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Increased frequency of nonconventional double positive CD4CD8 αβ T cells in human breast pleural effusions

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Breast cancer remains a leading cause of cancer-related death within the female population. Immunotherapy is expected to provide additional therapeutic benefits but has met so far limited success. This may be due in part to the poor understanding of immune responses to breast cancer. Although $CD4^+$ and $CD8^+$ T lymphocytes infiltrate these tumors, the phenotype and functions of these cells remain ill defined. This study was designed to investigate further about these questions, taking advantage of multiparameter flow cytometry on lymphocytes derived from peripheral blood, solid tumors, metastatic lymph nodes and pleural effusions samples of patients with breast cancer. Results showed that, in addition to conventional $CD4^+$ and $CD8^+$ αβ T cells, individual tumors and most pleural effusions contained significant fractions of unconventional double positive (DP) $CD4^+CD8^+$ αβ T cells. These DP T cells displayed the phenotype and cytotoxic potential of effector/memory activated $CD8^+$ T cells but differed essentially from these cells by a high production of IL-5 and IL-13. The increased frequency of DP T cells in advanced breast cancer and their high lytic potential and original cytokine profile suggest that this T-cell subset may play a specific role in the regulation of immune responses to human breast cancer.

Key words: tumor immunology; cellular immunology; T cells; immune regulation

Breast cancer is the first leading cause of cancer death within the female population, despite the fact that earlier diagnosis, conventional treatment improvement and targeted treatment development significantly increased its cure rate. Immunotherapy, including antibody-based treatments, cancer vaccines and adoptive transfer of selected or genetically modified tumor specific T cells may offer an additional therapeutic benefit to some patients.[1] To date, human breast cancer immunotherapy has not yet been developed successfully due in part to a limited understanding of the functional impact of the immune system in this cancer. Most studies on breast cancer concurred to establish a predominance of T cells and a paucity of B cells and NK cells among tumor associated lymphocytes (TAL).[2–7] However, further characterization of these T cells in term of phenotype, specificity and function remains to be addressed and a possible correlation between TAL amounts or quality and breast cancer prognosis.[2]

During the last decade, several nonconventional T-cell subsets were discovered on the basis of their function and/or phenotype. Among these, γδ TCR expressing T cells and invariant NKT have been partly characterized in human and mouse tumors, and in some studies, a regulatory function could be ascribed to these subpopulations.[8–11] Other human T-cell subsets have also been described in several pathological conditions on the basis of the coexpression of CD4 and CD8 [double positive (DP) T cells][12] or of the lack of both markers [double negative (DN) T cells].[13] Although antitumor activity mediated by DN T cells has been demonstrated,[14,15] no study has reported the presence of DP T cells in human cancer samples.

In the present study, we characterized the immune infiltrate in a series of tumor tissues and in malignant effusions of patients with breast cancers, compared the phenotype and functions of the different T-cell subsets and addressed their frequencies according to the stage of cancer.

Material and methods

Antibodies

The phenotype of cells was analyzed using monoclonal antibodies (mAbs) in conjunction with two- or three-color immunofluorescence. The mAbs used in this study include fluorescein isothiocyanate (FITC)-, phycoerythrin (PE)-, allophycocyanin (APC), peridin–chlorophyll–protein (PerCP) complex-conjugated reagents against CD3 , CD56, CD45RO, CD45RA, CD28, CD27, CD69, CCR7, CCR5, CD62L, CD25, CD152, cytotoxic T-lymphocyte antigen 4 (CTLA4), programmed death-1 (PD1), IFNγ, IL-2, IL-4, IL-5, IL-13, GM-CSF, TNFα, perforin, granzyme B, CD107a from BD Biosciences (Grenoble, France), CD4, CD8α, CD8β, TCRαβ, TCRγδ, CD94, CD161 (NKR-P1A), CD85j (ILT-2), KIR2DL1, KIR2DL3, KIR3DL1/3DS1 from Beckman Coulter (Roissy, France), NKG2A, NKG2C, NKG2D, GITR from R&D (Lille, France).

Patients and specimens

Peripheral blood were collected from healthy donors ($n = 11$) and from breast cancer patients ($n = 27$). Solid tumors ($n = 15$), invaded lymph nodes ($n = 5$) and malignant pleural effusions ($n = 16$) were collected from patients with breast cancer, all with formal consent.

