

Spectral Flow Cytometry Webinar Series

Watch our webinar series and learn how the ID7000™ system builds on Sony's experience with spectral analysis and simplifies many operations to advance the field of flow cytometry.



Watch Now

SONY



IL-2 Producing Memory CD4⁺ T Lymphocytes Are Closely Associated with the Generation of IgG-Secreting Plasma Cells

This information is current as of March 13, 2022.

Nicolle H. R. Litjens, Martin Huisman, Daniëlle Hijdra, Bart M. N. Lambrecht, Koert J. Stittelaar and Michiel G. H. Betjes

J Immunol 2008; 181:3665-3673; ;
doi: 10.4049/jimmunol.181.5.3665
<http://www.jimmunol.org/content/181/5/3665>

References This article **cites 39 articles**, 9 of which you can access for free at:
<http://www.jimmunol.org/content/181/5/3665.full#ref-list-1>

Why *The JI*? [Submit online.](#)

- **Rapid Reviews! 30 days*** from submission to initial decision
- **No Triage!** Every submission reviewed by practicing scientists
- **Fast Publication!** 4 weeks from acceptance to publication

**average*

Subscription Information about subscribing to *The Journal of Immunology* is online at:
<http://jimmunol.org/subscription>

Permissions Submit copyright permission requests at:
<http://www.aai.org/About/Publications/JI/copyright.html>

Email Alerts Receive free email-alerts when new articles cite this article. Sign up at:
<http://jimmunol.org/alerts>



IL-2 Producing Memory CD4⁺ T Lymphocytes Are Closely Associated with the Generation of IgG-Secreting Plasma Cells¹

Nicollé H. R. Litjens,^{2*} Martin Huisman,* Daniëlle Hijdra,[†] Bart M. N. Lambrecht,[‡] Koert J. Stittelaar,[‡] and Michiel G. H. Betjes*

The role of specific CD4⁺ T cell subsets in the induction of humoral immune responses in humans is largely unknown. In this study, the generation of hepatitis B surface Ag-specific CD4⁺ T lymphocytes following vaccination was closely monitored and characterized at the single-cell level. The appearance and absolute numbers of hepatitis B surface Ag-specific IL-2 producing effector memory CD4⁺ T lymphocytes was solely and tightly related to Ab titers reached. This relation remained present many years after vaccination. Subsequently, a relation was found between Ab titers and number of IL-2 producing memory CD4⁺ T lymphocytes for various other Ags. These observations matched the findings of an *in vitro* assay, using different T cell subsets to induce B cell differentiation into IgG-producing plasma cells. By depleting for IL-2 producing memory T cells, we demonstrated that these cells are important for B cell differentiation into IgG-producing plasma cells. Finally, blocking the action of IL-2 with an IL-2R- α Ab inhibited the differentiation of B lymphocytes into IgG-producing plasma cells. Based on these findings, we conclude that the development of Ag-specific IL-2-producing memory T cells appears to be essential for the development of IgG-secreting plasma cells in humans. *The Journal of Immunology*, 2008, 181: 3665–3673.

CD4⁺ T lymphocytes are important targets for vaccine development as they promote both cellular and humoral immune responses (1). Understanding the development and functions of Ag-specific memory CD4⁺ T lymphocytes is essential for designing new and improved vaccines.

T cell mediated memory requires differentiation and clonal expansion of naive T cells into effector T cells that contract and eventually will form an Ag-specific memory T cell population (2, 3). Recent developments in T cell research and the availability of various chemokine receptors, in particular CCR7, have enabled a more accurate dissection of the different T cell subsets. At least two different subsets can be distinguished within the CD4⁺ memory T cells using expression levels of CCR7 (4, 5). CD4⁺ memory T cells expressing the chemokine receptor CCR7 (central memory or reactive memory) are able to migrate to secondary lymphoid organs where they constitute a pool of long-lived cells. Upon re-encounter of the Ag, they develop more quickly into effector cells. CD4⁺ memory T cells lacking expression of CCR7 home to peripheral tissues where they can exert immediate effector function upon re-encounter of the Ag.

Little is known about the development and characteristics of Ag-specific memory CD4⁺ T lymphocytes induced by vaccination of humans, in relation to Ab titers induced. Vaccination of humans

for hepatitis B virus (HBV)³ with hepatitis B surface Ag (HBsAg) can be used as a model to study such a relation. HBV vaccination is T cell dependent and very effective in reaching adequate protection in >90% of the healthy vaccinees (6). Adequate protection seems to be related to type-1 cytokine responses involving IL-2 and IFN- γ and these cytokines were also shown to be produced by Ag-specific memory CD4⁺ T cell at the single cell level shortly after booster HBV vaccination (7). Thereafter, frequencies of HBsAg-specific CD4⁺ T cells are typically very low but can be reliably measured using an optimized protocol for analysis by flow cytometry (8). The aim of this study was to identify Ag-specific T cell responses that are related to the magnitude of the humoral response. The results show that IL-2 producing memory CD4⁺ T (Tmem) cells are closely associated with the development of an adequate humoral immune response, a relation that persists for many years after vaccination.

Materials and Methods

Study population

Fifteen healthy donors (eight females and seven males; mean \pm SD age is 43 \pm 14 years) were vaccinated against HBV using the standard vaccination protocol with recombinant hepatitis B vaccine (HBVAXPRO, Sanofi Pasteur MSD NV). The protocol consisted of a three-step vaccination with an injection (20 μ g/injection) in the left m. Deltoideus at time 0, 1, and 6 mo. In addition, healthy individuals who were vaccinated for HBV and tetanus toxoid in the past (5 to 25 years before) were included. Stable patients (n = 15) with end-stage renal disease (ESRD) receiving hemodialysis at the Erasmus Medical Center followed an intensified vaccination protocol with recombinant hepatitis B vaccine (40 μ g/injection and an extra booster dose; Refs. 9, 10) before entering the study.

*Department of Internal Medicine, Division of Nephrology, [†]Department of Pulmonary Diseases, and [‡]Department of Virology, Erasmus Medical Center, Rotterdam, The Netherlands

Received for publication February 5, 2008. Accepted for publication June 27, 2008.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

¹ This study was financially supported by a grant from the Dutch Kidney Foundation (Grant no. 1630008) and the Malpighi Foundation.

² Address correspondence and reprint requests to Dr. Nicollé Litjens, Department of Internal Medicine, Division of Nephrology, Room Ee 551, Erasmus Medical Center, P.O. Box 2040, 3000 CA Rotterdam, The Netherlands. E-mail address: n.litjens@erasmusmc.nl

³ Abbreviations used in this paper: HBV, hepatitis B virus; HBsAg, hepatitis B surface Ag; Tmem, total memory CD4⁺ T lymphocyte; ESRD, end-stage renal disease; moDC, monocyte-derived dendritic cell; Rs, Spearman's rho correlation coefficient; Tcm, central CD4⁺ T lymphocyte; Tem, effector CD4⁺ T lymphocyte.

