Novel Lipid Mediators of Innate Immunity and Inflammation

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This a transcription of the 'Alberto Sols Lecture' presented on Wednesday November 12, 2014 at the L Meeting of the Argentine Society of Biochemistry and Molecular Biology (SAIB) (Slide 1).

This cell is a human macrophage, derived from a blood monocyte, which is stained in blue with a protein of lipid metabolism called lipin-1 which localizes on the surface of these huge orange formations that tend to distribute on the periphery of the cytoplasm. These formations are lipid droplets and, as you can see, the macrophage contains many of them (Slide 2 – Untitled). If we take a closer look to these lipid droplets, what we see is something like that, a phospholipid monolayer decorated by a variety of proteins that encases a hydrophobic core composed, in macrophages, mainly of triglycerides (TAG) and cholesterol esters (CE). For many years, it was thought that these lipid droplets merely served as energy storage organelles to be used to meet the cellular demand of energy. Today we know that, in addition to this, lipid droplets serve a wide variety of biological roles. For the purposes of this talk, I have highlighted two of them, indicated here. In the first place, lipid droplets may function as platforms for signaling enzymes to dock and interact; this is particularly true for lipid signaling enzymes; lipin-1, as we have seen in the previous slide, cyclooxygenase-2, and cytosolic phospholipase $A_2\alpha$ (c$PLA_2\alpha$), all localize to this organelle. I will get back to c$PLA_2\alpha$ in a little while; this is the first enzyme in the eicosanoid cascasade, the one that opens the door to eicosanoid production by releasing free arachidonic acid (AA) from phospholipids [2,3]. In second place, lipid droplets have been found to play key roles in the development and progression of inflammatory metabolic disorders, of which the most common is cardiovascular disease (Slide 4 – Initiation of Atherosclerosis).

Atherosclerosis is a major cause for cardiovascular disease, and diabetes accelerates it [2]. Atherosclerosis is initiated by the abnormal activation of endothelial cells. Endothelial cells release a wide variety of products with inflammatory potential that may attract monocytes and favor the interaction of these monocytes with the endothelial cells, which results in the infiltration of the activated monocytes into the vessel wall. There, the monocytes will differentiate into macrophages and keep releasing proinflammatory mediators, thus perpetuating damage. Also, the macrophage will take up enormous amounts of lipids that have accumulated into that space (primarily cholesterol esters), store them into lipid droplets thus becoming foam cells, thus establishing an atheroma plaque. With time, smooth muscle cells from the tunica media will proliferate and reach the macrophage-rich area thus making things worse.

