Differential Impact of Age and Cytomegalovirus Infection on the γδ T Cell Compartment

Antoine Roux, Gisèle Mourin, Martin Larsen, Solène Fastenackels, Alejandra Urrutia, Guy Gorochov, Brigitte Autran, Catherine Donner, Daniel Sidi, Joyce Sibony-Prat, Arnaud Marchant, Marc Stern, Delphine Sauce and Victor Appay

J Immunol 2013; 191:1300-1306; Prepublished online 1 July 2013;
doi: 10.4049/jimmunol.1202940
http://www.jimmunol.org/content/191/3/1300

The Journal of Immunology is published twice each month by
The American Association of Immunologists, Inc.,
9650 Rockville Pike, Bethesda, MD 20814-3994.

Print ISSN: 0022-1767 Online ISSN: 1550-6606.

Differential Impact of Age and Cytomegalovirus Infection on the γδ T Cell Compartment

Antoine Roux,*,†,[1] Gisèle Mourin,*,[1] Martin Larsen,* Solène Fastenackels,* Alejandra Urrutia,* Guy Gorochov,* Brigitte Autran,*,‡ Catherine Donner,§ Daniel Sidi,¶ Joyce Sibony-Prat,‖ Arnaud Marchant,# Marc Stern,† Delphine Sauce,*,[1] and Victor Appay*,‡,[1]

γδ T cells represent a subset of unconventional T lymphocytes that are known for their reactivity against different pathogens and considered as intermediate mediators between adaptive and innate immunity. We provide in this paper further insights underlying the changes that affect the γδ T cell compartment with advanced age in humans. We show that both aging and CMV infection impact independently on the γδ T cell compartment. Most γδ T cells are significantly affected by age and present a decreased frequency in the elderly. The decline of the γδ T cell pool appears to be independent from the activity of the thymus, arguing in favor of an extrathymic site of γδ T cell production in humans. Of note, CMV infection, which is directly associated with the activation of the pool of Vδ2$^-$ γδ T cells, promotes nonetheless the inflation of this compartment throughout life. CMV seropositivity accentuates further the accumulation of highly differentiated lymphocytes in Vδ2$^-$ γδ T cell subsets with time, in contrast to Vδ2$^+$ γδ T cells, which maintain a less differentiated phenotype. This is similar to the effect of CMV on αβ T cells and suggests that γδ T cells may vary in differentiation phenotype according to distinct stimuli or pathogens. ***The Journal of Immunology*****, 2013, 191: 1300–1306.**

Advanced age is associated with a decline of the functional capacity of the immune system. This likely contributes to the increased susceptibility to and severity of infectious, cancerous, and autoimmune diseases, which characterize the elderly population. This deterioration, also referred to as immunosenescence, is reflected by alterations of whole organ function and of immune cell frequency and phenotype. The conventional αβ T cells have been extensively characterized in this context and appear to be particularly affected during the process of aging. Decreased numbers of naive cells and accumulation of highly differentiated oligoclonal memory populations are hallmarks of the CD8$^+$ and CD4$^+$ T cell pools in the elderly (1). The alterations of the αβ T cell compartment are directly related to the decline in T cell production associated with thymic involution as well as the recurrent mobilization of T cells upon infections with pathogens (2). In particular, the influence of CMV on the development of immunosenescence in humans is often highlighted and debated (3). CMV is a persistent and highly prevalent virus, which triggers particularly vigorous CD8$^+$ and CD4$^+$ T cell responses in its host. CMV-specific T cells harbor a late differentiation phenotype (CD27$^-$CD45RA$^+$), associated with a strong cytotoxic potential and proinflammatory profile, reflected by their capacity to produce cytokines such as IFN-γ and TNF-α (4). These cells can occupy up to 40% of the entire T cell memory pool (5), thus altering significantly the balance between the naive and memory T cell compartments. In donors with suboptimal T cell renewal, CMV is directly associated with exhaustion of the naive T cell pool and the development of an immunosenescence-like phenotype (6).

The alteration of unconventional T lymphocyte compartments with aging is instead poorly documented. Although γδ T cells represent only a small proportion of the total circulating T cell pool (0.5–6%) in humans, they are known for their strong proinflammatory nature and display reactivity against several pathogens (7, 8). They can respond to a number of stimuli (i.e., recognized by their TCR, NK-like receptors, as well as TLRs) and engage potent effector functions both in terms of cytokine and chemokine production capacity (e.g., IFN-γ, IL-17, and RANTES) and direct cytotoxicity (9). In the elderly, both the absolute numbers and percentages of total γδ T lymphocytes have been shown to decrease (10, 11). Interestingly though, subsets of γδ T cells appear to be differentially affected. The numbers of Vδ2$^+$ cells, which is the predominant population of circulating γδ T cells, decrease with age, in contrast to Vδ1$^+$ cells that remain stable (10, 11). The factors involved in the differential regulation of γδ T cells with aging are unclear. Our aim in this paper is thus to investigate further the fate of γδ T cell compartment overtime, with advanced age.

CMV infection is known to shape the γδ T lymphocyte compartment (12). Studies in organs or allogeneic stem cells transplanted patients indicate that γδ T cells are able to recognize

*INSERM Unité Mixte de Recherche S945, Infections and Immunity, Université Pierre et Marie Curie-Paris 6, Hôpital Pitié-Salpêtrière, 75013 Paris, France; †Service de Pneumologie, Hôpital Foch, 92151 Suresnes, France; ‡Assistance Publique-Hôpitaux de Paris, Groupe Hospitalier Pitié-Salpêtrière, Laboratoire d'Immunologie Cellulaire et Tissulaire, 75013 Paris, France; §Service de Cardiologie Pédiatrique, Hôpital Necker Enfants Malades, 75015 Paris, France; ¶Service de Gynécologie et Obstétrique, Hôpital Erasme, 1070 Brussels, Belgium; ‖Centre Hospitalier Intercommunal André Grégoire, Unité de Médecine Gériatrique Aiguë, 93105 Montreuil-sous-Bois, France; and #Institute for Medical Immunology, Université Libre de Bruxelles, 6041 Charleroi, Belgium

[1]A.R., G.M., V.A., and D.S. contributed equally to the work.

Received for publication October 22, 2012. Accepted for publication May 23, 2013.

This work was supported by Vaincre la Mucoviscidose. A.R. is supported by Vaincre la Mucoviscidose and the Fondation pour la Recherche Médicale. M.L. is supported by the Université Pierre et Marie Curie "EMERGENCE" program. A.M. is a senior research associate of the Fonds de la Recherche Scientifique (Belgium).

Address correspondence and reprint requests to Dr. Victor Appay or Delphine Sauce, INSERM Unité Mixte de Recherche S945, Infections and Immunity, Université Pierre et Marie Curie-Paris 6, Hôpital Pitié-Salpêtrière, 75013 Paris, France. E-mail addresses: victor.appay@upmc.fr (V.A.) or Delphine.Sauce@upmc.fr (D.S.)