Isolation of polyclonal cell populations (TIL, ILNL, PLEL and PBMC)

Solid tumor fragments of primary tumor or tumor invaded lymph node were mechanically disaggregated. The pleural effusion was centrifuged to collect cells. TAL of various origins: tumor infiltrating lymphocytes (TIL), tumor invaded lymph node lymphocytes (ILNL) and pleural effusion lymphocytes (PLEL) were isolated by culturing disaggregated tumor fragments or cells of liquid tumor into 24-well tissue culture plates with RPMI 1640 (Sigma-Aldrich) containing 8% human serum (local production), 100 U/mL penicillin, 100 μg/mL streptomycin (Sigma-Aldrich), 2 mM L-glutamine (Sigma-Aldrich) and 150 U/mL rIL-2 (Eurocetus, Rueil-Malmaison, France) for 10 to 14 days. These populations were then expanded by a single round of stimulation with phytohemagglutinin (PHA)-L (Sigma-Aldrich) in the presence of irradiated feeder cells (allogeneic lymphocytes and B-Epstein Barr virus B cells), as described.[16] The expanded lymphocytes were transferred into 6-well tissue culture plates with fresh medium to maintain a cell density of 0.5 to 1.5×10^6 cell/mL.

Peripheral blood mononuclear cell (PBMC) were isolated from blood by a Ficoll density gradient (Eurobio, Les Ulis, France).

Abbreviations: NKR, natural killer receptor; DP T cells, double positive T cells; DN T cells, double negative T cells; TAL, tumor associated lymphocytes; TIL, tumor infiltrating lymphocytes; ILNL, invaded lymph node lymphocytes; PLEL, pleural effusion lymphocytes.

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Immunofluorescence analysis

Cells (2×10^5) were stained with isotype controls or with three or four antibodies for 20 min at 4°C. Cells were then washed and 10^5 cells were acquired in the viable cells gate on a FACScalibur flow cytometer using Cellquest software (Becton Dickinson, Grenoble, France).

Analysis of intracellular cytokines and lytic markers by flow cytometry

Lymphocytes (2×10^5) were stimulated by OKT3, 5 μg/mL (Clinisciences), in 200 μL of RPMI 1640, 10% fetal calf serum (FCS) in the presence of Brefeldin A, 10 μg/mL (Sigma, St. Louis, MO) for cytokine analysis in round-bottom 96-well plates. The cultures were incubated for 6 hr at 37°C in 5% CO_2 humidified atmosphere. Cells were then stained at 4°C for 20 min, with anti-CD4 and anti-CD8 Abs for extracellular staining. For intracytoplasmic staining, cells were washed two times in 0.1% phosphate-buffered saline bovine serum albumin (PBS BSA), fixed 10 min at room temperature in a solution of PBS 4% paraformaldehyde (Sigma), washed again and stored at 4°C until labeling. Specific mAbs (cytokines, granzyme or perforine) were added to fixed cells and incubated for 30 min at room temperature.[17] Reagent dilutions and washes were made with PBS containing 0.1% BSA and 0.1% saponin (Sigma). After staining, cells were resuspended in PBS and 10^5 events were analyzed on a FACScalibur cytometer using Cell Quest Pro software. For analysis of lytic markers, TAL were not stimulated.

TAL stimulation and analysis of surface CD107a by flow cytometry

Lymphocytes (2×10^5) were stimulated by OKT3 (5 μg/mL) in 200 μL of RPMI 1640, 10% FCS in round-bottom 96-well plates. Anti-CD107a mAb was added during stimulation in each well. The cultures were incubated for 4 hr at 37°C in 5% CO_2 humidified atmosphere. Cells were then stained at 4°C for 20 min with anti-CD4 and anti-CD8 mAbs, washed two times in 0.1% PBS BSA and analyzed on a FACScalibur cytometer using Cell Quest Pro software.

Statistical analysis

Statistical analysis was done with InStat 2.01. Data were analyzed using Student–Newman–Keuls Multiple comparisons test or using Dunnett's test. $p < 0.05$ was considered significant.

