The role of gamma delta T lymphocytes in breast cancer: a review

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Running head and abbreviations:

Gammadelta T lymphocytes in breast cancer.

γδT lymphocytes – gammadelta T lymphocytes; MHC - major histocompatibility complex; TCR - T cell receptors; IFNγ – interferon gamma; TNFα – tumour necrosis factor alpha; ER – oestrogen receptor; PR – progesterone receptor; HER2 - human epidermal growth factor receptor 2; ICAM1 - intercellular adhesion molecule 1; AKT – protein kinase B; ERK - Extracellular Signal-regulated Kinase; STAT3 – Signal transducer and activator of transcription 3; PARP - Poly (ADP-ribose) polymerase; VEGF – Vascular endothelial growth factor; ENOS – endothelial nitric oxide synthase; TIL - tumour infiltrating lymphocyte; Treg – regulatory T lymphocyte, Th – helper T lymphocyte, DC – dendritic cell; TLR – toll-like receptor; TGF – transforming growth factor; IL - interleukin; GCSF – granulocyte-colony stimulating factor; IP-10 - interferon gamma-induced protein 10; DFS – disease-free survival; mRNA – messenger ribonucleic acid; pCR – pathological complete response; cT – clinical tumour stage; IPP - isopentyl pyrophosphate; Apppl - triphosphoric acid 1-adenosin-5’-yl ester 3-(3-methylbut-3-enyl)ester; HMBPP - (E)-4-hydroxy-3-methyl-but-2-enylpyrophosphate

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ABSTRACT

Gammadelta T (γδT) lymphocytes have provoked interest in oncology, particularly as regards their potential use in immunotherapy, because of their unique ability to recognise antigens without a requirement for major histocompatibility complex antigen presentation, and to quickly activate an anti-tumour response. However, work in some cancers has suggested that they also have pro-tumourigenic activity. Their role in breast cancer is unclear. This review outlines the evidence to date in *in vitro* studies, *in vivo* mouse models and in human studies regarding the role of γδT lymphocytes in breast cancer. We describe the seemingly opposing roles of the predominantly circulating Vγ9Vδ2^{+} subtype, which can suppress tumour growth through direct cytotoxicity, induction of apoptosis and inhibition of angiogenesis, and the predominantly tumour-infiltrating γδ1^{+} subtype which can promote tumour growth and spread through immunosuppressant effects. We summarise the evidence in breast cancer for the mechanisms of action of γδT lymphocytes and describe how factors in the tumour microenvironment may affect their function, polarising them towards a pro-tumourigenic, immune-suppressing role. We also describe the experience to date of γδT lymphocytes in immunotherapy for breast cancer and suggest the direction of work going forward, particularly as regards different breast cancer subtypes.

KEYWORDS

Gammadelta T lymphocytes; breast cancer; tumour microenvironment; prognosis; immunotherapy; bisphosphonates.

INTRODUCTION

Breast cancer is the most common cancer in females, and the second most common cause of cancer death in females in the UK. Despite significant advances in treatment over the last two decades, breast cancer still accounted for 11,433 deaths in the UK in 2014(1). Therefore, work is ongoing to develop more effective, targeted treatments, as well as reliable prognostic markers to help guide treatment regimen.

It is increasingly being recognised that the tumour microenvironment has an important role to play in the progression and dissemination of cancer(2, 3). The components of the tumour microenvironment, including fibroblasts, tumour-associated stroma and various immune cells, have different roles to play in the promotion or suppression of tumour growth. One small component of this microenvironment in humans is gamma delta T (γδT) lymphocytes, which are members of the immune system which display both innate and adaptive functions which vary between subtypes(4-6). Evidence of their cytotoxic activity against tumour cells in certain cancers, particularly cutaneous malignancy(7), as well as their ability to recognise tumour cells without the need for major histocompatibility complex (MHC) antigen presentation, has suggested that they may have a promising role in immunotherapy. However, more recent evidence in colorectal, ovarian and breast cancer has suggested that γδT lymphocytes may also have a pro-tumourigenic role(8-10). Several reviews over the last few years have described the role of γδT lymphocytes in cancer in general(11-15), but this review will summarise the evidence to date regarding γδT lymphocytes in breast cancer.

