

Original Article

Morphoproteomics provides support for TGF- β pathway signaling in the osteoclastogenesis and immune dysregulation of osteolytic Langerhans cell histiocytosis

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Abstract: Langerhans cell histiocytosis (LCH) has a challenging and still unclear pathogenesis. A body of literature points to impaired maturation of the lesional dendritic cells, and to immune dysregulation in the form of increased FoxP3 cells. Various cytokine abnormalities such as expression of transforming growth factor (TGF)- β have been reported, as well as abnormalities in lipid content in LCH cells. Morphoproteomic techniques were applied to identify the signal transduction pathways that could influence histogenesis and immune regulation in osteolytic LCH. Five pediatric cases of osteolytic LCH were examined, using antibodies against CD1a, S100, CD68, CD8, FoxP3, phosphorylated (p)-STAT3 (Tyr705), protein kinase C (PKC)- α , phospholipase (PL)D1, fatty acid synthase (FASN), and zinc finger protein, Gli2. Positive and negative controls were performed. A FoxP3(+)/CD8(+) cell ratio was calculated by counting the FoxP3+ and CD8+ cells in 10 high power fields for each case. There is induction of sonic hedgehog (SHH) mediators consistent with TGF- β signaling pathway through Smad3-dependent activation of Gli2, findings supported by the plasmalemmal and cytoplasmic expression of PKC- α and PLD1, and nuclear expression of Gli2, in lesional cells. The FoxP3+/CD8+ cell ratio is increased, ranging from 1.7-7.94. There is moderate cytoplasmic expression of FASN in most of the Langerhans cells, a finding that supports previously published phospholipid abnormalities in LCH and is consistent with PKC- α /PLD1/TGF- β signaling. With our study, we strongly suggest that the TGF- β cell signaling pathway is a major player in the pathogenesis of LCH, leading to non-canonical induction of nuclear Gli2 expression, thereby contributing to osteoclastogenesis in LCH histiocytes. It could also cause a state of immune frustration in LCH, by inducing the transformation of CD4(+)/CD25(-) cells into CD4(+)/FoxP3(+) cells. This coincides with the clinical evidence of a response to thalidomide in patients with osteolytic LCH, given its reported ability to reduce TGF-beta 1 and FoxP3 cells. Such TGF- β signaling in osteoclastogenesis and immune dysregulation, and the presence of FASN in the majority of cells, have additional therapeutic implications for osteolytic LCH.

Keywords: Morphoproteomics, TGF- β , signaling pathway, osteoclastogenesis, histogenesis, Langerhans cell histiocytosis

Introduction

Though Langerhans cell histiocytosis (LCH) is a disease mostly found in the pediatric population, it can also appear in adults. Distinct entities have been identified based on specific patterns of disease (monostotic and polyostotic). Approximately two thirds of the children with LCH have single-system disease (monostotic or

polyostotic lytic lesions of the bone, especially of the cranium) [1, 2].

Currently there are two main theories regarding the pathogenesis of LCH. There is a body of literature suggesting that it is a clonal disease. Arico *et al.* [3] studied the incidence of Langerhans cell histiocytosis in presumed (p) monozygotic (MT) and dizygotic (DT) twins in

Table 1. Study Population of Pediatric Cases with Osteolytic LCH.

| Case | Age | Sex | Type of lesion |
|------|-------|-----|---|
| 1. | 3 yo | M | Monostotic; left sphenoid wing |
| 2. | 9 yo | M | Monostotic; left iliac crest |
| 3. | 12 yo | F | Monostotic; pterygoid process |
| 4. | 5 yo | F | Monostotic, right fibular lesion |
| 5. | 2 yo | F | Polyostotic; palate and skull lesions; skin involvement present |

which one of the siblings was affected by the disease. He observed that four out of five sets of pMT developed the disease at close intervals, and had similar manifestations. One of the 3 DT observed developed the disease. In a study of 72 cases of LCH, Da Costa and colleagues [4] showed using immunocytochemistry that there is consistent p53 expression implying a mutation, but at that point the authors did not find a recurrent genetic abnormality. Specifically, they performed sequencing of exons 5 to 8 of the p53 gene and found no alterations in 7 cases analyzed. In 2010, Badalian-Very and co-workers [5] reported that thirty-five out of sixty-one studied cases of LCH (adult and pediatric population) had a recurrent BRAF V600E mutation. Seventeen out of the twenty-five (65.38%) pediatric cases of osteolytic LCH (eosinophilic granuloma) with only bone involvement had the mutation. However, phosphorylated (p)-extracellular signal-regulated kinase (ERK) and p-mitogen-activated protein kinase (MAP)-ERK Kinase (MEK) were expressed in all pediatric and adult cases, suggesting that there must be alternative mechanisms contributing to the pathogenesis of this disease.

