

Epigenetic Studies Revealed a Ghost Proteome in PC1/3 KD Macrophages under Antitumoral Resistance Induced by IL-10

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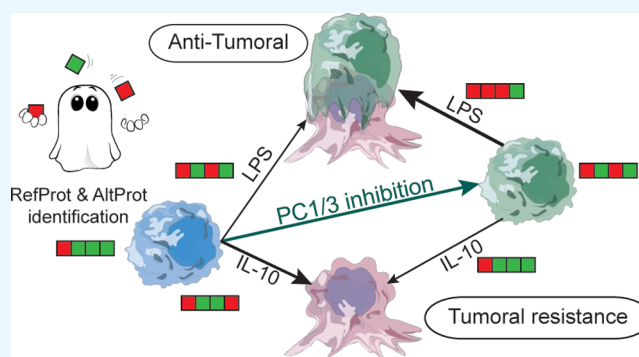


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ABSTRACT: Our previous investigation on macrophages has allowed us to show that the inhibition of the enzyme proprotein convertase (PC1/3) controls the activation of macrophages. We demonstrated that PC1/3 knockdown (KD) in macrophages exhibits an increased secretion of proinflammatory and antitumoral factors. In this biological context, we assessed the presence of histone modifications and the presence and contribution of a “ghost proteome” in these macrophages. We identified a set of alternative proteins (AltProts) that have a key role in the regulation of various signaling pathways. In this study, to further investigate the underlying mechanisms involved in the resistance of PC1/3-KD macrophages to anti-inflammatory stimuli, we have conducted a proteomic system biology study to assess the epigenome variation, focusing on histone modifications. Results from our study have indicated the presence of significant variations in histone modifications along with the identification of 28 AltProts, which can be correlated with antitumoral resistance under IL-10 stimulation. These findings highlight a key role of altered epigenome histone modifications in driving resistance and indicate that like the reference proteins, AltProts can have a major impact in the field of epigenetics and regulation of gene expression, as shown in our results.



INTRODUCTION

It is widely considered that eukaryote mature messenger ribonucleic acids (mRNAs) are monocistronic, leading to the translation of a single protein. According to the rules described by Kozak,¹ the coding mRNA sequence (CDS) corresponds to the longest nucleotide sequence in frame with a start (AUG) and stop codon, which is flanked by 5′ and 3′ UTRs and is 5′ capped. The CDS or reference open reading frame (RefORF) encodes the reference protein (RefProt). Nevertheless, based on these annotations, only 85–90% of good-quality proteomics data analyzed by MS/MS match theoretical peptides predicted from the RefProt database. Hence, 10–15% of the yet high-quality experimental peptides remain unassigned following reference database interrogation, falling through the prediction net. The reanalysis of these unmatched peptides against the alternative protein (AltProt) database has demonstrated that most orphan peptides originate from proteins encoded by alternative open reading frames (AltORFs), which were not predicted to be translated (thus absent from the reference databases)² but shown to play integral roles in mediating different molecular functions and biological processes. The data analysis of crosslinking mass spectrometry (XL-MS) experiments against a recently published human AltProt database revealed that hundreds of

AltProts (i) are in enough abundance to be detected from human cellular extracts and (ii) interact with RefProts involved in diverse cellular pathways.³ This observation is of major importance in the field of cancer biology and immunology. It implies that a given mRNA si/shRNA knockdown or CRISPR/Cas9 gene invalidation could trigger phenotypical changes independently of the canonical protein depletion. Such alterations could, in fact, be due to concomitant alterations of the AltProt levels, which is encoded by the same mRNA. In this context and based on our previous studies, we have decided to investigate the presence of such AltProts in macrophages under anti-inflammatory protumoral conditions correlated to a proteomic system biology platform to decipher the pathways involved. We decided to focus on proteomic histone modifications in order to evaluate the epigenetic alterations that can occur in the macrophage model, previously described in Duhamel et al. 2015.⁴

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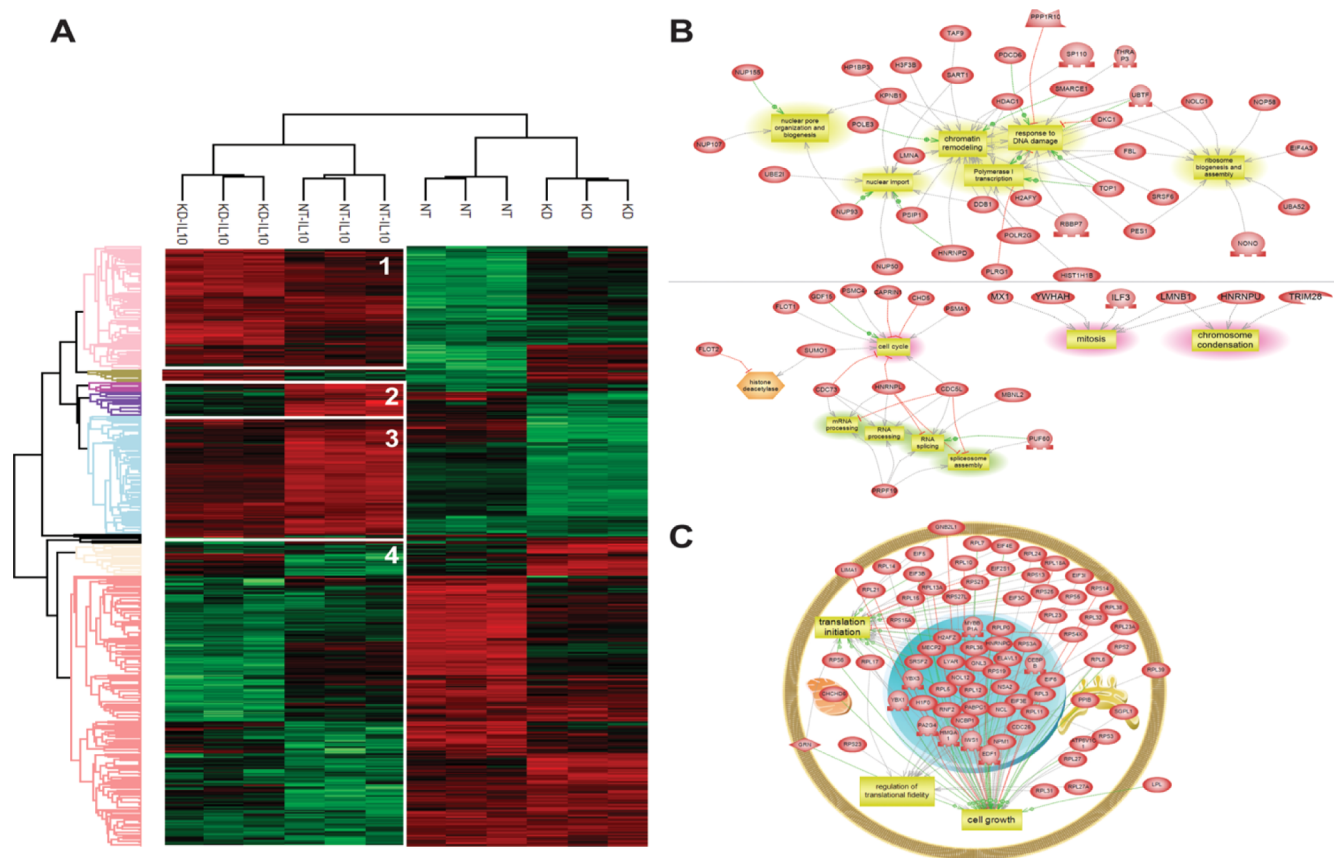