Results

Predominance of αβ T cells among $CD3^+$ human breast cancer infiltrating cells

Intratumoral cell infiltrate was analyzed by immunofluorescent staining and multicolor flow cytometry in solid tumors ($n = 15$), tumor invaded lymph nodes ($n = 5$) and pleuraleffusin/F86.7washem29-1Tfp11.89970TD(n)Tj/F81Tf0.77140TD(5)Tj/F41Tf1.106MIwa648-1.0559am-

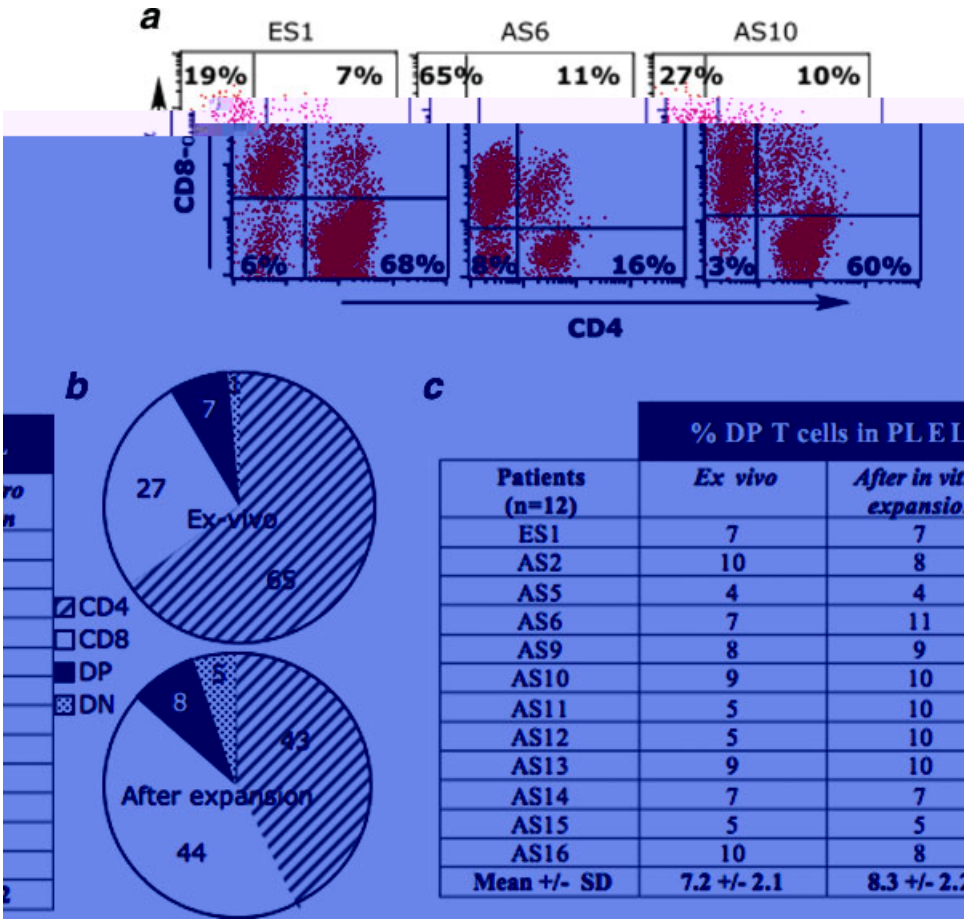

FIGURE 1 – Presence of DP T cells in PLEL derived from human breast cancer. (*a*) Surface expression of CD4 and CD8 on $CD3^+$ PLEL. Lymphocytes were analyzed after in vitro expansion by staining with mAb to CD3-APC, CD4-FITC, and CD8-PE and analyzed by flow cytometry. Panels show representative dot plots of CD4/CD8 expression on gated $CD3^+$ cells from three patients. (*b*, *c*) Impact of PHA-induced expansion on the relative frequency of DP T cells among $CD3^+$ PLEL. PLEL derived from pleural effusions of 12 breast cancer patients were analyzed freshly or after a short culture with PHA. The percentages of the four $CD3^+$ T cell subsets based on CD4 and CD8 expression are represented on the pie chart (*b*). The percentages of DP T cells in the 12 samples tested are indicated in the table (*c*). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

DP T cells (range from 1.9 to 16%) compared with blood (range from 0 to 2.5%).