Moreover, it is difficult to explain a pure clonal origin for a disease that, in some cases, regresses spontaneously and in other cases has a very aggressive behavior. Therefore, many researchers have concentrated on the study of the immunophenotype and chemokine expression of lesional cells in LCH. Fleming *et al.* [6] demonstrated the aberrant co-expression of CCR6 and CCR7 in twenty-four cases of LCH. Geissmann and colleagues [2], in an attempt to characterize the LCH cells, showed that these cells do not express CD83 and CD-Lamp (markers of mature dendritic cells), but that the majority of cells express CD14 (marker of immature dendritic cells). Senechal and co-workers reported on an expansion of

FoxP3 regulatory T cells in patients with LCH raising the possibility of dysregulation of the host immune system compromising its ability to eliminate LCH cells [7]. Additionally, osteoclasts, a consistent component of osteolytic LCH are regarded as a major contributor to osteolysis and the histogenesis of the osteolytic form of the disease.

Because of these existing data that support both clonal and dysmaturation/immune dysregulation-associated processes in the etiopathogenesis of osteolytic LCH, we decided to focus on defining the factors common to its histogenesis. In this context, we were guided by therapies with proven efficacy in osteolytic LCH, namely aminobisphosphonates that inhibit osteoclastic giant cells [8-14] and thalidomide, which inhibits transforming growth factor (TGF)- β signaling and downregulates T regulatory cells [15-19]. This focus is underscored by the previous report of Brown [20], noting that the latency-associated peptide of TGF- β 1 and osteoclastogenic interleukin (IL)-11[21], a downstream effector of the TGF- β signaling pathway [22] are expressed in osteolytic LCH. Thus, the objectives of this study are two-fold: firstly, to identify additional components of the TGF- β signaling pathway involved in osteoclastogenesis and T regulatory cell expansion in osteolytic LCH using morphoproteomics; and secondly, to develop possible therapeutic strategies that target these histogenetic processes.

Methods

With Institutional Board Review approval, four pathologists from the University of Texas-Health Science Center analyzed formalin-fixed, paraffin-embedded tissue from five pediatric cases (age ranging from 2-12) of LCH osteolytic lesions (**Table 1**). Histologic (hematoxylin&eosin; H&E) and immunohistochemical (S100

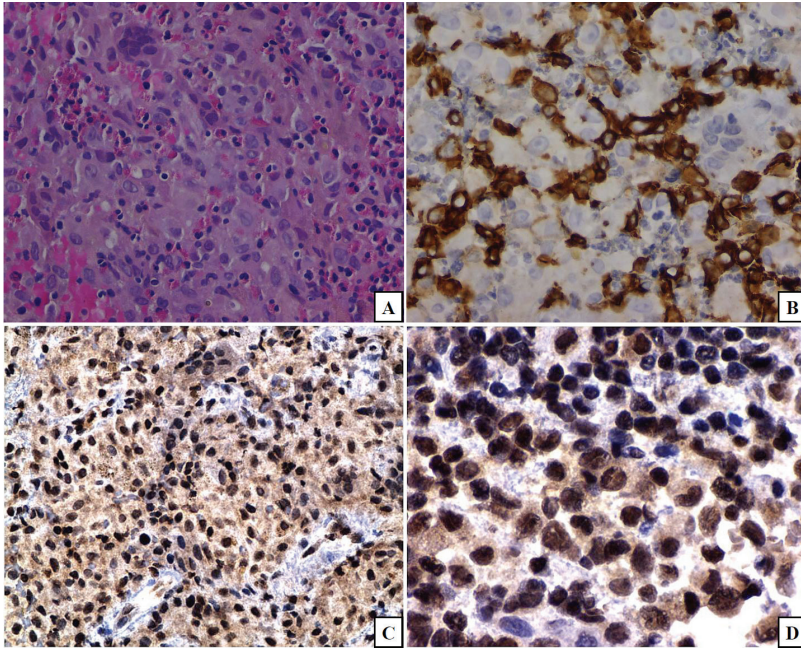


Figure 1. Hematoxylin-eosin (H&E) shows a proliferation of histiocytic cells, some with cleaved nuclei, nuclear grooves, admixed with multinucleated giant cells, including osteoclasts, and with an eosinophilic infiltrate (frame A). The lesional cells express CD1a (frame B). This pattern of expression is consistent with a diagnosis of Langerhans cell histiocytosis. Morphoproteomic application of a probe for glioma-associated oncogene protein (Gli)2 reveals nuclear expression in a majority of lesional Langerhans cells (frames C and D; DAB [brown] chromogen) with implications for TGF- β signaling and osteoclastogenesis. (original magnifications, x400 for A, B, and D and x200 for C).

