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ORIGINAL RESEARCH

Pro-necrotic molecules impact local immunosurveillance in human breast cancer

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ABSTRACT

Necrosis culminates in spilling cellular content through the permeabilized plasma membrane, thereby releasing potentially immunostimulatory molecules in the pericellular space of dead cells. Accordingly, molecules involved in necroptotic signaling, such as receptor-interacting serine/threonine-protein kinase 3 (*RIPK3*) and mixed lineage kinase-like (*MLKL*) have been found to stimulate anticancer immune responses in mouse models of chemotherapy. mRNAs encoding prominent pro-necrotic gene products (*RIPK1*, *RIPK3*, *MLKL*, *PGAM5* and *DFNA5*) were correlated with immune-related metagenes in several cancer types (breast, colorectal, lung, ovary, melanoma), revealing the strongest associations in breast cancer. In two independent breast cancer cohorts, the expression of *MLKL* and *DFNA5* was decreased at the mRNA levels in tumor as compared with normal tissues. Moreover, *MLKL* expression exhibited a strong positive correlation with genes reflecting the presence of B, NK and T lymphocytes in the tumor bed, in multiple distinct breast cancer subtypes. In contrast, the positive correlation between *RIPK3* and lymphoid cells was restricted to $HER2^+$ and triple negative/basal-like breast cancer. Moreover, the expression of *DFNA5*, which mediates post-apoptotic secondary necrosis, mostly correlated with the monocytic lineage and macrophages in ER^+/luminal A breast cancers. *MLKL* (and to some extent *RIPK1* and *RIPK3*) was strongly associated with the local expression of genes involved in interferon-α and interferon-γ responses. Altogether, these results support the idea that pro-necrotic signaling facilitates intratumoral immune responses in human breast cancer.

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Introduction

Although it had been initially thought that necrosis, a cell death modality culminating in plasma membrane permeabilization, would occur in a non-regulated fashion,[1] it has become clear that, at least in some cases, necrosis can occur in a highly regulated fashion following the activation of a series of pro-necrotic signaling molecules.[2] In the so-called necroptotic pathway, the central signaling module involves phosphorylation of the pseudokinase mixed lineage kinase-like (*MLKL*) by receptor-interacting serine/threonine-protein kinase 3 (*RIPK3*).[2,3] *RIPK3* is often activated by another kinase of the same family, *RIPK1*[4] and can associate with yet another putative pro-necrotic protein, namely, the mitochondrial protein phosphatase *PGAM5*.[5] *MLKL* associates with the plasma membrane and causes its permeabilization as a result of uncontrolled ion fluxes.[2,3,6] A homolog of *MLKL*, non-syndromic hearing impairment protein 5 (also known as deafness associated tumor suppressor, *DFNA5*), is activated in secondary necrosis (i.e. necrosis after apoptosis) as a result of its partial proteolysis by the pro-apoptotic caspase-3.[7]

Although it had been widely thought that apoptosis would be a non-immunogenic cell death modality, while necrosis would be immunogenic,[8] it turned out that the activation of caspases, which is a hallmark of apoptosis, may be required for immunogenic signaling when cell death is stimulated by chemotherapy or radiotherapy.[9-12] In addition, ER stress, autophagy and type-1 interferon signaling contribute to elicit immune responses to dead-cell antigens.[13-16] Recent research revealed that pro-necrotic molecules, in particular *RIPK3* and *MLKL*, can contribute to immunogenic cell death (ICD) signaling.[17-19] Of note, cancer cells often lose the expression of either *RIPK3* or *MLKL*,[20,21] which might increase their cell-intrinsic resistance to lethal signals and facilitate their escape from immunosurveillance. Altogether, these observations underscore the probable clinical relevance of ICD in determining the control of cancers by the immune system.

Breast cancer is under strong immunosurveillance,[22-25] and there is ample evidence that the suppression of ICD-relevant molecules and pathways has a negative prognostic impact in breast cancer patients, correlating with a poor ratio of $CD8^+$ cytotoxic T lymphocytes over $FOXP3^+$ regulatory T cells as a sign of poor local immunosurveillance.[14,26-30] However, it has not yet been investigated whether the expression of

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pro-necrotic gene products may affect immunosurveillance in patient samples. Here, we show that low expression of several pro-necrotic proteins, in particular RIP3, *MLKL* and *DFNA5*, may negatively affect the density of particular immune cell subtypes infiltrating breast cancers.

