Isopentenyl Pyrophosphate, a Mycobacterial Non-peptidic Antigen, Triggers Delayed and Highly Sustained Signaling in Human γδ T Lymphocytes without Inducing Down-modulation of T Cell Antigen Receptor*

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The Vγ9Vδ2 T cell subset, which represents up to 90% of the circulating γδ T cells in humans, was shown to be activated, via the T cell receptor (TcR), by non-peptidic phosphorylated small organic molecules. These phosphoantigens, which are not presented by professional antigen-presenting cells, induce production of high amounts of interferon-γ and tumor necrosis factor (TNF-α). To date, the specific signals triggered by these antigens have not been characterized. Here, we analyze proximal and later intracellular signals triggered by isopentenyl pyrophosphate (IPP), a mycobacterial antigen that specifically stimulates Vγ9Vδ2 T cells, and compare these to signals induced by the non-physiological model using an anti-CD3 antibody. During antigenic stimulation we noticed that, except for the proximal p56lck signal, which is triggered early, the signals appear to be delayed and highly sustained. This delay, which likely accounts for the delay observed in TNF-α production, is discussed in terms of the ability of the antigen to cross-link and recruit transducing molecules mostly anchored to lipid rafts. Moreover, we demonstrate that, in contrast to anti-CD3 antibody, IPP does not induce down-modulation of the TcR-CD3 complex, which likely results in the highly sustained signaling and release of high levels of TNF-α.

T cells expressing the γδ T cell receptor (TcR)1 represent in humans a relatively low T lymphocyte population and, particularly in peripheral blood, these cells account for only 1–5% of the circulating T cells (see Ref. 1 for review). In an adult the majority of these circulating T cells are classified as Vγ9Vδ2 subset (up to 90%). It has been shown that this γδ T cell subset dramatically increases during infection by intracellular pathogens of bacterial, viral, or parasitic origin (2–12). One of the particularities of the Vγ9Vδ2 T cells is to be activated by components identified as non-peptidic, phosphorylated small organic molecules (13–18). Some of these components have been purified from bacteria or parasites and are thought to be responsible for the in vivo expansion of Vγ9Vδ2 T cells during the acute phase of the infection process. There is so far no formal proof that these small molecules do bind to the Vγ9Vδ2 T cell receptor, however, transfer experiments of the Vγ9Vδ2 TcR in TcR-negative Jurkat cell mutants have provided strong evidence to suggest that the recognition of the phosphoantigens is mediated by TcR (17).

Stimulation of Vγ9Vδ2 T cells by phosphoantigens results mainly in the production of high amounts of interferon-γ (19–22) and tumor necrosis factor-α (TNF-α) (19, 23). However, to date, the specific signals that are triggered in Vγ9Vδ2 T cells upon stimulation with phosphoantigens have not been characterized. This aspect is of great importance for the possible pharmacological control of TNF-α release (24, 25) by these cells, because an overproduction of this cytokine could result in immunopathology (26).

In αβ T cells, it is well established that activation occurs as a result of multimolecular interactions between T cells and antigen-presenting cells (27–29). These interactions include the recognition of the peptide/major histocompatibility (MHC) complex by the T cell receptor and the binding of CD4 coreceptor to non-polymorphic regions on the MHC class II molecules. The cytoplasmic tail of CD4 associates with the Src family tyrosine kinase p56lck, which plays a key role in the early events of T cell activation. However, in the case of Vγ9Vδ2 T cell activation, the non-peptidic antigens do not need to be presented in the context of MHC molecules (30, 31). Moreover, it has been shown that Vγ9Vδ2 T cells do not express CD4 and poorly express CD8 (32, 33), which normally interact with MHC class II or class I molecules, respectively. Therefore, there is not, with αβ T cells, recruitment of these coreceptors, which stabilize TcR-ligand interaction and are essential for the formation of the "immunological synapse," which determines the extent and qualitative nature of the transduced signal (27, 34, 35).