[CMC715, Cell Marque, Rocklin, CA], CD1a [ab708, Abcam], and CD68 [MO814 Dako] studies were performed on all cases. Monoclonal antibodies against phospholipase D1 (PC-PLD1, 28314, Santa Cruz Biotechnology, Santa Cruz), protein kinase C- α (PKC- α , 8393 Santa Cruz), phosphorylated (p)-signal transducer and activator of transcription (STAT)3 phosphorylated on tyrosine 705 (8059 Santa Cruz), zinc finger protein Gli2 (ab26056 abCam), fatty acid synthase (FASN) (3180 Cell Signaling Technology), FoxP3 (ab10563, Abcam), and CD8 (ab4055, Abcam) were applied after tissue rehydration and antigen-retrieval. Using bright-field microscopy, the expression or absence of PLD1, PKC- α , p-STAT3 (Tyr705), Gli2, and FASN was assessed for each case, along with the subcellular compartmental distribution of these individual monoclonal antibodies. Positive and negative controls were run concurrently.

A FoxP3 (+)/CD8 (+) cell ratio was calculated by counting the FoxP3 (+) and CD8 (+) cells in 10 high power fields for each case.

Results

H&E evaluation of the tissue from the five cases examined showed destructive lesions com-

prised of a histiocytic population of cells with cleaved nuclei and nuclear grooves, admixed with multinucleated giant cells, including osteoclasts, and with an eosinophilic infiltrate. The histiocytic cells expressed S100, CD1a, and CD68 **Figure 1** depicts H&E and CD1a; S100 and CD68 although expressed are not depicted]. A diagnosis of osteolytic Langerhans histiocytosis was rendered for each of the five cases, based on the above findings. The zinc finger protein Gli2 was variably expressed in the majority of the nuclei of the lesional cells (**Figure 1**, frames C and D) and has implications in osteoclastogenesis (*vide infra*).

The majority of lesional Langerhans cells had moderate-intense cytoplasmic and plasmalemmal expression of PKC- α (**Figure 2A**) as well as mild-to-moderate expression of PLD1 (**Figure 2B**), with similar compartmentalization. There was focal mild cytoplasmic expression of these protein analytes in the multinucleated giant cells. FASN had moderate, variable cytoplasmic expression in the Langerhans cells and somewhat stronger expression in the multinucleated giant cells (**Figure 2C**).

In an attempt to investigate a possible immune-dysregulation in Langerhans histiocytosis, we counted the FoxP3+ (**Figure 3A**) and CD8+ (**Figure 3B**) cells in ten high power fields, and their ratio was increased in these cases, being