Among the many compounds secreted by endothelial cells that is, or has been the focus of our interest for so many years now: arachidonic acid (AA). Thus we took our human monocytes and exposed them to 10 μM AA, which is concentration of pathophysiological significance (Slide 5 – AA Induces Lipid Droplet Formation) [4]. Columns in the middle show the monocytes stained with DAPI to visualize their nuclei, and on the right column, you can see that monocytes exposed to this fatty acid produced lots of lipid droplets, stained in green with BODIPY. So these data provide an interesting concept, which is that the monocytes are bound to become a foam cell, and are starting to become one even before crossing the endothelial layer, even before to becoming an actual macrophage. This adds an interesting twist to the diagram shown in the previous slide, I believe. We also studied the effect of palmitic acid, a fatty acid that at much higher concentrations is proinflammatory [5]. However at 10 μM it did not induce any lipid droplet formation, thus suggesting that the AA effect is somewhat specific. Mass measurements confirmed that the AA-treated cells indeed produce elevated amounts of both TAG and CE (Slide 6 – AA Induces Neutral Lipid Formation) [4]. Of course, AA is a lipid (Slide 7 – AA Effects on Lipid Droplet Formation), so this elevated neutral lipid production could just occur as a consequence of a ‘passive’ incorporation of the fatty acid into neutral lipids. A second possibility is that AA actually activates the cells and thus neutral lipid production is the consequence of an ‘active’ signaling component which promotes the incorporation of other fatty acids in addition to AA. To distinguish between the two possibilities we used triacsin C, a compound that inhibits some members of the acyl-CoA synthetase family of enzymes and that, at least in monocytes, blocks the incorporation of the exogenous AA into neutral lipids (the ‘passive’ component) but not the incorporation of the endogenous fatty acids (the ‘active’ component). We took the monocytes and treated them with AA in the absence or presence of triacsin C (Slide 8 – TAG Fatty Acid Composition in Lipid Droplets) [4]. If you look on the left hand side, triacsin C blocked partially the production of TAG suggesting that the effect of AA works through both passive and active components. Now if you look on the right, this is the fatty acid profile of TAG, from left to right, myristic, palmitic, palmitoleic, stearic, oleic, linoleic and arachidonic acids. In the absence of triacsin C there is a huge incorporation of AA, however in the presence of the inhibitor this is totally prevented. Thus the inhibitor worked pretty nicely, but the important thing here is that no other fatty acid was affected by triacsin C; incorporation is the same whether or not triacsin C is present. So this highlights the active signaling component induced by AA and, because this is the phenomenon we are interested in and wish to characterize, from now on all the experiments include triacsin C. This slide shows the fatty acid profile of TAG and CE esters in the presence of triacsin C (Slide 9 – Fatty Acid Content of TAG and CE) [4]. The data on the left are the same as those in the previous slide. The profiles are very similar in qualitative terms in both cases, and of course there is no AA because of the presence of triacsin C. The important thing in this slide is the fatty acid in the purple box: palmitoleic acid. You can see there is very little in resting cells, and that it hugely increases in activated cells so that, in relative terms this is the fatty acid that increases the most. Now, for those in the the audience who work in atherosclerosis, diabetes, obesity, or lipid metabolism in general, you all know that palmitoleic acid is one of the “rising stars” of the field; it has been implicated in regulating inflammation; depending on conditions (species, concentration, origin) plamitoleic acid has been reported to exert anti- or pro-inflammatory actions; moreover, it has been suggested as well that this fatty acid functions as an adipokine, released by the adipose tissue to regulate lipid metabolism in liver. Thus our work adds to these results, and shows that activated monocytes synthesize palmitoleic acid and store it in significant quantities in the neutral lipids of lipid droplets.

I will get back to palmitoleic acid in a moment, but now please allow me to open a short parenthesis to discuss some data on the mechanism through which AA induces lipid droplets in the monocytes. That brings me back to cPLA$_2\alpha$, which is the enzyme depicted in this slide (Slide 10 – Group IVA Phospholipase A$_2$). The enzyme has two domains, on the left there is a C2 domain, a calcium and lipid-binding domain, and on the right the catlytic domain. Under resting conditions, the enzyme is cytosolic; however when activation occurs and intracellular Ca^{2+} rises, thanks to the C2 domain the enzyme associates to membranes, These red dots represent the calcium bound to the C2 domain, and this brings the catalytic domain in contact with the phospholipid substrate. What happens is that these two alpha helices open and this brings the catalytic side, in pink, in contact with the phospholipid. This enzyme shows a marked preference for phospholipids containing AA but it is important to emphasize that this does not mean that the enzyme cannot hydrolyze phospholipids without AA; it will, but a much slower rate. In blue there is an anionic binding site [6], which binds inositol lipids and phosphatidic acid and may function to support the interaction with the membrane and also for the enzyme to display full activity. Another important feature of this enzyme is that it is phosphorylated on Ser505, which is indicated with this grey dot in the figure. This phosphorylation is necessary for the enzyme to display full activity, and is carried out by members of the MAPK family, ERK, p38 and JNK [7]. Depending on cell type and stimulus the MAPK involved varies [2].