Recently we studied TNF-α release by Vγ9Vδ2 T cells when stimulated either with a monoclonal antibody directed against
the CD3 complex or with the mycobacterial phosphoantigen isopentenyl pyrophosphate (IPP) (36). We demonstrated that TNF-α production does not involve, as is the case in αβ T cells, CD28 costimulation. Moreover, we noticed that the cytokine production in Vα9Vδ2 T cells was highly delayed (∼10-h difference) when the cells were activated by a physiological phosphoantigen ligand (IPP) instead of anti-CD3 monoclonal antibody (mAb). This delayed cytokine production could be the result of a delayed triggered signaling or of the recruitment of different signaling molecules according to the stimulating agent used. In the present paper, we therefore studied the signals triggered in Vα9Vδ2 T cells upon stimulation with isopentenyl pyrophosphate, a physiological non-peptidic mycobacterial phosphoantigen known to be specifically mitogenic for Vα9Vδ2 T cells (14, 15), and compared these to the signals induced by anti-CD3 mAb. We show that the kinetics of the signals triggered upon IPP stimulation is quite different from that of the signals induced upon anti-CD3 mAb stimulation; the signals are largely delayed when the cells are stimulated with the non-peptidic antigen compared with those induced upon anti-CD3 mAb activation except for p56lck. But this delay cannot be assigned to the synthesis of de novo proteins. Moreover, we show that the majority of the phosphoantigen-induced signals, in contrast to the anti-CD3-triggered ones, are highly sustained and last for several hours. This long-lasting cell signaling observed with IPP stimulation is possibly related to the lack of induction of Trc-CD3 down-modulation that we demonstrate here.

**EXPERIMENTAL PROCEDURES**

**Chemicals and Reagents**—Recombinant IL2 (rIL2) was purchased from Chiron (Emeryville, CA), isopentenyl pyrophosphate (IPP) and enolase from Sigma Chemical Co. (St. Louis, MO), and LY 294002 from Calbiochem Corp. (Nottingham, UK). Anti-phospho-p42/44 MAPK antibody (Ab), anti-phospho-p38 MAPK Ab, anti-phospho-(Ser-473)-PKB Ab, and anti-PKB Ab were all purchased from Calbiochem Corp. (Nottingham, UK). Anti-phospho-p42/44 MAPK antibody (Ab), and recombinant LAT protein were from Upstate Biotechnology Inc. (Lake Placid, NY). Horseradish peroxidase-conjugated anti-mouse Ab, and recombinant LAT protein were from Immunotech (Marseille, France). UCHT1 (anti-CD3 monoclonal antibody (mAb)), anti-CD3, and anti-TcR Vα9 chain were produced by Vα9Vδ2 T cells stimulated either with anti-CD3 mAb or with an optimal dose of IPP over a broad time range, and activation of ERK1/ERK2 after 10 min at 37 °C. p56lck activity was measured by phosphorylation of the exogenous substrate enolase. Complexes were incubated in 50 μl of kinase assay buffer in the presence of 10 μg of acid-denatured enolase, 10 μCi of [γ-32P]ATP (6000 Ci/mm, PerkinElmer Life Sciences), and 4.5 μl unlabeled ATP. For ZAP-70 kinase assay, complexes were incubated in 25 μl of kinase buffer (100 μM Tris, pH 7.5, 125 mM MnCl2, 25 mM MnCl2, 2 mM EGTA, 250 μg Na3VO4, 2 mM dithiothreitol) in the presence of 4 μg of recombinant LAT protein, 10 μCi of [γ-32P]ATP (6000 Ci/mm, PerkinElmer Life Sciences), and 10 μl unlabeled ATP for 10 min at 37 °C. The reactions were stopped by addition of 2-mercaptoethanol-containing sample buffer and boiling. Radiolabeled proteins were then resolved on 10% SDS-PAGE, transferred to polyvinylidene difluoride membranes (Millipore), and then detected by autoradiography. Quantification of the phosphorylated bands reported in the results has been performed using a PhosphoImager Storm system driven by ImageQuant software (Molecular Dynamics, Sunnyvale, CA).