Prior to attempting a more extensive undertaking, we wanted to ascertain whether increased DP fractions were really present in the original material to exclude that it might represent a culture artifact. Thus, we looked for the presence of DP T cells directly *ex vivo* in the PLEL of 12 patients. As shown in Figures 1*b* and 1*c*, freshly isolated PLEL contained percentages of DP similar to those observed after expansion (7.2 *vs.* 8.3%, respectively). The unique alteration observed before and after expansion was a decrease in the ratio of SP $CD4^+$ to SP $CD8^+$ T cells going from 2.4 to 1, demonstrating a preferential *in vitro* expansion of CD8 T cells.

As *in vitro* manipulations did not alter the frequency of DP T-cell subpopulations, we then investigated whether the presence of these nonconventional cells varied with tumor stage. Using the AJCC classification for breast cancer based on TNM criteria, 3/34 patients were classified as stage I, 9/34 as stage II, 6/34 as stage III and 16/34 as stage IV. We analyzed the frequency of DP cells among TAL from tumors ($n = 14$; 3 stage I, 7 stage II and 4 stage III), from invaded lymph nodes ($n = 5$; 3 stage II and 2 stage III) and from pleural effusions ($n = 16$; all stage IV) from these 36 breast cancer patients (Fig. 2). Percentages of DP cells in pleural effusions from stage IV patients were statistically higher compared with invaded lymph nodes ($p < 0.05$) and even more when compared with tumors ($p < 0.01$). Furthermore, we showed a trend towards a higher percentage of DP T cells among TIL extracted from stage IIB patients in comparison with stage IIA patients (data not shown). Similar differences were observed between stages IIIB and IIIA patients.

In conclusion, breast tumor associated lymphocytes often contain significant fractions of nonconventional αβ DP T cells and the enrichment of this subset seems to correlate with tumor progression.

Phenotypic analysis of DP T cells derived from TAL

To address the potential role of DP PLEL, we performed an extensive phenotypic and functional analysis of these cells in comparison with SP PLEL. Six $CD3^+$ PLEL populations were analyzed for the expression of various T and NK cell markers, in association with the expression of CD4 and CD8 coreceptors. Fractions of DP and SP $CD8^+$ or $CD4^+$ T cells expressing these markers were summarized in Figure 3. Overall, the DP T-cell phenotype did not clearly differ from that of SP CD4 and CD8 T cells. DP T cells coexpressed high levels of CD45RO and low levels of CD45RA (Fig. 3 and data not shown). Forty and sixty % of DP T cells further expressed the activation markers CD25 and CD69, respectively. The majority of DP T cells expressed CD28, about one-third of them expressed CD27 and 20% expressed CD62L. Otherwise, similarly with SP T cells, most DP PLEL lacked glucocorticoid-induced tumor necrosis factor receptor family-related

gene (GITR), the lymph node-homing markers CCR5 and CCR7 and the negative regulatory receptors PD1 and CTLA-4.

Expression of NK receptors belonging to the immunoglobulin superfamily [KIR2DL1, KIR2DL3, KIR3DL1/3DS1, ILT-2 (Immunoglobulin-like Receptor 2)] and to the C-type lectin containing family (CD94, NKG2 members and NKR-P1A) was also examined in the three subpopulations. DP and SP CD8 populations showed a similar pattern of NKR expression, with low fractions of cells expressing ILT-2 (<10%), CD94 (<5%), NKG2-A (<5%) and NKR-P1A (15–20%) and high fractions of cells (approximately 50%) expressing NKG2-D. As expected, with the exception of NKR-P1A (mean value 25%), NK receptors were not expressed by the SP CD4 population. Therefore, overall DP PLEL exhibits a phenotype extremely similar to that of SP CD8 PLEL.