Abbreviations used in this article: BAL, bronchoalveolar lavage; PD-1, programmed death-1; YATEC, young adult thymectomized during early childhood.

www.jimmunol.org/cgi/doi/10.4049/jimmunol.1202940

CMV Ags and thus may be involved in the cellular immune response against this virus (13–16). Subsets of γδ T lymphocytes that are particularly mobilized in donors with apparent CMV replication usually express Vδ1, Vδ3, or Vδ5 and are usually assimilated to the Vδ2$^-$ γδ T cells for simplicity (12–17). Of note, the ligand recognized by a γδ TCR on CMV-infected stressed human cells has been identified recently (18). To address the potential relationship between age, CMV infection, and changes within the γδ lymphocyte compartment overtime, we have studied the frequency and phenotype of circulating Vδ2$^+$ and Vδ2$^-$ γδ T cells in donors grouped according to age and CMV infection status. γδ T cells were also analyzed in lung transplant patients characterized by active CMV replication (upon primary or chronic infection) posttransplantation. Our present data show that γδ T cell subsets are differentially influenced by age and CMV infection, which can explain the divergent changes affecting γδ T cell subsets.

Materials and Methods

Study subjects

Blood samples from 25 young (median = 23.96 y), 26 middle-aged (median = 37.12 y), and 28 old (median = 85.07 y) healthy adults were obtained for this study. Elderly individuals with malignancies, acute diseases, or advanced stages of severe chronic diseases, such as chronic inflammatory disease, atherosclerotic disease, congestive heart failure, poorly controlled diabetes mellitus, renal or hepatic disease, or chronic obstructive pulmonary disease, as well as individuals under immunosuppressive therapy were excluded from the study. We also studied young adults thymectomized during early childhood (YATEC; n = 22; median = 22.80 y). YATEC had complete removal of the thymus within 15 d after birth during open-heart surgery because of transposition of great vessels. Thymectomy was performed by total resection of both lobes for ease of surgical access to the heart and major vessels. Only donors with no residual cyanosis, transplantation or immunosuppressive therapy, cortisone therapy or hematologic disorders, and medication with drugs known to influence blood production in the bone marrow or the immune system were included. Pregnant women diagnosed with primary CMV infection were also studied (n = 10; median = 31.5 y). Diagnosis was based on documented seroconversion or increased titers of anti-CMV IgM when serostatus at the beginning of pregnancy had not been documented. Lung-transplanted patients (n = 45) undergoing primary or chronic CMV infection after lung transplantation were also included in the study (median = 48.78 y). Positive virus-specific PCR in total blood was used to establish active CMV replication in these patients. Posttransplant immunosuppressive treatment consisted of adapted doses of corticosteroid, calcineurin inhibitor and a purine synthesis inhibitor, with or without depleting Abs. Blood samples were obtained at months 2 or 4, and 9 or 12 following transplantation. All participants provided written informed consent. The study was approved by the Comité de Protection des Personnes of the Pitié Salpétrière Hospital (Paris, France), the Comité d'Ethique of the Hôpital Erasme (Brussels, Belgium), and the Comité d'Ethique of the Centre Hospitalier Universitaire Tivoli (La Louvière, Belgium). For each individual, PBMCs (isolated by density gradient centrifugation) were cryopreserved until use. CMV serology of healthy adults was performed on plasma samples using a Mastazyme-CMV serology kit (Mast Diagnostics, Merseyside, U.K.) or the ETI-CYTOK-G PLUS (Diasorin, Saluggia, Italy), according to the provider's recommendations.

In vitro stimulation

Cells were stimulated with CD3/CD28-coated beads (Life Technologies, Paisley, U.K.). PBMCs were then cultured for 10 d at 37°C and 5% CO_2 in RPMI 1640 medium supplemented with 10% human serum and 20 ng/ml IL-2 (R&D Systems, Minneapolis, MN). Unstimulated cells as well as cells stimulated for 3, 6, and 10 d were stained for activation markers.

Flow cytometry analysis

mAbs were obtained from the following vendors: 1) anti–Ki67-PE, anti–HLA-DR-PerCp-Cy5.5, anti–CD38-allophycocyanin, and anti–CD45RA-V450 (BD Biosciences, San Jose, CA); 2) anti–CD3-Cascade Yellow (DakoCytomation, Glostrup, Denmark); 3) anti–CD27-Alexa700, anti–CD25-BV785, and anti–programmed death-1 (PD-1)-allophycocyanin (BioLegend, San Diego, CA); and 4) anti–α*Pan* γδ-PE or -PE-Cy5 and Vδ2-FITC (Beckman Coulter, Villepinte, France). The viability dye Aqua (Invitrogen, Paisley, U.K.) was used to eliminate dead cells from the analysis. Staining for cell surface markers was performed as described previously (19). Data were acquired using either an LSRII or a Fortessa flow cytometers (BD Biosciences) and analyzed using FlowJo version 8.2 (Tree Star) and DIVA softwares (BD Biosciences).

Statistical analysis

Univariate statistical analysis was performed using GraphPad prism software. Groups were compared using the nonparametric Kruskal–Wallis or Mann–Whitney tests. The Wilcoxon test was used for comparison between time points. A p value < 0.05 was considered significant.

Results

CMV-dependent alteration of the γδ T cell compartment with age

Circulating γδ T cells were identified within the CD3$^+$ lymphocyte compartment in young (age: 18–25 y), middle-aged (age: 25–55 y), and old (age: >75 y) healthy donors (Fig. 1A). In line with previous findings (10, 11), we observed a decreased frequency of total γδ T cells with increasing age (Fig. 1B), which is reminiscent of the observations on the αβ T cells. However, this decrease was mainly evident in CMV-seronegative donors (Fig. 1C). The separation of the γδ T cell compartment according to Vδ2 expression revealed clear differences between the evolution of Vδ2$^+$ and Vδ2$^-$ γδ T cell subsets with aging (Fig. 1D). The Vδ2$^+$ T cell frequency was substantially reduced independently of the CMV serostatus. In contrast, the Vδ2$^-$ γδ T cell frequency decreased only in CMV-seronegative elderly, whereas it remained stable in the CMV-infected donors. Regardless of age, CMV-seropositive donors presented higher levels of Vδ2$^-$ γδ T cells, whereas the frequency of Vδ2$^+$ T cells was unaffected. The different fates of Vδ2$^+$ or Vδ2$^-$ γδ T cell subsets with aging depend therefore on the CMV serostatus. Although the majority of γδ T cells are significantly affected with age, CMV-seropositive individuals seem to maintain a robust and stable pool of Vδ2$^-$ γδ T cells throughout life.