**Cell Culture**—Peripheral blood mononuclear cells (PBMC) were isolated from healthy donors. Human γδ T lymphocytes were purified from PBMC, by positive immunoselection using anti-TcR Vα9 mAb and magnetic beads coated with anti-mouse IgG (Dynal, Cagnieux, France). After spontaneous detachment, γδ T cells were specifically activated in the presence of syngeneic monocytes, IPP (50 μM), and rIL2 (20 ng/ml). Human γδ1 T lymphocytes were purified from PBMC, by positive immunoselection using anti-γδ TCR Vα9 mAb and magnetic beads coated with anti-mouse IgG. After spontaneous detachment, γδ1 T cells were specifically activated in the presence of syngeneic monocytes, PHA (1 μg/ml) and rIL2 (20 ng/ml). Human peripheral blood-derived γδ T lymphoblasts were generated as described above and maintained in RPMI 1640 supplemented with 5% fetal calf serum (FCS), 5% human AB serum, 2 mM glutamine, and rIL2 (20 ng/ml) at 37 °C in 5% CO2 humidified atmosphere for 4 or 5 weeks.

**Preparation of Supernatants for Measurement of TNF-α Production**—γδ T cells (2 × 106 cells/ml) were cultured in 24-well tissue culture plates in RPMI 1640 supplemented with 5% FCS + 5% human AB serum in a total volume of 0.5 ml per well. When mentioned cells were pretreated with inhibitors (LY 294002, 5 μM) for 30 min at 37 °C, then stimulated with IPP (50 μM) or UCHT1 (2 μg). At different times, supernatants were harvested and assayed for TNF-α production in the supernatants using a human TNF-α ELISA kit (OptEIA set: human TNF-α, PharMingen, San Diego, CA) according to the manufacturer’s instructions.

**Cell Extract Preparation and Western Blot Analysis**—20 × 106 cells were stimulated at 37 °C by UCHT1 (10 ng/ml) or IPP (100 μM) for the indicated times. After stimulation, cells were lysed in 1 ml of lysis buffer containing 50 mM HEPES (pH 7.4), 150 mM NaCl, 10 mM NaF, 10 mM idoacetamide, 1% Nonidet P-40, 1 mM PMSF, 1 mM Na3VO4, and 1 μM/ml of each protease inhibitor (leupeptin, aprotinin, chymotatin). Proteins were concentrated by precipitation with 1.5 volumes of acetone. Proteins from 5 × 106 cells were separated by 10% SDS-PAGE, transferred to polyvinylidene difluoride membranes (Millipore) and probed for the indicated antigens, α-subunit (IPP. MAPK Ab (1:1000), anti-p38 MAPK Ab (1:1000), anti-phospho-p42/44 MAPK Ab (1:1000), anti-ERK2 Ab (1:1500), anti-phospho-(Ser-473) PKB Ab (1:1000), and anti-PKB Ab (1:1000). Immunoreactive bands were visualized with the chemiluminescence Western blotting system (Amershams Pharmacia Biotech).

**Immunoprecipitation**—Following stimulation, 20 × 106 cells (for p56lck immunoprecipitation) or 50 × 106 cells (for ZAP-70 immunoprecipitation and ζ chain) were lysed in 1 ml of lysis buffer. After cell lysis, p56lck, ZAP-70, or ζ chain were immunoprecipitated from clarified supernatants, respectively, with 4 μg of anti-p56lck Ab, 5 μg of anti-ZAP-70 Ab, or 10 μg of anti-ζ chain. Immune complexes were collected using protein A-Sepharose (Amershams Pharmacia Biotech, Uppsala, Sweden), washed three times with lysis buffer for ζ chain before loading on 15% SDS-PAGE, and revealed by Western blotting, or washed twice with washing buffer containing 20 mM Tris, pH 7.4, 140 mM NaCl, 1% Nonidet P-40, 500 μM Na3VO4, 1 mM PMSF, 1% aprotinin, and once with specific kinase assay buffer before performing kinase assay.