In this experiment we measured lipid droplet formation by flow cytometry (Slide 11 – Role of cPLA$_2\alpha$ in Lipid Droplet Formation). Thus the cells were treated or not with AA and treated afterward with BODIPY to stain the lipid droplets and, fluorescence was measured by flow cytometry. This shift to the right of the fluorescence of AA treated cells indicates that they have made lipid droplets. However, if we knock-down cPLA$_2\alpha$ by siRNA this fluorescence shift is not observed, indicating no lipid droplet formation. Thus, cPLA$_2\alpha$ is essential for the monocytes to make lipid droplets in the response to AA, which is an interesting conclusion because cPLA$_2\alpha$ is precisely the enzyme that mediates AA release from endogenous sources after receptor stimulation. Here, the situation is the other way around, exogenous AA activates cPLA$_2\alpha$ to produce lipid droplets. As indicated before, cPLA$_2\alpha$ is known to be phosphorylated in cells by members of the MAPK family and this phosphorylation activates the enzyme, so we studied next whether AA activates MAP kinases. We found that it activated p38, JNK, but not the ERKs (Slide 12 – AA Activates p38 and JNK) [4]. In accordance with these data, AA induces the phosphorylation of cPLA$_2\alpha$ (Slide 13 – AA Stimulates cPLA$_2\alpha$ Phosphorylation by both p38 and JNK) [4]. This phosphorylation is not affected by inhibitors of ERK, as expected since AA does not activate the ERKs. However, when both inhibitors are present, cPLA$_2\alpha$ phosphorylation is reduced at levels even lower than those found in unstimulated cells. Now, how does this relate with lipid droplet formation? (Slide 14 – Role of cPLA$_2\alpha$ in LD Formation) [4]. Here we have our monocytes with their nuclei stained in blue with DAPI. When treated with AA they make lipid droplets in green (BODIPY) and, in agreement with previous slides, if we block cPLA$_2\alpha$, in this case with a pretty well established inhibitor, pyrrophenone [8,9], lipid droplet formation is strongly inhibited, as expected. Now if we use the p38 inhibitor not very much happens, the cells still produce significant numbers of lipid droplets (Slide 15 – Role of cPLA$_2\alpha$ in LD Formation) [4]. The same occurs if we use the JNK inhibitor. However, if we use both at the same time, strong inhibition of lipid droplet formation is observed. Thus the same conditions that block cPLA$_2\alpha$ phosphorylation/activation lead to inhibition of lipid droplet formation. As a summary of this data, we show this model (Slide 16 – Simultaneous Activation of p38 and JNK by AA Activates cPLA$_2\alpha$), where AA activates p38 and JNK (but not ERK) and this two kinases act on cPLA$_2\alpha$ to activate it, so that the enzyme regulates lipid droplet synthesis. Two points to consider. First is that it is possible that p38 and JNK act both on cPLA$_2\alpha$ simultanously to activate it; however, since both kinases phosphorylate cPLA$_2\alpha$ on the same residue, this sems a bit odd, We hipothesize that maybe there is an intermediate kinase that is activated by both p38 and JNK, and this kinase is the one that directly phosphorylates cPLA$_2\alpha$. We are currently working in the lab to verify whether this hipothesis is correct. Second, how is cPLA$_2\alpha$ mediating lipid droplet formation? The answer is: we really do not know. We know however that cPLA$_2\alpha$ does not regulate the synthesis of neutral lipids, as shown in this slide, where inhibition of cPLA$_2\alpha$ with pyrrophenone does not modify TAG production in response to AA (Slide 17 – cPLA$_2\alpha$ Does Not Regulate Neutral Lipid Synthesis). Although we do not know what cPLA$_2\alpha$ is actually doing, we can always speculate with possible sites of action whitin a general mechanism for lipid droplet formation, thus we can speculate that some step of the formation, the budding of the lipid droplet out of the endoplasmic reticulum may be controlled by cPLA$_2\alpha$.

DGAT and ACAT, the terminal enzymes for TAG and CE biosynthesis, are homogeneously distributed along the endoplasmic reticulum and are able to synthesize a large amount of neutral lipid in response to different situations such as external lipid overload, endoplasmic reticulum stress or cellular activation. Initially, the neutral lipids formed are being accommodated into the hydrophobic space between the two leaflets of the endoplasmic reticulum up to a point where the two leaflets start to separate (Slide 18 –