THE IMMUNE FUNCTION OF γδT LYMPHOCYTES

γδT lymphocytes, identified in the mid-1980s(16, 17), make up 0.5-16% (average of 4%) of CD3+ cells in the blood and organised lymphoid tissues, and 10-30% in the intestine of healthy adult human subjects.(18). There are many different subtypes of γδT lymphocytes, with distinct phenotypic and functional properties, with some displaying more innate features and others more adaptive features(5). In humans, for example, $V\delta1^+$ T lymphocytes are the predominant subtype found in intestinal (along with Vδ3) and skin tissues while $V\delta2^+$ T lymphocytes make up the majority of those found in peripheral blood(18). Within these broad subtypes there are further specific subtypes. For example, a subset of Vδ1 and Vδ3 T lymphocytes found in the liver are distinct from those found in blood(19), and $V\delta2^+$ lymphocytes can be divided in to innate-like $V\gamma9^+V\delta2^+$ and adaptive $V\gamma9^-V\delta2^+$subsets (5).

γδT lymphocytes are characterised by T cell receptors (TCR) made up of γ and δ chains. In contrast to αβT lymphocytes, most do not display CD4 or CD8 co-receptors. Therefore, antigen recognition is not restricted to MHC molecules(18). They also express natural killer receptors (e.g. NKG2D) and thus, are sometimes considered to represent a link between the innate and adaptive immune systems(20). γδTCR recognise a diverse array of antigens including peptides, unprocessed proteins, sulfatides and phospholipids(17, 18). Furthermore, phosphoantigens have been identified as potent γδT lymphocyte stimulators(21). These pyrophosphate molecules are intermediates of the eukaryotic mevalonate, and the prokaryotic non-mevalonate pathways for isoprenoid synthesis. Examples include (E)-4-hydroxy-3-methyl-but-2-enylpyrophosphate (HMBPP), which is produced by microbes, and isopentyl pyrophosphate (IPP) which is overproduced by transformed eukaryotic cells. Recognition of these molecules by γδT lymphocytes is therefore key to their role in both anti-microbial and anti-tumour immunity(16). As γδT

lymphocyte activation is not reliant on antigen processing, it has been suggested that they can rapidly activate an immune response(18). However, there is some evidence in the context of cytomegalovirus and malaria infections, that γδT lymphocytes may be activated later in the immune response(22, 23). This response can be both in the form of direct cytotoxicity (through the perforin-granzyme pathway), and via stimulatory and regulatory effects on other components of the immune system, through secretion of cytokines such as IFNγ and TNFα, or by direct antigen presentation(21).

TECHNIQUES FOR THE ISOLATION AND EXPANSION OF γδT LYMPHOCYTES

The ability to isolate and expand γδT lymphocytes from human blood or tumour tissues facilitates further investigation of their actions. Examples of methods by which this can be achieved are as follows. Peripheral blood mononuclear cells are isolated from blood by centrifugation, then the cells are stimulated and cultured along with Il-2. αβT lymphocytes and CD4+ T lymphocytes can be removed using specific antibodies and magnetic bead selection, then the γδT lymphocytes are cloned(24). To obtain lymphocytes from tumour tissue the tissue is first minced and digested by enzymes to release the lymphocytes prior to culture and cloning(25). The DOT method, described by Almeida et al in a chronic lymphocytic leukaemia model, uses a two-step method whereby isolated γδT lymphocytes are cultured with anti-CD3 mAB, IL-4 and IFNγ followed by a second period of culture using IL-15 in place of IL-4. This resulted in selective expansion of cytotoxic $V\delta1^+$ T lymphocytes(26). Furthermore, a method which isolates T lymphocytes by encouraging their migration out of epithelial tissues has been described to obtain T lymphocytes from skin(27) and then modified for use in gut(28). Briefly, samples of skin or gut are cultured on matrices with IL-2 and IL-15 added to the medium, during which time the lymphocytes migrate out of

the tissue and can be harvested. It should be cautioned that the method used my affect what subtype is preferentially expanded.