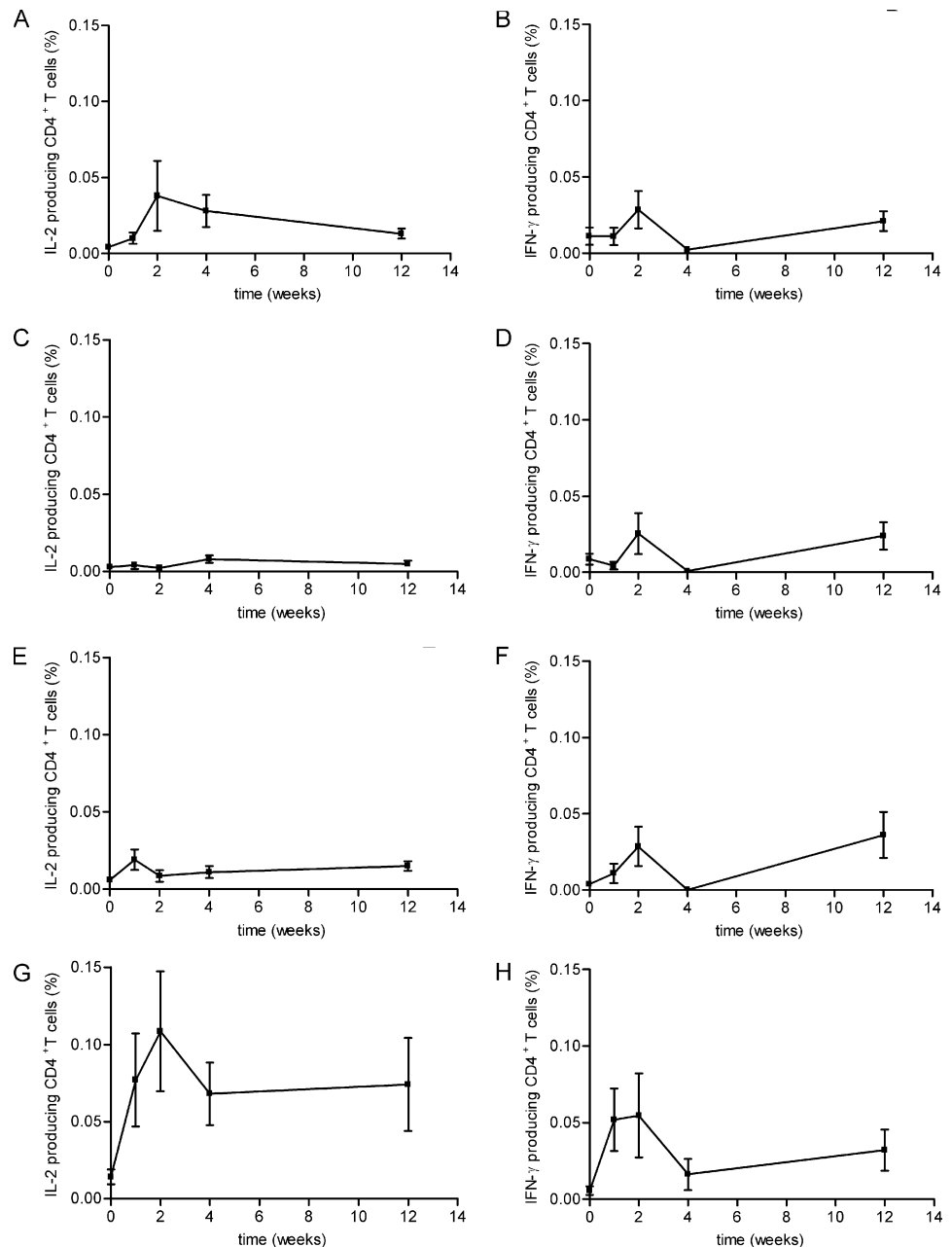


FIGURE 1. Development of HBsAg-specific IL-2 and IFN- γ producing CD4⁺ T lymphocytes in healthy HBV-vaccinees. Freshly isolated PBMC were stimulated with the combination of anti-CD28 and anti-CD49d and HBsAg for 6 h. Brefeldin A was added for the last 5 h of the incubation. Percentages of cytokine producing cells were determined within T cell subsets defined by expression of cell surface markers CD4, CD45RO, and CCR7. IL-2 and IFN- γ positive cells within the total CD4⁺ T cells (A and B), naive T cells (C and D), central memory T cells (E and F), and effector memory T cells (G and H) are shown. On the x-axis, the number of weeks after the last HBV vaccination is shown.

Study protocol

This study was approved by the medical ethical committee of the Erasmus Medical Center in Rotterdam, The Netherlands (Medical Ethical Committee no. 2005-265). It was conducted according to the principles of Declaration of Helsinki and in compliance with International Conference on Harmonization/Good Clinical Practice regulations. The healthy donors were followed up to 12 wk upon receipt of their last HBV vaccination step. At each interval (before and 1, 2, 4, and 12 wk after receiving the last booster) blood was drawn. PBMC were isolated, counted, and used for determination of quality and quantity of Ag-specific immune responses using various functional read-out systems. In addition, plasma was collected at each interval and stored at -20°C until analysis.

Isolation of PBMC

PBMC were isolated from 35 ml of venous blood by Ficoll-Paque plus (Amersham Biosciences AB) density gradient centrifugation (11) and resuspended in RPMI 1640 (Life Technologies) supplemented with 100 IU/ml penicillin, 100 $\mu\text{g/ml}$ streptomycin, 2 mM L-glutamine, and 10% heat-inactivated AB⁺ pooled human serum (further referred to as standard culture medium).

Intracellular cytokine staining

The protocol used to measure Ag-specific cytokine producing cells was optimized before (8). In brief, PBMC ($2.5 \times 10^6/\text{tube}$) were stimulated in duplicate with either costimulation alone (i.e., the combination of 1 $\mu\text{g/ml}$ anti-CD28 and 1 $\mu\text{g/ml}$ anti-CD49d) or with costimulation and a final concentration of 5 $\mu\text{g/ml}$ HBsAg (Fitzgerald Industries) for 6 h of which the last 5 h was in the presence of the cytokine secretion inhibitor Brefeldin A (Golgiplug; 1 μl per 10^6 PBMC; BD Pharmingen). In addition, PBMC were stimulated in the presence of costimulation with 37.5 IU/ml tetanus toxoid (SVM, Bilthoven) and 30 $\mu\text{g/ml}$ CMV Ag (Microbix Biosystems) to identify tetanus toxoid- and CMV-specific cytokine producing CD4⁺ T cells.

Following permeabilization, cells were stained with either FITC-labeled anti IL-2 (BD Pharmingen) or FITC-labeled anti IFN- γ (BD Pharmingen). Percentages of cytokine positive cells were determined by analyzing the samples on the FACSCalibur (BD Pharmingen), selecting cells that have a typical lymphocyte scatter pattern and are CD4⁺. Ag-specific T cells were characterized by staining the cell surface with PerCP-labeled anti-CD4, allophycocyanin-labeled CD45RO (both from BD Pharmingen) and PE-labeled CCR7 (R&D Systems) to dissect the different CD4⁺ T cell subsets

(12). To determine the extent of overlap for HBsAg-specific IL-2 and IFN- γ producing cells, a minor adjustment was made in the staining protocol, i.e., PE-Cy7 (instead of PE) -labeled CCR7 (BD Pharmingen) was used and PE-labeled IFN- γ (BD Pharmingen) was added to costain for IFN- γ -producing cells together with FITC-labeled IL-2. A similar analysis as applied for single positive cytokine producing cells was done on the FACSCanto II (BD Pharmingen) and using Diva 6.0 software (BD Pharmingen).

The percentage of cytokine-producing cells within a certain subset induced by HBsAg was corrected for the background signal, i.e., the percentage of cells that are induced to produce cytokines in response to costimulation alone. PBMC of healthy nonvaccinated individuals served as negative controls and showed no detectable signal for cytokine positive cells after coculture with HBsAg.

Ag-specific T cell proliferation

To determine Ag-specific proliferation, the protocol described by Verkade et al. (11) was used with minor adjustments. In brief, PBMC at a density of $1 \times 10^5/100 \mu\text{l}$ were transferred to a round-bottom-shaped 96-well plate (Nunc) in triplicates and stimulated for 6 days in the presence of $5 \mu\text{g/ml}$ HBsAg. Background proliferation was determined by stimulating cells with standard culture medium alone. Results are presented as stimulation indices, calculated by dividing the proliferation expressed as cpm induced by the stimulus by that observed in absence of the stimulus.

To more accurately investigate Ag-specific proliferation, the different subsets were sorted on a FACSaria (BD Pharmingen), using expression of cell surface markers as described above. The purity of the various subsets was determined using flow cytometry and cell samples were $>95\%$ pure. Subsequently, we cocultured the various subsets with autologous HBsAg-loaded mature monocyte-derived dendritic cells (moDC) or unloaded moDC as APC (11). The moDC were generated with GM-CSF and IL-4 using a cytokine mixture as maturation stimulus (11).

