane glycerophospholipids contain two fatty acids, but 20-25% do not, because in the sn-2 position they contain a fatty alcohol. And among these, there are those with a double bond conjugated to the oxygen of the ether bond: the plasmalogens. In innate immune cells, ethanolamine plasmalogens are very abundant.

Plasmalogens serve a number of important roles in physiology and pathophysiology (**Slide 3** – Biological Functions of Plasmalogens), For example, as they lack the sn-1 carbonyl, plasmalogen packing is easier and thus increases membrane rigidity; the presence of the vinyl ether bond makes them excellent ROS scavengers; they participate in a number of signaling pathways and, what is the most important for our interests, they consitute a major cellular reservoir of polyunsaturated fatty acids in cells of the immune system. This figure shows the fatty acid composition of the ethanolamine plasmalogens of murine peritoneal macrophages (**Slide 4** – Plasmalogen Fatty Acid Composition in Murine Macrophages). Depending on the nature of the fatty alcohol at sn-1, there are three kinds of plasmalogens: those that contain palmitic alcohol, oleic alcohol, and stearic alcohol. You can cleary see that, in all cases, almost everything here is polyunsaturates, with arachidonic acid being, by far, the most prevalent fatty acid. And if you take a look at the species distribution of arachidonic acid among all kinds of phospholipids in the macrophages, you can see that plasmalogens rank among the most enriched species (**Slide 5**

– Arachidonic Acid-Containing Species in Murine Macrophages). A large number of receptors signal by activating intracellular phospholipases A_2 that generate lipid messengers (**Slide 6** – Role of Phospholipase A_2 in Arachidonic Acid Release). Therefore, it would be logical to suggest that Plasmalogens Must Constitute a Major Source of Free AA During Activation (**Slide 7**), and this is the first story I want to talk about today.

For these studies we were lucky enough to count with plasmalogen-deficient cells, generously donated by Dr. Raphael Zoeller, from Boston University. This is biosynthetic route of plasmalogens in mammalian cells, with the first part in the peroxisome and the rest in the endoplasmic reticulum (**Slide 8** – Plasmalogen Biosynthesis in Animal Cells). We had two mutant cells of the RAW macrophage cell-line The first one, called RAW.108, lacks the first enzyme of the pathway (**Slide 9** – Plasmalogen Biosynthesis in Animal Cells), and the second, called RAW.12, lacks that same enzyme and also the final desaturase (**Slide 9** – Plasmalogen Biosynthesis in Animal Cells). The first thing we did was to verify by LC-MS that these cells indeed lack plasmalogens. You can see from the slide (**Slide 10** – Phospholipid Species Composition of Plasmalogen-Deficient Cells) that both mutants certainly lacked not only the plasmalogens but also the ether-PCs, as expected. The interesting thing here is that there is a compensatory elevation in the levels of diacylphospholipids carrying AA, so that…. (**Slide 11** – Phospholipid Fatty Acid Composition of Plasmalogen-Deficient Cells) the final result is that mutants contain the same levels of AA, and of other fatty acids, than normal cells. The phospholipid class distribution is also unchanged. Thus it seems that the mutants compensate their deficiency in ether phospholipids by making more diacyl phospholipids, something which, if you think about it, makes sense. So we measured AA release in these cells stimulated by a variety of stimuli and, to our surprise, the mutants released the same amounts of fatty acid as the normal cells. This was certainly unexpected (**Slide 12** – AA Release by Plasmalogen-Deficient Cells). Consistet with the data, mutants also produced the same eicosanoids as the normal cells, both in quantity and quality (**Slide 13** – Eicosanoid Production by Plasmalogen-Deficient Cells). So the obvious conclusion to these data is that cellular plasmalogen status has no influence on the AA mobilization response of the macrophages (**Slide 14**). We were quite surprised by this finding which, of course, left us with our question of what are plasmalogen good for unanswered.