Figure 1. (A) Proteomic analyses of histones from PC1/3 KD and NT macrophages treated or not treated with IL-10. The heatmap from the histone extracts using the EpiQuik total histone extraction kit followed by FASP digestion and shotgun proteomic analyses. (B) Systemic biology analyses of proteins identified and significantly overexpressed in KD cells under IL-10 treatment, represented by the cluster 1 in the heatmap. (C) Systemic biology analyses of proteins identified and significantly overexpressed in NT cells under IL-10 treatment, represented by the clusters 2 and 3 in the heatmap.

Macrophages are essential phagocytes of the innate immune system and are present in all organs and tissues of the body as resident cells. They play a critical role in both the maintenance of tissue homeostasis and inflammation regulation, performing vital tissue-specific functions and protecting the host from infection.⁵ These cells orchestrate both the innate immune responses and the adaptive immune responses.⁶ Both tissue-resident macrophages in homeostasis and activated macrophages under stimuli are driven by specific transcriptional changes and are controlled by complex cellular mechanisms. Among these upstream regulators, epigenetics now arises as a key driver of macrophage activity.⁵ Recently, we have demonstrated that an enzyme called proprotein convertase 1/3 (PC1/3) can control macrophage activation. PC1/3-knockout mice have high plasma levels of proinflammatory cytokines.⁷ Moreover, knockdown (KD) of PC1/3 in a rat macrophage cell line (NR8383) triggers high secretion of proinflammatory chemokines and cytokines.⁸ When stimulated with toll-like receptor (TLR) ligands (LPS and antitumoral drug Paclitaxel), PC1/3-KD macrophages are overactivated and their immune response is even more exacerbated.^{9–12} The secreted factors by PC1/3-KD macrophages post-TLR4 stimulation have shown to exhibit chemotactic and antitumor properties against breast, ovarian, and glioma cancer cell lines. In addition, PC1/3-KD macrophages were less-sensitive to IL-10, an anti-inflammatory cytokine, which is commonly produced in the tumor microenvironment to inhibit immune

cells, leading to antitumoral activation. Thus, the sensitivity to anti-inflammatory molecules and the length of TLR4 desensitization were reduced in these PC1/3-KD macrophages.^{9–12} However, during antitumoral immunotherapy, a repeated stimulation of TLR4 could reactivate PC1/3-inhibited macrophages even in a protumoral environment, whereas the underlying mechanisms involved are still under-investigated. In the present study, we hypothesized that its regulation can be mediated epigenetically with the contribution of AltProts, as we have shown previously in a different study.¹⁰

The aim of this study is to better understand the mechanisms involved in the resistance to IL-10 of PC1/3-KD macrophages. For that, we have investigated the epigenetic changes at the histone level, along with the variation of the reference and alternative proteins (AltProts). Finally, based on systemic biology analyses, we have correlated the proteins identified to the biological pathways in which they are involved.

RESULTS AND DISCUSSION

The analysis of the histone modifications such as acetylation/deacetylation and methylation/demethylation of lysine residues reveals the epigenetic modifications that can occur in macrophages in the context of tumor development. We thus focused our attention on such histone modifications in order to determine whether PC1/3 inhibition can modify the epigenetic

profile of macrophages, which could explain their IL-10 resistance.