B cell ELISPOT assay

The T cell-dependent B cell ELISPOT assay, first described by Lipsky (13, 14), was used to analyze the importance of different CD4 $^+$ T cell subsets for differentiation of memory B cells into Ab secreting plasma cells. In brief, the various CD4 $^+$ T cell subsets and CD19 $^+$ B cells, obtained from healthy volunteers 12 wks after HBV vaccination, were sorted using FACS Aria (BD Pharmingen). Subsequently, the CD4 $^+$ T cell subsets were treated with $10 \mu\text{g/ml}$ mitomycin C (Nycomed) at 37°C for 45 min and finally cocultured at $1 \times 10^5/\text{well}$ with 2.5×10^4 B cells on an anti-CD3 ($1 \mu\text{g/ml}$, RIV-9) coated 96-well plate for 6 days.

In addition, several variations to this protocol were applied to establish the role of IL-2-producing CD4 $^+$ T cells and IL-2. First, to investigate the importance of the IL-2 producing CD4 $^+$ T cells, Tmem were sorted and stimulated for 6 h on an anti-CD3 ($1 \mu\text{g/ml}$) and anti-CD28 ($2 \mu\text{g/ml}$, Serotec) coated plate. IL-2 secreting cells were isolated using the IL-2 secretion assay (Miltenyi Biotec) and the autoMACS (Miltenyi Biotec) according to the manufacturer's instructions. Sham-treated (control) Tmem in this assay underwent all procedures similar to the IL-2-depleted Tmem except for the use of an isotype-control instead of the IL-2 capture Ab. The IL-2-depleted Tmem as well as the sham-treated Tmem remained viable ($>90\%$ viability) and purity was $>98\%$. The positively selected fraction IL-2 secreting Tmem showed very poor viability ($<20\%$), a problem we were not able to solve, and we excluded the use of this cell fraction for experiments. Finally, all cell fractions were treated with mitomycin C before coculture with purified B cells.

Second, to study the effects of IL-2 in these experiments, we performed them in the presence of a monoclonal IL-2R- α blocking Ab (Zenapax, Hoffman-LaRoche) during the whole coculture method. This Ab inhibits the effects of IL-2 by preventing its interaction with the IL-2R- α -chain. For this purpose, purified Tmem were cocultured with purified B lymphocytes in presence of different concentrations of Zenapax (0 – $20 \mu\text{g/ml}$) for 6 days or similar concentrations of IgG1 (isotype control).

On day 5, the wells of a 96-well MaxiSorp immunoplate (Nunc) were coated overnight with either $5 \mu\text{g/ml}$ HBsAg, 37.5 lf/ml Tetanus Toxoid (SVM), $30 \mu\text{g/ml}$ CMV Ag (Microbix Biosystems), or with $44 \mu\text{g/ml}$ goat F(ab') $_2$ anti-human Ig (polyvalent) (Caltag Laboratories) at 4°C . The cells were harvested on day 6 and plated in triplicate at a density of $2.5 \times 10^4/\text{well}$ on the Ag-precoated plate and at a density of $1 \times 10^3/\text{well}$ if total IgG producing cell frequencies were determined. Following an overnight incubation at 37°C and $5\% \text{ CO}_2$, cells were lysed and debris was washed away using PBS/0.05% Tween 20. Subsequently, wells were incubated with $5 \mu\text{g/ml}$ biotinylated goat F(ab') $_2$ anti-human IgG (Biosource) for 1 h at 37°C followed by an 1 h incubation with labeled goat-anti-biotin Abs (U-CyTech Biosciences). Finally, Ab-producing cells were visualized us-

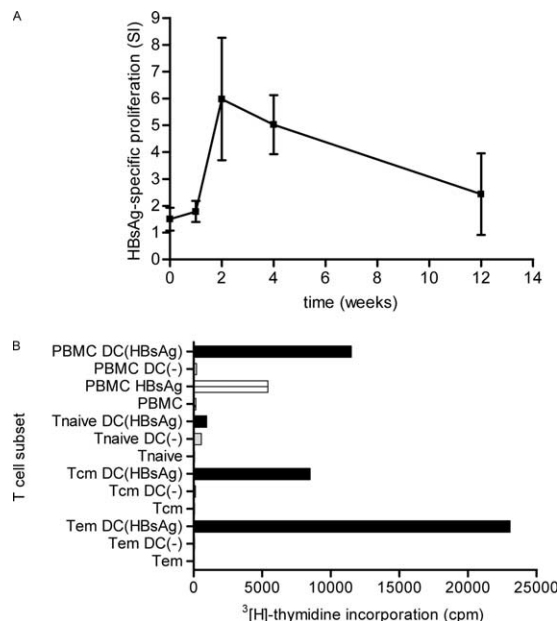


FIGURE 2. Kinetics of HBsAg-specific proliferation in healthy HBV vaccinees. At the various intervals, freshly isolated PBMC were stimulated with medium alone, HBsAg ($5 \mu\text{g/ml}$) for 6 days. At day 6, proliferation was determined by [^3H]thymidine incorporation and is expressed as a stimulation index (SI; A), calculated by dividing the HBsAg-induced proliferation by that induced by culture medium alone. On the x-axis, the number of weeks after the last HBV vaccination is shown. Twelve weeks following receipt of the last HBV vaccination, PBMC and the various CD4 $^+$ T cell subsets, sorted on a FACSaria, were cocultured at a density of $5 \times 10^4/\text{well}$ with 5×10^3 autologous HBsAg-loaded mature moDC and as negative controls: unloaded mature moDC or medium alone for 6 days. In addition, PBMC were also stimulated with HBsAg ($5 \mu\text{g/ml}$). At day 6, proliferation was determined by [^3H]thymidine incorporation and is expressed as cpm. On the x-axis, proliferation (median) is depicted in cpm and on the y-axis the unsorted PBMC and different T cell subsets are depicted (B). The black, gray, hatched, and open bars indicate stimulation with HBsAg-loaded mature moDC, unloaded mature moDC, HBsAg alone and medium alone, respectively. Experiments were performed in triplicate and median values of one experiment are depicted. Similar results were obtained with three different donors.

ing an activation solution (U-CyTech Biosciences) and an incubation in the dark at room temperature for 15–20 min. Color development was stopped using deionized water and spots were counted using an ELISPOT reader (BioSys).

Serum Ab measurements

Anti-HBsAg IgG titers were determined 4 wk after the last vaccination in plasma by the Department of Virology of Erasmus Medical Center (Rotterdam, The Netherlands) using the Enzyme Immuno Assay (AxSym, Abbott Diagnostics) according to the manufacturer's instructions. Anti-CMV titers were also determined by the Department of Virology as previously described (15). Finally, Ab titers for tetanus were determined at the Rijksinstituut voor Volksgezondheid en Milieu, as described previously (16).

Statistical analysis

Statistical analyses were performed using SPSS 11.5 for Windows. Data are means \pm SEM unless indicated otherwise. Nonparametric analyses were used to test whether significant changes were observed. In addition, to investigate whether relationships exist between the various immunological parameters Spearman's Rho correlation coefficients (Rs) were calculated. p values ≤ 0.05 were considered statistically significant.

