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Review Article

Human γδ T Cells and Tumor Immunotherapy

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Human Vγ2Vδ2 T cells recognize nonpeptide antigens derived from pathogenic microbes in a TCR-dependent manner, such as pyrophosphomonoester compounds from mycobacteria and malaria parasite and alkyl amines from *Proteus*, suggesting that this subset of γδ T cells is involved in infectious immunity. The precise recognition mechanism has been delineated using a site-directed mutagenesis strategy based on crystal structure of γδ TCR. On the other hand, several lines of evidence indicate that human γδ T cells are involved in tumor immunity. Although activated γδ T cells exhibit a cytolytic activity against most of tumor cells, only a small fraction of tumor cells, like Burkitt lymphoma cells and multiple myeloid cells, is recognized by human γδ T cells in a TCR-dependent manner. This implicates that human γδ T cells have two distinct pathways for anti-tumor immunity. One is a natural killer-like pathway and the other is a TCR-dependent pathway. Recently, it was shown that treatment of human tumor cells with nitrogen-containing bisphosphonates, therapeutic drugs for hypercalcemia in malignancy, generated antigenic structure on the surface of tumor cells, which could be recognized by human γδ T cells in a TCR-dependent manner. This tumor labeling system may lead to a novel strategy for cancer immunotherapy.
Key words γδ T cells, nonpeptide antigens, pyrophosphomonoesters, alkyl amines, nitrogen-containing bisphosphonates, TCR

INTRODUCTION

T lymphocytes consist of αβ and γδ T cells, which express αβ and γδ T cell receptor heterodimers, respectively[1-5]. αβ T cells recognize antigenic peptides in the context of major histocompatibility complex (MHC) class I or II molecules with the help of CD8 or CD4 molecules[6]. The crystal structure of trimer complex, consisting of TCR-αβ, peptide, and MHC, was solved and the precise recognition mechanism was visualized at the molecular level[7-10]. On the other hand, human γδ T cells recognize nonpeptide antigens like pyrophosphomonoesters[11] and alkyl amines[12,13] derived from microbial pathogens in a conventional MHC-independent manner[14-17]. Recently, nitrogen-containing bisphosphonate compounds known as therapeutic drugs for osteoporosis and hypercalcemia were shown to stimulate human γδ T cells[18-19]. This recognition is dependent on the presence of human tumor cells[20]. Thus, it is possible to develop a novel strategy for treatment of malignant cancer utilizing the mode of action by γδ T cells. In this review, we summarize the recent findings on the recognition of nonpeptide antigens by γδ T cells and discuss the possibility of immunotherapy for malignant cancer.

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Nonpeptide antigens

γδ T cells were first shown to be involved in the host defense against mycobacterial infection in leprosy patients and to be reactive against extracts from *Mycobacterium tuberculosis* as well as other pathogenic microbes such as malaria parasites[21,22]. Initially, it was recognized that the antigenic entity was nonproteinaceous, small, microbial products with the molecular weight of less than 3 kDa[23-26]. Then, γ-derivatives of nucleoside triphosphates[27,28] and isopentenyl pyrophosphate (IPP)-related compounds[29,30] were identified as antigenic substances in mycobacteria. Recently, more extensive efforts have been made to elucidate the bacterial metabolic pathways and it was demonstrated that (*E*)-4-hydroxy-3-methyl-but-2-enyl pyrophosphate was the most active metabolite present in pathogenic microbes[31,32]. In human cells, IPP is biosynthesized through the so-called mevalonate pathway, though microbial pathogens utilize a newly discovered pathway, a DOXP pathway, to synthesize the compound, in which (*E*)-4-hydroxy-3-methyl-but-2-enyl pyrophosphate is converted to isopentenyl pyrophosphate at the last catalytic step as shown in Fig. 1[33-35]. To date, a number of pyrophosphomonoesters have been chemically synthesized and examined for their structure-activity relationship[36-38]. According to these chemical studies, the core of antigenic entity is a methyl phosphate moiety and the existence of a carbon-carbon double bond, a hydroxyl group,

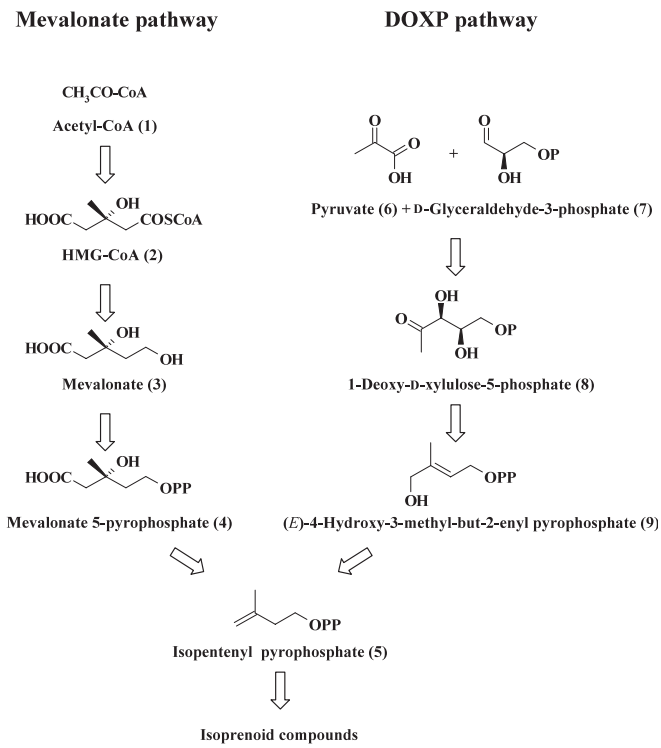

Fig. 1. Metabolic pathways for the biosynthesis of isopentenyl pyrophosphate. Isopentenyl pyrophosphate is a key metabolite in the biosynthesis of cholesterol, steroid hormones, bile acids, vitamins and so on in mammalian cells. This key molecule is biosynthesized through the mevalonate pathway, in which acetyl-CoA is the starting molecule and various metabolites including HMG-CoA, mevalonate, and mevalonate 5-pyrophosphate are sequentially synthesized. Recently, it was discovered that pathogenic microbes such as mycobacteria, malaria parasite, and *Helicobacter pylori* utilized a different pathway to biosynthesize isopentenyl pyrophosphate, so-called, the DOXP pathway. This pathway starts with pyruvate and D-glyceraldehyde-3-phosphate, from which 1-deoxy-D-xylulose-5-phosphate (DOXP), (*E*) -4-hydroxy-3-methyl-but-2-enyl pyrophosphate and so on are biosynthesized. On analyses of gene-disrupted mutants of the microbes and bioassay of chemically-synthesized compounds, the metabolite at the last step of this pathway, (*E*) -4-hydroxy-3-methyl-but-2-enyl pyrophosphate, was found to be one of the most bioactive nonpeptide antigens. Since this compound is produced by pathogenic microbes, this pyrophosphomonoester can be classified as pathogen-associated molecular patterns (PAMPs).