Histone Post-translational Modification Identification in PC1/3-KD Macrophages. Histones have been isolated from NT and PC1/3-KD macrophages after or without IL-10 treatment. In this context, the EpiQuik total histone extraction kit was used followed by post-translational modification (PTM) research using Epigentek's histone modification assay kit. We focused our attention on H3 PTM (Figures 1, S1 and Table S1). Compared to Nontarget (NT) control macrophages, PC1/3-KD macrophages present a high level of methylation of H3K27 and of H3K79. Mono-, di-, and trimethylation was detected especially for H3K27. Acetylation of H3K18 and H3K9 was also increased in PC1/3-KD compared to NT macrophages and the phosphorylation of H3ser28PH3S28P increased as well (Figures 1, S1 and Table S1). By contrast, a slight increase in mono- and dimethylation of H3K9 and H3K79 has been measured in NT cells (Figures 1, S1 and Table S1). After IL-10 treatment, H3K27 methylations largely decreased in PC1/3-KD cells compared to those without treatment. H3K79me₂, H3K9ac, H3K18ac, and H3ser28PH3S28P decreased in KD cells but are still high compared to those in NT cells. For NT cells, the IL-10 treatment increased H3K4me₁, H3K4me₂, H3K79me₂, H3K36me₁, and H3K36me₃. Only H3K36me₁ pops up after IL-10 treatment compared to NT control cells and PC1/3-KD cells (Figures 1, S1 and Table S1). Di- and trimethylation at H3K4, H3K36, and H3K79 triggers gene activation, with H3K4 trimethylation (H3K4me₃)-marking promoters and H3K36 and H3K79 methylations.^{5,18–20} Monomethylation of H3K4 is an activating mark unique to enhancers. H3K9 and H3K27 methylations are generally gene-repressive. However, H3K27me₃ is considered to be easily reversible and marks dynamically regulated genes. H3K9me₃ is a characteristic of heterochromatin, whereas H3K9me₂ is found more commonly at silent or lowly expressed genes in euchromatin.^{5,20} These data are in line with the fact that PC1/3-KD macrophages present a characteristic epigenetic code compared to NT macrophages with both H3K79 methylation and H3K9 acetylation, which are gene-activating markers, and H3K27 trimethylation, which is tightly associated with inactive gene promoters. H3ser28PH3S28P and H3K18ac are also detected at a high level in these PC1/3-KD cells. H3S28P and H3K18ac are known to activate transcription and antagonize polycomb silencing.⁵ However, under IL-10 treatment, H3K27 methylation falls, whereas the H3K79me₂ level remains high and H3K9ac and H3K18ac levels remain high as well. Histone acetylation generally correlates with gene activation.^{18,20} In order to detect the enzymatic proteins and the TFs (transcription factors) linked to these H3 PTM modifications, we performed shotgun proteomic analyses of the global cellular protein extract and then at a subproteome level after histone enrichment.

Global Proteome Analysis of NT and PC1/3-KD Cells Treated or Not Treated with IL-10. In order to evaluate the impact of immunosuppressive conditions on macrophages, we incubated the NT and KD cells with IL-10 (24 h). We have previously performed the shotgun proteome analyses of these cells under different conditions.⁴ However, we have not focused our attention on specific proteins, which can be involved in chromatin compaction and TFs (Table S1). Reanalyses of these previous data established the presence in NT cells of the enhancer Spi1, which controls the expression of

PU.1, required for tissue macrophage development. We also detected the Swi5-dependent recombination DNA repair protein 1 homolog (Sfr1), the heat shock 70 kDa protein 1-like (Hspa1l), the tRNA (cytosine-5)-methyltransferase, the Fidgetin-like protein 1, and the eukaryotic translation initiation factor 4E-binding protein 1 (4EBP1). In NT cells treated with IL-10, we identified the growth arrest- and DNA damage-inducible protein interacting protein 1 (GADD45 α), KH domain-containing, RNA-binding, signal transduction-associated protein 3 (Khdrbs3), double-strand break repair protein MRE11 (Mre11a), IL-1 β -induced lymphoid enhancer binding factor 1 (Lef1), DNA-binding TF that plays important roles in cancer, Nocturnin, SWI/SNF-related matrix-associated actin-dependent regulator of chromatin subfamily D member 2 (Smarcd2), serine/threonine protein kinase Nek6 (Nek6), elongation factor 1- α 2 (Efla2), and DnaJ (Hsp40) homologue, subfamily C, member 16 (Dnajc16). In PC1/3-KD cells, the WD repeat-containing protein 5 (WDR5), a common component of mixed lineage leukemia methyltransferase family members, which is important for histone H3 lysine 4 methylation (H3K4me), has been detected. Moreover, the protein quaking, Microphthalmia-associated TF (MITF) has been identified. In PC1/3-KD cells treated with IL-10, the exclusive proteins related to epigenetics are the DNA helicase MCM9, the zinc finger protein 830, and the regulator of pre-B-cell leukemia TFs (BPXs). The signature is clearly different between NT and PC1/3-KD macrophages and the IL-10 treatment impacts these cells differently. In order to further investigate this effect, we performed shotgun proteomic analyses at the level of the subproteome of the nucleus by performing a histone enrichment extraction procedure.

Histone Subproteome Analysis of NT and PC1/3-KD Cells Treated or Not Treated with IL-10. The cells were treated or not treated with IL-10 under the same conditions as mentioned before. Histones and total proteins were extracted before being subjected to shotgun proteome analyses followed by label-free quantification (LFQ). A total of 534 from the 1023 proteins identified showed a significant variation in abundance based on LFQ values after subjecting the data to multiple ANOVA tests with a *p*-value of 0.05 (Figure 1A, Table S1). A clear separation between treated and untreated cells can be observed. Four clusters are highlighted. Clusters 1–3 represent the proteins which are overexpressed in IL-10-treated cells compared to untreated ones. The proteins of the cluster 1 are more represented in PC1/3-KD cells treated with IL-10, while proteins of clusters 2 and 3 are more represented in NT cells treated with IL-10. Cluster 4 regroups proteins that are not expressed in NT and PC1/3-KD treated cells. In the cluster 1, we identified several histones (H1.4, H1.5, H2A.1, H2B, and H3f3b), histone deacetylase 1, histone-binding protein RBBP7, and non-POU domain-containing octamer-binding protein TFs (Taf6, purb), components of SWI/SNF chromatin-remodeling complexes (Smarc1 and Smarcd2), a component of the PAF1 complex (PAF1C) (Parafibromin, PHD finger-like domain-containing protein 5A), and also the GLYR1, which is a Putative oxidoreductase that is recruited on chromatin and promotes KDM1B demethylase activity. Systemic biology of the proteins contained in the cluster 1 reinforces the fact that the PC1/3-KD cells under IL-10 treatment are engaged in chromatin remodeling, response to DNA damage, regulation of DNA deacetylation, and transcription activation (Figure 1B).