Results and discussion

Impact of pro-necrotic molecules on immunosurveillance in different cancer types

Previous work by our group and that of others indicate that cell death occurring within established cancer can stimulate local immune responses[9,11,13,14,31-35] and that pro-necrotic signaling can contribute to this immunogenicity.[17-19] In a first step of this bioinformatics analysis, we took advantage from publicly available data, focusing on five frequent malignancies that are known to be under immunosurveillance (breast cancer, colorectal cancer, non-small cell lung cancer (NSCLC), melanoma and ovarian cancer). We determined the correlation of the mRNAs coding for prominent proteins involved in pro-necrotic signaling *(RIPK1, RIPK3, MLKL, PGAM5)* and secondary necrosis (*DFNA5*) with that of genes indicating the local presence of different stromal cell populations, based on the microenvironment cell populations-counter (MCP-counter) method, which facilitates estimating the absolute abundance of eight immune and two stromal cell populations.[36] As indicated by volcano plots in which the Spearman correlation values (ρ) were plotted against the p values, *MLKL* correlated positively with 6 out of 8 among the immune cell subtypes (T cells, CD8 T cells, CTL, NK cells, B cells, monocytic lineage and myeloid dendritic cells) in breast cancer with a rather low p value ($p < 10^{-30}$) (Fig. 1). This portion of extremely high positive correlations ($p < 10^{-30}$) dropped to 2 out of 8 (cytotoxic T lymphocytes and NK cells) for *MLKL* in the case of lung cancer and was not attained by any of the other cancer cell types (Fig. 1). Of note, *MLKL* and immune-related metagenes were not more abundant in breast cancer than in other cancer types (Fig. S1), a finding that excludes the breast-cancer-specific correlation between *MLKL* and immune subtypes is due to their high expression level. As a result, we decided to concentrate our subsequent analysis on breast cancer.

Reduced expression of pro-necrotic molecules in breast cancer

We determined the mRNA expression levels of *RIPK1, RIPK3, MLKL, PGAM5* and *DFNA5* in two major breast cancer-relevant databases, namely TCGA and molecular taxonomy of breast cancer international consortium (METABRIC[37]). We expected that such genes would be downregulated in malignancies due to their implication in cell-autonomous cell death pathways, as well as possibly in immunostimulatory signaling. When comparing the expression level of these genes in the total population of breast cancer patients, *DFNA5* was found to be significantly ($p < 10^{-8}$, one-sided Student t-test) downregulated (both in TCGA and METABRIC) and this downregulation was again significant ($p < 0.01$) for all subgroups of breast cancers (Fig. 2). A global tendency for downregulation was also found for *MLKL* ($p < 0.05$ in both TCGA and METABRIC) and *RIPK1* ($p < 0.05$ only in METABRIC). Subgroup analyses