γδT LYMPHOCYTES IN BREAST CANCER - IN VITRO AND MURINE STUDIES

In vitro studies of peripherally-derived γδT lymphocytes

Early *in vitro* studies demonstrated anti-tumourigenic activity of γδT lymphocytes in breast cancer cell lines. Bank et al first described the cytotoxicity of Vγ9Vδ2^{+} T cells, derived from normal peripheral blood, against the MCF7 breast carcinoma cell line(29). Similarly, Guo and co-workers used ex-vivo expanded γδT lymphocytes from healthy donors and showed Vδ2 lymphocytes to be cytolytic against several breast cell lines (the luminal A MCF-7 and T-47D lines, and the triple negative MDA-MB-231 line). Importantly, they also observed that they recognised and exhibited lytic activity against human breast cancer cells but not non-malignant cells. There was also a 75% reduction in lysis when co-cultured with a TCR antibody suggesting that their cytotoxicity is TCR-dependent(30). A caveat to this was however reported in 2017, that the antibodies to the TCR used in such studies may actually be inducing apoptosis of the γδT lymphocytes resulting in reduced cytotoxicity, rather than blockage of the TCR (31). A further study observed similar findings in that *in vitro* mouse-derived γδT lymphocytes were cytotoxic to tumour cells (the triple-negative 4T1 mammary adenocarcinoma cell line), but not the non-malignant fibroblasts(32). However, it is important to note that this latter study used mouse-derived γδT lymphocytes which represent a different subset to those found in humans, which may behave differently.

Later, more detailed work in different subtypes of breast cancer was carried out. Aggarwal and co-workers demonstrated dose-dependent inhibition of cell survivability and proliferation when tumour cells were co-cultured with human peripheral blood expanded

γδT lymphocytes. This was observed in all breast cancer cell lines used (MCF-7, HER2+ SkBr7, ER- MDA-MB-231), except for the BrCa-MZ01 cell line (ER/PR+), which conversely, showed increased proliferation with γδT lymphocytes. On further investigation, it was found that MICA/B (previously reported to engage with NKG2D receptor) was expressed on the sensitive cell lines but not the resistant one. Furthermore, although intercellular adhesion molecule 1 (ICAM1) was expressed on all cell lines, it was found to be up regulated in the sensitive cell lines and down regulated in the resistant cell line, suggesting that these molecules play a role in γδT lymphocyte cytotoxicity(33). A role for ICAM1 in tumour cell recognition is supported by evidence, in other cancer types, that it is a costimulatory ligand in endothelial protein C receptor-dependent recognition of tumour cell lines (HT29 colon cancer, K562 chronic myelogenous leukaemia and U937 lymphoma) by $V\delta2^-$ γδT lymphocytes(34).

The authors of the Aggarwal study suggest that γδT lymphocytes inhibit angiogenic signalling pathways and increase apoptosis. They noted that signalling pathways associated with cell survival, AKT and ERK, had reduced phosphorylation in the sensitive cell lines but this was up regulated in the resistant cell line. Furthermore, activation of pro-apoptotic signals such as PARP and cleaved Capsase 3 was significantly up regulated in sensitive cell lines suggesting that γδT lymphocytes induce apoptotic stress(33).

In vivo studies of peripherally-derived γδT lymphocytes

Some of the studies described above went on to further investigate their *in vitro* findings in murine models. Beck and co-workers found that their mouse-derived γδT lymphocytes localised to the tumour *in vivo* as long as the γδTCR was unblocked, providing additional evidence that this is a critical mode of tumour cell recognition(32)

In the Aggarwal et al study, when mice were injected *in vivo* with human-derived γδT lymphocytes and either the SkBr7 or the BrCa-MZ01 cancer cell line, tumour growth was reduced compared with those injected with the breast cancer cell line alone. This is particularly interesting in the case of the latter cell line, in view of the *in vitro* results detailed above, and the reasons for this variation in response were unclear. Similarly to the *in vitro* results, AKT, ERK and STAT3 phosphorylation were down regulated in the tumours of mice that were co-injected with γδT lymphocytes, compared to those injected with a cancer cell line only. Higher numbers of apoptotic cells were seen in these tumours and there were higher levels of cleaved Capsase 3. These tumours also showed down regulation of VEGF and ENOS when mice were injected with both tumour cells and γδT lymphocytes, as well as reduced angiogenesis(33).

