Lipid Droplets and Phospholipid Metabolism in Macrophages

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This a transcription of the talk delivered on Tuesday April 29, 2014 in Madrid on occasion of the *3rd Madrid Meeting on Dendritic Cells and Macrophages*, initially titled "Bioactive Lipids and Lipidomics in Innate Immunity and Inflammation" (Slide 1), and later re-titled "Lipid Droplets and Phospholipid Metabolism in Macrophages" (Slide 2).

What you have in this slide (Slide 3) is a monocyte-derived human macrophage [1], stained in blue with a protein of lipid metabolism called lipin-1, which localizes on the surface of these huge cytoplasmic formations that tend to distribute in the periphery of the cells. These formations are lipid droplets and, as you can see, macrophages have many of them. If we take a closer look at one of these lipid droplets, what we see is something like this (Slide 4 – Lipid Droplets): a phospholipid monolayer decorated with a variety of proteins and inside a hydrophobic core composed of triglycerides (TAG) and cholesteryl esters (CE). Well, for many years these lipid droplets were thought of only as storage organelles for neutral lipids to be mobilized in the case of energy needs. Today we know that, in addition to that storage role, lipid droplets serve a wide variety of roles in cell physiology. For the purposes of this talk I will only highlight two of them. In the first place, lipid droplets may serve as signaling platforms for signaling enzymes to dock and interact; this is particularly true for lipid signaling enzymes; cytosolic phospholipase $A_2\alpha$, cyclooxygenase-2 or lipin-1, all localize to this organelle. 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 5 – Initiation of Atherosclerosis).

Atherosclerosis is major cause for cardiovascular disease, and diabetes accelerates it [2]. Atherosclerosis is initiated by the abnormal activation of endothelial cells, which is produced e.g. by increased lipid in the blood (dyslipidemia) or sugar in blood (diabetes). 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 monocyte will differentiate into macrophage and will take up enormous amounts of lipids that have deposited into that space (primarily cholesterol esters), store them into lipid droplets thus becoming foam cells, and 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 may affect monocytes there is one that interests us in particular , actually it has been the focus of our interest for so many years now, and I bet that those in the audience who know me will have rapidly guessed what I am talking about: arachidonic acid (AA), of course. Endothelial cells secrete relative large amounts of this fatty acid (pathophysiological range 5-10 μM). Thus we took our human monocytes and exposed them to 10 μM AA, as I just said, the pathophysiological concentration [3] (Slide 6 – AA Induces Lipid Droplet Formation). Middle columns 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, and 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 [4]. 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 7 – AA Induces Neutral Lipid Formation). An important point not to forget is that AA is a lipid (Slide 8 – 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 [5] and that, in our system, 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 9 – TAG Fatty Acid Composition in Lipid Droplets). 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 10 – Fatty Acid Content of Triacylglycerol and Cholesteryl Esters). 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, hence we suspect if must bear some biological significance. For those in 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 [6]; it has been implicated in regulating inflammation [7, 8], and 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 [9]. 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.

What happens to phospholipids, which are the major reservoirs of fatty acids in cells? (Slide 11 – Phospholipid Fatty Acid Content of Human Monocytes). You can see here that there is also an increase of palmitoleic acid in phospholipids, not as noticeable as in neutral lipids, but a significant increase after all. So as a conclusion of this part of my talk (Slide 12 – 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.

I said before that palmitoleic acid is proinflammatory, so for you to see actual data, what this slide shows is an experiment where normal monocytes or monocytes loaded with palmitoleic acid are exposed to bacterial lipopolysaccharide, and the expression of various proinflammatory genes is measured (Slide 13 – Palmitoleic

Acid (16:1) as a Proinflammatory Lipid). We used lipopolysaccharide here just to obtain a very strong response. It is clear that the cells enriched in palmitoleic acid produced more proinflammatory cytokines after stimulation. 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, the palmitoleic active molecule should be 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. By using LC/MS [10-13], we set out to determine the phospholipids that contain palmitoleic acid under these conditions. But 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 (Slide 14 – Novel 16:1-PI Species That Appear After Activation (LC/MS)). Interestingly, all of them are phosphatidylinositol (PI) molecules. 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. 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 [14]. 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 15 – 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 16 – 1,2-Diarachidonoyl-sn-glycero-3-phosphoinositol), because we believe this lipid may be involved in the regulation of innate immune responses in macrophages [12].

I have no time to describe why and how we became interested in this lipid [10, 15], so let's just say that this is one of those lipids that increase after cell activation [10, 12] (Slide 17 – Stimulated Production of PI(20:4/20:4) in Macrophages) and tends to stay elevated, a behavior that is compatible with it being involved in signal transduction. Well, we took our lipid and made the complexes with the carrier, and added it to the cells (Slide 18 – Incorporation of PI(20:4/20:4) Into Cells). We let the cells rest and added the stimulus, zymosan in this case, which is a classical stimulus for macrophages, and studied various cellular responses (by the way these experiments were carried out with mouse peritoneal macrophages, no more human monocytes). Initially we focused on gene expression, because that is the most "fashionable" response one can measure, right?. 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 19 – PI(20:4/20:4) Does Not Regulate Gene Expression). 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 would have better to 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 20 – PI(20:4/20:4) Regulates Superoxide Anion Production). 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 21 – PI(20:4/20:4) Regulates Lysozyme Release).

So, as a conclusion of my talk, and this is my last slide (Slide 22 – 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 23 – Acknowledgments). And I thank you very much for your attention.

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