Essential Role of $cPLA_2\alpha$ in Inducing Positive Membrane Curvature) [10]. The nascent lipid droplet needs a local positive membrane curvature in the endoplasmic reticulum membrane baseline to grow up. Accumulation of lysophospholipids, which have a wedge shape conformation (in green in the slide), generates a local positive membrane curvature, beneficial in terms of nascent lipid droplet budding. Since lysophospholipids are generated by phospholipase A_2s, the key role that $cPLA_2\alpha$ appears to play in these stages of lipd droplet formation can be easily explained. Once the required positive curvature at the lipid droplet baseline is generated, more neutral lipids can be accommodated inside the phospholipid monolayer, either synthesized in the endoplasmic reticulum or directly in the forming lipid droplet, where the enzymes necessary for TAG and CE biosynthesis are localized. This is accompanied by the synthesis of phospholipid to maintain the appropriate phospholipid to neutral lipid and also by remodeling of phospholipid acyl chains. Phosphatidylcholine molecules on the lipid droplet surface are particularly enriched in monounsaturated fatty acids, which does not occur with the molecules at the endoplasmic reticulum. Once the lipid droplet buds from the endoplasmic reticulum membrane, conversion of the conical lysophospholipids into cylindrical phospholipids is necessary to establish a neutral curvature that confers stability and protects the hydrophobic core from lipolysis. Finally, before the lipid droplet is completely formed, a local negative curvature in the baseline of the endoplasmic reticulum membrane is required. This is promoted by the accumulation of phosphatidic acid (in pink in the slide), which induces a spontaneous strong negative curvature. Phosphatidic acid is thought to be generated primarily by phospholipase D.

Thus the general idea behind all this is that, there must be an intimate relationship between $cPLA_2\alpha$ and the shape of the endoplasmic reticulum by controlling the ratio of phospholipid to lysophospholipid. In support of this we have some experimental evidence that $cPLA_2\alpha$ may indeed regulate the structure of the endoplasmic reticulum. This is an electron microscopy experiment carried out in the lab of our collaborator Enrique Claro from the Autonomous University of Barcelona (Slide 19 – Inhibition of $cPLA_2\alpha$ Alters the Endoplasmic Reticulum Structure). Here you can see that the control cells have been activated to generate lipid droplets, look at them so nice, and the structure of the endoplasmic reticulum appears completely normal. However, if we use $cPLA_2\alpha$-deficient cells, no clear lipid droplets are observed and there is, according to the electron microscopy guy, clear signs of abnormal endoplasmic reticulum tubulovesicular structures.

Well with this we close the parenthesis and come back to plamitoleic acid again. We were here in this slide (Slide 20 – Fatty Acid Content of TAG and CE; repeat of slide 9). The next question that we want to answer is, what is the origin of ths palmitoleic plus other fatty acids? (Slide 21 – Origin of the Fatty Acids – Possibilities). There are two possibilities: first is that the fatty acids come from membrane phospholipids. If this is the case, then total cellular fatty acid should remain constant. The second possibility is that AA activates de novo fatty acid synthesis. In this case, total cellular fatty acid should increase. Thus we measured total fatty acids in cells and the result is clear, there is an increase in cellular fatty acids, thus indicating that AA indeed activates fatty acid de novo synthesis to make lipid droplets (Slide 22 – Total Fatty Acid Content of Human Monocytes) [4]. On the right we have the fatty acid profile of whole cells, that is neutral lipids plus phospholipids, and still under these conditions a significant increase in palmitoleic acid levels is observed.

Four genes control fatty acid synthesis in mammalian cells (Slide 23 – Expression of Genes Involved in de novo Fatty Acid Synthesis). These are acetyl-CoA carboxylase, that makes malonyl-CoA. Malonyl-CoA is used by fatty acid synthase to make palmitic acid, which can be either elongated to stearic or desaturated to palmitoleic acid. Stearic acid can be desaturated to oleic acid by the same desaturase that makes palmitoleic acid. We measured these four genes by qPCR and found that all of them were increased by AA in monocytes (Slide 24 – Expression of Genes Involved in de novo Fatty Acid Synthesis) [4]. So as a summary of this part of my talk (Slide 25 – Lipid Inflammatory Signals Regulate Cellular Lipid Metabolism), we believe that our data constitute an excellent example of a lipid proinflammatory signal, AA, acting on its target cell, the monocyte, to deregulate lipid metabolism, in this case increasing fatty acid synthesis. Among other things, this has the effect of increasing the cellular amount of palmitoleic acid, which can be sent to lipid droplets or exert other effects on the cells. If under these conditions palmitoleic acid exerted an antiinflammatory effect, we would have here a quite paradoxical situations: outside the cells a proinflmmatory lipid promotes the accumulation inside the cell of an antiinflammatory lipid.