In an earlier study, Beck et al had similarly reported that, in a syngeneic mouse model, when tumour-bearing mice injected with the 4T1 cell line were treated with ex vivo expanded murine γδT lymphocytes, a significant reduction in tumour growth was observed compared to untreated controls. Similar results were observed when human γδT lymphocytes were applied to a human xenograft model of breast cancer (using the 2LMP human breast cancer cell line) in the mice(32).

***In vitro* studies of tumour-infiltrating γδT lymphocytes**

The studies above used γδT lymphocytes from blood which, as previously mentioned, in humans are predominantly of the Vγ9Vδ2^{+} subtype. On the other hand, the γδT lymphocyte population of breast tumour-derived tumour infiltrating lymphocytes (TILs) have been shown to be predominantly (95%) of the Vδ1^{+} subtype, which make up 7.2-75.7% of breast tumour-derived TILs(25). In 2007, Peng et al showed that, *in vitro*, breast tumour-

derived TILs had an immune-suppressive role. They suppressed naïve T cell proliferation, as well as Il-2 production by CD4 and CD8 cells, and blocked dendritic cell (DC) maturation and cytokine secretion (25).

Since then, further work has attempted to elucidate the mechanisms by which γδT lymphocytes may promote tumour progression in breast cancer. Ye et al assessed the effect of human tumour-derived γδT lymphocytes on other immune cells *in vitro*. These γδT lymphocytes were functionally γδT regulatory (Treg) lymphocytes, which induced cell cycle arrest in cytotoxic T lymphocytes. Along with CD4 Tregs, they suppressed naïve T cell proliferation through induction of T cell senescence. It was observed that senescent CD4+ and CD8+ lymphocytes secreted large amounts of proinflammatory IL-6, IFNγ and TNFα, but also IL-10 and modified TGFβ1, suggesting a negative regulatory function. These senescent T lymphocytes also inhibited proliferation of functioning CD4+ T- lymphocytes. Furthermore, γδTreg lymphocytes inhibited proliferation of Th1 and Th17 effectors. Additionally, they induced senescence in DCs, which had a distinct deficiency in their maturation and co-stimulatory function, and lost their capacity to process and present antigens. Through investigation of ways to overcome this induction of T cell and DC senescence by γδT lymphocytes, the group demonstrated that toll-like receptor 8 (TLR8) signalling can prevent γδTreg-mediated senescence induction in functioning T- lymphocytes and DCs(35).

In vivo studies of tumour-infiltrating γδT lymphocytes

Both groups then tested their finding using *in vivo* mouse models. The Peng group confirmed the immune-suppressive role of breast tumour derived TILs observing that they suppressed the ability of CD8+ cells to kill tumour cells(25). Ye et al confirmed that γδTreg lymphocytes induced functioning T cell senescence, which in turn suppressed the

proliferation of functioning CD4+ lymphocytes. Furthermore they confirmed that manipulation of TLR8 signalling could prevent this senescence(35).

Investigation of IL-17 –secreting γδT lymphocytes

A 2015 study by Coffelt et al primarily investigated the role of neutrophils in breast tumour metastasis formation in mice. However, as a result of their investigations they demonstrated a requirement for the IL-17 - GCSF signalling cascade for neutrophil expansion. This was particularly required for the CD8-suppressing neutrophil phenotype to predominate, and drive metastases. When investigating the source of the IL-17, they found that splenic-derived γδT and CD4+ T lymphocytes both expressed IL-17A. However, only γδT lymphocyte depletion led to reduced IL17A and GCSF serum levels, reduced circulating neutrophils and a reversal of the CD8-suppressing neutrophil phenotype. Furthermore, depletion of γδT lymphocytes in the early phase of tumourigenesis in this mouse model reduced pulmonary and lymph node metastases but not primary tumour growth(36).