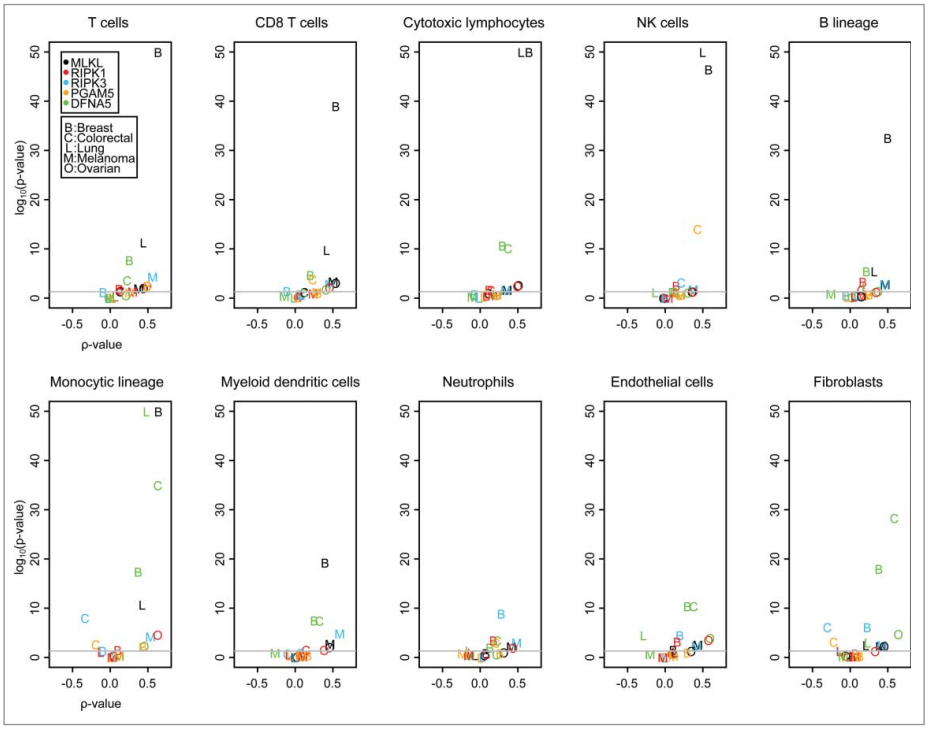

Figure 1. Volcano plots of Spearman correlation tests ("ρ value" *versus* p value). The Spearman tests were applied on correlations between selected genes (*MLKL*, *RIPK1*, *RIPK3*, *PGAM5* and *DFNA5*) and between activities of different immune cells, measured within MCP-counter. Datasets of different cancers (primary tumors) are considered: breast, colorectal, lung, melanoma, ovarian. p values are adjusted for each immune cell type following the Benjamini Hochberg method. The lowest p values are indicated as 10e-50.

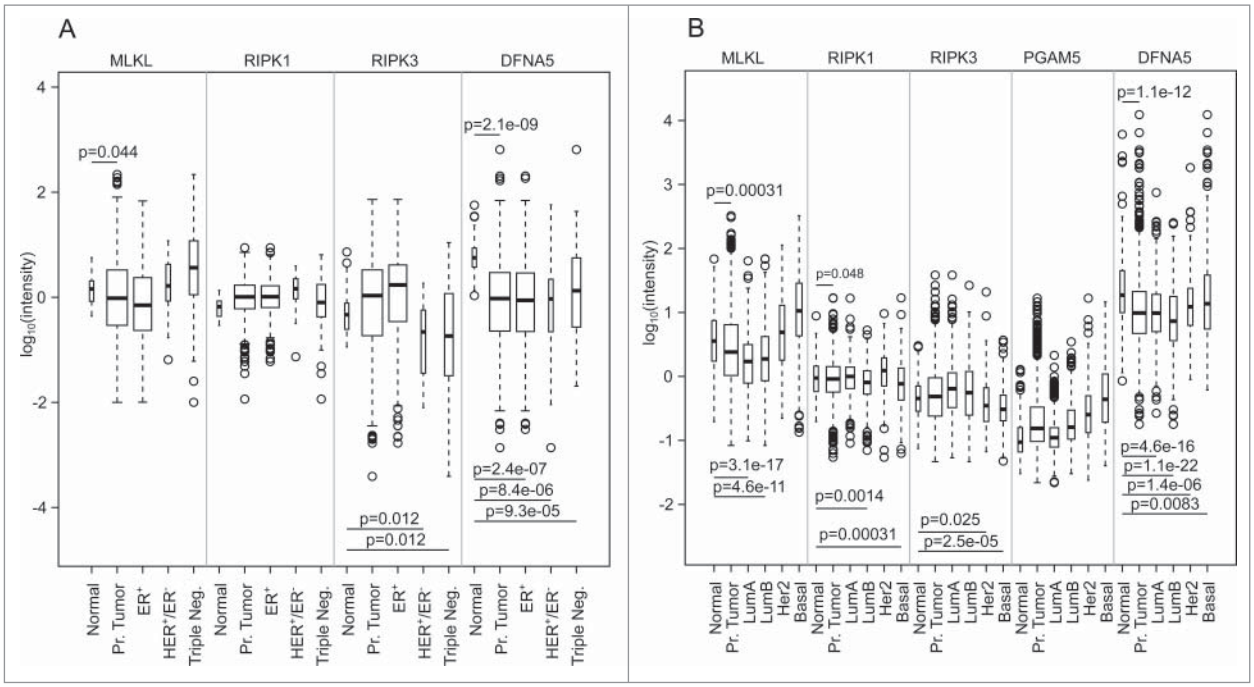

Figure 2. Expression of *MLKL*, *RIPK1*, *RIPK3*, *PGAM5* and *DFNA5* in breast cancer (normal tissue, primary [Pr.] tumors and tumor subgroups), from the TCGA data set (A) and the METABRIC data set (B). *p* values are indicated when the expression is significantly lower compared with normal tissue (one-sided t-test). TCGA breast cancer subgroups are slightly different than the usual ones because of missing information about tumor tissues. *PGAM5* expression is not available for TCGA data set because no probeset is available for this gene.