Among the cluster 1 (Figure 1B), we noticed proteins known to be involved in macrophage phenotype switch to an antitumor/proinflammatory phenotype, such as the chromo domain helicase DNA-binding protein 5 (Chd5), which is involved in the trimethylation of lysine 27 of histone 3 or the Parafibromin protein (cdc73) and Phf5a, which are a part of the PAF1C complex, which is involved in the trimethylation of lysine 4. Moreover, the Smarce1 and Smarcd2 are also known to be involved in the trimethylation and acetylation of lysines 4 and 9 on histone 3. The histones H2A and H1 are also present in this cluster. The proteins of this cluster which are overexpressed in PC1/3-KD macrophages with or without IL-10 stimulation appear to be involved in histone PTM, orienting the macrophages toward an antitumor phenotype. Furthermore, from the total protein extract, DNMT1 is also known to polarize macrophages to various antitumoral/proinflammatory phenotype stimuli (Table S2). Systemic biology analyses of the proteins contained in the clusters 2 and 3 showed enrichment in proteins involved in “translation initiation, regulation of the translation”, and the “cell growth” pathways.

In the clusters 2 and 3, which are specific to NT-IL-10 cells and which contain some common proteins with KD-IL-10 cells (Figure 1A,C, Table S2), we identified the SET protein which is involved in the methylation of lysines 4 and 36 on H3 histones. From total extract proteins, JMJD6, Sirt 2, and HDAC1 are factors known to be involved in the protumoral/anti-inflammatory macrophage phenotype (Table S1).¹³ JMJD6 forms a protein complex with p53 and catalyzes p53 protein hydroxylation, leading to p53 inactivation and colon cancer cell proliferation and survival.¹⁴ In the cluster 4, specific to KD-IL-10 cells, proteins involved in chemokine signaling pathway and proinflammatory factors have been identified, that is, Gnb1, Gnai1, Gnai2, rapa1, rapb1, Fcer1g, coronin 1B, CD36, and CD44 (Figure S1).

From the dataset, we focused our attention on PTMs that can occur on histones in order to determine whether PC1/3 inhibition can modify the histone profile in macrophages, which could explain their IL-10 resistance (Table S2). We identified H1.4K21me, H1.4K63me, H1.4K64me, H1.4S2P, H1.4S36P, H1.4S55P, H1.4S58P, H1.4S65P, H1.4W4P, H1.5W4P, H1.5S18P, H3K24ac, H3K80me, H4K80me, H3K24me, H3R54me, H3K57ac, H3R84me, H3K123ac, H3Y55P, and H4R79me (Table S2). From the list of these histones, we performed quantitative analyses across the different cells and under different conditions. Only H1.4K63me, H1.4S58P, H1.4S55P, H1.5S18P, H2B1K35me, H3K123ac, H3K80me, and H3R84me showed quantitative expression differences (Figure 2). The heatmap showed two branches, one separating KD cells from the other conditions (Figure 2). In the second branch, NT cells are separated from NT and KD cells treated with IL-10. Except one sample, NT-IL-10 and KD-IL-10 are also separated (Figure 2). In KD cells, none of the histones with specific PTM are overexpressed. H3K123ac is overexpressed only in NT cells. H2B1K35me, H3R84me, H1.4S58P, H1.4S55P, and H1.4K63me are overexpressed in two of the three KD-IL-10 cells (Figure 2). Epigentek's histone modification assay kit was used and we focused our attention on H3 PTM (Figure S2). Compared to nontarget (NT) control macrophages, PC1/3 KD macrophages present a high level of methylation of H3K27 and of H3K79. Mono-, di-, and trimethylation was detected especially for H3K27. Acetylation of H3K18 and H3K9 was also

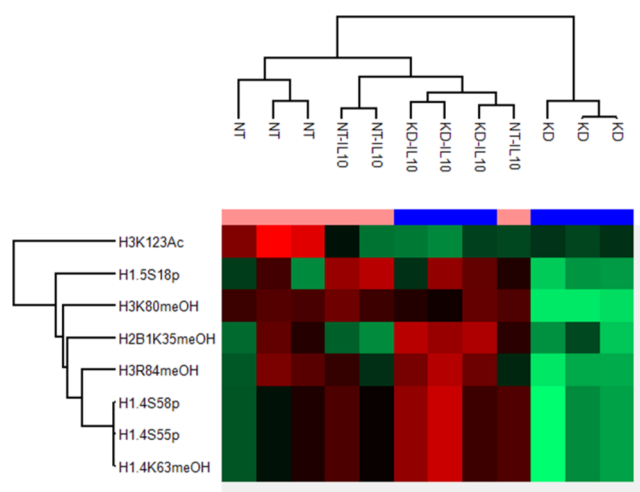


Figure 2. Heatmap of histones with specific PTM extracts using the EpiQuik total histone extraction kit followed by FASP digestion and shotgun proteomic analyses.

increased in PC1/3 KD compared to that in NT macrophages and the phosphorylation of H3S28P increased as well (Figure S2). By contrast, a slight increase in mono- and dimethylation of H3K9 and H3K79 has been measured in NT cells (Figure S1). After IL-10 treatment, H3K27 methylations largely decreased in PC1/3 KD cells compared to those without treatment. H3K79me₂, H3K9ac, H3K18ac, and H3S28P decreased in KD cells but are still high compared to those in NT cells. For NT cells, the IL-10 treatment increased H3K4me₁, H3K4me₂, H3K79me₂, H3K36me₁, and H3K36me₃. Only H3K36me₁ pops up after IL-10 treatment compared to NT control cells and PC1/3-KD cells (Figure S1). Di- and trimethylation at H3K4, H3K36, and H3K79 triggers gene activation, with H3K4 trimethylation (H3K4me₃)-marking promoters and H3K36 and H3K79 methylations.^{5,13–15} Monomethylation of H3K4 is an activating mark unique to enhancers. H3K9 and H3K27 methylations are generally gene-repressive. However, H3K27me₃ is considered to be easily reversible and marks dynamically regulated genes. H3K9me₃ is a characteristic of heterochromatin, whereas H3K9me₂ is found more commonly at silent or lowly expressed genes in euchromatin.^{5,15} These data are in line with the fact that PC1/3 KD macrophages present a characteristic epigenetic code compared to NT macrophages with both H3K79 methylation and H3K9 acetylation, which are gene-activating markers, and H3K27 trimethylation, which is tightly associated with inactive gene promoters. H3S28P and H3K18ac are also detected at a high level in these PC1/3 KD cells. H3S28P and H3K18ac are known to activate transcription and antagonizes polycomb silencing.⁵ However, under IL-10 treatment, H3K27 methylation falls, whereas H3K79me₂ and H3K9ac and H3K18ac remain high. Histone acetylation generally correlates with gene activation.