Other studies have also suggested that γδT lymphocytes serve as an important source of IL-17, which in addition to the mechanism described above, can also promote tumour growth by suppression of tumour cell apoptosis and by induction of angiogenic factors(37). However, while IL-17- secreting γδT lymphocytes are common in mice, they are extremely rare, particularly in health, in humans(38). Because of this, most studies of γδT17 lymphocytes have been carried out in mice rather than humans, and it is currently not clear how similar the murine and human subsets are, and therefore how far findings in mouse studies can be applied to humans(38). In humans there is evidence that a highly inflammatory milieu is required for polarisation to an IL-17-secreting phenotype(15). IL-17 has been shown to be associated with more aggressive tumours and worse outcomes in

breast cancer, but it can be produced by a number of immune cells other than γδT lymphocytes, including Th17 lymphocytes and macrophages(37). Therefore, it is currently uncertain how significant the role of IL-17 -secreting γδT lymphocytes is in human breast cancer.

In summary, both *in vitro* and *in vivo* studies demonstrate that different subtypes of γδT lymphocytes have seemingly opposing effects on the growth of breast tumours. Vγ9Vδ2+ T lymphocytes, the subtype found in peripheral blood, display cytotoxicity towards breast tumour cells, increase apoptosis and inhibit angiogenic signalling pathways. These actions may be breast cancer subtype dependent, rely on specific signalling pathways, be TCR-dependent, and change depending on MICA/B and ICAM1 expression levels. Conversely, $V\delta1^+$ T lymphocytes, the predominant subtype found in breast tumours, demonstrate pro-tumour activity through a number of immune-suppressing effects. These include suppression of naïve T cell proliferation and DC maturation via induction of senescence, which in turn inhibits proliferation of functioning T lymphocytes and DCs, which lose their capacity to process and present antigens. Furthermore, $V\delta1^+$ T lymphocytes also secrete immunosuppressant cytokines.

γδT LYMPHOCYTES IN BREAST CANCER - HUMAN STUDIES

There is a comparative paucity of translational studies in humans utilising tissue or blood samples. A 2009 study took blood samples from 38 patients with a new diagnosis of breast cancer and compared them to blood samples from 79 age-matched healthy controls. They found the proportion of γδT lymphocytes in the circulating blood of healthy controls to be 1.6 times higher than that in the breast cancer patients ($p=0.002$)(39). As an extension to this, later studies have observed that higher T stage (a measure of the extent of the tumour)

is correlated with lower circulating Vγ9Vδ2^{+} T lymphocytes in breast cancer patients(40). In the 2009 study, expanded γδT lymphocytes from breast cancer patients produced significantly higher quantities of IL-6 and TNFα but less IFNγ ($p<0.05$) than those from healthy subjects, and had a higher percentage of effector CD27- cells than CD27+ memory cells. The authors also noted that the γδT lymphocytes from the cancer patients released significantly less granzyme B, which is one mechanism by which γδT lymphocytes exert their cytotoxicity, than those from healthy donors ($p<0.02$). While there was generally higher cytotoxicity from the cells of healthy controls, this did not reach statistical significance(39). The reduced levels of circulating γδT lymphocytes in patients with tumours compared to healthy individuals, and with increasing stage, seen in these translational studies may provide some support to the *in vitro* and *in vivo* observations of the localisation of these cells to tumour, as described earlier. Alternatively, this observation may represent an effect of the tumour on γδT lymphocytes as it progresses, reducing their anti-tumoural efficacy. Alternatively, patients with low circulating γδT lymphocytes may be more susceptible to breast cancer.

In contrast, previously described experimental studies by Peng et al and Ye et al suggested that γδT lymphocytes in the tumour microenvironment promoted tumour growth(25, 35). These results were supported by Ma et al who compared tumour tissue from 81 patients to paired normal tissue. They found that numbers of γδT lymphocytes in the tissue positively correlated with higher T stage, positive lymph node status and HER2 expression. Patients with higher numbers of γδT lymphocytes in tumour tissue had worse recurrence free survival (HR 34.68; 95% CI: 4.79-250.88; p=0.0004) and worse overall survival (HR 3.34; 95% CI: 1.21-9.25; p=0.002). Furthermore, these findings were not subtype specific(10).