For you to see actual data indicating that palmitoleic may have proinflammatory properties under these settings, what this slide shows is an experiment where normal monocytes or monocytes loaded with palmitoleic acid are exposed to bacterial lipopolysaccharide, and activation of the inflammasome (IL-1β production) is measured (Slide 26 – Palmitoleic Acid (16:1) as an Anti-inflammatory Lipid?). We used lipopolysaccharide here just to obtain a very strong response [11]. It is clear that the cells enriched in palmitoleic acid produced less IL-1β after stimulation. As a positive control we used 22:6, an omega-3 fatty acid that is known to inhibit the inflammasome. Palmitoleic acid inhibited IL-1β at levels comparable to that of 22:6. Now, in this experiment palmitoleic is not floating around as a free fatty acid; it has been taken by the cells and incorporated into various cellular lipid classes. So, whatever the mechanism for this increased production of cytokines is, it is likely that the palmitoleic active molecule is a lipid ester and, because the overwhelming majority of the palmitoleic acid is in phospholipids, we speculate that this bioactive entity is a phospholipid that contains palmitoleic acid. So we set out to determine the nature of this phospholipid. By using GC/MS we determined first the distribution of palmitoleic acid between phospholipid classes, phosphatidylinositol, phosphatidylethanolamine, phosphatidylcholine, and phosphatidylserine in resting and AA-treated monocytes (Slide 27 – 16:1-Contaning Phospholipid Classes (GC/MS)). In resting cells the richest class in 16:1 is phosphatidylcholine, but this changes in the activated cells. In qualitative terms, the class that increases the most is phosphatidylinositol. There is little in resting but a clear increase is seen in actvated cells. This fact, along with a technical reason that I will mention later, made us focus on this class of phospholipids. By using LC/MS [12-16] we separated the molecular species of phosphatidylinositol (Slide 28 – Novel 16:1-PI Species That Appear After Activation (LC/MS)). To restrict the search and obtain only a few hits, we focused only on those palmitoleic phospholipids that showed up in activated cells but did not occur in resting cells. By doing this, we got two, maybe three species. The first one contains not one but two palmitoleyl lateral chains. The second one, which increased way more than the first one after activation, contains palmitoyl and palmitoleyl chains. Finally, there is maybe a third one, which contains stearic in addition to palmitoleic. Problem with this one is that it is isobaric with this other which is a major one. Isobaric means that they both have the same mass to charge ratio, thus we cannot resolve them with our machine. Thus at this point we cannot tell how much of this increase, if any, is actually due to the palmitoleic-containing lipid. Never mind, we still have two excellent candidates for our studies. So our strategy from now on is to make these lipids in the lab, to introduce them into the cells, and see what happens. Well, for doing this it is really fortunate that these lipids are of the inositol class, because inositol lipids are anionic, and anionic lipids can be transfected into cells just like you transfect DNA or RNA [17]. This is the technical reason I was referring to before. Thus, by using lipofectamine, lipofectin, or anything on that sort, you name it, you can get the lipid inside the cells and study its effects on a number of cell functions (Slide 29 – Intracellular Delivery of Anionic Phospholipids). At this point I have to stop the palmitoleic acid story here because we still have no data to show but, please let me talk instead for the rest of my talk about an unusual phospholipid, also a PI molecule, which does not contain palmitoleic acid, but two arachidonoyl tails (Slide 30 – 1,2-Diarachidonoyl-sn-glycero-3-phosphoinositol), because we believe this lipid may be involved in the regulation of innate immune responses in macrophages [14]. These experiments were conducted in murine macrophages, so from now to the end of the talk I will refer to these cells.