Ghost proteome Identification. lncRNAs play a role in the programming of histone modifications, silencing or activation. HOTAIR, for example, is a 2.2 kilobases lncRNA in the HOXC locus and can recruit the polycomb-repressive complex 2 (PRC2, comprised of SUZ12, EED, and the lysine methyltransferase EZH2) to its target gene and methylate H3K27 *via* EZH2.¹⁶ We recently demonstrated that ncRNA and lncRNA code some new proteins, so-called AltProts, forming the ghost proteome.^{3,17} AltProts represent the

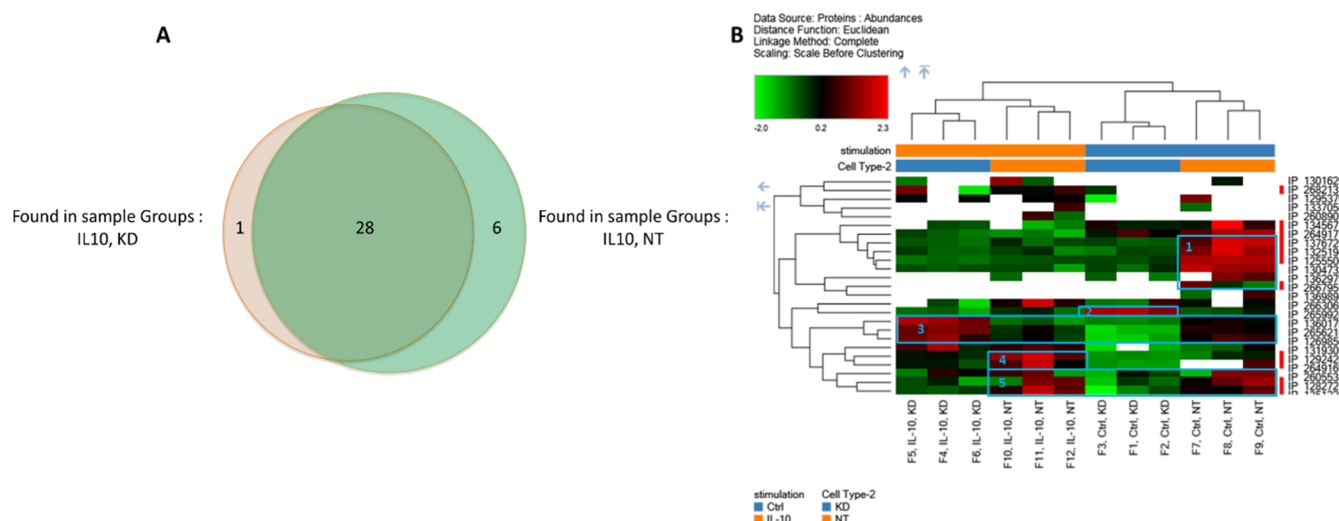


Figure 3. Result of AltProt identification in the KD- IL-10 compared to NT- IL-10 and control for each condition. (A) Venn diagram comparing the AltProt identification on KD IL-10 and NT IL-10; few AltProts are specific to each condition and majority are shared. (B) Heatmap of the AltProt for P -value > 0.05, comparing all types of stimulation and cells; if lots of AltProts are shared by KD IL-10 and NT IL-10, the quantification presents some variation.

invisible part of the proteome because they are currently not described. In fact, mass spectrometry proteomics strategies are based on the identification of proteins by comparison with databases.^{18–23} However, it has been shown that many proteins have not yet been referenced in these databases, in particular, because of the rules used to predict the reference proteins. Thus, AltProts represent proteins of lower molecular weight than RefProts and are derived from regions of the RNA described as noncoding. AltProts can be found in the regions 5'UTR and 3'UTR or even shifting on the reading frameworks (CDS) in +2 or +3. Proteins from these regions do not share common sequences with the RefProts produced by the same mRNA and are therefore not isoforms. In this context, we looked for such proteins in the histone subproteome of macrophages. A total of 40 AltProts have been identified, for example, 36 in NT control and NT-IL-10: 30 are common between the two conditions, two are specific to the control conditions, and four are specifically expressed in NT-IL-10 (Figure S2). A total of 31 are expressed by KD and KD-IL-10 cells: 27 are common between the two conditions, two are specific to the control conditions, and two are specifically expressed in KD-IL-10 (Figure S2). A total of 10 AltProts are in common between all cell types and conditions. Finally, 28 AltProts are common between PC1/3 KD and NT cells under IL-10 stimulation, one is specific to KD-IL-10 and six are specific to NT-IL-10 (Table S3, Figure 3A). The heatmap (Figure 3B) presents five clusters of significantly variable AltProts. We observe in a first cluster six AltProts (IP_1376729, IP_1325194, IP_1255506, IP_1304732, IP_1362979, and IP_2667951) having a higher abundance in the NT-Ctrl conditions; the expression of these AltProts therefore seems specific to the NT type. However, after IL-10 treatment, a drop-in expression of these AltProts is noticed to reach the same level as PC1/3 KD and KD-IL-10 cells. These six AltProts could be linked to the signaling pathways directed by PC1/3 and IL-10. We also observe the presence of an AltProt (cluster2) (IP_2659924) overexpressed under the KD-Ctrl conditions, which means that this AltProt is the result of PC1/3 inhibition, but ultimately inhibited after IL-10 treatment. In the third cluster, three AltProts (IP_1360175, IP_2656216,