But the 'odd' behavior of AA-containing plasmalogens –of AA-containing ethanolamine glycerophospholipids in general–, does not stop here. If you look at a lipidomic analysis of the content of AA-containing species after stimulation, you will see that PC and PI species decrease with time, which is to be expected, because the PLA_2s are doing their job. Importantly however, the PE species, including he plasmalogens, do not seem to change greatly (**Slide 15** – Changes in AA-containing Species); they go their own way. And not only here, but also there (**Slide 16** – Delineating the Origin of Prostaglandins). By using two different isotopes and playing with the times of exposure, it is possible to differentiate the cellular pools of AA by class, by calculating an H/C ratio. I am not gonna get into the details of this because it is a bit complicated; just believe me that this can be done. Then we compared these ratios with those of the prostaglandins formed on stimulation of the macrophages. The idea is that the H/C ratio of the products should match that of the H/C of the precursors and, if so, we can establish precursor/product relationships. The H/C ratio for PGE_2 was very close to that of PC, strongly suggesting that PC was indeed the principal originator of the AA being converted to this PG. As for 6-keto-$PGF_{1\alpha}$, its H/C ratio was intermediate between those of PC and PI, consistent with a contribution of both phospholipid classes. Note in contrast the high H/C value for free AA, which was intermediate between that of PE and those of PC and PI, suggesting that free AA had been derived from all phospholipid classes.

To understand this 'odd' behavior of PE, we have to mention that there is a huge metabolic difference between PC and PI on one hand, and PE on the other hand, regarding the utilization of AA (**Slide 17** – The Sequential Incorporation of AA Into Phospholipids).When AA is provided to the cells, it is rapidly incorporated into PC and PI, but not in PE. AA is slowly incorporated into PE at the expense of PC, via CoA-independent transacylase (CoA-IT). Shown in real data, you can see this again in this time-course. It so happens that there is a compound that is capable of stopping this transfer from PC to PE almost completely. It is called SK&F98625 (**Slide 18** – The Sequential Incorporation of AA Into Phospholipids). Although this compound is of course not devoid of unspecific effects, we realized that its use could provide us with a nice tool to investigate the role of

AA in PE. With the inhibitor, we would have cells whose AA levels in PE are much lower than normal, and this could be useful under certain conditions.

Here I will open a short parenthesis to introduce you briefly to ferroptosis (**Slide 19** – Ferroptosis: a phospholipid-regulated form of cell death). This is a non-apoptotic form of cell death that depends on iron. Unlike other forms of cell death, ferroptosis does not use a specific protein effector, like a pore-forming protein or a receptor. Rather, lipid oxidation and the resultant membrane damage is the cause. Under normal conditions cells have mechanisms to control lipid peroxidation, which occurs either enzymatically via LOX or spontaneously. The major defensive effector is glutathione peroxidase 4 (GPX4). When this enzyme fails, phospholipid peroxides accumulate and, in the presence of iron, peroxyl radicals are generated, and they help propagate a radical chain reaction. As I said, cells possess several mechanism to counteract lipoperoxide accumulation, GPX4, Fe^{2+} levels, glutathione levels, etc. But there is one that, for me, stands above the rest: the cellular PUFA status, that is, how many PUFAs the cells have. This leads to the mechanism of AA entry into cells, that is, incorporation into PC, and subsequent remodeling into PE.

What is the interesting thing of this in relation to my talk? Well, it so happens that the phospholipids that acts as the (major/only) executioners of ferroptosis are precisely the PE molecules that become peroxidized. Accepting this, we reasoned that in cells treated with the CoA-IT inhibitor, we could have a very nice system to test and confirm a role for AA-containing PE species in innate immune cells. This figure shows ferroptotic cell death induced by RSL-3, a direct inhibitor of GPX4, in cells treated or not with the CoA-IT inhibitor. These are RAW264.7 macrophages. It seems pretty clear that cells treated with the inhibitor display resistance to RSL3-induced ferroptosis (**Slide 20** – Inhibition of AA Remodeling Prevents Ferroptosis). So, in all what these results are telling us is that AA accumulates so much in PE species, especially the plasmalogens, to endow the cells with a powerful and efficient means to die gracefully (**Slide 21**). This, of course, in addition to or independently of a role as free fatty acid providers for eicosanoid regulation.