Primary CMV infection is associated with strong activation of Vδ2$^-$ γδ T cells

The maintenance of the Vδ2$^-$ γδ T cell pool in the elderly owing to CMV infection is supported by previous observations showing expansions of Vδ2$^-$ γδ T cells in transplant patients with evidence of CMV replication (13–16). To further support the possibility that CMV may mobilize and promote the maintenance of Vδ2$^-$ γδ T cells, we studied the frequency and activation levels of Vδ2$^+$ and Vδ2$^-$ γδ T cells in pregnant women with primary CMV infection and in chronically infected donors. As early as primary infection, CMV-infected subjects presented a significant increase in Vδ2$^-$ (but not Vδ2$^+$) γδ T cells compared with noninfected controls (Fig. 2A). Because CD38 expression is a robust marker of activation on αβ T cells in vivo (20), we tested whether it could also be used to assess γδ T cell activation levels. To validate the use of CD38 as a marker of γδ T cell activation, expression kinetics of CD38 along with other markers associated with αβ T cell activation (i.e., CD25, HLA-DR, and PD-1) were analyzed on γδ T cells upon in vitro polyclonal stimulation (Fig. 2B). Stimulation with immobilized anti-CD3/CD28 Abs resulted in the sequential upregulation of CD25, HLA-DR, and PD-1 and CD38 on γδ T cell surface (Fig. 2C), supporting the use of these markers for γδ T cell activation. We thus quantified the ex vivo expression of CD38 (as well as HLA-DR, PD-1, and Ki67, a marker associated with cell cycling) on donor Vδ2$^+$ and Vδ2$^-$ cells upon primary CMV infection (Fig. 2D). Strongest expressions of CD38, together with HLA-DR, PD-1, and Ki67, were found on Vδ2$^-$ (but not Vδ2$^+$)

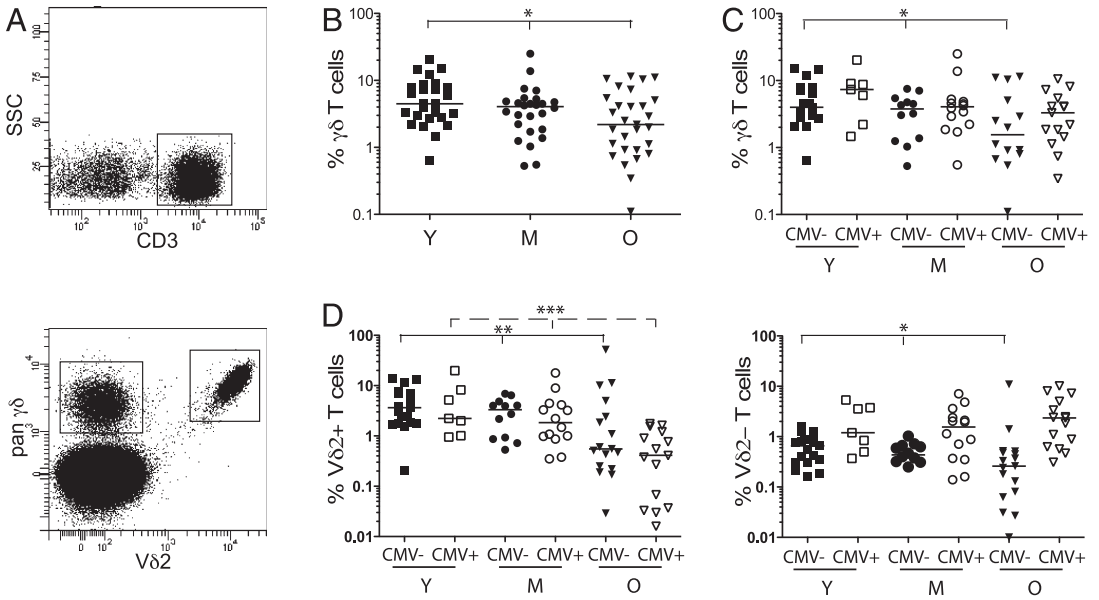

FIGURE 1. Changes in Vδ2$^+$ or Vδ2$^-$ γδ T cell subset frequency with age and CMV infection. (**A**) Representative FACS stainings of Vδ2$^+$ and Vδ2$^-$ γδ lymphocytes within the CD3$^+$ T cell compartment. Percentage of γδ T cells in young (Y, $n = 26$), middle-aged (M, $n = 26$), and old (O, $n = 28$) adults (**B**) and according to CMV serostatus (**C**). (**D**) Percentage of Vδ2$^+$ (*left panel*) or Vδ2$^-$ (*right panel*) γδ T cells in young (Y), middle-aged (M), and old (O) adults, according to CMV serostatus. Bars indicate the median. The Mann–Whitney or Kruskall–Wallis tests were used for comparing two groups or three or more groups, respectively. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

cells from donors with primary CMV infection in comparison with noninfected CMV or chronically infected controls (Fig. 2E). Overall, these data support the robustness of CD38 surface expression as a marker of γδ T cell activation and show that primary CMV infection results in strong activation of Vδ2$^-$ γδ T cells.

Parallel CD8$^+$ αβ or Vδ2$^-$ γδ T cell mobilization by active CMV replication

We next studied patients undergoing lung transplantation as a model to study the impact of active CMV replication in primary and chronic infection settings. CMV-seropositive lung transplant patients typically undergo a number of CMV replication episodes in the months following transplantation. Patients were divided into three groups according to their CMV donor/recipient serostatus: no CMV infection (D$^-$/R$^-$ patients), primary CMV infection (D$^+$/R$^-$ patients), and chronic CMV infection (D$^-$/R$^+$ and D$^+$/R$^+$ patients). To evaluate the effect of active CMV replication, analyses of Vδ2$^+$ and Vδ2$^-$ γδ T cells were performed at two time points posttransplantation: between 2 and 4 mo (usually before CMV replication episodes) and between 9 and 12 mo (after possible CMV replication episodes) posttransplantation. Primary or chronic CMV infection was associated with evident increases in CD38 expression on the surface of Vδ2$^-$ γδ T cells over time, whereas no changes in CD38 expression were observed in the Vδ2$^+$ cell subset (Fig. 3A). Moreover, Vδ2$^-$ cell activation was stronger in patients presenting evidence of CMV replication (i.e., positive CMV PCR) posttransplantation (Fig. 3B). Of note, 9–12 mo posttransplantation, Vδ2$^-$ cell activation levels paralleled those of CD8$^+$ αβ T cells in CMV-infected patients (Fig. 3C). Kinetics of Vδ2$^-$ or CMV-specific CD8$^+$ T cell (identified with tetramers) activation were in fact very similar posttransplantation (Fig. 3D). Finally, both primarily ($p = 0.0007$) and chronically ($p = 0.03$) CMV-infected patients presented significant expansion of Vδ2$^-$ γδ T cells overtime (Fig. 3E), which resembles the inflation phenomenon of CMV-specific CD8$^+$ T cells described previously (21). In contrast, CMV infection had no impact on Vδ2$^+$ γδ T cell levels (data not shown). One year after transplantation, CMV-infected lung transplant patients displayed higher levels of circulating Vδ2$^-$ γδ T cells compared with CMV-uninfected donors ($p = 0.01$). Overall, the activation and expansion of the Vδ2$^-$ γδ T cells driven by CMV likely promote the maintenance of this subset with advanced age.