indicated that *RIPK3* was only downregulated in $HER2^{+}/ER^{-}$ and triple-negative breast cancers (in TCGA) and $HER2^{+}$ and basal-like breast cancers (METABRIC). Hence, analyses of two independent cohorts (Fig. 2) indicate that breast cancers tend to reduce the expression level of major pro-necrotic mediators.

To understand the mechanisms through which the expression of pro-necrotic genes is diminished in breast cancer compared with normal tissue, we determined promoter methylation (according to Koo et al.[21] methylation of *RIPK3* represses necrosis in cancer), mutations and copy number variations. Of

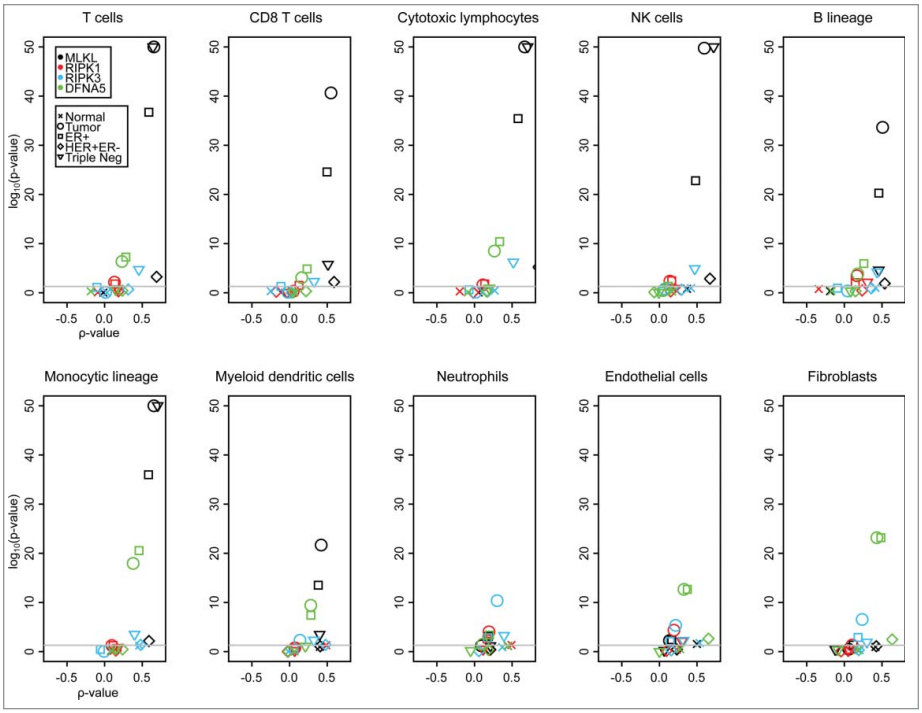

Figure 3. Volcano plots of Spearman correlation test ("ρ value" and associated *p* value) in TCGA breast cancer (normal tissue, primary [Pr.] tumor and the indicated tumor subgroups). The Spearman tests were applied on correlation between selected genes (*MLKL*, *RIPK1*, *RIPK3*, *PGAM5* and *DFNA5*) and between the activities of different immune cells, measured within MCP-counter, in TCGA breast cancer data set. Subgroups are slightly different than the usual ones because of missing information. *PGAM5* expression is not available for the TCGA data set because there is no associated probeset. *p* values were adjusted for each immune cell type (Benjamini-Hochberg method). The lowest *p* values are indicated as 10e-50.

note, mutations affecting these genes were infrequent, while a reduction in copy numbers was observed in <2% of breast cancers (Fig. S2, from cBioPortal, http://www.cbioportal.org,[38,39]). DNA hypermethylation did not affect any of the pro-necrotic genes investigated here (Fig. S3, from Wanderer, http://maplab.imppc.org/wanderer/,[40]). As a result, the mechanisms that account for the relative downregulation of *MLKL*, *RIPK3* and *DFNA5* in breast cancer tissue remain elusive. Nevertheless, we did not find any significant effect of the expression of these genes on overall survival.