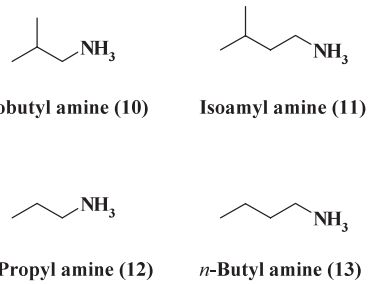

Fig. 2. Structures of alkyl amine compounds capable of stimulating human γδ T cells. Although human γδ T cells were initially found to recognize negatively-charged pyrophosphomonoester compounds, oppositely-charged alkyl amine compounds turned out to be bioactive in stimulating the same subset of γδ T cells. Chemical structures of the representative alkyl amine antigens are shown, in which isobutyl amine, isoamyl amine, *n*-propyl amine, and *n*-butyl amine are known to be produced by *Proteus monganii*, *Yersinia enterocolitica*, *Bacteroides fragilis*, and *Trichinella pseudospiralis*, respectively. It was demonstrated that hydrocarbon chains with 2 to 5 carbon atom units could stimulate human γδ T cells, reminiscent of pyrophosphomonoester antigens, implicating that alkyl amines were also recognized by TCR-γδ in a fashion similar to pyrophosphomonoester antigens.

and a halogen atom greatly influence the bioactivity[39]. Furthermore, the stimulating activity of the compounds can be predicted using an appropriate algorithm based on computational chemistry methods used in drug design, comparative molecular field analysis and pharmacophore modeling[40].

In the course of screening for other classes of nonpeptide antigens, alkyl amine compounds were accidentally found to activate the same subset of human γδ T cells. In contrast to pyrophosphomonoesters bearing negative charges, alkyl mines are positively-charged in structure as indicated in Fig. 2. Alkyl amines are also produced by pathogenic microbes including *Proteus*, *Bacteroides*, and *Clostridium*, suggesting that human γδ T cells are involved in the host defense mechanisms against pathogenic microbes. Since TCR-γδ is generated as a result of gene rearrangement, γδ T cells are classified as a family of immune cells of adaptive immunity[41]. When it comes to antigens, however, both pyrophosphomonoesters and alkyl amines are categorized as pathogen-associated molecular patterns (PAMPs), characteristic for innate immunity[42]. In addition, γδ T cells secrete a variety of lymphokines, which mediate the activation and differentiation of other cells of adaptive immune system[43]. Taken together, human γδ T cells serve as a bridge between innate immunity and adaptive immunity in structure as well as functions as summarized in Table 1.

TCR-dependent recognition

Conventional αβ T cells utilize TCR-αβ to recognize antigenic peptides[44]. Thus, it was essential to determine whether or not the recognition by γδ T cells of the nonpeptide antigens was dependent on TCR-γδ. To examine the TCR-dependency, a Jurkat transfectant system was employed, in which TCR-negative Jurkat cells, J.RT3-T3.5, were transfected with vectors harboring cDNAs for TCR-γ chain and TCR-δ chain together with a neomycin-resistant gene. When signal was delivered from TCR-γδ, the Jurkat cells produce

Table 1. Immunological character of human γδ T cells. Human γδ T cells can be categorized as cells in innate immunity in that they recognize pathogen-associated molecular patterns (PAMPs), and also classified as cells in adaptive immunity, since they express TCR-γδ, a product of gene rearrangement. In adddtion, upon recognition of the PAMPs, γδ T cells secrete a variety of lymphokines that mediate the differentiation and activation of cells in adaptive immunity, indicating that γδ T cells serve as a bridge between innate immunity and adaptive immunity structurally as well as functionally.

	Innate immunity	γδ T cells	Adaptive immunity
Species	Invertebrates Vertebrates	Vertebrates	Invertebrates Vertebrates
Cells	Macrophages Dendritic cells NK cells	γδ T cells	αβ T cells B cells
Gene rearrangement	No	Yes	Yes
Antigens	PAMPs (LPS, Peptide glycans, Lipoproteins)	PAMPs (PP esters, Alkyl mines)	Peptides Carbohydrates Chemicals

IL-2 in a dose-dependent manner[45]. As shown in Fig. 3, both pyrophosphomonoesters and alkyl amines are recognized by the γδ-Jurkat cells, demonstrating that the microbial nonpeptide antigens are recognized by γδ T cells in a TCR-dependent manner[46]. It is worth to note that microbial metabolites classified as PAMPs are recognized by TCR-γδ, a product of gene rearrangement characteristic for adaptive immunity.

Tumor recognition

Some γδ T cells express CD56 and NKG2D on their cell surface, which are known to be expressed on natural killer cells. In fact, highly activated γδ T cells exhibit a lytic activity against a variety of tumor cells[47]. On analyses using the above Jurkat transfectant system, most of the tumor cells are killed by γδ T cells in a TCR-independent manner, while the recognition of a small fraction of tumor cells including PRMI8226, a multiple myeloma cell line, and Daudi, a Burkitt lymphoma cell line, is dependent on TCR-γδ[48]. That is, γδ T cells elicit a tumoricidal activity in two different mechanisms, TCR-independent natural killer-like activity and TCR-dependent cytototoxic activity. These tumoricidal activities might be involved in the immunosurveillance system against naturally occurring cancer cells[49]. Because of the accumulation of granules in the cytoplasm, perforin is supposed to be the major mediator in the cytotoxicity[50].

Recently, it was demonstrated that γδ T cells were upregulated in some patients with renal cell carcinoma[51]. The proportion of patients with an elevated number of γδ T cells (10% or more) increased with increasing the stage of the disease up to stage III. Interestingly, the level of γδ T cells tended to decrease after surgery. While the proportion of γδ T cells in tumor infiltrating lymphocytes (TILs) was not different from that in PBMC, the level of activation of the γδ T cells in TILs was significantly higher than that of PBMC. On analyses of CDR3 regions of TCR-γ and TCR-δ chains, the junctional sequences were extremely skewed and the most frequently occurring TCR-γ chains were shared in two patients examined, indicating that γδ T cells recognized a specific antigen expressed on renal cell carcinomas in a TCR-dependent manner. Although the entity of the antigen has not been elucidated yet, the observation supports the hypothesis that γδ T cells are involved in the immunosurveillance system in humans. It is well known that spontaneous regression sometimes takes place in patients with renal cell carcinoma[52]. Although the mechanism for the regression remained elusive, it is possible that γδ T cells secreting IFN-γ play a critical role in the spontaneous regression.