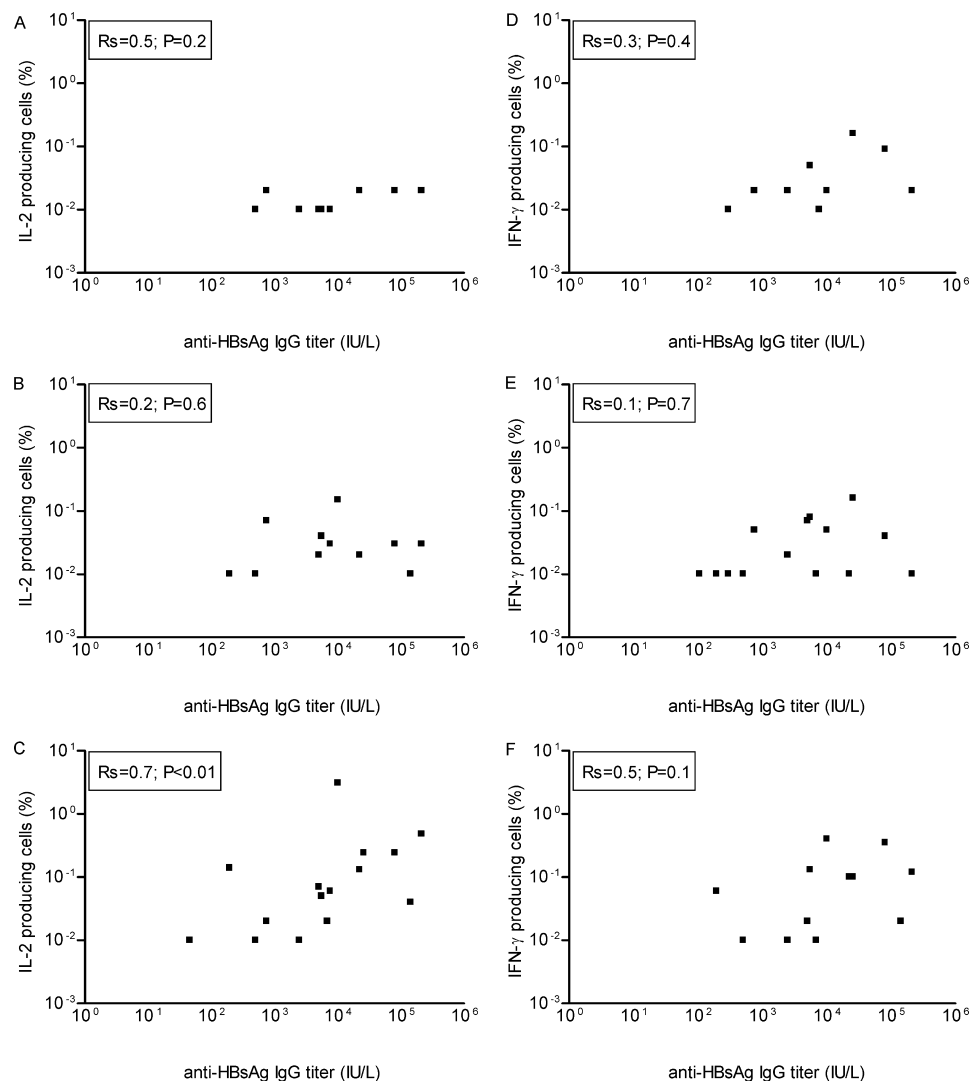


FIGURE 3. HBsAg-specific IL-2⁺ effector memory, but not IFN- γ ⁺ CD4⁺ T cells correlate with anti-HBs titers. Peak responses for HBsAg-specific IL-2 (A–C) and IFN- γ (D–F) producing cells (%) were associated with anti-HBsAg titers, measured 4 wk following the last HBV vaccination. On the x-axis, anti-HBsAg titers (IU/liter) are depicted and on the y-axis, percentages of HBsAg-specific IL-2 respectively IFN- γ producing naive CD4⁺ T lymphocytes (A and D), Tcm (B and E), and Tem (C and F) are displayed.

Results

IL-2 and IFN- γ positive HBsAg-specific CD4⁺ T cells show different kinetics and subset distribution after booster vaccination

Both IL-2 and IFN- γ positive HBsAg-specific CD4⁺ T cell responses could be readily detected after the last vaccination (Fig. 1, A and B).

The percentage of IL-2-positive cells showed on average the highest response at 2 wks within the effector memory CD4⁺ T (Tem) cells (Fig. 1G; $0.11 \pm 0.04\%$), which was preceded by a maximal response within the central memory CD4⁺ T (Tcm) cells at 1 wk (Fig. 1E). No significant responses were observed in the naive T cell fraction (Fig. 1C). At 12 wk the median absolute numbers of IL-2 positive HBsAg-specific CD4⁺ T cells were 1.1 (range $0\text{--}4.2$) $\times 10^4$ /liter in the Tcm and 1.2 ($0\text{--}14.7$) $\times 10^4$ /liter in the Tem.

HBsAg-specific IFN- γ -producing CD4⁺ T cells were found in all subsets and followed a similar pattern within these subsets. A maximal response was observed 2 wk after vaccination. In absolute numbers, almost half of the IFN- γ positive T cell response was observed in the naive T cell fraction at 2 and 12 wk. At 4 wk, a sharp decrease was noticed in the numbers of HBsAg-specific IFN- γ -producing CD4⁺ T cells when compared with that at 2 or 12 wk ($p < 0.05$), resulting in almost absent signal within the naive (Fig. 1D) and central memory (Fig. 1F) subset.

At 12 wk after vaccination, the average percentage of IL-2 and IFN- γ -producing HBsAg-specific CD4⁺ T cells was similar (0.01 and 0.02%) (Fig. 1, A and B, respectively).

IL-2 and IFN- γ producing Tem are associated with Ag-specific proliferation

The kinetics of HBsAg-specific proliferation (Fig. 2A) followed those of the cytokine producing cells and the highest response was noted at 2 to 4 wks following vaccination. Next, we investigated Ag-specific proliferation of the different CD4⁺ T cell subsets at 12 wk following receipt of the last HBV vaccination and noticed that Tem cells accounted for the major part of HBsAg-specific proliferation (Fig. 2B). In accordance with this observation, we could only find a significant correlation between HBsAg-specific proliferation and the numbers of IL-2 positive Tem ($R_s = 0.5$; $p < 0.01$) and to a lesser extent IFN- γ ($R_s = 0.4$; $p < 0.01$) producing CD4⁺ Tem.

Generation of IL-2 positive Tem is important for obtaining protective Ab titers

Anti-HBsAg titers amounted to 5350 IU/liter (range: 46.4–213770 IU/liter). The anti-HBsAg titers correlated positively with maximal HBsAg-specific proliferation ($R_s = 0.6$; $p < 0.05$), and most specifically with maximal (peak) percentages of HBsAg-specific IL-2 positive effector memory CD4⁺ T lymphocytes (Fig. 3C; $R_s =$

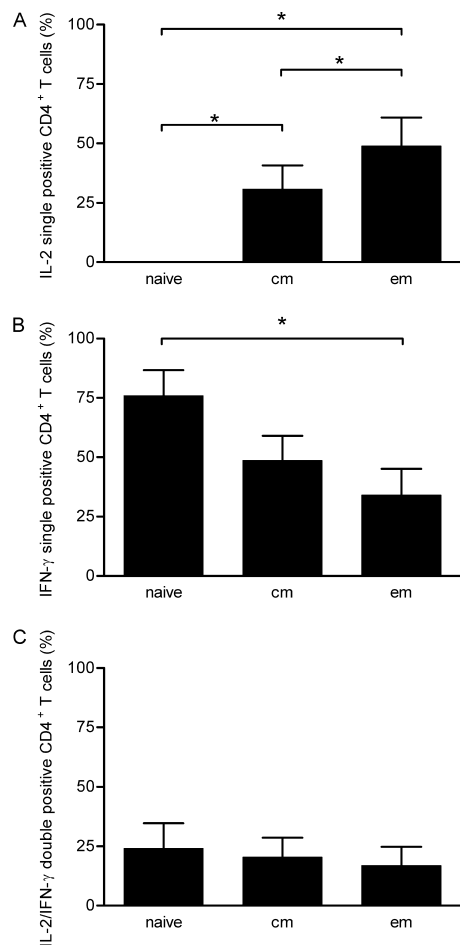


FIGURE 4. IL-2 single and not IL-2/IFN- γ double positive effector memory CD4⁺ T cells account for most of the HBsAg-specific CD4⁺ T cell response. At 12 wk following receipt of the last HBV vaccination, percentages of HBsAg-specific cytokine producing cells being either single IL-2 (A), single IFN- γ (B) positive, or positive for both IL-2 and IFN- γ (C) were determined. The percentage of single- and double-positive cells of the total sum of cytokine positive cells was calculated for the different CD4⁺ T cell subsets. Results of 16 HBV-vaccinated healthy individuals are shown. *, $p < 0.05$.