**In Vitro Kinase Assay**—For p56lck kinase assay, complexes were resuspended in 50 μl of specific kinase buffer (50 μM Pipes, pH 7.5, 10 μM MgCl2, 10 μM MnCl2, 100 μM EGTA, 250 μM Na3VO4, 1 mM PMSF, 10 μM Na3VO4, 2 mM dithiothreitol) in the presence of 5 μCi of [γ-32P]ATP (6000 Ci/mm, PerkinElmer Life Sciences) and incubated for 10 min at 37 °C. p56lck activity was measured by phosphorylation of the exogenous substrate enolase. Complexes were incubated in 50 μl of kinase assay buffer in the presence of 10 μg of acid-denatured enolase, 10 μCi of [γ-32P]ATP (6000 Ci/mm, PerkinElmer Life Sciences), and 4.5 μl unlabeled ATP. For ZAP-70 kinase assay, complexes were incubated in 25 μl of kinase buffer (100 μM Tris, pH 7.5, 125 mM MgCl2, 25 mM MnCl2, 2 mM EGTA, 250 μg Na3VO4, 2 mM dithiothreitol) in the presence of 4 μg of recombinant LAT protein, 10 μCi of [γ-32P]ATP (6000 Ci/mm, PerkinElmer Life Sciences), and 10 μl unlabeled ATP for 10 min at 37 °C. The reactions were stopped by addition of 2-mercaptoethanol-containing sample buffer and boiling. Radiolabeled proteins were then resolved on 10% SDS-PAGE, transferred to polyvinylidene difluoride membranes (Millipore), and then detected by autoradiography. Quantification of the phosphorylated bands reported in the results has been performed using a PhosphorImager Storm system driven by ImageQuant software (Molecular Dynamics, Sunnyvale, CA).

**RESULTS**

**Production of TNF-α by Anti-CD3 mAb- or IPP-stimulated Vα9Vδ2 T Cells**—We previously established that high amounts of TNF-α are produced by Vα9Vδ2 T cells when stimulated with either anti-CD3 mAb or with the physiological antigen IPP (36). As shown in Fig. 1, upon anti-CD3 mAb stimulation, TNF-α is produced very early with its maximum reached after 3 h, whereas maximum production induced by IPP only occurs after 16 h. This delay between the two stimulation processes could reflect either a recruitment of different signals or a difference in the kinetics of the triggered signals. To test these hypotheses, we analyzed the kinetics of the extracellular regulated kinase (ERK) and p38, two mitogen-activated protein kinase (MAPK) pathways directly involved in TNF-α production by Vα9Vδ2 T cells stimulated either with anti-CD3 mAb or with IPP (36).

**Study of ERK Activation**—Purified Vα9Vδ2 T cells were stimulated with either anti-CD3 mAb or with an optimal dose of IPP over a broad time range, and activation of ERK1/ERK2 was studied. As shown in Fig. 2A, phosphorylation of ERK1/
ERK2 upon anti-CD3 mAb activation is very rapid (maximum reached at 5-min stimulation) and decreases but lasts at a high degree for around 30 min. After 30-min activation, the intensity is reduced to a very low phosphorylation level even though it still remains detectable after 2 h. In contrast, when the cells are stimulated with IPP, the phosphorylation/activation of ERK1/ERK2 begins to be faintly detectable after 1 h and reaches a plateau after 3 h, which lasts for at least one more hour. After 6 h, even though the phosphorylation signal begins to decrease, it still remains high.

Previous studies have shown that IPP induces cell proliferation and cytokine release in γδ T cells but not in other subsets of γδ T cells (13–15, 17, 23). However, we could not rule out the possibility that, even if IPP was not able to trigger biological responses in γδ T cells, which do not express the γδ TcR complex, it could trigger intracellular signals. To investigate this, we studied ERK2 activity in γ1δ1 T cells (which are another important subset of γδ T cells in human blood). As shown in Fig. 2B, anti-CD3 stimulation induces a strong and rapid ERK2 activation in γ1δ1 T cells; however, IPP is not able to trigger any ERK2 activation in these cells following either short or prolonged stimulation.

We also wondered if the observed delay in IPP-induced ERK2 activation could be assigned to a necessity to synthesize de novo proteins. As shown in Fig. 2C, pretreatment with cycloheximide, a protein synthesis inhibitor, does not modify the activation of ERK2 induced by IPP. In addition we confirmed that cycloheximide efficiently blocks protein synthesis at the concentration used in these experiments (10 μg/ml; data not shown).