Positive correlation between the expression of pro-necrotic mediators and the immune infiltrate

MLKL stood out for positive correlations with many different stromal cell types, in particular T cells, $CD8^+$ T cells, cytotoxic T lymphocytes, NK cells, B cells, monocytic cell and myeloid dendritic cells (but not neutrophils, endothelial cells and fibroblasts), both in TCGA (Fig. 3) and in METABRIC (Fig. 4), irrespective of whether all breast cancers were analyzed together or whether they were analyzed as subgroups. Such positive (though more moderate) correlations were also found for *RIPK3*, but mostly in the subgroups of triple-negative breast cancers (TCGA, Fig. 3) and basal-like tumors (METABRIC). *DFNA5* exhibited positive correlations with several lymphoid and myeloid subpopulations across the entire breast cancer cohort (Fig. 3, 4), as well as in $Her2^+$ (TCGA, Fig. 3) and luminal A breast cancers (METABRIC, Fig. 4). Of note, in normal tissue, no significant correlations were found between mRNAs coding for pro-necrotic molecules and those indicating the presence of immune cells in the TCGA (Fig. 3), while such associations were found in several incidences (and in particular for *MLKL*) in the METABRIC cohort (Fig. 4), perhaps reflecting the fact that in TCGA "normal" tissue is from patients without cancer, while in METABRIC "normal" tissues has been retrieved from cancer patients, adjacent to the malignant tumor. Irrespective of this discrepancy, it appears that the expression levels *MLKL*, *RIPK3* and *DFNA5* often correlate with distinct immune cell subtypes. These positive correlations suggest that the abundance of immunologically relevant cell types is globally reduced in cancer tissues as compared with normal ones, as this applies to the expression of necrosis-associated genes. Indeed, immune cell type activities are lower in tumor tissues as compared with normal breast tissues in the METABRIC data set. However, this tendency was not found for the TCGA data set (Fig. S4). The reason for this discrepancy is not clear, yet may be linked to the disparate definition of "normal" tissue for the 2 data sets.

At a next step, we determined whether the positive correlations obtained by means of the MCP counter[36] (Fig. 3, 4) could be reproduced using other methods for extracting information on the abundance of immune cell subsets from microarray data, namely CIBERSORT (which informs on the relative rather than the absolute abundance of immune subsets in a tissue)[41] and IMMUNOME (which yields data on the absolute abundance of immune subsets, implemented in Ref.[42] based on Ref.[43]). *MLKL* expression strongly correlated with multiple different immune cell subtypes, both according to the MCP and the immunome methods. The correlation between *MLKL* expression and particular