Well, it seems we may have found out something that plasmalogens are useful for. As our studies on the role of plasmalogens in ferroptosis are quite preliminary, let me move to another story, this one with human macrophages to seek for more plasmalogen roles in innate immunity (**Slide 22** – Macrophage Polarization and Phagocytosis). Depending on the environment, macrophages can be in two different, opposing states of activation, which is called polarization (**Slide 23** – Polarized Activation of Macrophages). On the one hand, there is the classic or M1 response, which leads macrophages to acquire a proinflammatory phenotype and is triggered by stimuli such as LPS or IFNγ, and leads to the secretion of proinflammatory cytokines such as those indicated in the figure. On the other hand there is the M2 or alternative, anti-inflammatory, pro-resolving response, IL-4 is the typical stimulus and finally leads to the positive regulation of anti-inflammatory genes such as IL-10, TGFα or ARG-1 among others.

For those who do not know me, I am a phospholipase A_2 guy who enjoys trying to find new roles for these enzymes in cells. So we decided to study the effect on macrophage polarization on the many phospholipase A_2 genes that these cells express. We stimulated the cells with LPS + IFNγ to obtain M1 macrophages and IL-4 to obtain M2 macrophages (**Slide 24** – Expression of PLA_2s During Human Macrophage Polarization) and then we analyzed by qPCR the expression of the different genes. And what we found was unexpected. Only one phospholipase A_2 varied significantly, $sPLA_2$-V, and it did so under M2 conditions. Immunoblot analysis confirmed that the induction of the $sPLA_2$-V gene leads to a time-dependent increase in the protein (**Slide 25** – Induction of $sPLA_2$-V Protein During Macrophage Activation by IL-4).

What are the functional consequences of this IL-4-induced increase in $sPLA_2$-V? One of the most evident functional characteristics of M2-polarized macrophages is their increased phagocytic capacity. Therefore, we analyzed the ability of these cells to phagocytose zymosan, which is a homogenate of the cell wall of the yeast S. cerevisiae in the presence and absence of $sPLA_2$-V (**Slide 26** – $sPLA_2$-V Depletion Inhibits IL-4- Stimulated Zymosan Phagocytosis). As can be seen on the slide, IL-4 treated cells phagocytose much more zymosan than untreated cells. When cells deficient in $sPLA_2$-V were used, such an increase is not observed. The next slide shows the opposite experiment, the effect of overexpressing $sPLA_2$-V on the phagocytic capacity of cells (**Slide 27** –

sPLA$_2$-V Overexpression Increases Zymosan Phagocytosis). It can be seen that, simply by overexpressing the enzyme, the phagocytic capacity of the cells increases as much as if the cells had been treated with IL-4, thus making IL-4 treatment unnecessary to observe the same level of response.

sPLA$_2$-V is an enzyme, so it seems logical to assume that the effects of this enzyme on phagocytosis are related to changes in the cellular phospholipid content **(Slide 28** – Do these effects correlate…). So we carried out an extensive lipidomic analysis of these cells by LC-MS trying to find differences that could help explain the results. Just to cut the story short and not bore you with lots of lipidomic data, I will move directly to the meaty stuff, which is the analysis of lysophospholipids. Let me remind you that lysophospholipids are phospholipids specifically produced phospholipases A (**Slide 29** – Lysophospholipid Species in IL-4-Treated Human Macrophages). No differences between IL-4-treated versus control cells were observed; however, when we examined the sPLA$_2$-V-depleted cells a very striking change was observed, highlighted by the red arrows. The levels of some 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 30** – LysoPE levels are maintained by sPLA$_2$-V in IL-4-treated cells).

So, what is the biological consequence of this finding? To answer this we went back to our phagocytosis assay and what we wanted to determine is whether adding exogenous lysoPE had any effect on phagocytosis (**Slide 31** – LysoPE 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 32** – LysoPE 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,. Thus 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 33** – LysoPE Restores Phagocytosis in sPLA$_2$-V-Deficient Cells. Bacteria). The next slide shows that this effect of lysoPE is specific, since it is not reproduced by any other lysophospholipid, lysoPC or lisoPI (**Slide 34** – LPC and LPI Do Not Restore Phagocytosis in sPLA$_2$-V-Deficient Cells). Therefore, as a conclusion to this part of the talk, we have seen that lysoPE plays a key role in increasing the phagocytic activity of M2-polarized macrophages and that this metabolite is produced by sPLA$_2$-V (**Slide 35** – LysoPE is involved in IL-4-induced phagocytosis).