Study of p38 MAPK Activation—We similarly studied activation of p38 kinase in Vγ9Vδ2 T cells that have been stimulated either with anti-CD3 mAb or with IPP. Fig. 3A shows that, as for ERK activation, p38 MAPK phosphorylation appears to be delayed in IPP stimulation compared with anti-CD3 stimulation. The kinetics are very similar to that of ERK1/ERK2 with the maximum activation in IPP stimulation occurring 2 h after triggering and lasting as a plateau for at least 4 more hours. In anti-CD3 mAb stimulation, the maximum is already reached within 5 min and then decreases, but the activated form remains elevated for at least 2 h. As we have shown for ERK2 MAPK, IPP does not trigger p38 activation in γ1δ1 T cells (Fig. 3B) and cycloheximide pretreatment does not modify IPP-induced p38 activation in γδ92 T cells (Fig. 3C).

Study of p56\lck Activation—We questioned whether the delay observed for ERK and p38 kinase activation, which are later signals in the transduction cascade, could be due to a difference in the triggering of a signal directly related to TcR/CD3 ligation. For that purpose, we studied activation of the Src family kinase p56\lck, which represents one of the earliest events in αβ T cell stimulation. Fig. 4A shows that the immunoprecipitated p56\lck, in the presence of [γ-\32P]ATP, displays a 60-kDa-shifted band in anti-CD3 stimulation, which corresponds to the conversion of p56\lck to an Lck form phosphorylated on Ser-59 (37, 38). This hyperphosphorylation was already observed in αβ T cells stimulated with anti-CD3 mAb or phorbol 12-myristate 13-acetate (39) and was shown not to be concomitant with an increase of the kinase activity but rather to be accompanied by a decrease in the kinase activity (40–43). Similarly, in our experiments, we could not detect, through phosphorylation of enolase used as exogenous substrate, any activity of p56\lck in anti-CD3 stimulated γδ T cells.

In IPP stimulation (Fig. 4B) conversion of p56\lck to a slower migrating form also exists but occurred along with an increased intensity of the p56 band (as a control we checked that the amounts of immunoprecipitated p56\lck loaded on the gel were similar in each sample; data not shown). This increased inten-
sity of the p56<sub>Lck</sub> band reflects autophosphorylation of the kinase and its activation. Indeed, kinase activity was detectable through phosphorylation of enolase. It has to be noted that activation of p56<sub>Lck</sub> in IPP-stimulated cells is rapid (detectable at 5 min) and peaked at 30–45 min. Therefore, because p56<sub>Lck</sub> activation in IPP stimulation is high and rapid, it can hardly be accountable for the delay observed in the MAPK late signals.

**Study of ZAP-70 Kinase Activity**—It is generally accepted that immunoreceptor tyrosine-based activation motifs (ITAM) of the signal-transducing subunits of CD3, as well as the ζ chain, are phosphorylated by Lck, making them competent to associate with zeta-associated protein (ZAP)-70 (reviewed in Ref. 28). Once recruited, ZAP-70 is activated through its phosphorylation by Lck and then is able to recruit and phosphorylate its own substrates, SLP76 and a linker for activation of T cells (LAT) (28, 29, 44). Because we observed a rapid activation of p56<sub>Lck</sub> upon IPP stimulation, we questioned whether the ζ chain and ZAP-70 are also rapidly activated. First, we immunoprecipitated the ζ chain proteins and studied their phosphorylation in samples from unstimulated or stimulated cells. We were unable to detect by Western blot, using an anti-phosphotyrosine Ab, phosphorylation of this protein in either unstimulated or IPP-stimulated samples. However, we showed that in the immunocomplex, ZAP-70, is coprecipitated with the ζ chain and the amount of coprecipitated ZAP-70 is higher in the immunocomplex from anti-CD3- or IPP-stimulated cells than from unstimulated-cells. This indicates that the ζ chain may also be more phosphorylated in these samples (Fig. 5A). Following stimulation with IPP, the maximum amount of coprecipitated ZAP-70 protein is observed after 30-min IPP stimulation. Moreover, we studied ZAP-70-induced phosphorylation using recombinant LAT as an exogenous substrate. As shown in Fig. 5B, recombinant LAT is phosphorylated by immunoprecipitated (IP)-ZAP-70 from cells stimulated by IPP. However, as for ERK and p38, phosphorylation of LAT is largely delayed compared to activation of p56<sub>Lck</sub>; indeed phosphorylation is detectable only with IPP-ZAP-70 from 30-min-activated cells and lasts as a plateau with IP-ZAP-70 from at least 2-h-activated cells. As a control, LAT appears to be highly phosphorylated with IP-ZAP-70 from cells activated for 5 min with anti-CD3 mAb. The delayed phosphorylation of recombinant LAT thus demonstrates that ZAP-70 is activated tardily in IPP stimulation.