Phenotypic alterations of the γδ T cell compartment with aging

Like αβ T cells, phenotypic analyses of the γδ T cell compartment can be based on the surface expression of CD27 and CD45RA (Fig. 4A), separating the γδ T cell compartment into distinct subsets, known to have different functional capacities (22–24). We observed changes in the distribution of these subsets associated with aging (Fig. 4B): the γδ T cell compartment in the elderly was characterized by an increased representation of cells with a CD27$^-$CD45RA$^+$ phenotype ($p = 0.0025$), commonly associated with late memory αβ T cell differentiation. Previous work shows that CMV infection results in the increase of CD27$^-$ CD45RA$^+$ cells within the Vδ2$^-$ γδ T cell compartment, in line with observations on CD8$^+$ T cells (15, 25). We therefore characterized the phenotype of Vδ2$^+$ and Vδ2$^-$ γδ T cell subsets separately, taking into consideration donor CMV serostatus (Fig. 4C). The analysis highlighted the high degree of heterogeneity within the γδ T cell compartment, with two main observations. First, Vδ2$^+$ and Vδ2$^-$ γδ T cell subsets presented intrinsic phenotypic differences, independently of age or CMV infection, characterized by a bias toward CD27$^-$CD45RA$^-$ and CD27$^-$ CD45RA$^+$ phenotypes for Vδ2$^+$ and Vδ2$^-$ γδ T cells, respectively (even in seronegative donors or young adults). Second, although the phenotype of Vδ2$^+$ γδ T cells remain largely unchanged over time, CMV infection ($p = 0.011$), but also advanced age ($p = 0.038$), was independently associated with an increase in CD27$^-$CD45RA$^+$ cells in the Vδ2$^-$ γδ T cell compartment. These observations were further supported by phenotypic analyses of Vδ2$^+$ and Vδ2$^-$ γδ T cells in lung transplant patients with primary or chronic CMV infection (Fig. 4D): in CMV-uninfected patients, Vδ2$^-$ γδ T cells were mainly CD27$^-$CD45RA$^+$, a phenotypic distribution that was further accentuated with CMV infection.

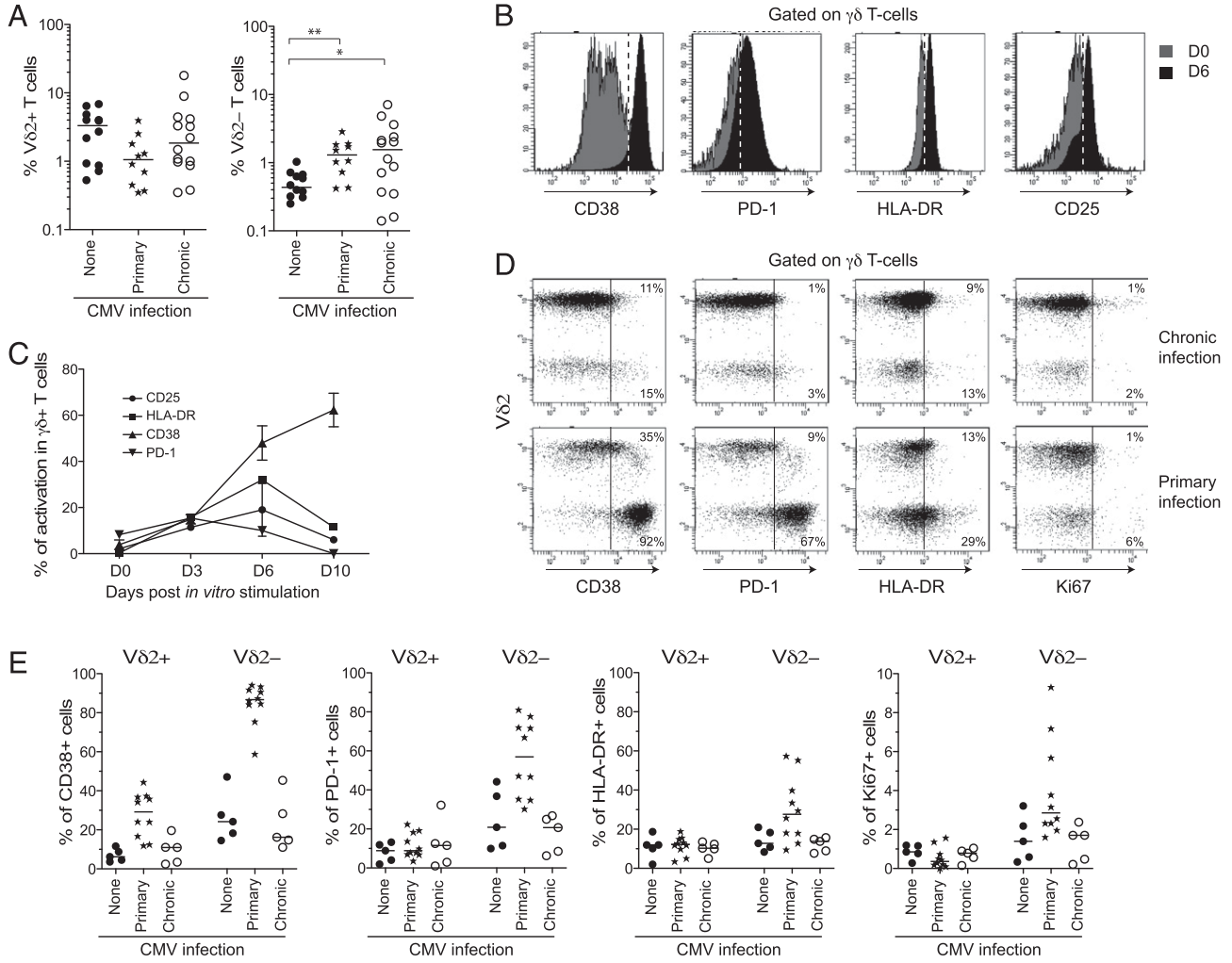

FIGURE 2. Activation of Vδ2$^-$ γδ T cells upon primary CMV infection. (**A**) Percentage of Vδ2$^+$ (*left panel*) or Vδ2$^-$ (*right panel*) γδ T cells in middle-aged subjects presenting no (n = 11), primary (n = 10), or chronic (n = 14) infection with CMV. Bars indicate the median. The Mann–Whitney U test was used for comparing groups. *$p < 0.05$, **$p < 0.01$. (**B**) Representative FACS stainings showing CD38, PD-1, HLA-DR, and CD25 overlay expression on γδ T cells at days 0 and 6 poststimulation with anti-CD3/CD28 Abs in vitro. The cutoff line is set to highlight the effect of in vitro activation on γδ T cells but does not represent a threshold for marker expression positivity. (**C**) Kinetics of CD38, CD25, HLA-DR, and PD-1 on γδ T cells upon in vitro stimulation with anti-CD3/CD28 Abs. (**D**) Representative FACS stainings showing CD38, PD-1, HLA-DR, and Ki67 expression on Vδ2$^+$ or Vδ2$^-$ γδ T cells from a CMV chronically infected donor and a subject with primary CMV infection. The cutoff line is set to highlight the effect of in vivo CMV primary infection on γδ T cells but does not represent a threshold for marker expression positivity. Numbers indicate the percentages of Vδ2$^+$ or Vδ2$^-$ γδ T cells expressing the different activation markers. (**E**) CD38, PD-1, HLA-DR, and Ki67 expression on Vδ2$^+$ or Vδ2$^-$ γδ T cells in middle-aged subjects presenting no (n = 5), primary (n = 10), or chronic (n = 5) infection with CMV. Bars indicate the median.