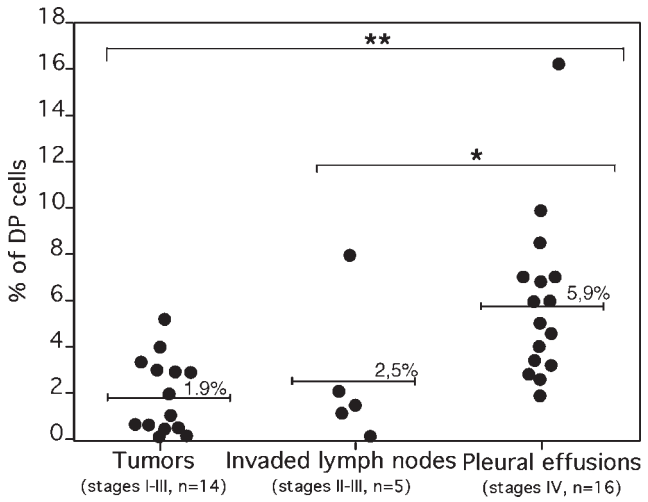

Figure 2 – DP cells frequency in breast cancer patients. TIL (n = 14), ILNL (n = 5) and PLEL (n = 16) from cancer patients at different stages were analyzed after *in vitro* expansion by three-color flow cytometry using antibodies specific for CD3, CD4 and CD8. Percentage of DP cells on gated CD3 cells in each sample was examined and mean values (horizontal lines) are presented. *$p < 0.05$; **$p < 0.01$ by Student–Newman–Keuls Multiple Comparisons test were mentioned.

Functional analysis of DP T cells derived from TAL

We then analyzed by flow cytometry the lytic capacity and the cytokine secretion profile of DP T cells derived from solid tumors (n = 4) and from pleural effusions (n = 5) compared with SP $CD4^+$ and $CD8^+$ T cells. Because results did not differ as a function of the TAL origin, data were pooled in Table II.

The cytolytic potential was estimated by measuring intracellular stores of granzyme and perforin and CD107a exocytosis upon stimulation by anti-CD3.[19] In these conditions, fractions of DP T cells as high as those of SP $CD8^+$ T cells expressed granzyme and perforine and surface CD107a. In comparison, SP $CD4^+$ T cells were nearly negative.

The cytokines profiles of the TAL subsets were determined by intracellular cytokines labeling (Table II). Mean fractions of TAL subsets secreting TNF-α, IFN-γ and GM-CSF were similar (58, 43 and 36%). In contrast, the percentages of IL-2, IL-4, IL-5 and IL-13 secreting cells were statistically higher among DP cells than among SP cells. This was especially clear for IL-5 and IL-13 ($p < 0.01$) with respectively a mean of 25 and 63% of DP T cells secreting these cytokines whereas these percentages did not exceed 8 and 40% in the SP subpopulations. Therefore, DP T cells have a lytic potential as high as SP CD8 TAL and exhibit a higher capacity to produce most cytokines especially IL-5 and IL-13.

Discussion

This study reports for the first time increased frequencies of the unconventional DP $CD4^+CD8^+$ αβ T cell subset among breast cancer TAL.

In the majority of breast cancer samples, we documented, in addition to the classical αβ T cells, the presence of unconventional T-cell subsets, such as γδ, DN and DP T cells. The presence of infiltrating γδ T cells has already been reported in various types