Similarly, Ye et al, in a cohort of 46 patients, found significantly increased numbers of Vδ1$^+$ T lymphocytes in fresh breast tumour tissues compared to paired normal tissue, with higher expression in late stage tissues compared to earlier stages. They then investigated reasons for this finding *in vitro*, and found that supernatants from breast cancer tissues and cell lines induced significant migration of Vδ1$^+$ T lymphocytes. This effect was not seen for melanoma or colorectal cancers. Furthermore, no difference in chemotactic activity was observed between the different breast cancer subtypes. This activity seemed to be mediated via IP-10 secreted by the breast cancer cells, as a neutralising antibody to IP-10 abolished the chemotaxis. In human breast tissue, Vδ1$^+$ T lymphocytes surrounded IP-10 expressing cancer cells. In a murine model, when human Vδ1$^+$ T lymphocytes were injected, they accumulated at the tumour site within 3-10 days whereas γδ2$^+$ and CD4+ T cells did not. The IP-10 antibody again inhibited chemotaxis and led to enhanced CD8-mediated anti-tumour immunity(41).

In a small study, which focussed on triple negative breast cancer, Hidalgo and co-workers demonstrated that the lymphocytic infiltrate of these tumours contained many γδT lymphocytes, compared to normal breast tissue. They compared ductal and medullary breast cancer tissue and found differences in the distribution of γδT lymphocytes between the two tumour types; they were more frequently located in the stroma of the ductal specimens whereas they were more typically located in the tumour parenchyma and at the invasive tumour cell margin of medullary tumours(42).

In contrast, a more recent study by Bense et al seems to suggest a more favourable role for γδT lymphocytes in breast cancer, in patients treated with chemotherapy. Their initial dataset analysed gene expression profiles of tumours from 7270 newly diagnosed

non-metastatic breast cancer patients (prior to any treatment) by performing in-silico analyses. They used CIBERSORT (a method for characterising the cell composition of tissues from their gene expression profiles(43)) to estimate the fractions of 22 immune cell types. In 611 patients who underwent neoadjuvant therapy, they found that a higher fraction of γδT lymphocytes was associated with a higher pathological complete response (pCR) rate (OR 1.55; 95% CI:1.01-2.38; p=0.046). In 846 patients who received either neoadjuvant or adjuvant chemotherapy they reported prolonged DFS (HR 0.68; 95% CI 0.48-0.98; p=0.040), independent of receptor status, and improved overall survival in the HER2 positive subtype (HR 0.27; 95% CI:0.10-0.73; p=0.009). It should be noted that a significant proportion (75.7-88.5%) of the immunohistochemistry-derived receptor status were not given in pathology data so these were inferred using mRNA expression. Therefore, the subtype-specific results of this study are not directly comparable to the others included in this review(44). In addition, it should be cautioned that there is some evidence of potential overlap of gene signatures using CIBERSORT between γδT lymphocytes and other immune cells such as CD4, CD8 and natural killer cells(45).

These few studies, in correlation with the *in vitro* and murine studies of $V\delta1^+$ T lymphocytes, suggest that tumours may actively recruit this subtype of γδT lymphocyte that correlates with higher disease stage and worse prognosis, adding supporting evidence to their pro-tumourigenic activity. This is in contrast to the $V\gamma9V\delta2^+$ subtype, which decreases in number and shows reduced anti-tumour effectiveness as tumour stage increases, suggesting that the tumour may act to reduce the numbers and effectiveness of this subtype in the circulation. It should be noted however that these studies were carried out in small cohorts and therefore larger studies are required to validate these results.

Work both in breast cancer and in a variety of other cancers, however, tells us that the situation is not as 'black and white' as there being simply a pro-tumour $V\delta1^+$ subtype and an anti-tumour $V\gamma9V\delta2^+$ T cell subtype. As Zhao et al comprehensively describe in their recent review, γδT lymphocytes display a degree of functional plasticity. Regardless of the configuration of their γδ chains, functionally they can be polarised to different subtypes, whether that be cytotoxic cells, γδTh lymphocytes, or FOXP3+ Treg lymphocytes, depending on the stimulus of various cytokines. It seems that the constituents of the tumour microenvironment may polarise γδT lymphocytes towards more pro-tumourigenic functional subtypes(15).