Recognition of infected cells

In addition to tumoricidal activity, γδ T cells exhibit a cytototoxic activity against cells infected with bacteria and viruses. Mycobacteria are typically engulfed by macrophages, though not digested by enzymes such as hydrolases. Instead, they reside in phagosomes and remain alive for a long period of time. According to previous reports, macrophages infected with mycobacteria are efficiently recognized by γδ T cells[53]. It is noteworthy that the infected cells are targeted by the γδ T cells with Vγ2Vδ2-bearing TCR, the same subset of γδ T cells, which recognize microbial nonpeptide antigens. Based on this fact, it is likely that highly bioactive pyrophosphomonoester metabolites secreted from mycobacteria are somehow translocated and displayed on the surface of macrophages to γδ T cells. Another possibility is

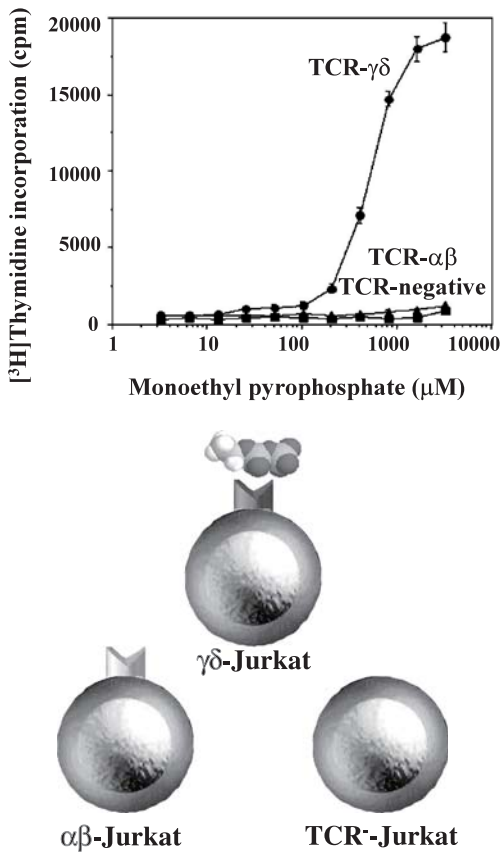

Fig. 3. TCR-dependent recognition of nonpeptide antigens by human γδ T cells. A wild type Jurkat cell line expresses TCR-αβ on the cell surface and a TCR-β gene-deficient Jurkat mutant cell line, J.RT3-T3.5, displays no detectable TCR-αβ/CD3 complex. After transfection of the TCR⁻-Jurkat cell line with expression plasmids for Vγ2-bearing TCR-γ chain and for Vδ2-bearing TCR-δ chain, together with neomycin-resistant plasmid, the Jurkat mutant cell line expresses TCR-γδ and produces IL-2 upon engagement of the TCR-γδ with its ligand. When three Jurkat cell lines, αβ-Jurkat expressing TCR-αβ, TCR⁻-Jurkat expressing no detectable TCR, and γδ-Jurkat expressing TCR-γδ, were challenged by one of the pyrophosphomonoester antigens, monoethyl pyrophosphate, a significant production of IL-2 was detected only in γδ-Jurkat by utilizing the standard CTLL-2 proliferation assay. This clearly showed that γδ T cells recognize nonpeptide antigens in a TCR-dependent manner. It was also confirmed that γδ T cells recognized alkyl amine antigens in a TCR-dependent manner using the same Jurkat transfectant system.

that mycobacterial infection induces the accumulation of endogenous isopentenyl pyrophosphate and its derivatives in the target cells, which are displayed to γδ T cells. It is also possible that unidentified proteinaceous molecules are induced by mycobacterial infection and recognized by γδ T cells. Although the precise mechanisms for the recognition remain to be elucidated, it is critical that γδ T cells induce apoptosis in the infected cells[54]. It is well demonstrated that mycobacteria can be killed during the course of apoptotic death of host cells, while they are intact when the host cells undergo necrosis[55]. Thus, it is physiologically important for γδ T cells to induce apoptosis in the target cells for the eradication of intracellularly infected microbial pathogens.

Virally infected cells were also shown to be lyzed by the same subset of γδ T cells. When cells are infected with *Herpes simplex* virus, γδ T cells exhibited a cytotoxic activity against the infected cells in a TCR-dependent manner[56]. The antigenic entities displayed on the surface of the target cells have not been elucidated yet, and several mechanisms have been proposed to explain this cytotoxicity. One is that viral infection provokes the accumulation of isopentenyl pyrophosphate and its derivatives in the target cells, which might directly stimulate γδ T cells. The other one is that viral infection elicits the de novo synthesis of unidentified membrane molecules as a danger signal and the nascent molecules are recognized by the γδ T cells. Moreover, viral products themselves might be recognized by TCR-γδ. Although the mechanisms for the γδ T cell recognition of the cells infected with intracellular pathogens remain enigmatic, the usage of particular TCR-γδ repertoires and TCR-dependency are evident in the recognition. In summary, γδ T cells exhibit a cytotoxic activity against microbially infected cells as the first-line of defense and produce a variety of lymphokines to provoke the adaptive immunity, in which the recognition of nonpeptide antigens might play a pivotal role in initiating the responses.

Recognition mechanisms

Since it was formally established that TCR-γδ was involved in the recognition of nonpeptide antigens derived from pathogenic microbes, it was necessary to solve the crystal structure of the heterodimer for the elucidation of the mechanism for γδ T cell recognition of nonpeptide antigens. Because both the extracellular domains of Vγ2-bearing TCR-γ chain and Vδ2-bearing TCR-δ chains were not properly expressed in *E. coli*, several codons preferably used for mammarian tRNA were replaced with those for *E. coli*, then, X-ray analysis was carried out. Based on the crystal structure of TCR-γδ, there was a positively-charged pocket on the surface of TCR, formed by γK109 of CDR3γ and δR51 of CDR2δ[57]. Because pyrophosphomonoester antigens bear a negatively-charged pyrophosphate moiety, these positively-charged side chains might play an essential role in the recognition[58]. In addition, previous sequence studies indicated that δL97 of CDR3δ was well conserved in Vγ2Vδ2-bearing T cells derived from healthy adults, suggesting that δL97 was selected under the pressure of nonpeptide antigens produced by pathogenic microbes[59]. Thus, the side chain of δL97 might also be involved in the recognition.