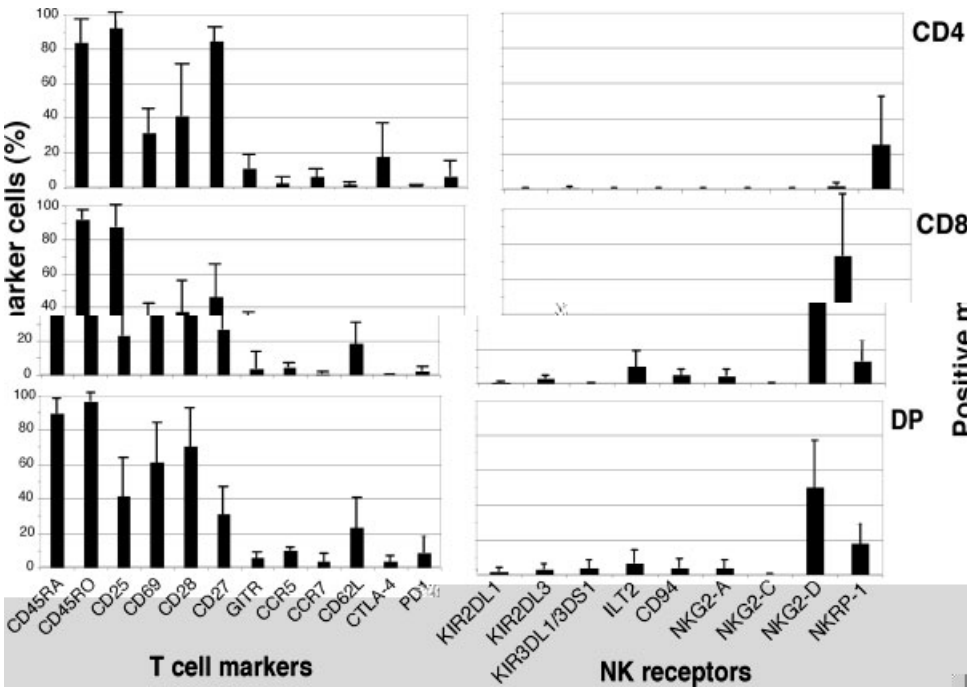

Figure 3 – Phenotypic characterization of CD4/CD8 T lymphocyte subsets. The mean percentage of cells with the indicated marker is shown for each subpopulation of six TAL. Error bars indicate SD.

TABLE II – COMPARISON OF FUNCTIONAL ACTIVITIES OF DP T CELLS WITH THAT OF SP SUBPOPULATIONS BY FACS ANALYSIS

	DP	CD4	CD8
Cytokine Production[1]			
TNF-α	66 ± 22	58 ± 24	65 ± 20
IFN-γ	52 ± 34	36 ± 34	49 ± 39
IL-2	39 ± 17	20 ± 14*	23 ± 13
IL-4	38 ± 13	23 ± 13*	24 ± 14
IL-5	25 ± 16	6 ± 6**	8 ± 8**
IL-13	63 ± 11	37 ± 14**	40 ± 13**
GM-CSF	47 ± 18	37 ± 14	33 ± 14
Lytic Potential[2]			
Perforin	59 ± 33	8 ± 7**	51 ± 31
Granzyme	73 ± 23	47 ± 35	71 ± 24
CD107a	37 ± 25	5 ± 3**	26 ± 18

[1]For cytokine production analysis, data are expressed as mean % of intracellular cytokine secreting cells in response to anti-CD3 stimulation ($n = 9$).–[2]For expression of intracellular cytotoxic mediators, TAL populations were fixed, permeabilized and stained for perforin and granzyme. For detection of T cell degranulation, TAL populations were analyzed for CD107a mobilization following polyclonal stimulation. Data are expressed as mean % of marker positive cells ($n = 4$). Significance increase of cytokines production or lytic potential by DP T cells was evaluated by Dunnett's test.
*$p < 0.05$; **$p < 0.01$.

of cancer[8] with a potent cytotoxic antitumor effector activity.[20–22] On the contrary, in a recent study, a suppressor role has been attributed to a γδ1 T-cell population among lymphocytes infiltrating breast tumors.[10] The fraction of γδ T cells that we found in breast cancers and effusions was highly variable, with a given effusion containing approximately 70% of these cells. Nevertheless, on average the fraction of these cells remained low and not significantly different from that present in healthy donor or patient PBMC. Multiparametric cytometry analysis further showed that DN TAL essentially corresponded to γδ T lymphocytes whereas all DP T cells were αβ T cells.

Nonconventional DP $CD4^+CD8^+\alpha\beta$ T cells were found enriched in individual breast cancer TAL and systematically in PLEL. We showed that the fraction of this subpopulation was very low in PBMC derived from healthy donors or patients (whatever the stage of the cancer). Consequently, our data support the accumulation of DP T cells in metastatic pleural effusions of patients with breast cancer but not in circulating blood. We also documented that the fractions of these cells were statistically higher in pleural effusions (*i.e.* stage IV) compared with invaded lymph nodes ($p < 0.05$) and solid tumors from stages I to III patients ($p < 0.01$). In addition, based on TNM classification, stage B tumors (>5 cm) contained more DP T cells than stage A tumors (<5 cm), suggesting, together with the highest enrichment in stage IV PLEL, that these cells progressively accumulated in tumor samples during cancer progression. Although DP T cells have never been described in human cancers, several investigators have reported their presence in small amount in circulating blood of healthy individuals.[23,24] Their frequency is considerably increased in some autoimmune[25–28] and infectious diseases.[29,30]

DP T cells seem in fact to represent diverse subpopulations. At least three subsets of DP T cells can be distinguished on the basis of their level of CD4 and CD8 expression and by the expression of CD8 αα homodimer or αβ heterodimer.[12,31] The DP T cells that we observed in breast tumor samples were predominantly $CD4^{low}CD8^{high}$ and CD8αβ. This profile differentiates them from the $CD4^{high}CD8^{low}$ DP T cells described in Hodgkin lymphoma,[31] Kawasaki disease[32] and in intestinal inflammatory bowel disease[33] and from intestinal DP T cells because these cells express the CD8αα homodimer.