To deepen into these effects of sPLA$_2$-V, we thought it could be important to investigate the compartmentalization of lysoPE synthesis, that is, in which part of the cell this lysoPE is produced or, in other words, to determine the subcellular localization of the enzyme. that is producing it. For this purpose we used cells transfected with sPLA$_2$-V bound to EGFP (**Slide 36** – sPLA$_2$-V Does Not Translocate to the Phagosome in Human Macrophages). In the resting cell it can be seen that the cytoplasm is full of green dots that probably represent secretory granules. When we treat the cells with zymosan to start phagocytosis, the green dots of sPLA$_2$-V in the cytoplasm disappear most likely because the enzyme is being secreted, but we do not see any accumulation of green around the particles, suggesting that the enzyme enzyme does not significantly interact with the phagosome. This is something that surprised us, since previous work by others had shown that sPLA$_2$-V translocates to the phagosome in mouse macrophages. Another notable difference between human and mouse cells.

This behavior of sPLA$_2$-V is also in stark contrast with the behavior of another major phospholipase A$_2$ present in these cells, the cPLA$_2\alpha$ which does translocate to the phagosome (**Slide 37** – cPLA$_2\alpha$ Translocates to the Phagosome in Human Macrophages). These are EGFP-cPLA$_2\alpha$ transfected human macrophages, exposed to zymosan in red. The movement of the enzyme into the phagosome can be clearly seen, which is even better appreciated in pseudocolor. And since we are dealing with cPLA$_2\alpha$, we will say that this enzyme is phosphorylated/activated by MAP kinases at Ser^{505} and, if what we transfect is a mutant cPLA$_2\alpha$ that cannot be phosphorylated because we have replaced Ser505 with an Ala, the enzyme does not translocate at all (**Slide 38** –

S505A cPLA$_2$α Mutant Does Not Translocate to the Phagosome), which demonstrates the importance of translocation for this enzyme to manifest its functionality. In the upper part of the figure, the translocation of the enzyme is shown, as in the previous one. But if an S505A mutant is used, which prevents phosphorylation, the enzyme does not translocate.

What is cPLA$_2$α doing in the phagosome? The results we have in this regard are certainly surprising (**Slide 39** – cPLA$_2$α Inhibition Modifies the Pattern of Phagosome Internalization). A macrophage is shown at the top after 2 h of exposure to zymosan; the phagocytosed particles appear to be concentrated inside the cell, around the nucleus. However, if we use pyrrophenone, a cPLA$_2$α inhibitor, there are many particles around the nucleus, but there are also many scattered around the cytoplasm. And if we use cells deficient in cPLA$_2$α by siRNA, we see the same thing, many cells scattered around the cytoplasm. Here on the right is the quantification. With all this we can show a scheme like the one shown in this slide (**Slide 40** – Distinct Roles for sPLA$_2$-V and cPLA$_2$α in Regulating Phagocytosis), which indicates that there are two phospholipase A$_2$s involved in phagocytosis in human macrophages. On the one hand, the sPLA$_2$-V that we do not know exactly where it is working. We speculate perhaps in the plasma membrane, near the phagosome, where it hydrolyzes PE to generate lysoPE, which is necessary to regulate the spread or quantity of ingested particles. On the other hand, we have cPLA$_2$α, which interacts with the phagosome and somehow regulates the internalization of the particles. And one last question that I would like to address in this topic is if these two enzymes interact, if there is some kind of cross-talk between them. And the answer is yes, there is interaction. If we take cells deficient in sPLA$_2$-V by siRNA, put them to phagocytose zymosan and examine the phosphorylation status of cPLA$_2$α, we find that there is a significant decrease; here on the right is the quantification. So this is very interesting because if sPLA$_2$-V regulates cPLA$_2$α phosphorylation, and cPLA$_2$α phosphorylation is important for translocation to the phagosome, sPLA$_2$-V is regulating cPLA$_2$α translocation to the phagosome (**Slide 41** – sPLA$_2$-V Depletion by siRNA Inhibits cPLA$_2$α Phosphorylation). As I said before, cPLA$_2$α is phosphorylated by MAP kinases, so it is possible that sPLA$_2$-V regulates MAP kinase activation. I will get back to this in a moment. Therefore as a conclusion to this part, we see that there are two phospholipases regulating phagocytosis but their function is not redundant; one regulates the extension of the process and the other internalization (**Slide 42** – Two Distinct Phosholipase A$_2$s Regulate Phagocytosis in a Non-Redundant Manner).