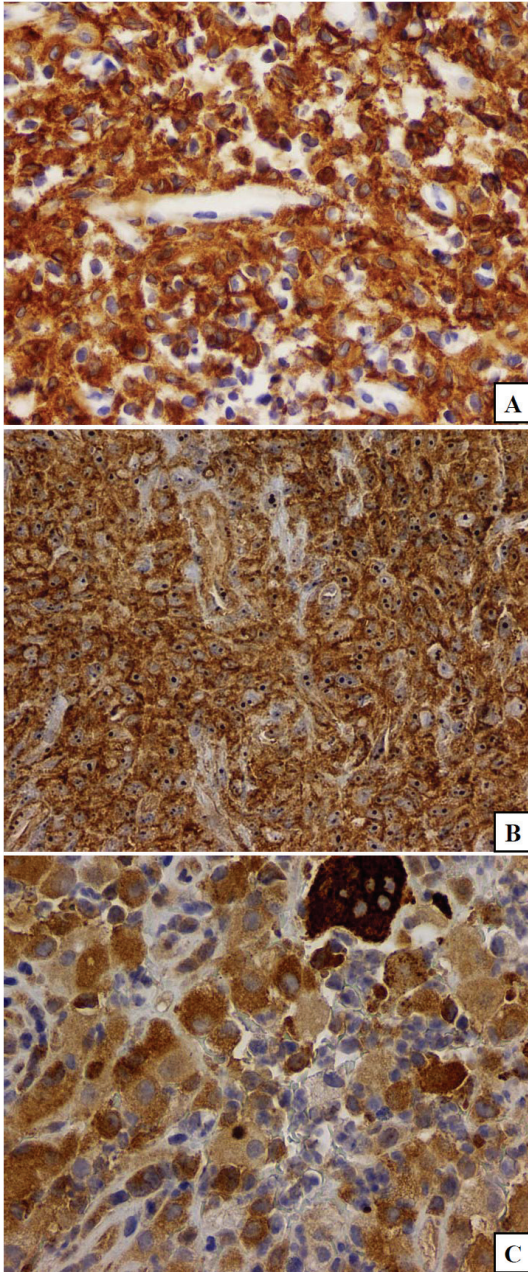


Figure 2. PKC-alpha (frame A) and phospholipase D1(PLD1;frame B) share a similar subcellular distribution on the plasmalemmal and in the cytoplasmic compartments of the majority of lesional cells. Fatty acid synthase (FASN;frame C) has variable intensity of cytoplasmic expression in the majority of the lesional cells. (original magnifications, x200 for A and B, x400 for C).

the highest (7.94:1) in case 5, in which there were polyostotic skull lesions and skin involvement with a poor response to treatment. The lowest FoxP3/CD8 ratio observed was 1.7:1. Correspondingly, p-STAT3 (Tyr 705) was

expressed focally in the nuclei of lesional Langerhans cells but it was not observed in the multinucleated giant cells (**Figure 3C**). A negative immunohistochemical control is included in **Figure 3D**.

Discussion

Morphoproteomics utilizes morphology to determine the subcellular compartmentalization of both phosphorylated and non-phosphorylated protein analytes in evaluating their state of activation. Furthermore, by combining such findings with both the correlative expression of other proteins and the nature of companionate cells in the lesional microenvironment, morphoproteomics can provide clues to histogenesis, lesional biology, and potential therapeutic options [23, 24].

Our morphoproteomic findings in the lesional cells of LCH to include nuclear Gli2 expression, plasmalemmal and cytoplasmic PKC- α and PLD1, and cytoplasmic FASN coincide with previous observations of TGF- β 1 expression [20, 25] and pathway signaling in LCH. Specifically, the effects of TGF- β 1 signaling include osteoclastogenesis through IL-11 and Gli2-associated parathyroid hormone-related protein production [21, 22, 26-31]. Furthermore, the expression of IL-11 was previously demonstrated in LCH by Brown RE [20] and Andersson BU *et al.* [32], and its role in osteoclastogenesis was reviewed by Guk KD and co-workers [33], with the comment that this role was very well defined *in vitro*. Also, IL-11 signals through JAK/STAT pathway [34], and together with TGF- β 1 pathway signaling [35] stimulate the genesis of FoxP3+ lymphocytes [36-40], contributing to the immune dysregulation of LCH. Correspondingly, we found an increased FoxP3+ to CD8+ ratio of lymphocytes in the five cases of LCH that we studied, and the ratio appeared to coincide with the aggressiveness of the disease. (The only case with extensive involvement of the cranium, with extension to the skin, had a ratio of FoxP3+/CD8+ lymphocytes of 7.94:1.) Such evidence of immune dysregulation was previously reported by Senechal *et al.* [7], who described a consistent expansion of T regulatory cells in biopsies from 40 patients with LCH. Furthermore, it accords with the gene expression profile in LCH lesion CD3+ cells of an activated regulatory T cell phenotype with