With this in mind, the challenge being tackled by various groups is how to apply the characteristics of γδT lymphocytes in the clinical setting, in particular how to exploit their anti-tumourigenic effects without promoting their immune-suppressant characteristics.

THERAPEUTIC APPLICATIONS OF γδT LYMPHOCYTES

There has been increasing interest in the use of bisphosphonates in breast cancer treatment. A 2011 review summarises the clinical evidence for improved outcomes of patients treated with bisphosphonates, such as zoledronate, in several cancers but particularly in breast cancer in the neoadjuvant, adjuvant and metastatic settings. This evidence seems to be most marked in ER positive disease(46). The mechanisms behind the efficacy of bisphosphonates are not fully understood but one aspect may include immunomodulation via activation of $V\gamma9V\delta2^+$ T lymphocytes.

Investigating the mechanism of action of bisphosphonates against breast tumours, Dhar and co-workers showed that lysis of MCF-7 tumour cells pre-treated with bisphosphonates is mediated by γδTCRs and is partially NKG2D-dependent. They also

observed that γδT lymphocytes appeared to crawl over the surface of untreated tumour cells but were unable to form stable conjugates and lyse cells, whilst the zoledronate-treated cells were surrounded by γδT lymphocytes, forming tight conjugates, and they lysed the tumour cells in a matter of seconds (47). Another study which pre-treated tumour cells with risedronate demonstrated that IFNγ secreted by γδT lymphocytes activates ICAM1 in luminal (ER positive) breast cell lines, which then leads to tumour recognition by ICAM engagement and subsequent tumour growth reduction(48)

As well as increasing their anti-tumour efficacy, bisphosphonates have been shown to stimulate Vγ9Vδ2$^+$ T lymphocyte expansion *in vitro*. Benzaid et al demonstrated, in *in vitro* and *in vivo* murine models, that treatment with zoledronate led to intracellular accumulation of isopentyl pyrophosphate (IPP) and triphosphoric acid 1-adenosin-5'-yl ester 3-(3-methylbut-3-enyl)ester (ApppI) in luminal breast cancer cell lines (uptake of zoledronate was reduced in basal cell lines) which led to increased proliferation and mobilisation of Vγ9Vδ2$^+$ T lymphocytes, with increased cytotoxicity(49).

Lending support to the theory that γδT lymphocytes are important to the efficacy of bisphosphonate treatment, as suggested by these *in vitro* studies, are the results of a phase 1 trial of zoledronate. 10 metastatic breast cancer patients were administered zoledronate in combination with IL-2, and peripheral blood Vγ9Vδ2$^+$ T lymphocyte numbers were measured at regular intervals. They found that three quarters of patients surviving past 12 months showed robust Vγ9Vδ2$^+$ T lymphocyte numbers whereas the 3 patients who died between months 3-11 did so after a decline in Vγ9Vδ2$^+$ T lymphocytes (50).

Sugie et al investigated the importance of the frequency of bisphosphonate administration in cancer therapy. They monitored the effect on Vγ9Vδ2$^+$ T lymphocyte

numbers in the peripheral blood of breast cancer patients after four weekly zoledronate injections and found that cell numbers progressively declined after each dose. Therefore, they postulated that the frequency/dosage interval of bisphosphonate administration might account for some of the variability in treatment response seen in the major clinical trials. They suggested that less frequent zoledronate infusion might be better than intensive zoledronate therapy at promoting the Vγ9Vδ2$^+$ T lymphocyte effects in early stage breast cancer patients(40).

The ability of bisphosphonates to stimulate Vγ9Vδ2$^+$ T lymphocytes has also led to interest in using Vγ9Vδ2$^+$ T lymphocytes themselves as immunotherapy, however, clinical trials in various cancers have shown lower response rates than hoped with an average response ratio of 21%(15). There are two methods to perform immunotherapy using these cells, either by stimulating the population of Vγ9Vδ2$^+$ T lymphocytes to expand *in vivo*, similarly to the studies above, or by adoptive transfer where the patient's own Vγ9Vδ2$^+$ T lymphocytes are expanded *ex vivo* using bisphosphonate stimulation prior to treatment.