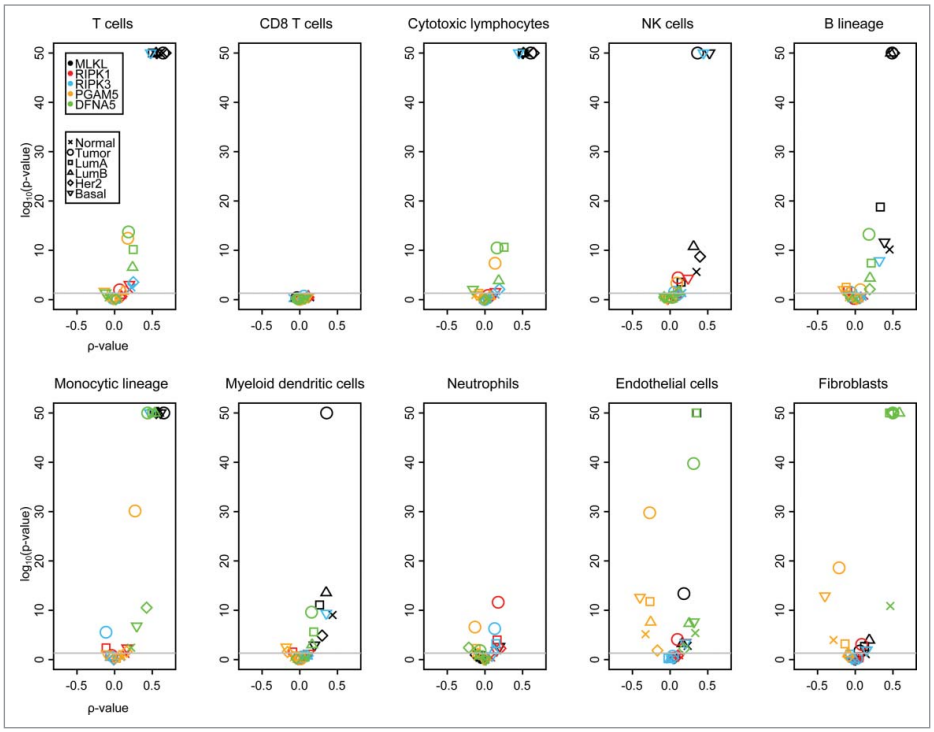

Figure 4. Volcano plots of Spearman correlation test ("ρ value" and associated p value) in METABRIC breast cancer (normal tissue, primary [Pr.] tumor and the indicated tumor subgroups). The Spearman tests were applied on correlation between selected genes (*MLKL*, *RIPK1*, *RIPK3* and *DFNA5*) and between activities of different immune cells, measured within MCP-counter, in the METABRIC breast cancer data set. p values were adjusted for each immune cell type (Benjamini-Hochberg method). The lowest p values are indicated as 10e-50. Note that $CD8^+$ T cells are uniquely defined by the CD8A gene and that the probe detecting this gene may be inappropriate in the METABRIC data set.

immune subsets was less impressive when the CIBERSORT method was used, yet appeared clear for follicular helper T cells, memory B cells, M1 macrophages and γ/δ T cells (Fig. 5). Analyses of the levels of *MLKL* expression found in distinct immunological cell subtypes (from "The Human Protein Atlas", http://www.proteinatlas.org/,[44-47] and from the "Immunological Genome Project", https://www.immgen.org/,[48]) suggest a particular strong expression in the spleen and in stem cell subpopulations (Fig. S5), yet revealed no clear overlap with the correlations found in breast cancer (Fig. 5). The correlation between *RIPK3* and metagenes corresponding to different immune subtypes was only detectable by MCP and IMMUNOME but not CIBSERSORT analyses. With respect to *DFNA5*, the positive correlations across distinct breast cancer types were only concordant for one particular immune subset, namely monocytic cells (MCP) and macrophages (IMMUNOME) (Fig. 5). However, the fact that *DFNA5* is particular abundant in granulocytes (Fig. S5) might explain its association with neutrophil infiltration in breast cancers (Fig. 5). Altogether, these results suggest that a few particular associations may reflect functional interactions between pro-necrotic molecules and immune cell subsets in human breast cancer. The CIBERSORT analysis differs from the MCP and IMMUNOME analyses, probably because CIBERSORT estimates the relative ratio of different immune cell types, while MCP and IMMUNOME methods estimate the absolute abundance of immune effectors.

Positive correlation between the expression of pro-necrotic mediators and molecular processes

In a final step, we sought to correlate the expression levels of pro-necrotic molecules with annotated molecular processes rather than with specific immune subpopulations in breast cancer transcriptomes (Fig. 6). The strongest enrichment that was fully reproducible (between the TCGA and the METABRIC data sets) affected the interferon-α and interferon-γ responses, both of which exhibited a strong correlation with *MLKL* expression and, to a lower degree, with that of *RIPK1* and *RIPK3* (Fig. 6). Both interferon responses are well-known to play a major role in general immunosurveillance[49] and in particular in the immune control of breast cancer,[15,16] strongly supporting the idea that pro-necrotic molecules (and in particular *MLKL*) favor local antitumor immune responses.