In order to examine this hypothesis, alanine substitution

was employed in the Jurkat transfectant system[60]. Then, γK108 and γK109 of CDR3γ, δR51 of CDR2δ, and δL97 of CDR3δ were replaced with alanine, respectively, to give constructs for γK108A, γK109A, δR51A, and δL97A as indicated in the top panel of Fig. 4. Each one-point mutant was paired with the corresponding wild type TCR-γ or TCR-δ chain and four Jurkat cell lines expressing mutant TCR-γδ were established. These transfectants produced a high level of IL-2 into the culture medium in response to anti-CD3 monoclonal antibody, equivalent to the Jurkat cells with wild type TCR-γδ, suggesting that signaling pathway in all the transfectants was normal.

When challenged by one of the pyrophosphomonoester antigens, monoethyl pyrophosphate, the production of IL-2 was totally abolished in γK108A, δR51A, and δL97A mutants, although the response was diminished to about one third in γK109A mutant. However, the substitution of γK109 with the oppositely-charged glutamic acid resulted in the complete loss of the recognition[61]. Taken together, γK108A, δR51A, and δL97A seem to play a critical role in the recognition of pyrophosphomonoester antigens, and γK109 is partially involved in the responses. On the basis of the results, the recognition mechanism by γδ T cells of pyrophosphomonoesters can be depicted as shown in the middle panel of Fig. 4. A negatively-charged inorganic phosphate molecule is located in the positively-charged surface formed by γK108 and δR51. When a negatively-charged pyrophosphomonoester antigen approaches to the site, the inorganic phosphate can be expelled from the surface and instead the pyrophosphomonoester antigen can interact with the positively-charged side chains of γK108 and δR51. At the same time, a hydrophobic bridge can be formed between a hydrocarbon chain of the nonpeptide antigen and a side chain of δL97. In this case, γK109 might be involved in the interaction to some extent.

As for alkyl amine antigens, the IL-2 secretion was completely hampered in γK108A, δR51A, and δL97A mutants in response to one of the alkyl amine antigens, isobutyl amine, while γK109A mutant produced IL-2 as much as the wild type TCR-γδ. This indicates that γK109 is not involved in the recognition of alkyl amine antigens. A possible recognition mechanism is shown in the bottom panel of Fig. 4. As in the case of pyrophosphomonoseter antigens, an inorganic phosphate molecule is located in the positively-charged surface consisting of γK108 and δR51. The inorganic phosphate is not, however, expelled from the position and rather serves as a bridge between a positively-charged alkyl amine and positively-charged side chains of γK108 and δR51. Then, the hydrocarbon chain of alkyl amine antigen interact with the side chain of δL97.

Since γK109 is located in the center of the positively-

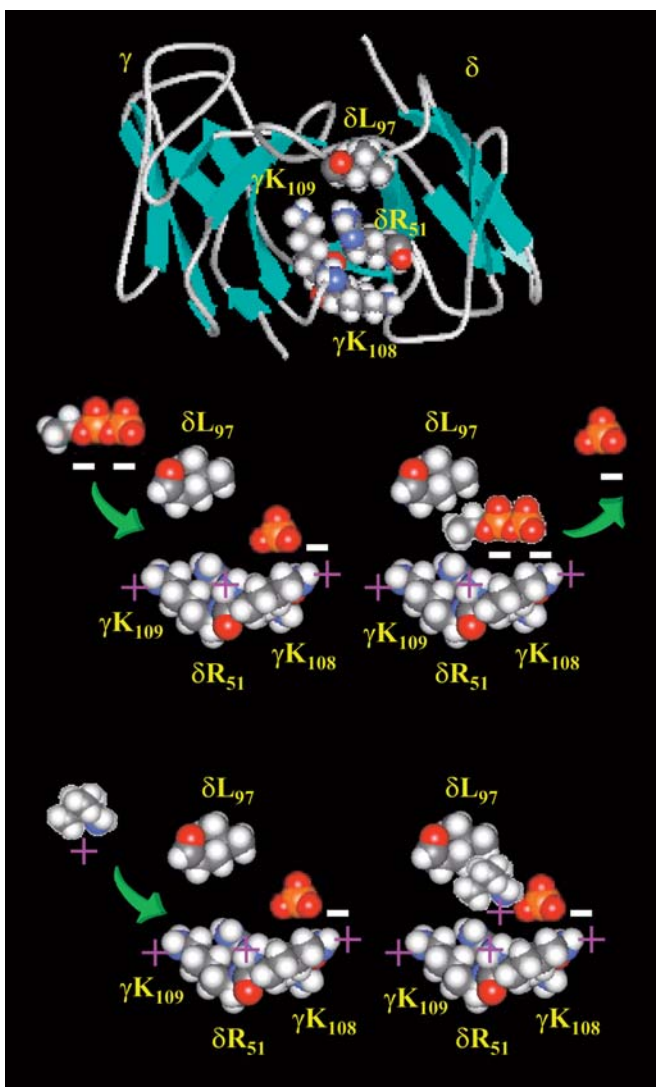

Fig. 4. Model for the recognition of nonpeptide antigens by TCR-γδ. The top panel illustrates the top view of the TCR-γδ, with the amino acid residues possibley involved in the recognition of nonpeptide antigens being highlighted as the CPK model. Each amino acid residue, γK108, γK109, δR51, or δL97, was replaced by alanine and the mutated TCRs were examined for their ability to recognize nonpeptide antigens using Jurkat transfectant system. For pyrophosphomonoester antigens, γK108, δR51, and δL97 played an essential role in the recognition and γK109 was partially involved in the interaction. The middle panel depicts the possible model for the interaction between pyrophosphomonoester antigens with the TCR surface consisting of the three essential amino acid residues plus γK109. In the recognition of alkyl amine antigens, the three amino acid residues, γK108, δR51, and δL97, are also prerequisite, though γK109 are not likely to be essential. As shown in the bottom panel, an environmental inorganic phosphate seems to serve as a charge-bridge between positively-charged alkyl amine antigens and positively-charged γK108 and δR51 residues. In both classes of antigens, the side chain of δL97 residue might interact with the hydrophobic hydrocarbon chain of the nonpeptide antigens.

charged pocket on the surface of TCR-γδ, the amino acid residue is initially considered as the most essential factor in the recognition of the nonpeptide antigens. In addition, it can be postulated that γK108 does not play an essential role in the recognition, because the side chain of this residue extends in the opposite direction compared to γK109 and δR51. On the contrary, γK108, but not γK109, is likely to be critical in the recognition mechanism. If this is the case, the edge, but not the center, of the positively-charged pocket on the surface of TCR-γδ might interact with nonppetide antigens directly or through a bridge of negatively-charged inorganic phosphate.