Overall, DP T cells and SP $CD8^+$ T cells infiltrating breast cancer samples had an identical phenotype. This phenotype indicates that DP T cells are at a stage intermediate between central and effector memory ($CD45RA^{low}CD45RO^{high}CD62L^{+/-}CCR7^-CD27^{+/-}CD28^+$) according to Klebanoff *et al.*[34] Higher fractions of DP than of SP T cells, however, expressed CD25 and CD69. This suggests that DP T cells either are activated more strongly or downregulate activation more slowly than SP T cells.

Finally, NK receptors expression by DP T cells did not differ them from SP T cells. NK receptors can be expressed by T lymphocytes and this expression may regulate their effector functions.[35,36] About half DP T cells express the NKG2D receptor, already shown to be constitutively expressed and to function as costimulatory by most cytotoxic cells both $CD8^+$ T cells and NK.[37,38] About 20% of DP T cells expressed also the C-type lectin receptor NKR-P1A (CD161), for which a differential regulation of NK and T cell functions has been reported.[39,40] Interestingly, $CD4^+CD161^+$ T cells have been shown enriched in cancer patients which we also observed in this study and, a regulatory role could be ascribed to these cells.[41] Finally, the two inhibitory receptors ILT2 and CD94-NKG2A were expressed by similar low fractions of DP T cells and SP $CD8^+$ T cells. A contribution of these receptors in limiting tumor specific T-cell responses by TIL has been documented in various tumors, especially for ILT2, in breast tumors.[42–44]

Of interest, it has been shown that DP T cells play an important regulatory role in inflammatory bowel disease[33] and may contribute to the adaptative immune response during viral infections.[45] However, little is known regarding their function in cancer, except for one study describing the capacity of DP T cells infiltrating a cutaneous T-cell lymphoma to exert a tumor-specific HLA Class I restricted lysis.[46] In agreement with what was observed for intestinal DP T cells, breast tumor associated DP T cells have an overall higher capacity to produce cytokines than SP T cells.[47,48] Upon CD3 activation, high fractions of DP T cells produced, in a decreasing order, TNF-α, IL-13, IFN-γ, GM-CSF, IL-2, IL-4 and IL-5. A statistical analysis revealed that the percentages were clearly higher among SP T cells for IL-5 and IL-13 ($p < 0.01$) and, in a lower range, for IL-2 and IL-4. IL-5 is best known as a Th2 cytokine involved in eosinophil maturation and function and in B cell growth and antibody production. A role of this cytokine in the induction of cytotoxic T lymphocytes *in vivo* has also been described.[47] IL-13 is a Th2 cytokine with similar biological activity to IL-4. Recent studies showed a role of IL-13 in negative regulation of the immune response against tumors and a direct effect on the growth of tumor cells.[49,50] A fraction of human NKT has also been shown to secrete high amounts of IL-5 and IL-13.[18,50,51] Therefore, secretion of these two cytokines by DP TAL suggest that these cells might play, at the effector level, roles similar to those of NKT. Nonetheless, DP and NKT likely have different ligands because DP do not express the NKT invariant TCR Vα24 and in addition lack CD56. Breast tumor associated DP described here were also characterized by a very high cytotoxic potential.

In conclusion, this is to our knowledge, the first report about the presence of significant fractions of DP T cells in human tumors. The potential role of these cells in advanced breast tumors remains to be addressed. Present results showed that DP TAL display high lytic potential and high cytokine production ability, especially IL-5 and IL-13. Because the increase of DP TAL is observed in advanced breast cancer, it can be speculated that these cells might play a significant role in regulating immune responses to human breast cancer.

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