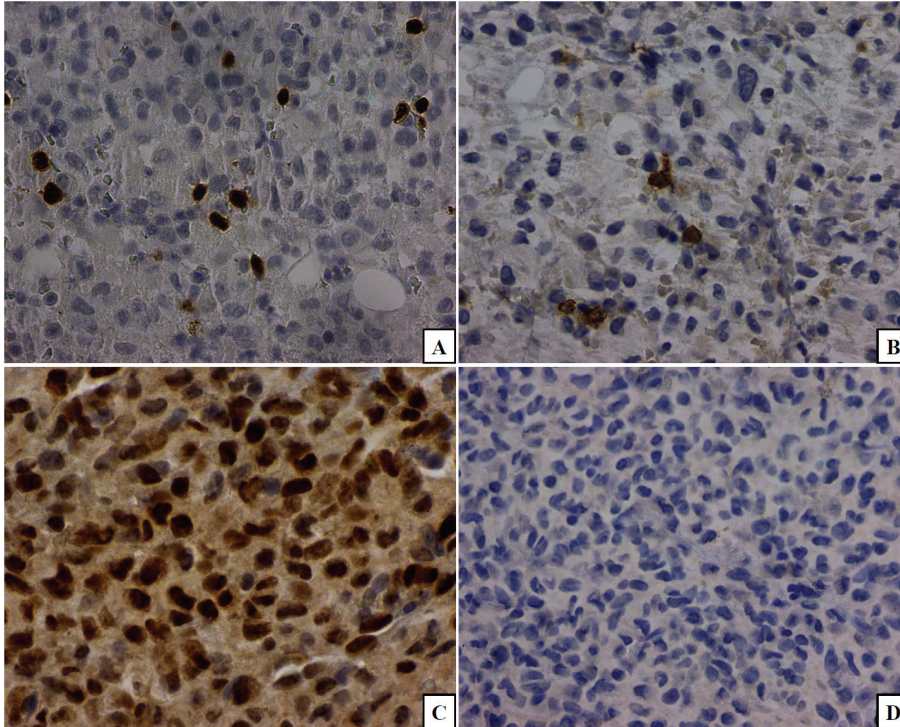


Figure 3. The calculated ratio of FoxP3+/CD8+ lymphocytes, revealed an increased number of FoxP3+ cells (frame A) compared to CD8+ cells (frame B). The highest ratio corresponded to the case with the most aggressive clinical course (case 5). Relatedly, phosphorylated (p)-signal transducer and activator of transcription (STAT3) (Tyr 705) is variably expressed in nuclei of the lesional Langerhans cells (frame C). A negative control is depicted in frame D. (original magnifications, x400 A-C and x200, D).

increased expression of *FOXP3* gene [41]. In addition, our finding of the expression of *FASN* could reflect TGF- β signaling given the latter's apparent role in inducing *FASN* [42, 43]. Moreover, it provides a correlate with the original observation of preferential lipid metabolism in histiocytosis X [44, 45]. The expression and role of fatty acid synthase (*FASN*) has not been reported in LCH to date, as per a review of the National Library of Medicine's Medline Data Base. We noticed variable expression of *FASN* in all cases, and this finding has possible pathogenetic implications with regard to PKC- α /PLD1/TGF- β signaling, as well as therapeutic implications (see schematic and legend for **Figure 4**; *vide infra*). As previously stated, the role of *Gli2* in osteoclastogenesis is well known [26], and its expression in lesional Langerhans cells provides a correlate for TGF- β signaling via the non-canonical TGF-beta {Smad3}*Gli2* signaling pathway in the genesis of multinucleated osteoclast-like cells in LCH [26-31]. Again, based on a review of the National Library of Medicine's Medline Data Base, our study appears to be the first to demonstrate the nuclear expression of *Gli2* in osteolytic LCH. The central role of TGF- β pathway signaling in incorporating these and previous observations in the osteoclastogenesis and immune dysreg-

ulation of osteolytic LCH is represented in the schematic in **Figure 4** and detailed in the corresponding legend.

Finally, the combination of morphoproteomic and morphometric findings in the study, although based on a small series of cases, raise possible therapeutic options that would combine to inhibit TGF-beta pathway signaling and immune dysregulation, the latter by targeting T regulatory (FoxP3+) cells. This is also summarized in the schematic (**Figure 4** and legend).

In conclusion, this study reinforces the importance of an activated TGF- β pathway in the biology and histopathology of osteolytic LCH, with the immediate effects to include osteoclastogenesis and expansion of T regulatory cells. It also raises possible therapeutic strategies to target this central TGF- β signaling in the treatment of osteolytic LCH.