Nitrogen-containing bisphosphonates

Recently, it was discovered that nitrogen-containing bisphosphonate compounds like pamidronate, ibandronate, alendronate, neridronate, risedronate, and zoledronate utilized as therapeutic drugs for osteoporosis and hypercalcemia in malignant cancer could stimulate the same subset of human γδ T cells as shown in Fig. 5. The first indication of the antigenicity of the nitrogen-containing bisphosphonates in γδ T cells came from the in vitro culture of peripheral blood mononuclear cells (PBMC), based on the fact that nitrogen-containing bisphosphonates had structural similarity to both pyrophosphomonoesters and alkyl amines, in which the bisphosphonate moiety and the amino group in nitrogen-containing bisphosphonate compounds correspond to the pyrophosphate residue in pyrophosphomonoesters and the amino group in alkyl amines, respectively, as illustrated in Fig. 6. When PBMC derived from healthy adult volunteers were incubated in the presence of pamidronate, selective expansion of Vγ2Vδ2-bearing T cells took place and the secretion of IFN-γ was induced[18]. Interestingly, non-nitrogen-containing bisphosphonates like etidronate and chlorodonate failed to stimulate the γδ T cells, consisting with the findings that the substitution of the backbone structure, P-O-P, in pyrophosphomonoester antigens with P-C-P resulted in the abrogation of the stimulatory activity. This suggests that nitrogen atom in nitrogen-containing bisphosphonates is essential for the activation of γδ T cells. The substitution of P-C-P backbone with P-C also abolished the stimulating activity of the nitrogen-containing bisphosphonates, formally establishing that both nitrogen atom and bisphosphonate moiety played an important role in the stimulatory activity in the compounds. Based on the analysis of a number of synthetic bisphosphonate compounds, the structural features of antigenic entity was revealed by the algorithm utilized in the examination of pyrophosphomonoesters. It is noteworthy that the pharmocophore of nitrogen-containing bisphosphonates are different from that of pyrophosphomonoesters, implying different targets for the two antigenic substances[62].

Then, the *in vivo* antigenicity of nitrogen-conatining bisphosphonates was demonstrated by observation of patients

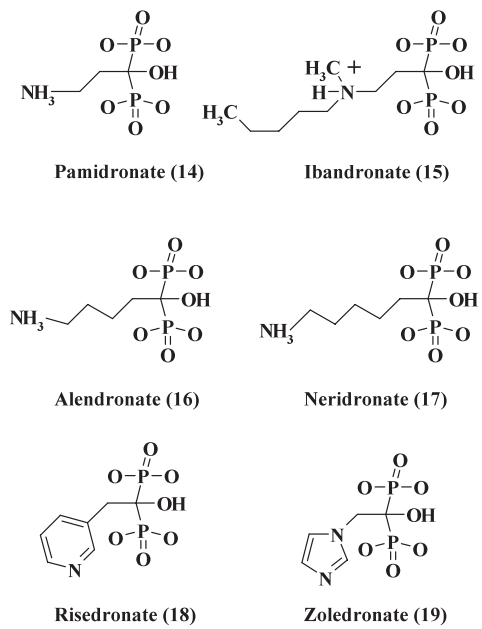

Fig. 5. Structures of representative bisphosphonate antigens capable of stimulating human γδ T cells. Bisphosphonate compounds were originally synthesized and used in industry as corrosion inhibitors or complexing agents in the textile. Then, the compounds were developed as drugs for use in bone diseases, based on the concept that their chemical analog, inorganic pyrophosphate, inhibited calcium phosphate precipitation. Since the P-C-P structure allowed chemists to synthesize a variety of derivatives, a number of bisphosphonates were examined for their biological activity. Bisphosphonate compounds of the first generation like etidronate and chlodronate were found to inhibit bone resorption moderately. When an amino group was added to the aliphatic chain, the efficacy was greatly improved, implicating that the second generation bisphosphonates can be used clinically. Furthermore, cyclic genimal bisphosphonates containing a nitrogen atom in the ring, so-called, the third generation bisphosphonates, turned out to be the most active drugs as inhibitors of bone resorption. It is noteworthy that the second generation and the third generation drugs can stimulate human γδ T cells. In representative bisphosphonates, pamidronate, ibandronate, alendronate, and neridronate are classified as the second generation bisphosphonates, and risedronate and zoledronate are the third generation compounds.

who received pamidronate. It was well known that intravenous treatment of Paget's disease with pamidronate often resulted in the occurrence of flu-like symptoms, fever, and myalgia[63]. According to a carefully controlled double blind placebo study, 20- 30% of patients suffered such symptoms related to pamidronate infusion. Interestingly, the adverse effects were observed mostly within 48 hours of the first pamidronate infusion and this lasted for less than 24 hours. In fact, no acute-phase responses were reported after the third or fourth administration. In 14 patients who had been treated

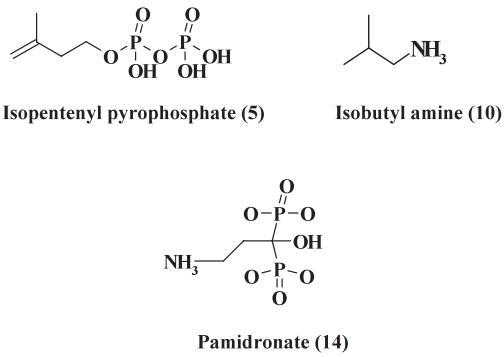

Fig. 6. Comparison of the structures of nonpeptide antigens. So far, three classes of compounds are known to be biologically active in stimulating human γδ T cells, pyrophosphomonoesters, alkyl amines, and nitrogen-containing bisphosphonates. When three representative compounds, isopentenyl pyrophosphate, isobutyl amine, and pamidronate, were compared, the P-C-P moiety in the bisphosphonate is similar to the P-O-P moiety in the pyrophosphomonoester and the aliphatic amine moiety is shared by the bisphosphonate and the alkyl amine, indicating that pamidronate has characters similar to pyrophosphomonoesters as well as alkyl amines. However, mutational analyses demonstrate that the mode of recognition of pamidronate is closer to that of alkyl amines rather than pyrophosphomonoesters, while both the nitrogen-atom and the P-C-P moiety are required for bioactivity in nitrogen-containing bisphosphonates.

with pamidronate, the sencond infusion did not cause the acute-phase responses even after 120- 160 days from the initial administration, indicating that pamidronate had certain effects on immune cells[64].