Sugie et al investigated Vγ9Vδ2$^+$ T lymphocyte proliferation using the second method, *ex vivo* expansion. They observed that Vγ9Vδ2$^+$ T lymphocyte proliferation in the presence of zoledronate and IL-2, as well as production of IFNγ and TNFα, was correlated to the initial Vγ9Vδ2$^+$ T cell frequency in the patient's blood. These observations suggest that immunotherapy may have limited effectiveness in patients with low circulating Vγ9Vδ2$^+$ T lymphocyte levels. However, when they added IL-18 to zoledronate and IL-2, they found enhanced proliferative response and cytokine production, regardless of the initial Vγ9Vδ2$^+$ T lymphocyte frequencies. They therefore concluded that, in this context, Vγ9Vδ2$^+$ T cell immunotherapy is feasible for both early and late stage breast cancer(40).

The same group registered a phase II clinical trial in 2014 with the primary aim of assessing the add-on effect of a single dose of zoledronate with preoperative letrozole (a competitive inhibitor of the enzyme aromatase, used in the treatment of breast cancer to block the conversion of androgens to oestrogens in postmenopausal women) in cT1/2N0M0 ER positive, Her2 negative breast cancer, but with the secondary aim of investigating the changes in frequency of Vγ9Vδ2^{+} T lymphocytes after the administration of zoledronate. It will also assess whether higher levels of peripheral Vγ9Vδ2 T lymphocytes pre-treatment are predictive of a better response(51).

It has been suggested that bisphosphonates and Vγ9Vδ2^{+} T lymphocytes in combination may have clinical utility in metastatic bone disease in breast cancer, as the benefits of the actions of bisphosphonates on bone may be exploited in addition to the effects on γδT lymphocyte proliferation and function already described. A recent study demonstrated that, following pre-treatment of the mice with zoledronate, ex vivo expanded human Vγ9Vδ2^{+} T lymphocytes localised to the tumour in bone. Furthermore, multiple infusions of Vγ9Vδ2^{+} T lymphocytes reduced bone tumour growth, and this effect was potentiated following zoledronate pre-treatment. These mice also had the lowest volume of pulmonary metastases and reduced osteolysis was also noted, suggesting a promising role for this combination therapy in bone metastases, permitting both reduction in bone degradation and reduction in tumour burden(52).

The use of γδT lymphocytes in combination with other agents has also been investigated. One study showed that treatment of Her2 positive cell lines with γδT lymphocytes in combination with trastuzumab resulted in increased efficacy of the trastuzumab compared to trastuzumab or γδT lymphocytes alone. This also held true in

xenografts of this cell line in mice, showing reduced tumour growth in those treated with the combined therapy(53).

In addition to the therapeutic applications of γδT lymphocytes outlined above, some evidence summarised in this review, regarding correlations between numbers of γδTILs and cancer outcome measures, suggests a potential role for γδT lymphocytes as a predictive biomarker which could stratify patients for more aggressive systemic therapy.

CONCLUSIONS

In summary, γδT lymphocytes show unique potential in cancer immunotherapy due to their ability to recognise tumour cells independently of MHC antigen presentation and to rapidly initiate an immune response, bridging the innate and adaptive immune systems. They do this through direct cytotoxicity, secretion of cytokines such as TNFα and IFNγ that stimulate other components of the immune response, inhibition of angiogenesis, and through antigen presentation. However, functional plasticity where certain cytokines in the tumour microenvironment drive γδT lymphocytes towards an immunosuppressive role, has resulted in limitations to the effectiveness of immunotherapy to date. To elicit the full potential of this therapy, the goal of optimising the γδT lymphocyte subtype to an anti-tumour role, and manipulating the tumour microenvironment to promote rather than inhibit this purpose must be pursued. Perhaps uniquely in breast cancer, early work has suggested that the effectiveness of γδT lymphocytes against tumours may vary in different molecular subtypes and therefore, work in breast cancer must be designed to fully evaluate effects within the different subtypes.

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