In this slide there is the full profile of AA-containing phospholipids of murine macrophages (Slide 31 – AA-Containing Phospholipids in Resting Macrophages) [14]. In red there are the choline phospholipids, in green the etanolamine phospholipids, in yellow the inositol lipids, and in pink the serine phospholipids. By class on the right, the richest class is the green, followed by the red. Lesser amounts in yellow and pink. Well what happens when we stimulate the cells? Of course AA will be liberated and the amount of these species will decrease. We used zymosan, which is a classical stimulus for macrophage activation [18,19]. This is what we have in this slide (Slide 32 – AA-Containing Phospholipids in Zymosan-Stimulated Macrophages) [14]. Most of the red phospholipids decrease significantly, as it does one of the yellow ones. The pink vary little and the green do not change at all. But this is well described so no point here. The points is, you see two lipids that actually increase, not decrease. The first one, red, is PC(20:4/20:4), but again this is no new, as this phospholipid was described some 25 years ago. However a second one, the inositol equivalent, PI(20:4/20:4) also increases and we believe this is really new stuff [12, 14, 20]. So we proceeded to characterize it. We found that only zymosan and its opsonized variant induced clear formation of this lipids; other did induce very little or nothing at all (Slide 33 – Stimulated Production of PI(20:4/20:4) in Macrophages), so its production seems to be stimulus-dependent. This lipid increases linearly with time after zymosan stimulation and tends to stay elevated, a behavior that is compatible with it playing a role in activation (Slide 34 – Stimulated Production of PI(20:4/20:4) [12]. Interestingly, if we do this experiment in the presence of pyrrophenone, which inhibits $cPLA_2\alpha$, production of this lipid is almost completely abolished, so yes, production of this lipid is another cellular function controlled by $cPLA_2\alpha$. Just for the sake of comparison, this the time-course of the major species PI(20:4/20:4) (Slide 35 – Stimulated Production of PI(20:4/20:4) [12]. This lipid goes down and pyrrophenone increases its levels, it does not decrease it. More, to verify whether formation of this lipid is of pathophysiological consequence we sought for it in a simple animal model of inflammation, mouse peritonits (Slide 36 – Production of PI(20:4/20:4) in Mouse Peritonitis) [14]. In this model, we inject zymosan in the peritoneum and this will result in the efflux of phagocytic cells, primarily neutrophils Thus we collect cells from the peritoneum at different times and measure PI(20:4/20:4), which you can see it clearly increases. On the opposite, this other species, PI(18:0/20:4), decreases. So this lipid could be pathophysiologically important and thus studied its function in cells. We prepared the lipid in the lab and introduced it in cells using the strategy I described before (Slide 37 – Incorporation of PI(20:4/20:4) Into Cells). We made the complexes, gave them to the cells, waited, stimulated with zymosan and looked for responses. Initially we focused on gene expression, because that is the most "fashionable" response one can measure. So we stimulated the cells, either untreated or loaded with PI(20:4/20:4) and measured the expression of various genes by qPCR (Slide 38 – PI(20:4/20:4) Does Not Regulate Gene Expression) [14]. Zymosan induced significant increases which were the same in control and in PI(20:4/20:4)-loaded cells. Also, the lipid did not do anything on its own. So it is clear that PI(20:4/20:4) does not regulate gene expression, which was quite a disappointing finding. However it got us thinking that perhaps we better look at short-term, acute responses. And among these, what a better response to measure than production of reactive oxygen intermediates, superoxide anion in this case? (Slide 39 – PI(20:4/20:4) Regulates Superoxide Anion Production) [14]. We stimulated the macrophages with either PMA or zymosan and obtained nice responses, which were significantly increased when PI(20:4/20:4)-loaded cells were used. Granted, the increases are not very impressive; however, when we used cells loaded with an irrelevant lipid, no increase was appreciated. More importantly, when we assayed another immediate response, that is, secretion of lysosomal hydrolases lysozyme, we observed again a significantly increased response when PI(20:4/20:4)-loaded cells were used (Slide 40 – PI(20:4/20:4) Regulates Lysozyme Release) [14].

So, as a conclusion of my talk, and this is my last slide (Slide 41 – Novel Lipid Mediators of Macrophage Activation), we have described novel lipid mediators of phagocyte activation, palmitoleic acid and PI(20:4/20:4). The fun starts now in the lab, as we have to define pathways and effectors impacted upon by these mediators. Just to conclude, I would like to thank all the people in my lab who have been involved in these projects, and to my collaborators, Dr. M. Balboa from my institute, and Dr. E. Claro from Barcelona, and also to our sponsors, thanks to whom our lipidomics work can continue without interruptions (Slide 42 – Acknowledgments). And I thank you very much for your attention.