Based on the clinical implication and the in vitro observation, 10 patients who received pamidronate infusion were examined for γδ T cells[18]. As expected, 4 patients suffered from the adverse effects and had an increase in the γδ T cell population in peripheral blood. Then, the acute-phase reaction assessed by the increase in body temperature correlated with the increase in the γδ T cell population. In addition, subsequent pamidronate infusions did not cause the increase in body temperature and the change in the γδ T cell population, suggesting that the adverse events were attributable to cytokines like IFN-γ produced by the γδ T cells activated by pamidronate.

On close examination of the antigenicity elicited by pamidornate, it was confirmed using the Jurkat transfectant system that the recognition of nitrogen-containing bisphosphonate compounds was dependent on TCR-γδ like other naturally occurring nonpeptide antigens, pyrophosphomonoesters and alkyl amines[61]. Mutational analyses, however, indicated that the mode of recognition of pamidronate was more similar to that of alkyl amines than that of pyrophosphomonoesters, because γK108A, δR51A, and δL97A failed to respond to pamidronate, while γK109A recognized the nitrogen-containing bisphosphonate efficiently, which was comparable to the wild type TCR-γδ [60]. Moreover, pamidronate failed to induce proliferative responses of γδ T cells in the secondary responses in the absence of proper antigen-presenting cells, although a vigorous expansion and IFN-γ production were observed in pyrophosphomonoester antigens[48].

In addition, it was demonstrated that there was an intrinsic difference between pyrophosphomonoester antigens and nitrogen-containing bisphosphonates. When PBMC were incubated in the presence of pamidronate, a remarkable expansion of γδ T cells were observed as in the case of pyrophosphomonoester antigens. When adherent cells including macrophages and dendritic cells were depleted from PBMC, the nonadherent cell preparation did not respond to pamidronate, although a significant response was observed in pyrophosphomonoester antigens[48]. This indicates that, unlike pyrophosphomonoester antigens, which do not require specific antigen-presenting cells, macrophages or dendritic cells were essential in the recognition of pamidronate by γδ T cells in the primary responses. Interestingly, primed γδ T cells failed to respond to pamidronate even in the presence of macrophages and dendritic cells, consisting with the in vivo observation that the first administration of pamidronate causes the acute-phase reactions, while γδ T cells population did not change after subsequent pamidronate infusions, that is, the patients did not suffer adverse reactions[48].

Requirement of tumor cells in recognition of pamidronate

In the course of study on the mechanism for recognition of macrophages pretreated with pamidronate, macrophage-like cells, osteoclast-like giant cells derived from patients, were examined for the ability to stimulate γδ T cells upon pretreatment with pamidronate. Surprisingly, γδ T cells recognized the osteoclast-like giant cells in the primary responses as well as in the secondary responses. The osteoclast-like giant cells seemed to be tumor cells, suggesting that tumor cells pretreated with pamidronate could stimulate γδ T cells. Then, a variety of tumor cells pretreated with pamidronate were tested for stimulatory activity in γδ T cells in the secondary responses. As listed in Table 2, most of the tumor cells could induce a proliferative response in γδ T cells[20]. It was, then, confirmed by the Jurkat transfectant system that the recognition was TCR-dependent.

Species specificity

To analyze further the recognition mechanism, tumor cells originated from animals other than humans were examined for their stimulating activity[65]. As listed in Table 3, none of the animal tumor cells could induce the production of IFN-γ in γδ T cells. Interestingly, human γδ T cells were not activated by even monkey tumor cells pretreated with pamid-

Table 2. Induction of tumoricidal activity in human γδ T cells by the pretreatment of human tumor cells with pamidornate. A variety of human tumor cells pretreated with medium only or pamidronate were incubated with γδ T cells and examined for the specific lysis (%) at the effector/target ratio of 20 : 1. It is worth to note that most of the cancer cell lines exhibited increased susceptibility to γδ T cells upon pretreatment with pamidronate. Several human tumor cells lines, however, failed to stimulate γδ T cells even after the pamidronate pretreatment, suggesting that unidentified membrane molecules were involved in the recognition.

Tissue	Cell	% Specific lysis (E/T=20)	
		Unpulsed(%)	Pulsed(%)
Gastric cancer	MKN1	48.10	91.0
	MKN28	28.20	55.6
	MKN74	5.30	15.2
	MKN45	6.90	3.10
	GCIY	7.90	36.1
	KATOIII	4.20	34.8
Cholangiocell carcinoma	SkchA1	0.20	25.6
	MzchA1	2.20	31.4
	MzchA2	15.40	67.3
	1TKB	2.54	46.8
	2TKB	14.50	66.0
	24TKB	9.40	64.6
	KMBC	1.90	7.60
	HuCCT1	6.90	56.7
	TFK1	4.20	34.5
	RBE	11.00	30.2
Pancreatic cancer	MiaPaca2	6.80	24.3
	KP4-1	6.00	41.7
	KP4-2	8.00	57.1
	KP4-3	17.20	53.6
	PK1	4.20	41.4
	PK8	8.80	78.4
	AsPC1	3.00	30.6
Colon cancer	LoVo	17.80	36.7
	HT29	4.30	42.2
	CACO2	5.00	20.6
	SW403	11.20	22.8
	WiDr	2.20	24.4
	DLD-1	2.40	27.8
	COLO302	0.80	26.6
Renal cell carcinoma	786-0	7.10	58.9
	ACHN	4.10	11.2
	Caki-1	6.10	67.3
	Caki-2	8.40	12.6
	UOK111	7.50	61.4
	UOK121	37.70	90.9
	VMRC-RCW	24.30	61.8
	VMRC-RCZ	0.10	28.0
	A-704	0.40	10.1
Embryoma of the kidney	G-401	2.90	16.6
	G-402	3.40	22.5
	293	3.30	18.1
APL	HL-60	9.00	14.6
Lymphoma	U-937	20.90	39.4
	SCC-3	8.40	33.5