Analyses of γδ T cells in bronchoalveolar lavage from lung transplant patients showed also CMV-independent differences between Vδ2$^+$ and Vδ2$^-$ γδ T cell phenotypes (data not shown), thus reinforcing the distinctions between these cells beyond circulating blood. Altogether, the phenotypic change observed in the total γδ T cells in the elderly (i.e., increased CD27$^-$CD45RA$^+$ cells) can therefore be mainly attributed to changes in the Vδ2$^-$ γδ T cell compartment with age or CMV infection.

Thymic output independent decrease of γδ T cell frequencies

Aside from the maintenance of the Vδ2$^-$ γδ T cell frequency in relation to CMV infection, the size of the γδ T cell compartment decreases with aging, like for αβ T cells. The latter evokes the possibility that thymic involution and decreased thymic output with advanced age may be the cause of the reduced γδ T cell frequency. To address this point, we analyzed the absolute counts of Vδ2$^+$ and Vδ2$^-$ γδ T cells (in comparison with naive and memory αβ T cell counts) in young adults (18–26 y old) who were thymectomized shortly after birth in the setting of cardiac surgery (in comparison with age-matched controls, middle-aged and elderly donors). YATEC represent an informative group to evaluate the importance of the thymus beyond the production of the initial T cell stock and to assess the long-term consequence of reduced thymic output, independently from age. As expected, CMV infection was generally associated with increased numbers of memory αβ as well as Vδ2$^-$ γδ T cell subsets in all settings (i.e., likely related to the expansion of "CMV induced T cells" in these subsets) (Fig. 5A, 5B). However, although both naive or memory αβ T cell frequencies presented similar alterations in the elderly and YATEC, the number of γδ T cells was more affected by advanced age than by thymectomy. In fact, the absolute counts of Vδ2$^+$ and Vδ2$^-$ γδ T cells were not reduced in YATEC and remained equivalent to those observed in young or middle-aged controls (Fig. 5A). Overall, this suggests that the maintenance of the γδ T cell pool is less dependent on the thymus than the αβ T cell pool and that the reduction in the absolute counts of γδ

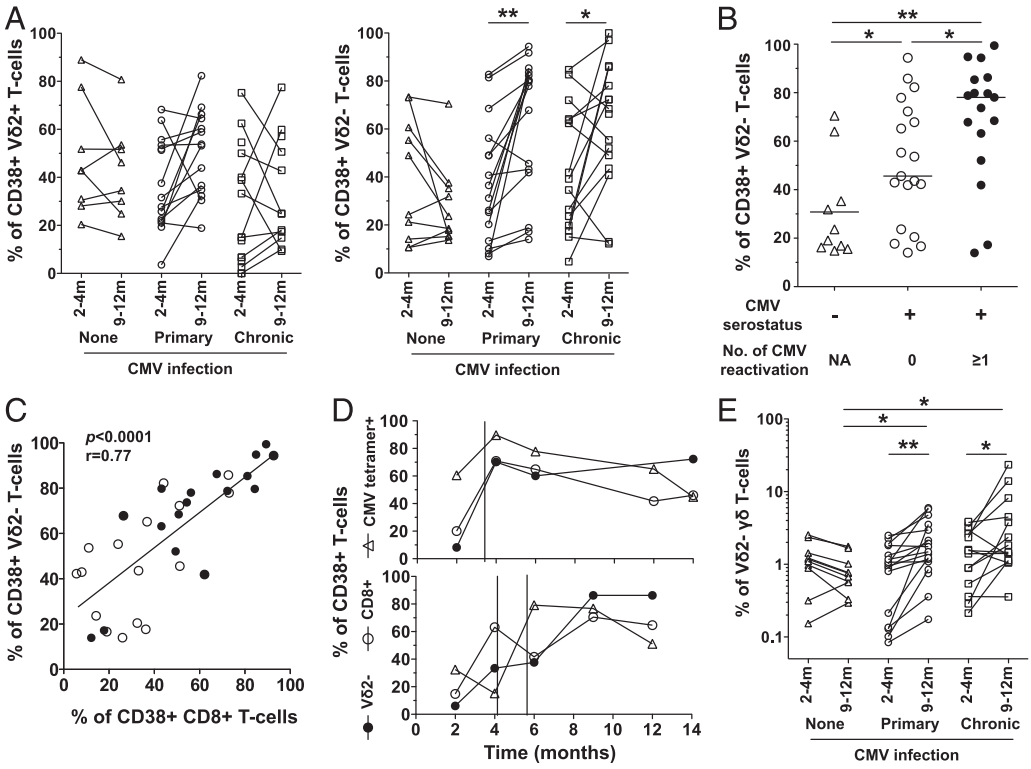

FIGURE 3. Inflation of the γδ T cell compartment with active CMV replication. (**A**) Percentage of $CD38^{+}V\delta2^{+}$ (*left panel*) and $V\delta2^{-}$ (*right panel*) T cells in patients with primary ($n = 14$), chronic ($n = 14$) CMV infection, or uninfected ($n = 10$), early (+2–4 mo) and late (+9–12 mo) posttransplantation. The Wilcoxon test was used for comparison between time points. $*p < 0.05$; $**p < 0.01$, respectively. (**B**) Percentage of activated $V\delta2^{-}$ T cells at a late time point (+9–12 mo) posttransplantation in CMV-seronegative patients (△), CMV-seropositive patients without viral reactivation (○), and with one or more viral reactivation (i.e., positive CMV-specific PCR in total blood during posttransplantation follow-up) (●). The Mann–Whitney *U* test was used for comparison. (**C**) Correlation between activation levels in $V\delta2^{-}$ γδ and $CD8^{+}$ T cells in CMV infected lung transplant patients (+9–12 mo post-transplantation), with evidence (●) or not (○) of active CMV replication. The Spearman's rank test was used to determine the correlation. (**D**) Parallel changes in CD38 expression levels on $V\delta2^{-}$ γδ T cells and total or CMV-specific $CD8^{+}$ T cells (identified using HLA-A*0201 pp65-NV9 tetramers) from two representative patients over the posttransplant follow-up period. Vertical lines denote episodes of CMV replication. (**E**) Percentage of $V\delta2^{-}$ γδ T cells in patients with primary, chronic CMV infection, or uninfected, early (+2–4 mo) and late (+9–12 mo) posttransplantation.

T cells in the elderly may involve other factors than thymic involution.