0.7; $p < 0.01$). This maximal response was for most individuals at 2 wk, but 21% of the healthy controls showed the IL-2 Tem peak response at 1 (7%) or 4 wk (14%) after vaccination. Such a relation could not be found for any other HBsAg-specific IL-2 positive (Fig. 3, A and B) or IFN- γ positive (Fig. 3, D–F) T cell subset, respectively.

In addition, we determined the percentages of single IL-2 and single IFN- γ positive and IL-2/IFN- γ double positive cells in the different CD4⁺ T cell subsets at 12 wks following HBV vaccination for 16 healthy volunteers. Single IL-2 positive cells were noticed predominantly within the Tem when compared with Tcm or naive CD4⁺ T lymphocytes (Fig. 4A), whereas double positive cells were observed at a similar frequency in all subsets (Fig. 4C). The median (range) absolute number of single IL-2⁺ Tem was 1.4 (0 – 41.2) $\times 10^4$ /L and significantly higher ($p < 0.05$) than that of single IL-2⁺ Tcm 0.9 (0 – 25.4) $\times 10^4$ /L. Again, absolute numbers of the total, single and double-positive IL-2 or IFN- γ producing CD4⁺ T cell subsets were related to the humoral immune response. Only total IL-2 positive ($R_s = 0.5$; $p = 0.03$) and IL-2 single positive ($R_s = 0.6$; $p = 0.02$) Tem were significantly associated with anti-HBsAg titers.

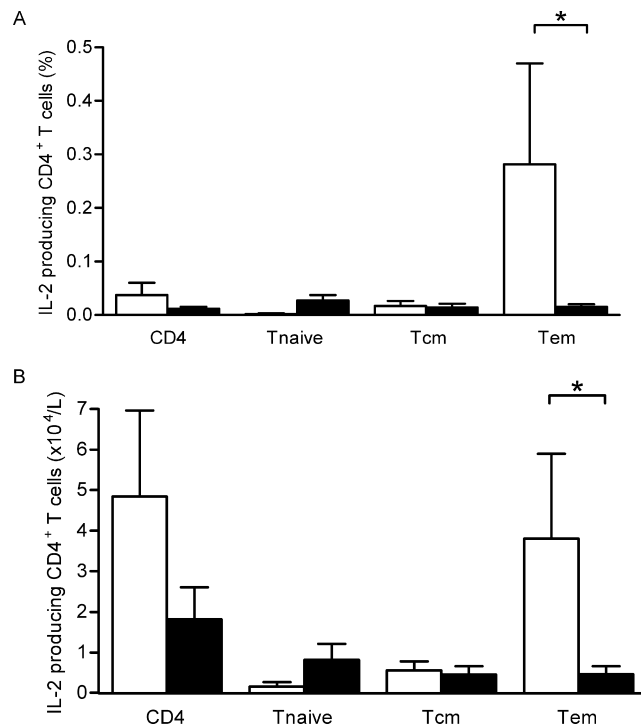


FIGURE 5. HBsAg-specific IL-2 producing effector memory CD4⁺ T cells are low or even absent in ESRD patients. Mean and SEM percentages (A) or absolute numbers (B) of HBsAg-specific IL-2 producing CD4⁺ T cells were determined two wks following receipt of the last HBV vaccination step for 15 healthy volunteers and 15 ESRD patients. The open symbols represent the healthy volunteers and the closed, the ESRD patients. *, $p < 0.05$.

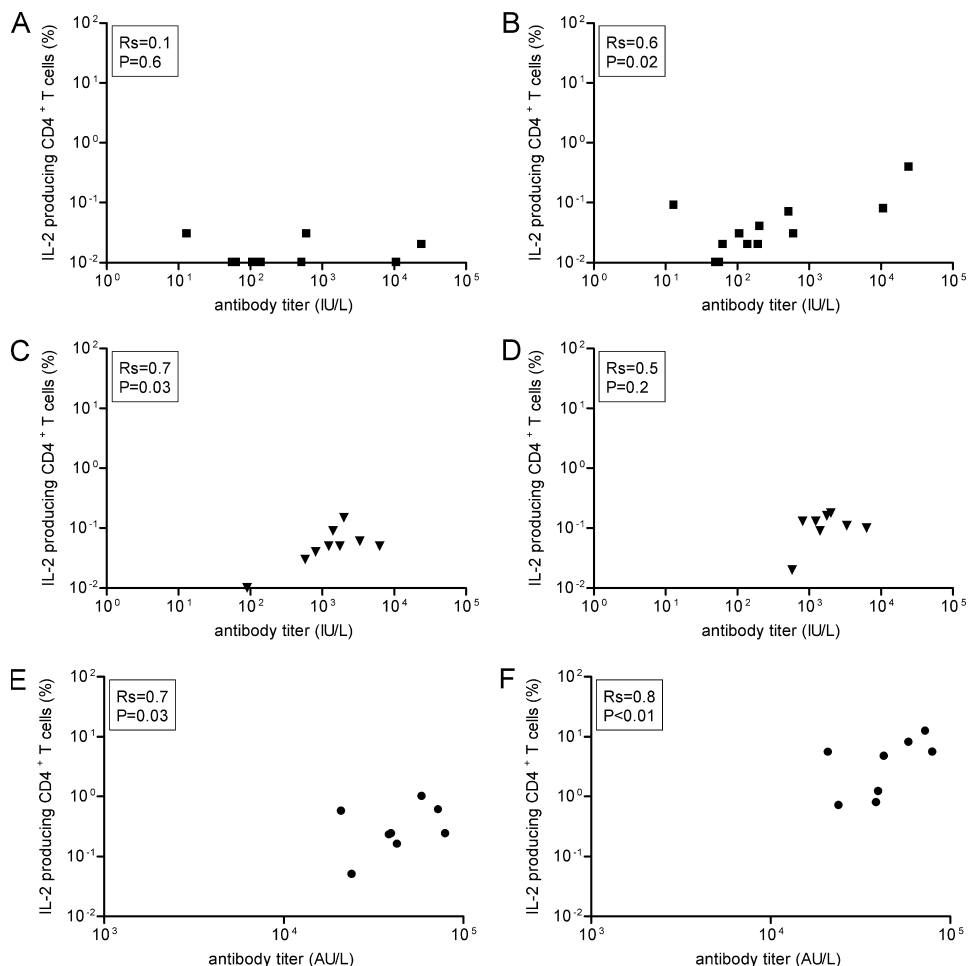
The clinical significance of this relation between IL-2 producing Tem and anti-HBsAg titers was supported by findings upon analysis of the HBsAg-specific CD4⁺ T cell responses in patients with ESRD. These patients are known to be poor responders to T cell dependent vaccination Ags like HBsAg (17). Two weeks after the last HBV vaccination, the percentages (Fig. 5A) and absolute numbers (Fig. 5B) of IL-2 and IFN- γ positive (data not shown) CD4⁺ T cells in patients were lower although not reaching significance ($p = 0.2$) when compared with healthy individuals. However, low to undetectable IL-2 positive Tem ($p = 0.02$) were found (median: 0.19×10^4 /liter; range: 0 – 2.4×10^4 /liter) in association with low to absent Ab titers (median 33.9 IU/liter; range: 0 – 672.8 IU/liter).