And now for the last part of the talk, we return to our plasmalogen-deficient cell lines in order to dig a bit deeper into the role of this class of phospholipids. RAW cells are not very good at phagocytosing zymosan particles, so, as was done with previous experiments with human macrophages, it is necessary to opsonize the stimulus to get good responses. The mutants phagocytosed zymosan to a much lesser extent than normal cells, which is not particularly surprising (**Slide 43** – Phagocytosis of Zymosan by Plasmalogen-Deficient Cells). And guess what happens when the phagocytosis assay is performed with cells that have been treated with lysoPE (lysoplasmalogen vs lysophosphatidylethanolamine), in this case? (**Slide 44** – LysoPlsEtn Increases Phagocytosis in Plasmalogen-Deficient Cells). Yes, the answer is fully restored. These experiments are also notable because, under the same conditions, (acyl) lysoPE has no effect, so it is clear that the vinyl ether bond here is crucial.

As I said before, plasmalogens have profound effects on the biophysical properties of membranes, as they end to rigidize those in which they are abundantly present. To define this better we carried experiments to analyze the membrane fluidity of RAW 264.7 and RAW.108 cells by FRAP using confocal laser microscopy (fluorescence recovery after photobleaching), to analyze membrane fluidity (**Slide 45** – Analysis of Membrane Fluidity in Macrophages - FRAP). Basically what you do here is burn a defined area of the cell membrane and then measure the recovery of fluorescence over time. The more fluid a membrane is, the less time it will take to recover fluorescence. It can be seen here that the mutant cells recover more fluorescence than the normal ones, thus indicating that their membranes are more fluid. From these measurements a "mobile fraction (fluorescence recovery)" can be calculated such that the higher this value, the more fluid the membrane. When RAW.108 cells were exposed to lysoPlsEtn but not lysoPtdEtn, the mobile fraction of the membrane decreased, reaching values similar to those of normal RAW 264.7 cells. These data demonstrate that increased plasmalogen levels in

RAW.108 cells reduce cell membrane fluidity down to levels found in cells showing normal plasmalogen levels (**Slide 46** – Analysis of Membrane Fluidity in Macrophages - FRAP). That is, the plasmalogens contribute to rigidify the membranes.

And finally, again in line with the above, treatment of RAW.108 cells with lysoPlsEtn increased the phosphorylation/activation of p44ERK and p42ERK kinases, suggesting that plasmalogens enhance intracellular signaling originating from phagocytic receptors, which may be decisive for optimal phagocytosis (**Slide 47** – Lysophospholipid Effects on MAPK Signaling). And I brought this here because, as said before, we know that cPLA$_2$α phosphorylation is somehow regulated by sPLA$_2$-V, which in turn hydrolyzes plasmalogens, which in turn regulate MAP kinases. So this provides a working model to address at a molecular level the interactions between these two phoapholipases during phagocytosis.

So, as a conclusion to my talk, we can say that, contrary to all expectations, the presence or absence of plasmalogens in cells does not exert any influence on the eicosanoid production response of macrophages. But on the other hand, plasmalogens seem to be key to phagocytosis, and most likely too for ferroptosis. Plasmalogen levels determine properties of membrane lipids that may be essential for an adequate phagocytic response. So there is some kind of biological specificity that we need to keep characterizing (**Slide 48** – Biological specificity).

To conclude, thank the "plasmalogen crew" of my laboratory... (**Slide 49** - Acknowledgments). Thank you also to our collaborators and sponsors (**Slide 50** - Acknowledgments). A comprehensive list of significant papers from our laboratory, directly related to the topic under discussion, follows.

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