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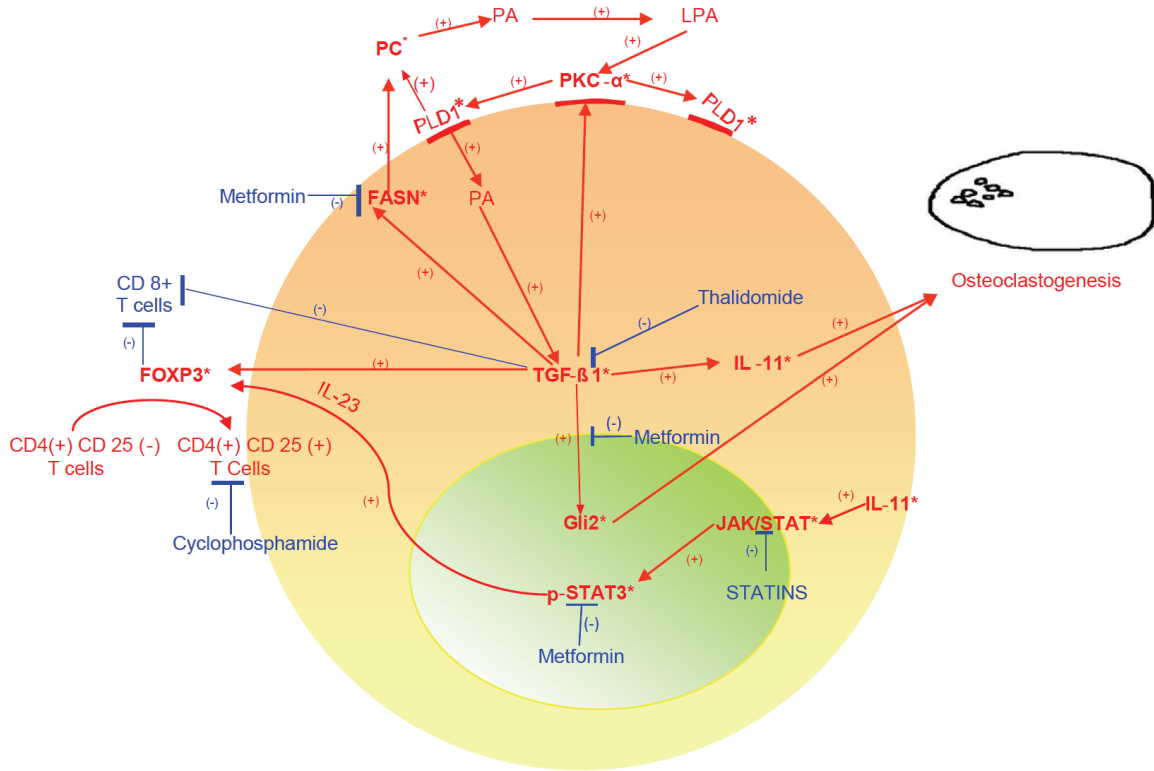


Figure 4. Schematic depicting a central role for transforming growth factor (TGF)- β 1 signaling in osteoclastogenesis and immune dysregulation of osteolytic Langerhans cell histiocytosis (LCH). Specifically, TGF- β 1 expression [20, 25] and signaling in osteolytic LCH correlates with and could lead to: 1) interleukin (IL)-11 [20, 22, 28, 32] and in turn, both osteoclastogenesis [21, 33] and the activation of signal transducer and activator of transcription (STAT)3 pathway [34], evidenced by nuclear expression of phosphorylated (p)-STAT3 (Tyr 705) and subsequent expansion of the FoxP3 + population via IL-23 [36, 37, 41]; 2) nuclear expression of glioma-associated oncogene protein (Gli)2 [26, 30] leading to the formation of parathyroid hormone-related protein (PTHrP) and then to activation of RANKL with osteoclastogenesis [26, 31, 46, 47]; 3) fatty acid synthase (FASN) expression with lipogenesis to include phosphocholine (choline-phospholipids have been described in LCH [44]) and phosphatidylcholine [45], the substrate for phospholipase (PL)D1 with subsequent phosphatidic acid (PA) and lysophosphatidic acid (LPA) formation and in turn to complex activation of protein kinase C (PKC)- α , PLD1 and TGF- β 1 [48-57]; 4) activation of PKC- α [58-60]; and 5) downregulation of CD8+ cell-associated immune surveillance [61] and with activation of T regulatory (FoxP3+) cells [35, 38-40]. Targeted therapeutic possibilities in this context include metformin to: 1. Activate AMPK with inhibition of TGF- β {Smad3}-associated Gli2 and IL-11 signaling [26-31, 62, 63] and in turn, inhibition of PTHrP and RANKL signaling [46, 64, 65] with reduced osteoclastogenesis; 2. Downregulate FASN [66] leading to reduced substrate for the PLD1/PKC- α /TGF- β signaling axis; and 3. Inhibit STAT3 activation at tyrosine 705 [67]. Moderators of TGF- β pathway signaling related to immune dysregulation include: thalidomide, which has shown efficacy in blocking the TGF- β pathway [16-19] and clinically, in treating osteolytic LCH [15]; and metronomic cyclophosphamide, which targets T regulatory cells [68]. *Indicates protein analytes identified in osteolytic LCH.

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