**Study of PKB Phosphorylation**—Several papers have shown that TNF-α production in several cell types, including T cells, is dependent on phosphoinositide 3-kinase (PI3K) activation (45, 46). Moreover, in αβ T cells, it was demonstrated that TcR engagement results in rapid phosphorylation of Tyr-685 in the p85 subunit of PI3K (47), and that this phosphorylation and the consequent activation of PI3K have been attributed to Lck. We therefore studied whether, in Vγ9Vδ2 T cells, TNF-α is also dependent on PI3K activation. As shown in Fig. 6A, TNF-α production induced with either IPP or anti-CD3 mAb is inhib-
shown that sustained signaling results from prolonged T cell receptor occupancy (51–53). We therefore questioned whether the long-lasting signaling triggered in IPP stimulation compared with that induced during anti-CD3 stimulation could be parallel to a difference in the rate of TcR down-modulation. We therefore stimulated γδ T cells with either anti-CD3 mAb or with IPP. The cells were paraformaldehyde-fixed at different times after stimulation and analyzed for TcR expression using FITC-conjugated anti-Vγ9 mAb. As shown in Fig. 7, TcR is down-modulated in a time-dependent manner upon anti-CD3 stimulation whereas, with IPP, it remains unmodified. In parallel, as a control of stimulation efficacy, we analyzed ERK phosphorylation, which showed the same kinetics as that presented above (data not shown).

**DISCUSSION**

The present paper studies signals triggered in Vγ9Vδ2 T cells by IPP, a physiological antigen specific for this T cell subpopulation. It has first to be noted that, in contrast to in vitro studies of αβ T cell activation by physiological antigen (51), those of Vγ9Vδ2 T cell stimulation do not require that the antigen be presented by antigen-presenting cells. The IPP-induced signals were compared with those triggered by the non-physiological model, using an anti-CD3 mAb. It appears that signals triggered by IPP leading to TNF-α release were delayed compared with those induced by anti-CD3 mAb, and this delay cannot be assigned to the synthesis of de novo proteins as shown in the experiments in the presence of cycloheximide. In contrast, the Src family kinase p56lck, the activation of which represents one of the earliest events in T cell stimulation (28, 54), appears to be triggered very early in IPP-stimulated γδ T cells. Moreover, its enzyme activity can be detected through its autophosphorylation and by phosphorylation of enolase used as exogenous substrate. In αβ T cells, p56lck enzyme activation was shown to occur upon TcR-CD3 ligation, but this was demonstrated in cell lines (mostly Jurkat cells) (41, 55). In primary αβ T cells, engagement of the TcR-CD3 complex by anti-CD3 mAb leads to hyperphosphorylation of p56lck, observed in SDS-PAGE as a slower migrating band (60 kDa) (37, 38), with no increase but even a decrease in kinase activity (40–43). Autophosphorylation activation of the kinase in primary αβ T cells is, however, detectable when co-receptors CD4 or CD8 are engaged by interacting components such as anti-CD4 mAb or human immunodeficiency virus external glycoprotein gp120 in CD4+ cells (56). In this case, p56lck activation is detectable early (5 min after activation), as is the case in IPP stimulation of γδ T cells. In Vγ9Vδ2 T cells, similarly to what happens in αβ T cells, anti-CD3 stimulation leads to the appearance of a 60-kDa band, but there is no visible increase in enzymatic activity. Signal triggering in anti-CD3 stimulation, leading to activation of downstream signaling pathways, can involve, as has been postulated in αβ T cells, another Src family kinase, p55fyn, which has been shown to be directly associated with the T cell receptor complex (57, 58). The fact that Vγ9Vδ2 T cells do not express CD4 and express CD8 poorly (the cells we used in the present study were CD4-CD8-; data not shown) and that IPP stimulation leads to p56lck activation could suggest that this antigen, in addition to engaging the TeR-CD3 complex, could also engage another cell surface molecule as a co-receptor. Such an hypothesis that responsiveness of Vγ9Vδ2 T cells is modulated by the expression of an (unknown) molecule with a co-receptor-like function is similar to that described for CD4 and CD8 co-receptors in αβ T cells recently put forward by Bürk and co-workers (59). p56lck has been described in αβ T cells to phosphorylate ITAM on the CD3 ε chain and TcR ζ (60) chain, rendering them competent for recruitment of the Syk family kinase ZAP-70.
Subsequent phosphorylation activation of this kinase triggers the downstream signaling pathways regulating the transcription of genes essential for cytokine production. In IPP stimulation, the events that are directly dependent on p56Lck activation, i.e. recruitment and activation of ZAP-70 and PI3K as well as later signals like ERK and p38 kinase, appeared delayed in comparison to that induced in anti-CD3 stimulation. This delay is likely not attributable to a slow rate in IPP-induced phosphorylation of raft-associated proteins, which leads to triggering of tyrosine phosphorylation of the TcR by anti-CD3 cross-linking. However, aggregating cells with an anti-PKB Ab after Ab stripping. This experiment is representative of three experiments.