Discussion

Aging is associated with significant alterations of both the adaptive and innate arms of the immune system. The present study focuses on γδ T cells, which are considered intermediate mediators between adaptive and innate immunity. We provide in this paper further insights underlying the changes that affect the γδ T cell compartment with advanced age. We show that both aging and CMV infection impact independently on the γδ T cell compartment. This dual effect provides an explanation for the discrepancy in the changes observed between $V\delta2^{-}$ and $V\delta2^{+}$ γδ T cells in elderly (10, 11). Although the frequency of $V\delta2^{+}$ γδ T cells

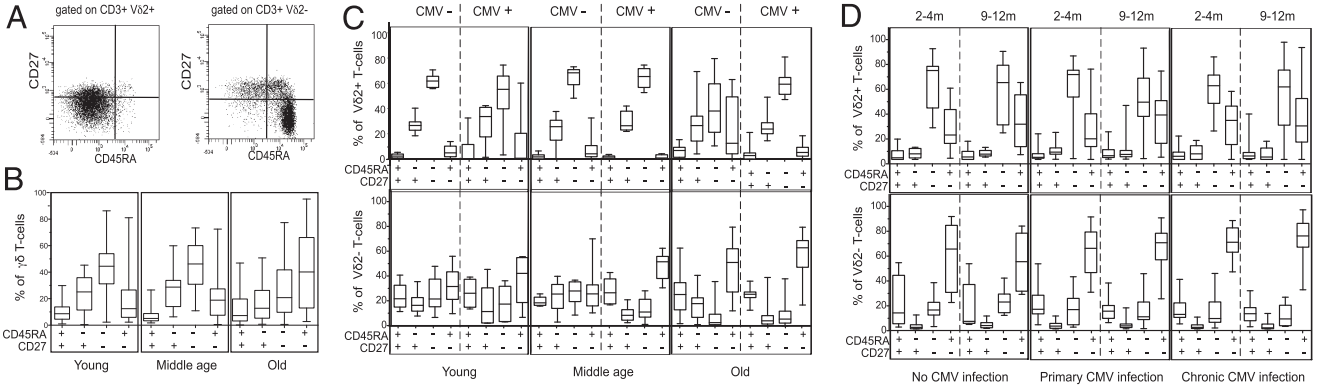

FIGURE 4. γδ T cell phenotypic changes with aging or CMV infection. (**A**) Representative FACS stainings of CD27 and CD45RA expression on $V\delta2^{+}$ (*left panel*) or $V\delta2^{-}$ (*right panel*) γδ T cells. (**B**) γδ T cell phenotype in young ($n = 18$), middle-aged ($n = 18$), and old ($n = 28$) adults. (**C**) Phenotypic distribution of $V\delta2^{+}$ (*upper panel*) or $V\delta2^{-}$ (*lower panel*) T cells in young ($n = 18$), middle-aged ($n = 18$), and old ($n = 28$) adults according to the CMV serostatus. (**D**) Distribution of $V\delta2^{+}$ (*upper panel*) or $V\delta2^{-}$ (*lower panel*) T cells in lung transplant patients with primary ($n = 14$) and chronic ($n = 14$) CMV infection or uninfected ($n = 10$), early (+2–4 mo), and late (+9–12 mo) posttransplantation.

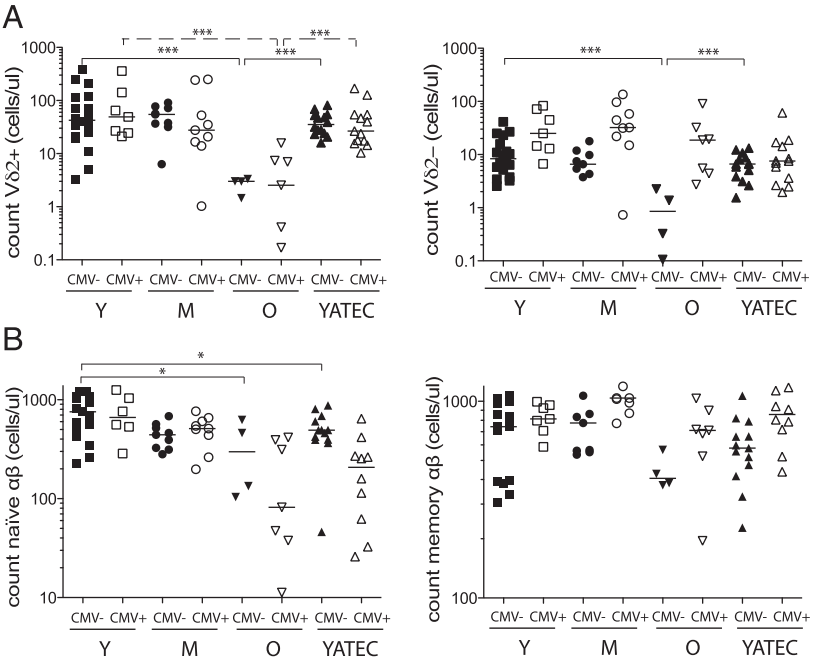

FIGURE 5. Thymus-independent production of γδ T cells. Absolute counts of Vδ2$^+$ (*left panel*) or Vδ2$^-$ (*right panel*) T cells (**A**) and naive (*left panel*) or memory (*right panel*) αβ T cell counts (**B**) in young (Y, $n = 24$), middle-aged (M, $n = 17$), old (O, $n = 11$) adults, and YATEC ($n = 23$), separated according to CMV serostatus. Bars indicate the median. The Mann–Whitney U test was used for comparison. $*p < 0.05$, and $***p < 0.001$, respectively.

declines in all individuals with advanced age, Vδ2$^-$ γδ T cell numbers only decrease in CMV-seronegative donors. In CMV-seropositive donors, the frequency of Vδ2$^-$ γδ T cells is likely preserved overtime as the result of the strong mobilization of the Vδ2$^-$ γδ T cell pool because of persistent CMV infection. This phenomenon is reminiscent of the so called memory T cell inflation described for αβ T cells in the context of infections with persistent viruses, in particular CMV (26). Age and CMV infection are also associated with phenotypic changes, affecting primarily the Vδ2$^-$ γδ T cell compartment (i.e., increased representation of CD27$^-$CD45RA$^+$ cells), whereas the phenotype of the Vδ2$^+$ γδ T cells remains remarkably stable overtime. Altogether, our data highlight the distinct fates of Vδ2$^+$ and Vδ2$^-$ γδ T cells with aging and the close connection to the CMV serostatus.