and IP_1269850) have a higher level of expression in the KD-IL-10, whereas under other conditions, their expression is either repressed or not changed, so this points out three AltProts specific to PC1/3 KD conditions overexpressed under IL-10 treatment and leading to the hypothesis of a role in tumor resistance. In the fourth cluster, two AltProts (IP_1292426 and IP_2649169) are overexpressed only under the NT-IL-10 conditions. In this case, PC1/3 inhibition is not involved in the production of these AltProts in macrophages, but they can be implicated in macrophage resistance under IL-10 treatment. Finally, a fifth cluster is visible, grouping three AltProts overexpressed in both the NT-Ctrl and also in the NT-IL-10. Here again, these AltProts are clearly specific to macrophages independently of PC1/3 inhibition. The different clusters show a variable amount of AltProts between different conditions including PC1/3 inhibition or no PC1/3 inhibition and resistance or no resistance to IL-10 treatment, which can be schematized in Figure 4.

Of the 40 AltProts identified, seven (Table 1) have attracted attention because they have significant identifications but also homology rates below 80: one is a noncoding RNA (IP_1295370), two are localized at the 5'UTR (IP_1292426 and IP_2663069), three are situated in the 3'UTR (IP_1345677, IP_2656216, and IP_1269850), and one is in the CDS at +2 (IP_1360175). IP_1360175, IP_2656216, IP_1295370, and IP_1269850 are expressed 2–3 fold higher in KD under IL-10 stimulation compared to those in NT cells under IL-10 stimulation. By contrast, IP_2663069 and IP_1292426 are expressed 1.5–2 fold higher in NT cells under IL-10 stimulation than those in KD cells under IL-10 stimulation (Tables 1 and S3). The ncRNA is linked to the serine/threonine protein kinase WNK1 (WNK lysine deficient protein kinase 1), which is known to be involved in fluid-phase pinocytosis of LDL by macrophages,²⁴ and in cancer, this protein interacts with PI3K-AKT, TGF- β , and NF- κ B signaling and plays its unanticipated role in the regulation of angiogenesis.^{25,26}

Considering the reference protein, Dennd4c from which mature RNA contains in its 5'UTR the IP_1292426, this

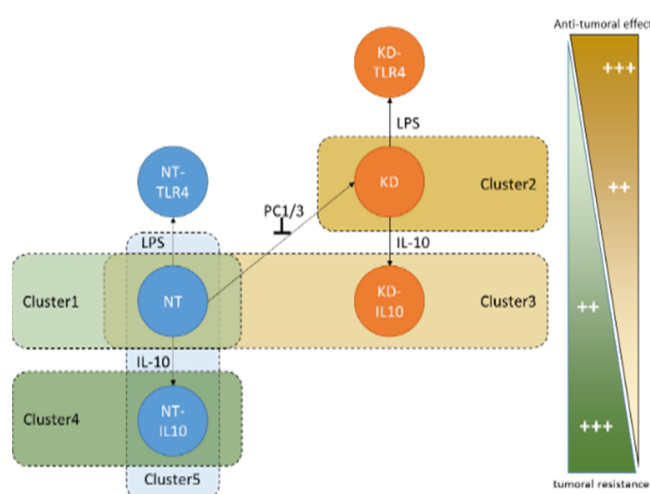


Figure 4. Schematic representation of the influence of PC1/3 inhibition and IL-10 treatment in the balance between tumor resistance and antitumoral effects according to what we previously published with LPS and paclitaxel and TLR4 stimulation.^{11,12} In this equilibrium, clusters of AltProts identified are represented, with the aim to understand the influence of these AltProts in the pathways involving both PC1/3 inhibition and IL-10 treatment.

protein is known to be involved in the M2 macrophage phenotype.²⁷ The ubiquitin-activating enzyme E1-like protein/ubiquitin-activating enzyme 7 (UBE1L/UBA7) is the activating enzyme of interferon-stimulated 15 kDa protein (ISG15). This protein is known to be involved in innate immune response, especially toward viral infection.^{28–30} ISG15 is an antiviral protein³¹ and the role of UBA7 is primordial in its activity. The presence of AltUba7 can be of real importance in the modulation of antiviral response. In KD cells treated with IL-10, AltUba7 is overexpressed, which can contribute to glioma tumor proliferation inhibition, as we previously noticed through antiviral activity.¹² For the AltNtpcr, the Ntpcr is also overexpressed in the M1 macrophage phenotype.²⁷ AltGdf1, which is the highest-overexpressed protein in KD cells treated with IL-10, comes from the reference protein Gdf1 (growth differentiation factor 1) and is known to be a novel mediator of macrophage infiltration in obesity.³²

CONCLUSIONS

Taken together, in our work, we have established that NT-IL-10 cells express proteins involved in methylation of lysine 4 and 36 of H3 histones along with nucleic factors JMJD6, Sirt 2, and HDAC1 known to be altered in the protumoral/anti-inflammatory macrophage phenotype. By contrast, KD-IL-10 cells express factors involved in the chemokine signaling

Table 1. Seven Principal AltProt Identification, Shared by KD and NT Cells under Stimulation of IL-10 or Not, Accession ID is According to the OpenProt Database, Location on the RNA Producing the AltProt Identified, Original Gene Coding for the AltProt but Currently Named by the RefProt Coding, Finally the Ratio of LFQ Obtained for Both Kinds of Cell, Ratio of (IL-10 NT)/(Control), (IL-10 KD)/(Control), and Finally (IL-10 KD)/(IL-10 NT).^a