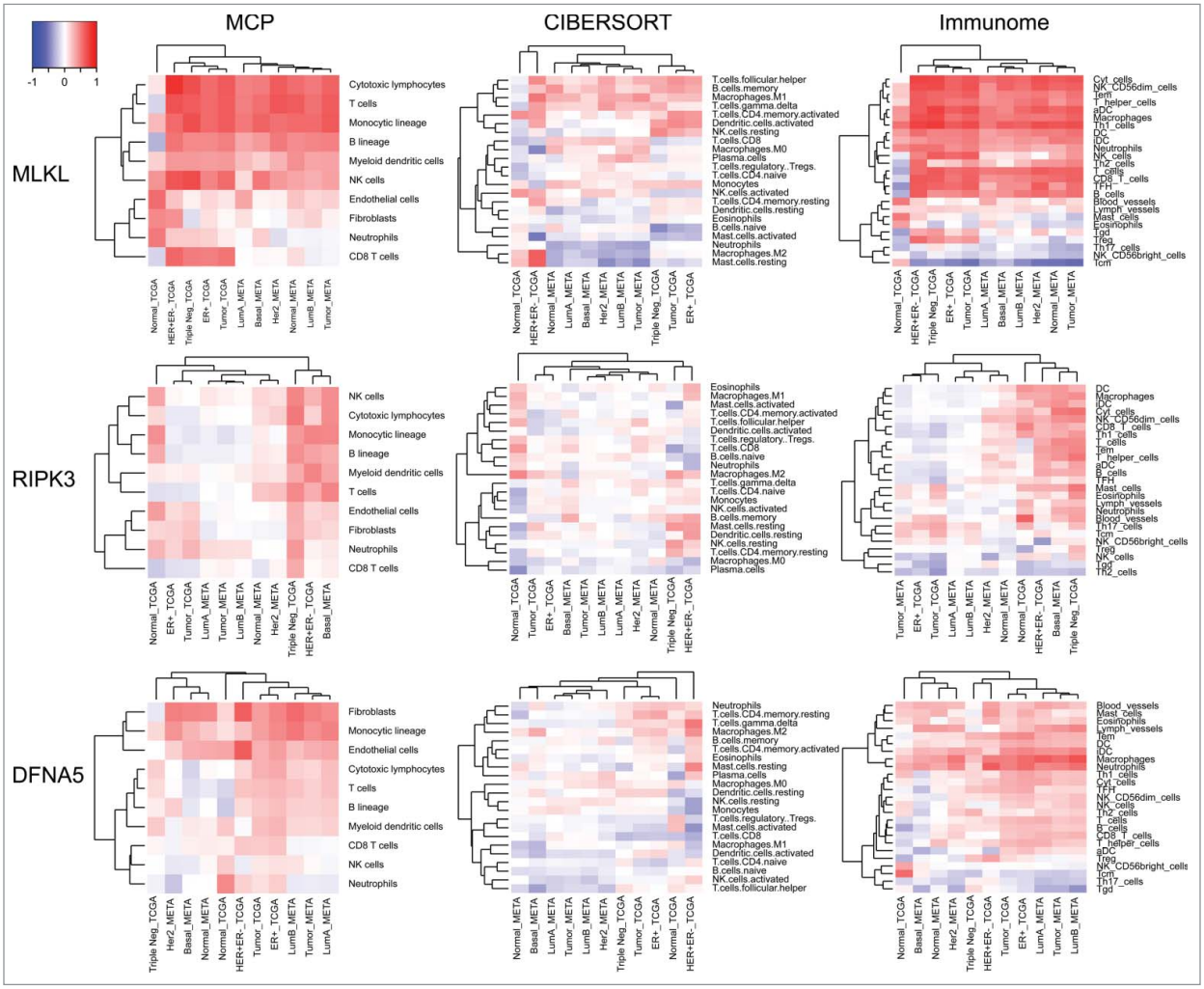

Figure 5. Heatmap representation of Spearman correlation coefficients. Correlations between three genes (*MLKL*, *RIPK3* and *DFNA5*) and between activities of different immune cells are indicated. Immune cell densities were estimated by three different methods: MCP-counter, CIBERSORT and IMMUNOME. Datasets are from TCGA and METABRIC breast cancer microarrays (normal tissue, all primary tumors and tumor subgroups).