IL-2 producing Tmem remain closely related to Ab titers many years after antigenic exposition

Next, we examined the relation between IL-2-producing effector memory CD4⁺ T lymphocytes and the persistence of Ab titers many years after vaccination. For this purpose, Ag-specific IL-2-producing CD4⁺ T lymphocytes were enumerated following stimulation with HBsAg, the vaccination Ag tetanus toxoid or viral Ags from latent CMV infection (Fig. 6).

Although the median time post vaccination was 9 years (ranging from 1 to 19 years), a positive association between IL-2 producing Tem (Fig. 6B), but not Tcm (Fig. 6A) and anti-HBsAg titers was noticed. For tetanus toxoid, of which the last vaccination was 15 to 25 years ago, a positive relation was observed for IL-2-positive Tcm (Fig. 6C) but not Tem (Fig. 6D). Ab titers to CMV were positively related to the percentage of IL-2-positive CMV-specific Tcm (Fig. 6E) and Tem (Fig. 6F). Of note is the observation that higher Ab titers (CMV Ag > tetanus toxoid > HBsAg) correlated

FIGURE 6. IL-2 producing memory CD4⁺ T cells are important for maintenance of (steady state levels of) Ab titers. Percentages of IL-2 producing cells were determined of 8–10 healthy volunteers previously vaccinated for HBV (squares; *A* and *B*) and Tetanus (triangles, *C* and *D*) and seropositive for cytomegalovirus (CMV; circles; *E* and *F*). IL-2 responses were associated with Ab titers measured in plasma samples collected at the time of study. On the x-axis, Ab titers (IU respectively AU/liter) are depicted and on the Y-axis, percentages IL-2 producing central memory (*A*, *C*, and *E*) and effector memory (*B*, *D*, and *F*) CD4⁺ T cells are displayed.



with the presence of higher numbers of IL-2-positive memory CD4⁺ T cell responses for each of the Ags.

IL-2-producing Tmem are important for differentiation of Ag-specific memory B lymphocytes into Ab secreting cells

The close correlation between IgG Ab titers and the numbers of circulating IL-2-positive Ag-specific memory T cells suggested a direct role of these cells for induction of IgG producing plasma cells.

We used a T cell-dependent B cell ELISPOT assay to analyze the potential of naive CD4⁺ T lymphocytes, Tcm, and Tem for inducing differentiation of memory B lymphocytes into Ab-secreting cells for HBsAg (at 12 wk following last vaccination), tetanus toxoid, and CMV Ag. For nonspecific IgG production (plates coated with anti-Ig, Fig. 7*A*) and all Ags tested, the naive T cell fraction had little capacity to induce Ab-secreting cells. In line with the ex vivo data, a dominant role for CD4⁺ Tem over Tcm was observed for supporting the development of Ab secreting cells specific for HBsAg (Fig. 7*B*). An equal contribution of CD4⁺ Tcm and Tem for inducing memory B lymphocytes to differentiate into tetanus toxoid- (Fig. 7*C*) and CMV-specific IgG (Fig. 7*D*) secreting cells was observed.

Finally, we depleted the Tmem for IL-2 secreting cells and tested the capacity of this fraction to induce differentiation of memory B lymphocytes into Ab-secreting cells. Only the IL-2 depleted Tmem fraction, and not the sham-treated Tmem, did reduce the capacity of memory B lymphocytes to differentiate into Ab-secreting cells (Fig. 8*A*). Next, we performed this test in the presence of an IL-2R- α blocking mAb to prevent effects induced by

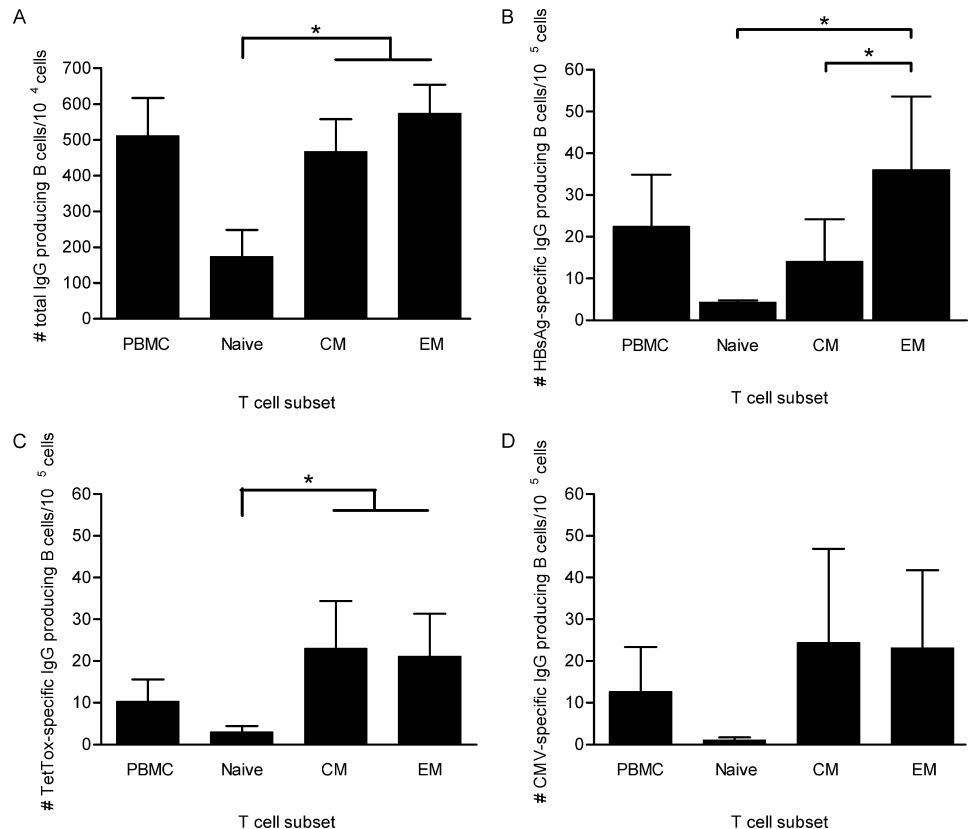
IL-2. Increasing concentrations of the Ab resulted in a substantial inhibition (on average 84% at 20 μ g/ml) of the development of IgG-producing plasma cells (Fig. 8*B*, only 20 μ g/ml of the isotype is shown). The results are in accordance with the ex vivo data and indicate that IL-2 secretion by Tmem is critically involved in the generation of IgG secreting plasma cells from memory B cells.

Discussion

In this study, we have analyzed, at the single-cell level, the development and characteristics of Ag-specific CD4⁺ T cells following protein vaccination in humans in relation to the generation of IgG-secreting plasma cells.

Recent studies have indicated that IL-2 producing Ag-specific T cells dominate the cytokine response after protein vaccination in humans (18, 19). Similar to others (20, 21), we could not reliably detect Ag-specific T cells positive for Th type-2 cytokines like IL-4 and IL-10 (our unpublished data), and therefore focused on the detection of IL-2 and IFN- γ -positive HBsAg-specific T cells. The responses of IL-2 and IFN- γ -positive HBsAg-specific T cells were similar for the total CD4⁺ T cells, but the IL-2⁺ cells dominated the response in the effector memory subset. In support of the differentiation pathway proposed by Sallusto (4, 22) and Seder (23), peak responses for IL-2 producing Tcm preceded those observed for Tem lymphocytes. However, the kinetics for HBsAg-specific IFN- γ -producing CD4⁺ T cells was similar in all subsets, and these cells almost completely disappeared from the circulation at 4 wk after vaccination. Our data are only observational, and

FIGURE 7. Memory CD4⁺ T cells are important for inducing memory B cell differentiation toward Ab secreting cells. PBMC and the various CD4⁺ T cell subsets, sorted on a FACSARIA, were mitomycin C-treated according to the protocol described by Lipsky et al. (13) and cocultured at a density of 1×10^5 /well with 25×10^3 autologous B-lymphocytes in anti-CD3 ($1 \mu\text{g}/\text{ml}$)-coated 96-well plates. At day 6, cells were harvested and transferred to a 96-well ELISPOT plate either coated with Ig (A), HBsAg (B), Tetanus toxoid (C), or CMV lysate (D). Cells were obtained from healthy volunteers 12 wk after HBV vaccination. On the x-axis, the unsorted PBMC and the different T cell subsets are depicted whereas on the y-axis, numbers of either total IgG or Ag-specific IgG producing cells per 10^4 and 10^5 cells, respectively are displayed. Results shown are means (and SEM) of six different donors. *, $p < 0.05$.



therefore cannot identify the mechanisms underlying these remarkable differences in time for the presence of IL-2 and IFN- γ -positive cells. However, they clearly show that the results of any study of Ag-specific T cells after vaccination may change substantially within an interval of 4 wk.