ronate, while monkey γδ T cells expanded when stimulated with nonpeptide antigens[66]. In addition, Jurkat cells expressing the wild-type TCR-γδ also failed to secrete IL-2 in response to these animal tumor cells pretreated with pamidronate, indicating that the recognition by human γδ T cells of tumor cells pretreated with nitrogen-containing bisphosphonate antigens was species-specific. Based on these results, several models for the recognition mechanism can be hypothesized as shown in Fig. 7. The most straightforward one is that pamidronate nonspecifically attaches to the surface of human tumor cells and then recognized by TCR-γδ with the help of unidentified human-specific costimulatory signals. The second one is that unidentified human-specific molecules present pamidronate to TCR-γδ. The third one is that pamidronate taken up by tumor cells inhibits farnesyl pyrophosphate synthase[67-70], resulting in the accumulation of the upstream metabolites such as isopentenyl pyrophosphate[71,72], which can stimulate γδ T cells as illustrated in Fig. 8. In fact, it was demonstrated by quantum chemical calculations that nitrogen-containing bisphosphonates acted as carbocation transition state analogs for the enzyme[73]. The fourth one is that pamidronate induces unidentified molecules like stress-inducible proteins that might be recognized by γδ T cells. So far, all the mechanisms are likely and they are not mutually exclusive. Thus, further studies are required to elucidate the precise mechanism for the recognition by Vγ2Jγ1.2Vδ2-bearing T cells of human tumor cells pretreated with nitrogen-containing bisphosphonates.

Tumor immunotherapy

On the basis of the above results, it is possible to develop novel strategies for treatment of patients with malignant cancer. The simplest one is to expand γδ T cells in vivo by infusion of pamidronate. While the strategy is simple, several different mechanisms for tumorigenicity can be expected because of the complicated modes of action by γδ T cells. One is that γδ T cells are stimulated with pamidronate-pulsed macrophages, then the activated γδ T cells exhibit a TCR-independent cytotoxicity against tumor cells. Since this type of cytotoxicity is similar to, so-called, a natural killer activity and largely nonspecific, it is difficult to evaluate the efficacy in vivo. When tumor cells themselves express a specific antigen for γδ T cells, like RPMI8226 and Daudi[74], the γδ T cells activated by pamidronate-pulsed macrophages might elicit a cytolytic activity against the multiple myeloma cells and the Burkitt lymphoma cells in a TCR-dependent manner. A more interesting mechanism is that pamidornate is first displayed on tumor cells or the drug induces the accumulation of antigens like isopetenyl pyrophosphate or the expression of an identified molecule on the surface of tumor cells. Then, γδ T cells exert a cytotoxic activity in a TCR-dependent manner against the tumor cells expressing the antigenic substances.

Table 3. Failure in IFN-γ production by human γδ T cells in response to animal tumor cells pretreated with pamidronate. Human γδ T cells were challenged by a variety of animal tumor cell lines pretreated with medium only or pamidronate and examined for their intracellular accumulation of IFN-γ. It is evident that none of the animal tumor cell could stimulate human γδ T cells even after the pretreatment with pamidronate, indicating that the recognition was species-specific.

Species	Cell	Tissue	Unpulsed (%)	Pulsed (%)
Mouse	FM3A	Mammary gland	0.11	0.08
	MMT060562	Mammary gland	0.76	0.21
	CCRF-S-180II	Muscles	0.10	0.15
	MethA	Fibrosarcoma	1.13	0.26
	P3U1	Myeloma	0.90	0.60
	SP2/0	Myeloma	0.68	1.23
	J774A.1	Myeloma	1.14	1.23
	J558L	Myeloma	1.11	1.51
	NIH/Ras	Fibrosarcoma	0.94	0.40
	B16	Melanoma	0.80	0.88
	F9	Teratocarcinoma	0.39	0.73
	Lcell	Fibraosarcoma	1.59	0.71
Rat	MSK	Osteosarcoma	4.35	3.74
	Y3-Ag1.2.3	Myeloma	1.38	1.26
	L-2	Lung	2.18	4.01
	NRK-49F	Kidney	0.84	1.18
Hamster	V79-6TG	Lung	0.64	0.72
	CHO-K1	Ovary	2.11	2.15
	RPMI1864	Melanoma	3.10	0.50
	BHK-21	Kidney	0.50	0.95
	CHL/IU	Lung	1.89	2.80
Bovine	CKT-1	Kidney	0.57	0.26
	HH	Carotida Artery	2.61	2.80
Rabbit	SIRC	Cornea	0.68	0.38
Monkey	BS-C-1	Kidney	0.58	1.38
	VERO	Kidney	0.46	0.35
	CV-1	Kidney	0.44	0.83
	JCT-12P.3(F)	Kidney	0.27	0.35
Pig	PK	Kidney	1.23	3.28
Dog	MDCK	Kidney	0.53	0.75
Cat	CRFK	Kidney	0.90	0.26
Marsupial	PtK2	Kidney	0.39	1.14
Chicken	LMH	Liver	0.59	0.33
Indian Muntajak	Indian Muntajac	Skin	0.65	0.44

Because macrophages and dendritic cells fail to stimulate primed γδ T cells in the secondary responses even after the pretreatement with pamidornate[20], this mechanism seems to be predominant after the second pamidronate infusion. It was previously observed that the infusion of bisphosphonates induced the inhibition of osteolytic bone matastasis of breast cancer[75,76]. It is, therefore, important to examine carefully the effect of nitrogen-containing bisphosphonates on tumor regression in patients administered with the drugs.