The analysis of global γδ T cell frequency and phenotype suggests that γδ and αβ T cell pools experience similar changes with advanced age, characterized by a decreased absolute number and a bias toward CD27$^-$CD45RA$^+$ cells. The dissection of the γδ T cell compartment into different subpopulations shows nonetheless that the situation is complex. As discussed above, not only Vδ2$^+$ and Vδ2$^-$ γδ T cell subsets present distinct evolution with age in relation to CMV infection, but they emerge as clearly different subsets, in particular with regards to their phenotype, which is in line with previous studies showing distinct expression of CD27 and CD45RA on these γδ cell subsets (15, 22–25). This is mostly independent from CMV or age, although both factors reinforce this distinction. The reason for the different phenotype of Vδ2$^+$ and Vδ2$^-$ γδ T cell subsets, with CD27$^-$CD45RA$^-$ and CD27$^-$CD45RA$^+$ predominance, respectively, is unclear. One hypothesis may be though that these differences in phenotype reflect the unequal differentiation of γδ T cells according to the type of stimulus or pathogen they react to. The ligands for Vδ2$^+$ γδ T cell TCR are known to be microbial or autophosphoantigens (27), whereas Vδ2$^-$ γδ T cells are more likely to be specific for Ags present during infections with viruses, in particular CMV. The type of antigenic stimulation or pathogenic environment may thus drive γδ T cells to distinct differentiation stages. This is reminiscent of the situation in CD8$^+$ αβ T cells, where CMV-specific cells adopt a highly differentiated phenotype (CD27$^-$CD45RA$^+$) in contrast to cells specific for other viruses displaying an early/intermediate differentiation phenotype (CD27$^{+/-}$CD45RA$^-$) (19). Eventually, the apparent phenotypic discrepancy between Vδ2$^+$ and Vδ2$^-$ γδ T cells may actually not reflect an intrinsic difference between these subsets but rather the different outcome of their mobilization by distinct stimuli and pathogens, as observed in the case of αβ T cells. Our investigation of the γδ T cell phenotype also revealed that several markers usually associated with αβ T cell activation (e.g., CD38, PD-1, HLA-DR, CD25, and Ki67) can also be used to assess the activation status of γδ T cells. In particular, monitoring CD38 expression on the surface of γδ T cells may be particularly useful to follow the activity of these cells in vivo. Of note, gene expression profile analyses of γδ T cells from newborns with primary CMV infection performed by Vermijlen et al. (12–17) shows that CD38 upregulation is part of a transcriptional program characterizing γδ T cells activated following congenital CMV infection (D. Vermijlen, personal communication). In this study, we observed that the upregulation of CD38 on Vδ2$^-$ γδ T cells was closely related to active CMV replication (during primary infection or in lung transplant patients) and was also directly correlated with activation levels of CD8$^+$ αβ T cells in this context.

Parallels between γδ and αβ T cell compartments have nonetheless some limits. Our observations in thymectomized donors suggest that the decline of the γδ T cell pool with advanced age may be independent from the reduced thymic activity, because YATEC present normal levels of γδ T cells. The main organ of γδ T cells production remains a matter a debate, in particular when comparing mouse and human. In mice, studies have shown that the thymus is essential for the maturation and generation of γδ T cells (28, 29). However, the presence of γδ (and not αβ) T cells in athymic patients suffering from DiGeorge syndrome (30–32) argues in favor of an extrathymic site of γδ T cell production, which is in line with our observations as well as previous works supporting extrathymic generation of the human TCRγδ repertoire (33). Although it is clear that γδ T cells can be produced in the thymus, it is possible that the latter may only be a secondary or expandable pathway of γδ T cell generation. The thymus is necessary for the development of αβ T cells, which undergo negative and positive selection in this organ. In contrast, γδ T cells mature early without the need for this double-selection process and may

thus be generated in the absence of the thymus. The decrease in γδ T cell numbers with advanced age (i.e., Vδ2$^+$ γδ T cells and Vδ2$^-$ γδ T cells in CMV-seronegative donors) may eventually be due to a general reduction of lymphopoiesis in the elderly who are characterized by decreased number of not only T cells but also B cells and NK cells. It is possible that this may relate to the alteration of upstream compartments, like CD34$^+$ hematopoietic progenitors. Overall, the present data support further the distinction between γδ T cell and αβ T cell development in humans.

In conclusion, our study provides new insights into the alteration of the γδ T cell compartment with aging. The differences between Vδ2$^+$ and Vδ2$^-$ γδ T cells illuminate the complexities of the factors driving the changes occurring during advanced age, in particular the impact of CMV in this process. Although the development and exact role of γδ T cells in immunity are not fully understood, alterations of this compartment may be part of the process of immunosenescence, associated with the decline of the immune competence in the elderly.

Acknowledgments

We thank the patients and staff of the Department of Geriatrics of the Montreuil Hospital, the Service of Pneumology of the Foch Hospital, and the Pediatric Cardiology Department at the Necker Hospital as well as the healthy volunteers participating in this study. We also thank Sandra Lecomte for expert technical assistance and David Vermijlen for constructive discussions.

Disclosures

The authors have no financial conflicts of interest.