Accession	P S M	Loc ati on	Protein Sequence	G e n e	Transcript accession	Abu ndan ce Ratio : (IL10 , NT, Sam ple) / (Ctrl, NT, Cont rol)	Abun danc e Ratio : (IL10 , KD, Sam ple) / (Ctrl, KD, Cont rol)	Abu ndan ce Ratio : (IL10 , KD, Sam ple) / (IL10 , NT, Sam ple)
IP_1360175	6	CDS	MWWSKMLNISSERWKQSTLPSPQGNFQGSQHVQIGKGLGCP STKLPLDLLFPKLYSSGPWRGPLLFRDHYHE	Gdf1, Cers1	TA=NM_001044240.2,ENSRNOT000000 27275.6,ENSRNOT00000092417.1,NM_ 001044230.2	0,646	3,13	3,192
IP_2656216	8	3'UTR	MLIHYYTKSLNRHHPKQIVHTGSEGVFRDEV	Klhl29	TA=XM_008764550.2, XM_017594070.1	1,092	2,226	1,54
IP_1345677	2	3'UTR	MLGPEYHPVHRVLCPPRTPYSCACSLSKF	Ntpcr	TA=NM_001134573.1,ENSRNOT000000 26965.7	0,619	0,812	1,45
IP_1269850	2	3'UTR	MGDMGTWGREREGSRLACLSASRGWGHFLKPQEYGTSSLE KKPKNFIVSSWRAGSEVADIIVDNRLAASVHEGCKS	Uba7	TA=ENSRNOT00000084429.1	1,055	1,801	1,433
IP_1295370	11 4	ncRNA	MDSNTALQEVLTAAIHDLARGIRKAAKALDKRQAHLCVLASD CDEPMYVVLVEALCAEHQINLIKVDNKKLGEVWVGLCKIHREE KPRKVVGCSVVVDYKGESQAKDVIEEYFKCKK	AC10 6932. 1	TA=ENSRNOT00000052241.3	0,852	2,92	1,107
IP_1292426	16	5'UTR	MGQAVWRCVSWTCSYHSCVTMTCCRLESLNVRV	Denn d4c	TA=ENSRNOT00000082596.1, XM_01759 3922.1	2,177	1,492	0,701
IP_2663069	1	5'UTR	MEGSRPRAPLLLYPWVLAFLMAAFSALSPVSPTLSCPRLTAAI SKDSLGPVSGQIEKYILSGSSWGLTSLRC	Adcy7	TA=XM_017601390.1	1,422	0,597	0,467

^aIn the gradient, blue to red indicates the under to overabundance under IL-10 conditions.

pathway and proinflammatory factors. Interestingly, Kllh29, Uba7, Ntpcr, and Dennd4c are proteins specifically expressed in alveolar macrophages, which is in concordance with our NR8383 cell lines, which are lung alveolar macrophages. Thus, the AltProts identified are clearly signatures of such alveolar macrophages and demonstrate their contribution in regulating vital immunological pathways.

Experimental Section Reagents. The rat alveolar macrophage NR8383 cell line (CRL-2192) was obtained from ATCC (USA). LPS-EB ultrapure acquired from Invivogen. Ham's F12K medium, phosphate-buffered saline (PBS), puromycin, and foetal bovine serum (FBS) were provided from Life Technologies (Milan, Italy).

Culture of NR8383 PC1/3-KD and NT Cell Lines. PC1/3 knockdown in NR8383 macrophages was performed using lentiviral transduction as described previously.⁴ NR8383 PC1/3-knockdown (PC1/3-KD) and NR8383 nontarget (NT) shRNA cell lines were cultured in Ham's F12K medium supplemented with 15% FBS and 12 $\mu\text{g}/\text{mL}$ puromycin. Culture was performed at 37 °C in a humidified atmosphere (5% CO_2).

Total Protein Extraction. Six sterile well plates of 2×10^6 NR8383 PC1/3-KD and NT cells were plated and starved overnight in Ham's F12K medium supplemented with 2% FBS. Cells were treated during 24 h with 20 ng/mL IL-10 in serum-free medium or left untreated. After stimulation, cells were collected, washed once with ice-cold PBS, and then lysed with RIPA buffer (150 mM NaCl, 50 mM Tris, 5 mM EGTA, 2 mM EDTA, 100 mM NaF, 10 mM sodium pyrophosphate, 1% NP40, 1 mM PMSF, and 1 \times protease inhibitors) for total protein extraction. Sonication for 30 s were performed three times and cell debris were removed by centrifugation (20,000g, 10 min, 4 °C). Supernatants were collected and protein concentrations were measured using a Bio-Rad protein assay kit, according to the manufacturer's instructions.

Histone Extraction. NT or PC1/3-KD cells were washed once with ice-cold PBS and then treated with the EpiQuik total histone extraction kit.

Analysis of Post-translational Changes on Histones. EPIGENTEK's EpiQuik histone H3 modification multiplex assay kit (colorimetric) is used according to the manufacturer's protocol. A total of 100 ng of histone extraction was used for the assays.

Proteomic Analyses. Shotgun bottom-up analysis was applied. Each sample was normalized to obtain a final concentration of 1.5 $\mu\text{g}/\mu\text{L}$ per sample. An equal volume of DTT 0.1 M was added and then incubated for 40 min at 56 °C to reduce disulfide bridge. Using the FASP method,¹⁴ the samples were processed. Briefly, a filter with a nominal molecular weight limit of 30,000 (Amicon Ultra-0.5 30k, Millipore) is used, the samples are transferred into the filters, and the alkylation step was done using IAA solution (0.05 M) for 20 min in the dark at room temperature. Three washes are performed with ammonium bicarbonate before digestion was carried overnight with LysC/trypsin (40 $\mu\text{g}/\text{mL}$ in 50 mM Tris-HCL solution at pH 8) at an incubation temperature of 37 °C. The filters containing the digests were then centrifuged and rinsed using 50 μL of saline solution (0.5 M); the enzyme activity was stopped with 10 μL TFA 5% for each tube. Enrichment and desalting were then performed for each sample with a ZipTip C-18 (Millipore) before undergoing LC-MS/MS analysis.