As a matter of fact, several clinical trials have already

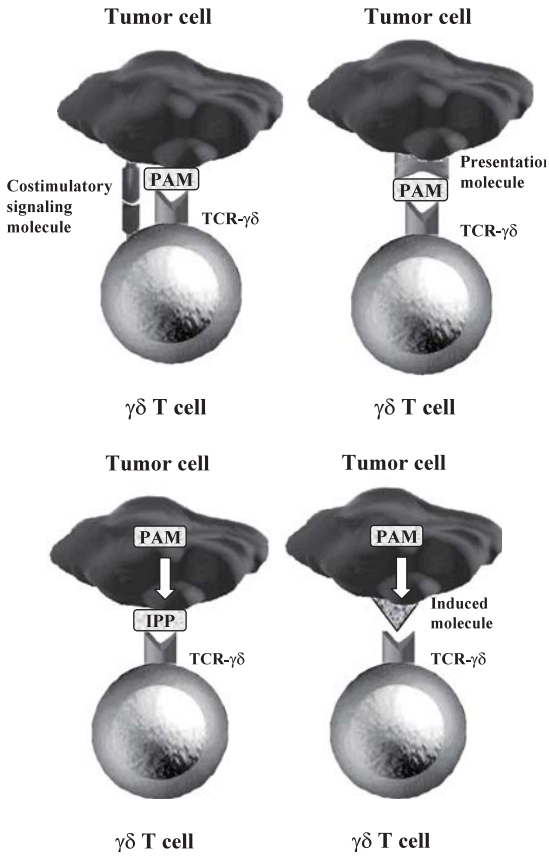

Fig. 7. Schematic representation of the models for the recognition of tumor cells pretreated with pamidronate by human γδ T cells. While γδ T cells recognize human tumor cells pretreated with pamidronate (PAM), the recognition mechanism remains enigmatic. These models were not mutually exclusive and therefore it is possible that several mechanisms are operating at the same time.

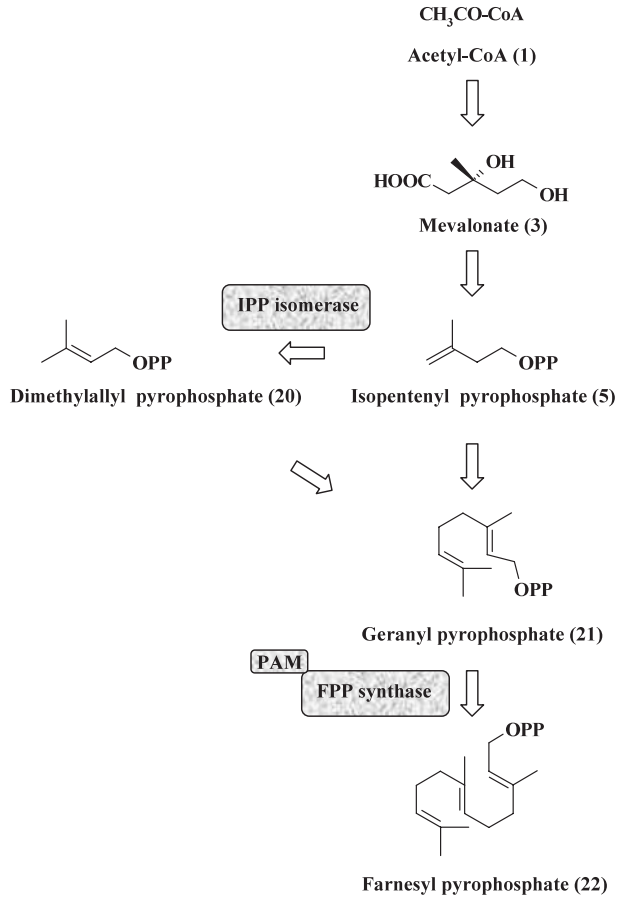

Fig. 8. Possible mechanism for the accumulation of isopentenyl pyrophosphate and its derivatives in tumor cells pretreated with pamidronate. Isopentenyl pyrophosphate is a key metabolite in isoprenoid biosynthesis. Isopentenyl pyrophosphate is converted to dimethylallyl pyrophosphate by the action of isopentenyl pyrophosphate isomerase (IPP isomerase), and geranyl pyrophosphate is generated from isopentenyl pyrophosphate and dimethylallyl pyrophosphate. Then, the nascent geranyl pyrophosphate is further converted to farnesyl pyrophosphate by the action of FPP synthase. Enzymatic analyses demonstrated that pamidronate (PAM) inhibited FPP synthase. In addition, the enzyme could also be inhibited by most of other nitrogen-containing bisphosphonates. Recently, it was further found that IPP isomerase could be inhibited by certain nitrogen-containing bisphosphonates. Thus, it is possible that the pretreatment of tumor cells with nitrogen-containing bisphosphonates results in the accumulation of isopentenyl pyrophosphate and other biologically active pyrophospahomonoester metabolites.

been made to confirm this novel strategy for cancer. It was reported that a significant proliferation of γδ T cells was observed in 55% of patients with refractory/relapsed lymphoma or myeloma and a partial regression was observed in 33% patients, when pamidronate and IL-2 were administered[77]. In this clinical trial, patients were selected by previous *in vitro* culture of PBMC with pamidronate plus IL-2. Thus, the patient selection is crucial in this therapy. As for adverse reactions, some patients suffered from low-grade fever and chills, though the side effects might be due to IL-2 infusion and only transient, indicating that this treatment schedule is well tolerated.

Since the third generation nitrogen-containing bisphosphonate compounds have recently been commercially available, a preclinical study using one of the drugs, zoledronate, was carried out[78]. The purpose of this study was to evaluate the in vivo effect of the zoledronate infusion on the effector function of γδ T cells. In this study, zoledronate was administered in patients with metastatic cancer every three weeks and the character of γδ T cells was examined by in vitro stimulation with isopentenyl pyrophosphate before and after the infusion. According to the report, the zoledronate infusion resulted in the enhanced IFN-γ production upon stimulation

with nonpepide antigens, indicating that zoledronate could be used for the treatment of matastatic cancer. For confirming the anti-tumor effect by the infusion of nitrogen-containing bisphosphonate drugs plus IL-2, more intense clinical studies are required.

Although more complicated and technically more elaborate, compared to the in vivo infusion of pamidronate, the in vitro expansion of γδ T cells and the subsequent infusion of the activated γδ T cells plus nitrogen-containing bisphosphonates may also lead to a novel strategy for cancer immunotherapy. Although nonpeptide antigens were not employed, a pioneering study on adaptive immunotherapy using γδ T cells was carried out in patients with advanced cancer[79,80]. PBMC derived from patients were expanded by anti-CD3 antibody and IL-2 and the resultant γδ T cells were transferred into patients with cancer. According to this study, there is a positive correlation between the patients' survival and initial counts of γδ T cells, indicating that γδ T cells might play a critical role in this immunotherapy. Since we now have a protocol for expanding human γδ T cells effectively using pyrophosphomonoester antigens, it is possible to prepare a large number of γδ T cells in vitro for adaptive immunotherapy. Basically, both the γδ T cell transfer and the infusion of nitrogen-containing bisphosphonates proved to be well tolerated. Thus, it is worth to perform the novel immunotherapy using highly active pyrophosphomonoester antigens and nitrogen-containing bisphosphonate drugs in patients with malignant cancer.

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