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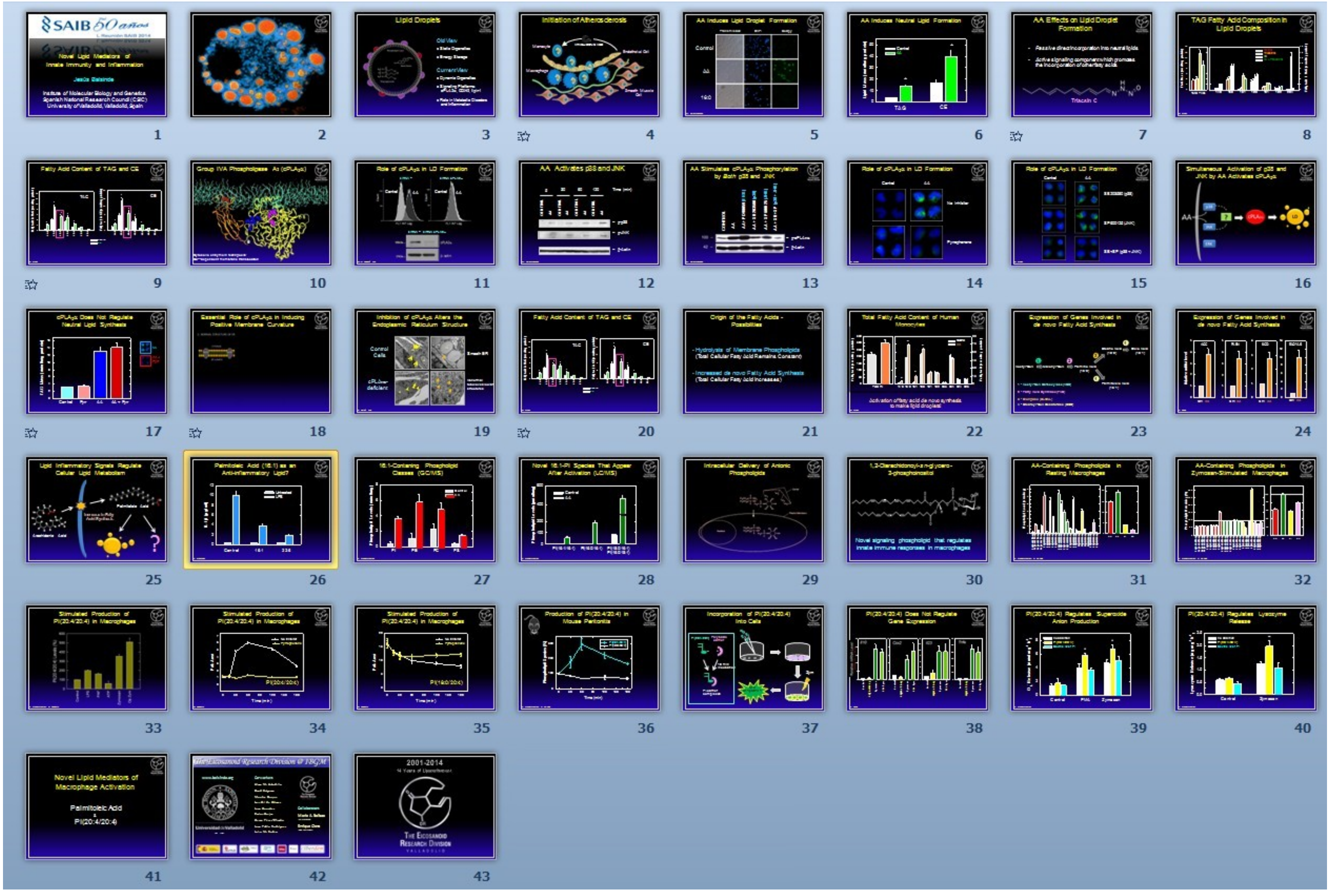
SAIB 50 años
Novel Lipid Mediators of Innate Immunity and Inflammation
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