As a possible consequence, the rate for the physical recruitment of a sufficient number of engaged TcR-CD3 complexes to colocalize with the rafts anchored transducing molecules is slower than that occurring in anti-CD3 stimulation, leading to a delay in cell signaling (ZAP-70, PI3K, MAPKs) and TNF-α release. Of course, the results we present herein are in vitro results obtained with purified Vγ9Vδ2 T cells, and it cannot be totally ruled out that in vivo the antigens could be presented by other cell types through cell surface molecules not yet determined. According to such an hypothesis, the kinetics of the triggered signals could be faster. To investigate this point, we studied TcR-CD3 down-modulation in human peripheral blood-derived Vγ9 T cells. Human peripheral blood-derived Vγ9 T cells were stimulated for the indicated times by UCHT1 (10 μg/ml) or IPP (100 μM). Total cellular proteins were separated on 10% SDS-PAGE and revealed by Western blot analysis using an anti-phospho-(Ser-473) PKB Ab (which specifically reveals the phosphorylated form of PKB) and reprobed with an anti-PKB Ab after Ab stripping. Each analysis has been repeated at least three times.
behave as a monovalent antigen and thus could engage several TCR-CD3 complexes together. In this case, the kinetics of the triggered signals could be faster. To test this hypothesis, we compared the kinetics of TNF-α production by Vγ9Vδ2 T cells induced by a non-peptidic antigen such as IPP and by a whole pathogen. We chose to use as a pathogen a strain of *Brucella*, which we have shown produces a non-peptidic antigen that is able to stimulate γδ T cells (62). TNF-α production that we measured was lower in supernatants from cells stimulated with gentamicin-killed bacteria than from those stimulated with IPP (probably due to the lower concentration of non-peptidic antigen present on the surface of the bacteria compared with the IPP concentration that we used), but the kinetics of TNF-α production was identical (data not shown).

One of the striking features concerning IPP-induced signals, is that they are highly sustained compared with those induced by anti-CD3 mAb or with those described in αβ T cells stimulated either with anti-CD3 mAb or physiological antigens presented in the context of MHC molecules. It has been shown that sustained signaling can be related to TCR occupancy (51). Here we demonstrated that, in contrast to anti-CD3 mAb, IPP does not induce down-modulation of the TcR–CD3 complex. There-
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J. Biol. Chem. 2001, 276:15961-15967. doi: 10.1074/jbc.M008684200 originally published online February 13, 2001

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