References

1. Dorshkind, K., E. Montecino-Rodriguez, and R. A. Signer. 2009. The ageing immune system: is it ever too old to become young again? *Nat. Rev. Immunol.* 9: 57–62.
2. Nikolich-Zugich, J. 2008. Ageing and life-long maintenance of T-cell subsets in the face of latent persistent infections. *Nat. Rev. Immunol.* 8: 512–522.
3. Koch, S., R. Solana, O. Dela Rosa, and G. Pawelec. 2006. Human cytomegalovirus infection and T cell immunosenescence: a mini review. *Mech. Ageing Dev.* 127: 538–543.
4. Appay, V., R. A. van Lier, F. Sallusto, and M. Roederer. 2008. Phenotype and function of human T lymphocyte subsets: consensus and issues. *Cytometry A* 73: 975–983.
5. Sylwester, A. W., B. L. Mitchell, J. B. Edgar, C. Taormina, C. Pelte, F. Ruchti, P. R. Sleath, K. H. Grabstein, N. A. Hosken, F. Kern, et al. 2005. Broadly targeted human cytomegalovirus-specific CD4$^+$ and CD8$^+$ T cells dominate the memory compartments of exposed subjects. *J. Exp. Med.* 202: 673–685.
6. Sauce, D., M. Larsen, S. Fastenackels, A. Duperrier, M. Keller, B. Grubeck-Loebenstein, C. Ferrand, P. Debré, D. Sidi, and V. Appay. 2009. Evidence of premature immune aging in patients thymectomized during early childhood. *J. Clin. Invest.* 119: 3070–3078.
7. Poccia, F., C. Agrati, F. Martini, M. R. Capobianchi, M. Wallace, and M. Malkovsky. 2005. Antiviral reactivities of γδ T cells. *Microbes Infect.* 7: 518-528.
8. Bonneville, M., R. L. O'Brien, and W. K. Born. 2010. γδ T cell effector functions: a blend of innate programming and acquired plasticity. *Nat. Rev. Immunol.* 10: 467–478.
9. Hayday, A., and R. Tigelaar. 2003. Immunoregulation in the tissues by γδ T cells. *Nat. Rev. Immunol.* 3: 233–242.
10. Argentati, K., F. Re, A. Donnini, M. G. Tucci, C. Franceschi, B. Bartozzi, G. Bernardini, and M. Provinciali. 2002. Numerical and functional alterations of circulating γδ T lymphocytes in aged people and centenarians. *J. Leukoc. Biol.* 72: 65–71.
11. Michishita, Y., M. Hirokawa, Y. M. Guo, Y. Abe, J. Liu, K. Ubukawa, N. Fujishima, M. Fujishima, T. Yoshioka, Y. Kameoka, et al. 2011. Age-associated alteration of γδ T-cell repertoire and different profiles of activation-induced death of Vδ1 and Vδ2 T cells. *Int. J. Hematol.* 94: 230–240.
12. Déchanet, J., P. Merville, F. Bergé, G. Bone-Mane, J. L. Taupin, P. Michel, P. Joly, M. Bonneville, L. Potaux, and J. F. Moreau. 1999. Major expansion of γδ T lymphocytes following cytomegalovirus infection in kidney allograft recipients. *J. Infect. Dis.* 179: 1–8.
13. Déchanet, J., P. Merville, A. Lim, C. Retière, V. Pitard, X. Lafarge, S. Michelson, C. Méric, M. M. Hallet, P. Kourilsky, et al. 1999. Implication of γδ T cells in the human immune response to cytomegalovirus. *J. Clin. Invest.* 103: 1437–1449.
14. Lafarge, X., P. Merville, M. C. Cazin, F. Bergé, L. Potaux, J. F. Moreau, and J. Déchanet-Merville. 2001. Cytomegalovirus infection in transplant recipients resolves when circulating γδ T lymphocytes expand, suggesting a protective antiviral role. *J. Infect. Dis.* 184: 533–541.
15. Pitard, V., D. Roumanes, X. Lafarge, L. Couzi, I. Garrigue, M. E. Lafon, P. Merville, J. F. Moreau, and J. Déchanet-Merville. 2008. Long-term expansion of effector/memory Vδ2-γδ T cells is a specific blood signature of CMV infection. *Blood* 112: 1317–1324.
16. Knight, A., A. J. Madrigal, S. Grace, J. Sivakumaran, P. Kottaridis, S. Mackinnon, P. J. Travers, and M. W. Lowdell. 2010. The role of Vδ2-negative γδ T cells during cytomegalovirus reactivation in recipients of allogeneic stem cell transplantation. *Blood* 116: 2164–2172.
17. Vermijlen, D., M. Brouwer, C. Donner, C. Liesnard, M. Tackoen, M. Van Rysselberge, N. Twité, M. Goldman, A. Marchant, and F. Willems. 2010. Human cytomegalovirus elicits fetal γδ T cell responses in utero. *J. Exp. Med.* 207: 807–821.
18. Willcox, C. R., V. Pitard, S. Netzer, L. Couzi, M. Salim, T. Silberzahn, J. F. Moreau, A. C. Hayday, B. E. Willcox, and J. Déchanet-Merville. 2012. Cytomegalovirus and tumor stress surveillance by binding of a human γδ T cell antigen receptor to endothelial protein C receptor. *Nat. Immunol.* 13: 872–879.
19. Appay, V., P. R. Dunbar, M. Callan, P. Klenerman, G. M. Gillespie, L. Papagno, G. S. Ogg, A. King, F. Lechner, C. A. Spina, et al. 2002. Memory CD8$^+$ T cells vary in differentiation phenotype in different persistent virus infections. *Nat. Med.* 8: 379–385.
20. Giorgi, J. V., Z. Liu, L. E. Hultin, W. G. Cumberland, K. Hennessey, and R. Detels. 1993. Elevated levels of CD38$^+$CD8$^+$ T cells in HIV infection add to the prognostic value of low CD4$^+$ T cell levels: results of 6 years of follow-up: the Los Angeles Center, Multicenter AIDS Cohort Study. *J. Acquir. Immune Defic. Syndr.* 6: 904–912.
21. Karrer, U., S. Sierro, M. Wagner, A. Oxenius, H. Hengel, U. H. Koszinowski, R. E. Phillips, and P. Klenerman. 2003. Memory inflation: continuous accumulation of antiviral CD8$^+$ T cells over time. *J. Immunol.* 170: 2022–2029.
22. Dieli, F., F. Poccia, M. Lipp, G. Sireci, N. Caccamo, C. Di Sano, and A. Salerno. 2003. Differentiation of effector/memory Vδ2 T cells and migratory routes in lymph nodes or inflammatory sites. *J. Exp. Med.* 198: 391–397.
23. De Rosa, S. C., J. P. Andrus, S. P. Perfetto, J. J. Mantovani, L. A. Herzenberg, L. A. Herzenberg, and M. Roederer. 2004. Ontogeny of γδ T cells in humans. *J. Immunol.* 172: 1637–1645.
24. Caccamo, N., S. Meraviglia, V. Ferlazzo, D. Angelini, G. Borsellino, F. Poccia, L. Battistini, F. Dieli, and A. Salerno. 2005. Differential requirements for antigen or homeostatic cytokines for proliferation and differentiation of human Vγ9Vδ2 naive, memory and effector T cell subsets. *Eur. J. Immunol.* 35: 1764–1772.
25. Couzi, L., V. Pitard, S. Netzer, I. Garrigue, M. E. Lafon, J. F. Moreau, J. L. Taupin, P. Merville, and J. Déchanet-Merville. 2009. Common features of gammadelta T cells and CD8$^+$ αβ T cells responding to human cytomegalovirus infection in kidney transplant recipients. *J. Infect. Dis.* 200: 1415–1424.
26. O'Hara, G. A., S. P. Welten, P. Klenerman, and R. Arens. 2012. Memory T cell inflation: understanding cause and effect. *Trends Immunol.* 33: 84–90.
27. Thedrez, A., C. Sabourin, J. Gertner, M. C. Devilder, S. Allain-Maillet, J. J. Fournié, E. Scotet, and M. Bonneville. 2007. Self/non-self discrimination by human γδ T cells: simple solutions for a complex issue? *Immunol. Rev.* 215: 123–135.
28. Hayday, A. C., and D. J. Pennington. 2007. Key factors in the organized chaos of early T cell development. *Nat. Immunol.* 8: 137–144.
29. Ciofani, M., and J. C. Zúñiga-Pflücker. 2007. The thymus as an inductive site for T lymphopoiesis. *Annu. Rev. Cell Dev. Biol.* 23: 463–493.
30. Musiani, P., S. Valitutti, F. Castellino, L. M. Larocca, N. Maggiano, and M. Piantelli. 1990. Intrathymic deficient expansion of T cell precursors in Down syndrome. *Am. J. Med. Genet. Suppl.* 7: 219–224.
31. McLean-Tooke, A., D. Barge, G. P. Spickett, and A. R. Gennery. 2008. Immunologic defects in 22q11.2 deletion syndrome. *J. Allergy Clin. Immunol.* 122: 362-367, 367 e361-364.
32. Kanaya, Y., S. Ohga, K. Ikeda, K. Furuno, T. Ohno, H. Takada, N. Kinukawa, and T. Hara. 2006. Maturational alterations of peripheral T cell subsets and cytokine gene expression in 22q11.2 deletion syndrome. *Clin. Exp. Immunol.* 144: 85–93.
33. Parker, C. M., V. Groh, H. Band, S. A. Porcelli, C. Morita, M. Fabbi, D. Glass, J. L. Strominger, and M. B. Brenner. 1990. Evidence for extrathymic changes in the T cell receptor γ/δ repertoire. *J. Exp. Med.* 171: 1597–1612.