MS Data Acquisition. The analyses was performed in an Easy-nLC 1000 nano-UPLC chromatograph (Thermo Scientific) in the reverse phase in a preconcentration column (75 μm ID \times 2 cm, 3 μm , Thermo Scientific) and an analytical column (Acclaim PepMap C18, 75 μm ID \times 50 cm, 2 μm , Thermo Scientific) interfaced with a nanoelectrospray ion source on an Q-Exactive Orbitrap mass spectrometer (Thermo Scientific). A linear gradient of acetonitrile in 0.1% formic acid (5–35%, for 2 h) at 300 nL/min flow rate was used to separate peptides. The MS parameter was set to a resolution of 70,000 full width at half maximum (fwhm), a mass range of m/z 300–1600, an AGC of 3×10^6 ions, and a maximum injection time of 120 ms. The MS/MS was performed in the independent data mode, defined to analyze the 10 most intense ions of MS analysis (Top 10). For MS/MS was set to a resolution of 17,500 fwhm, a mass range of 200–2000 m/z , an AGC of 5×10^4 ions, a maximum injection time of 60 Ms, and an isolation window of 4.0 m/z .

Data Analyses. All data were analyzed using MaxQuant software version 1.5.8.3.³³ Proteins were identified based on the interrogation of the *Rattus norvegicus* database (Uniprot, release April 2017, 7983 entries).³⁴ Digestion parameters are set for trypsin with a maximum of two missed cleavages. Variable modifications are defined for oxidation of methionine and N-terminal protein acetylation and fixed modification was set for the Carbamidomethylation of cysteine. The LFQ was defined as a quantification method. Identification parameters were set on mass tolerance, 6 ppm was selected for the MS mode, 20 ppm fragmentation data of MS/MS tolerance and false discovery rate (FDR) were adjusted at 1%, and at least one unique peptide was used. The statistical analysis of identified proteins was performed using Perseus software version 1.5.2.6.³⁵ The matrix was filtered by removing the potential contaminant, reverse and only identified by site. Then, the samples were grouped into four categories: NT, NT-IL-10, KD, and KD-IL-10. Statistical analysis was performed by ANOVA test with a p -value of 5%. Hierarchical clustering and the profile plot were performed and visualized by Perseus.³⁵ For the AltProts, Proteome Discoverer V2.3 (Thermo Scientific) is used; the protein database was downloaded from OpenProt (<https://openprot.org/>)² and included RefProt, novel isoforms and, AltProts predicted from both Ensembl and RefSeq annotations (Rnor_6.0.84, Rnor_6.0) for a total of 293,303 entries.³⁶ The following processing and consensus parameters are used: Trypsin as the enzyme, two missed cleavages, methionine oxidation as variable modification, and carbamidomethylation of cysteines as static modification, Precursor Mass Tolerance: 10 ppm and Fragment mass tolerance: 0.1 Da. The validation was performed using a percolator with the protein-strict FDR set to 0.001%. A consensus workflow was then applied for the statistical arrangement, using the high-confidence protein identification. Results are filtered to maintain the master protein and high-confidence protein FDR.

The mass spectrometry proteomics data have been deposited to the ProteomeXchange consortium via the PRIDE partner repository with the dataset identifier PXD020610.

Subnetwork Enrichment Pathway Analysis. Using Elsevier's Pathway Studio (version 11.0//Elsevier) and the Ariadne ResNet database,³⁷ all connection and relation between the differentially expressed proteins under all conditions were depicted. The subnetwork enrichment analysis

(SNEA) algorithm was used in the identified proteins, NT or KD with or without IL-10 treatment for detecting the statistically significant altered biological pathways in which the identified proteins are involved. Nonrandom connection between two categorical variables organized by a specific relationship was detected by the algorithm using Fisher's statistical test. Also, this algorithm starts by creating a central "seed" from all the relevant identities in the database and from this point builds connections based on the relationship between entities and the seed.³⁷ The SNEA algorithm compares the subnetwork distribution to the background distribution using one-sided Mann–Whitney *U* test and calculates a *p*-value, thus representing a statistical significance between different distributions. In all analyses, the GenBank ID was used to form experimental groups based on the different conditions present for analysis. Finally, based on biological processes and molecular function, the pathway networks were reconstructed with its associated targets.³⁷ In addition, we performed a targeted protein pathway analysis using the protein list identified in the different clusters.

■ ASSOCIATED CONTENT

■ Supporting Information

The Supporting Information is available free of charge at <https://pubs.acs.org/doi/10.1021/acsomega.0c02530>.

Shotgun proteomic data from NT and PC1/3 KD cells after treatment or no treatment with IL-10 and shotgun proteomic data of histone extracts of NT and PC1/3 KD cells treated or not treated with IL-10. List of PTMs identified on histones after shotgun proteomic analysis. List of identified ghost proteins in histone extracts from NT and PC1/3 KD cells after treatment or no treatment with IL-10. Quantification of post-translational changes on H3 Histones. ELISA test against post-translational changes of the H3 histones according to Epigentek's histone modification assay kit. Venn diagram of the repartition to the identification for the different sample conditions. (PDF)

Authors' details (XLSX)

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■ Author Contributions

MS.; methodology, T.C., M.S. M.D., F.R., F.K., S.A., and I.F.; Validation, T.C. and O.B.; formal analysis, M.; investigation, I.F., F.R., M.D., B.O., T.C., and M.S.; resources, I.F. and M.S.; data curation, T.C., M.D., and M.S.; writing—original draft, T.C., F.R., I.F., and M.S.; writing—review and editing, T.C., M.D., F.K., and M.S.; supervision M.S. project administration, M.S.

■ Notes

The authors declare no competing financial interest.

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