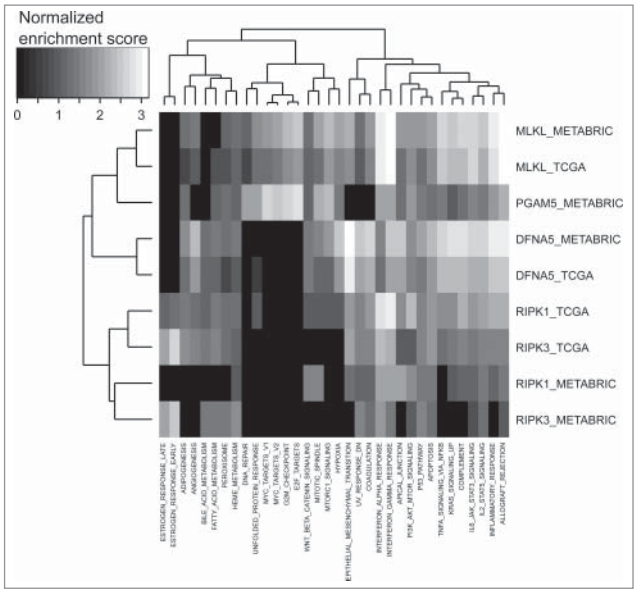

Figure 6. Heatmap representation of GSEA hallmark enrichment scores. GSEA analysis was based on Spearman correlation coefficients, between genome-wide expression and between selected genes (*MLKL*, *RIPK1*, *RIPK3*, *PGAM5*, *DFNA5*), in the two breast tumor data sets TCGA and METABRIC. *PGAM5* expression is not available for the TCGA data set because there is no associated probeset.

Concluding remarks

The bioinformatics analyses that we performed on two independent breast cancer cohorts revealed a marked tendency to reduce the expression of the two central mediators of necroptosis (*RIPK3* and *MLKL*) as well as that of the central mediator of secondary necrosis (*DFNA5*) in breast cancers compared with adjacent tissues. The molecular mechanisms of this relative downregulation remain elusive because the frequency of promoter hypermethylation, mutation or allelic loss of the corresponding genes/loci was too small to have a statistical impact on gene expression. Importantly, it appeared that high expression of *MLKL* (and to a less extent that of *RIPK3*) correlated with an enhanced density of multiple effectors of the cellular immune system including myeloid and lymphoid elements, perhaps reflecting the fact that these types of cells often are expressed in an "organized" (highly correlated) fashion in breast cancer.[42,50,51] In contrast, *DFNA5* expression was particularly well-correlated with that of myeloid cells, in particular macrophages. These correlations were partially dependent on the breast cancer subtype. For instance, *RIPK3* correlated with the lymphoid infiltrate in particular in $HER2^+$ and triple negative/basal-like breast cancer, perhaps reflecting the fact that these classes of mammary carcinomas is under particularly strong immunosurveillance.[52]

The precise mechanisms accounting for poor immune infiltration in mammary tumors with reduced expression of pro-necrotic gene products remain elusive. Preclinical experiments performed on *RIPK3*- or *MLKL*-deficient cancers revealed that such tumors were relatively unable to recruit immune cells post-chemotherapy, likely due to the absence of cellular release of ATP (which acts on purinergic receptors to attract myeloid cells into the tumor bed) and *HMGB1* (which acts on toll-like receptor-4 to induce the activation/maturation of dendritic cell precursors).[18] Furthermore, *RIPK3*- or *MLKL*-deficient cancer cells showed a reduced activation of interferon response genes upon *in vitro* exposure to anthracyclines,[18] and similarly reduced expression of *MLKL* (and to some extent *RIPK1* and *RIPK3*) in breast cancer correlated with a diminished expression of genes involved in interferon-α and interferon-γ responses. Future preclinical work as well as clinical studies must determine whether measures to compensate for such defects (e.g. ATPase inhibitors to increase extracellular ATP levels, synthetic toll-like receptor-4 agonists, stimulators of the interferon response) may overcome the obstacle to immunosurveillance that results from deficient expression of pro-necrotic molecules.

Materials and methods

Datasets

The two biggest data sets of breast cancer microarrays, which are publicly available, were used are as follows: TCGA Breast cancer and METABRIC.[37] For the latter, when a gene has several probesets, we used the one with the biggest variance. For other cancers, we used the biggest microarrays data set that has at least one probeset for *MLKL* and one for *DFNA5*: a multi-cancers data set (http://www.intgen.org/, "Bittner") for colorectal cancer, a lung cancer data set,[53,54] a skin cancer datataset[55] for melanoma and an ovarian cancer data set.[56] The multi-cancer data set was also used for producing Fig. S1.

Immune infiltrate estimation

We used the R-package associated with MCP-counter[36] to estimate the density of infiltration by distinct immune cell types from microarray data. CIBERSORT[41] and IMMUNOME (metagenes associated with immune cells defined in Ref.[42] based in selected genes/probeset from Ref.[43]) were also used.

Gene set enrichment of correlation

We ranked the genes/probesets according to the Spearman correlation coefficient (with *MLKL*, *RIPK1*, *RIPK3*, *PGAM5*, *DFNA5* in various cases) and applied GSEA[57] to these lists ("GseaPreranked"). Moreover, we used the "h.all.v5.2.symbols.gmt [hallmarks]" gene set database.

Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

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