Analysis of the Ag-specific T cell responses shortly after HBV vaccination revealed that IL-2-positive Tem were closely related to both the Ag-specific proliferative response, and the serum levels of Ab achieved. A more detailed analysis involving the extent of overlap of IL-2 and IFN- γ producing CD4⁺ T cells in the different CD4⁺ T cell subsets confirmed that only IL-2 single positive Tem and not those also producing IFN- γ were related to Ab titers. In addition, the data of the B cell ELISPOT indicated that effector memory type CD4⁺ T cells have a superior capacity for inducing differentiation of memory B cells into IgG secreting plasma cells. Therefore, this study links effector memory type CD4⁺ T cells to generation of plasma cells, a function previously unrecognized. In vivo, interaction of memory B and T cells will take place in the lymphoid tissues, for which expression of CCR7 on the T cell surface is required for entrance. As the effector memory T cells do not express this molecule, they will not home to these sites. However Tem may overcome this problem as they are able to transiently increase the expression of CCR7 on their cell surface upon activation (24).

Even many years after vaccination, we could find a significant correlation between circulating HBsAg-specific IL-2 positive Tem and Ab titers. Maintenance of Ab titers is thought to be Ag independent, as there are no convincing arguments for continuous stimulation by antigenic cross-reactivity as the underlying mechanism. Currently, two not mutually exclusive hypotheses exist, to explain long-lived Ab responses. The first suggest that they are maintained by long-lived plasma cells in the bone marrow that secrete Abs for extended periods (25, 26). The second hypothesis states that memory B cells are continually differentiating into plasma cells in an

Ag-independent manner due to bystander or polyclonal activation (27). Recently, bystander activation was also suggested as a possible mechanism for maintenance of Ag-specific T cells (28–30). Our data are not in contradiction with the hypothesis of bystander activation as the mechanism that supports maintenance of the immunological memory of T and B cells. However, the close relation between IL-2 memory T cells and Ab titers, observed even decades after vaccination, dictates that the process of bystander activation has a proportional effect on T and B cells, thereby keeping the mutual ratio intact. As such, this may be beneficial for maximal effective reactivation of the immune response upon re-encounter of the Ag.

For tetanus toxoid and whole CMV Ag, a correlation was found between the Ab titers and respectively IL-2 positive Tcm and IL-2 positive Tcm and Tem. Apparently, expression of the CCR7 molecule on IL-2 positive memory T cells that are related to induction of plasma cells varies with the type of Ag. The observed difference with respect to the relation of different memory CD4⁺ T cell subsets with Ab titers may be associated with the way Ag is detected by the immune system and the distinct signals that are induced by the different types of Ags (23, 31, 32). With respect to the type of Ag, CMV, for example, is a persisting Ag and thus continuously able to trigger the immune response. This might explain the observed association of both IL-2 producing Tcm and Tem with Ab titers. This is distinct from HBsAg and tetanus toxoid, both vaccination Ags and both cleared upon re-encounter. In agreement with our findings, a preferential expansion of the Tcm compartment as compared with the Tem subset was observed upon re-encounter of the Tetanus Toxoid (33).

For the total IgG production in vitro, which essentially includes all memory B cell responses, both Tcm and Tem appeared equally effective. Depletion of IL-2 secreting Tmem showed that these cells are in vitro important for the generation of IgG secreting plasma cells. The effect of depletion was not maximal, which may

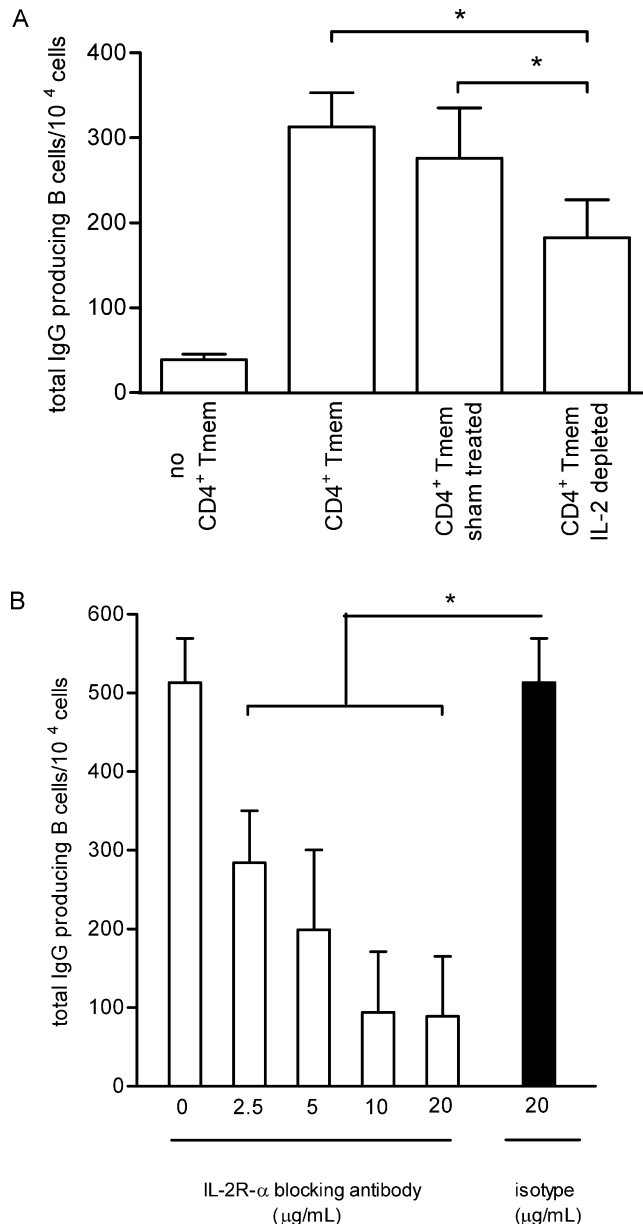


FIGURE 8. IL-2 is essential for memory B cell differentiation toward Ab secreting cells. Sorted memory CD4⁺ T cells (Tmem) were stimulated with plate-bound anti-CD3 and anti-CD28 for 6 h. Thereafter, IL-2 secreting cells were depleted from the Tmem fraction using the IL-2 secretion assay. Sorted autologous B lymphocytes at a density of 25×10^3 /well were cocultured without or with the unfractionated Tmem, sham-treated Tmem or Tmem depleted for IL-2 secreting cells in anti-CD3 coated 96-well plates. At day 6, the number of IgG producing B cells was established using an ELISPOT assay. The numbers of IgG producing cells per 10^4 B cells are displayed on the y-axis (A). In addition, mitomycin C-treated Tmem were cocultured with B lymphocytes in continuous presence of different concentrations of an IL-2R- α blocking mAb (\square) or 20 μ g/ml IgG1 (isotype control, \blacksquare). At day 6, the number of IgG producing B cells was established using an ELISPOT assay. The numbers of IgG producing cells per 10^4 B cells are displayed on the y-axis (B). Results shown are means (and SEM) of six different donors. *, $p < 0.05$.

be caused by the presence of a few remaining IL-2 secreting Tmem in the depleted cell fraction. The result of the IL-2 receptor blocking experiment showed that the action of IL-2 is essential for differentiation of memory B lymphocytes to IgG producing plasma cells.

The results from this study closely fit to what is known about the role of IL-2 in the final differentiation pathway of B cells into Ab secreting cells. The majority of memory B cells expresses the IL-2 receptor (34) and late in a primary immune response the IL-2 secreted by Ag-activated Th cells binds to high affinity IL-2 receptors on Ag-activated B cells (35). IL-2 then acts on two crucial regulators of B cell development, the Pax5 gene encoded transcription factor B cell lineage-specific activator protein and Blimp-1. IL-2 down-regulates B cell lineage-specific activator protein RNA expression in a concentration-dependent manner, relieving the repression of the Ig J chain gene, and rapidly induces transcripts of the Blimp-1 gene (36, 37). Blimp-1 is a member of the zinc finger-containing family of DNA-binding proteins, and is now recognized as the master switch for the terminal differentiation of B cells into plasma cells (38). In accordance with these findings, the presence of T cells can be replaced by exogenous IL-2 in an in vitro model of Ag-specific T cell-dependent differentiation of B cells to plasma cells (39). Therefore, a functional role of IL-2 secreted by Ag-specific T cells in the generation of plasma cells seems plausible and readily explains why the lack of IL-2 positive HBsAg-specific T cells after vaccination is related to a poor anti-HBsAg Ab titer.

In summary, our data show a critically important role for IL-2 secretion by memory CD4⁺ T cells for the induction of IgG producing plasma cells after antigenic stimulation, a relation that may persist for decades without re-exposure to the specific Ag.

Acknowledgments

We thank Reinier van der Linden of the Department of Cell Biology (Erasmus Medical Center, Rotterdam, The Netherlands) for performing additional sorting experiments. Furthermore, we also thank the diagnostic section of the Department of Virology (Erasmus Medical Center, Rotterdam, The Netherlands) and the Rijksinstituut voor Volksgezondheid en Milieu (RIVM, Bilthoven, The Netherlands) for determining Ab titers.

Disclosures

The authors have no financial conflict of interest.

References

- Abbas, A. K., K. M. Murphy, and A. Sher. 1996. Functional diversity of helper T lymphocytes. *Nature* 383: 787–793.
- Sprent, J., and C. D. Surh. 2001. Generation and maintenance of memory T cells. *Curr. Opin. Immunol.* 13: 248–254.
- Zinkernagel, R. M. 2000. On immunological memory. *Philos. Trans. R. Soc. Lond B Biol. Sci.* 355: 369–371.
- Sallusto, F., D. Lenig, R. Forster, M. Lipp, and A. Lanzavecchia. 1999. Two subsets of memory T lymphocytes with distinct homing potentials and effector functions. *Nature* 401: 708–712.
- Sallusto, F., J. Geginat, and A. Lanzavecchia. 2004. Central memory and effector memory T cell subsets: function, generation, and maintenance. *Annu. Rev. Immunol.* 22: 745–763.
- Zanetti, A. R., A. Mariano, L. Romano, R. D'Amelio, M. Chironna, R. C. Coppola, M. Cuccia, R. Mangione, F. Marrone, F. S. Negrone, A. Parlato, E. Zamparo, C. Zotti, T. Stroffolini, and A. Mele. 2005. Long-term immunogenicity of hepatitis B vaccination and policy for booster: an Italian multicentre study. *Lancet* 366: 1379–1384.
- Stubbe, M., N. Vanderheyde, M. Goldman, and A. Marchant. 2006. Antigen-specific central memory CD4⁺ T lymphocytes produce multiple cytokines and proliferate in vivo in humans. *J. Immunol.* 177: 8185–8190.
- Litjens, N. H., M. Huisman, C. C. Baan, C. J. van Druningen, and M. G. Betjes. 2008. Hepatitis B vaccine-specific CD4⁺ T cells can be detected and characterized at the single cell level: Limited usefulness of dendritic cells as signal enhancers. *J. Immunol. Methods* 330: 1–11.
- DaRoza, G., A. Loewen, O. Djurdjev, J. Love, C. Kempston, S. Burnett, M. Kiai, P. A. Taylor, and A. Levin. 2003. Stage of chronic kidney disease predicts seroconversion after hepatitis B immunization: earlier is better. *Am. J. Kidney Dis.* 42: 1184–1192.
- Propst, T., A. Propst, K. Lhotta, W. Vogel, and P. Konig. 1998. Reinforced intradermal hepatitis B vaccination in hemodialysis patients is superior in antibody response to intramuscular or subcutaneous vaccination. *Am. J. Kidney Dis.* 32: 1041–1045.
- Verkade, M. A., C. J. van Druningen, L. M. Vaessen, D. A. Hesselink, W. Weimar, and M. G. Betjes. 2007. Functional impairment of monocyte-derived dendritic cells in patients with severe chronic kidney disease. *Nephrol. Dial. Transplant* 22: 128–138.

12. Litjens, N. H., C. J. van Druningen, and M. G. Betjes. 2006. Progressive loss of renal function is associated with activation and depletion of naive T lymphocytes. *Clin. Immunol.* 118: 83–91.
13. Lipsky, P. E. 1980. Staphylococcal protein A, a T cell-regulated polyclonal activator of human B cells. *J. Immunol.* 125: 155–162.
14. Hirohata, S., D. F. Jelinek, and P. E. Lipsky. 1988. T cell-dependent activation of B cell proliferation and differentiation by immobilized monoclonal antibodies to CD3. *J. Immunol.* 140: 3736–3744.
15. Betjes, M. G., N. H. Litjens, and R. Zietse. 2007. Seropositivity for cytomegalovirus in patients with end-stage renal disease is strongly associated with atherosclerotic disease. *Nephrol. Dial. Transplant* 22: 3298–3303.
16. von Hunolstein, C., H. Aggerbeck, N. Andrews, G. Berbers, F. Fievet-Groynne, P. A. Maple, R. M. Olander, M. Raux, and A. Tischer. 2000. European seroepidemiology network: standardisation of the results of diphtheria antitoxin assays. *Vaccine* 18: 3287–3296.
17. Verkade, M. A., W. J. van de, M. Klepper, L. M. Vaessen, W. Weimar, and M. G. Betjes. 2004. Peripheral blood dendritic cells and GM-CSF as an adjuvant for hepatitis B vaccination in hemodialysis patients. *Kidney Int.* 66: 614–621.
18. De Rosa, S. C., F. X. Lu, J. Yu, S. P. Perfetto, J. Falloon, S. Moser, T. G. Evans, R. Koup, C. J. Miller, and M. Roederer. 2004. Vaccination in humans generates broad T cell cytokine responses. *J. Immunol.* 173: 5372–5380.
19. Divekar, A. A., D. M. Zaiss, F. E. Lee, D. Liu, D. J. Topham, A. J. Sijts, and T. R. Mosmann. 2006. Protein vaccines induce uncommitted IL-2-secreting human and mouse CD4 T cells, whereas infections induce more IFN- γ -secreting cells. *J. Immunol.* 176: 1465–1473.
20. Goncalves, L., B. Albarran, S. Salmen, L. Borges, H. Fields, H. Montes, A. Soyano, Y. Diaz, and L. Berrueta. 2004. The nonresponse to hepatitis B vaccination is associated with impaired lymphocyte activation. *Virology* 326: 20–28.
21. Vingerhoets, J., G. Vanham, L. Kestens, G. Penne, G. Leroux-Roels, and P. Gigase. 1994. Deficient T-cell responses in non-responders to hepatitis B vaccination: absence of TH1 cytokine production. *Immunol. Lett.* 39: 163–168.
22. Sallusto, F., A. Langenkamp, J. Geginat, and A. Lanzavecchia. 2000. Functional subsets of memory T cells identified by CCR7 expression. *Curr. Top Microbiol. Immunol.* 251: 167–171.
23. Seder, R. A., and R. Ahmed. 2003. Similarities and differences in CD4+ and CD8+ effector and memory T cell generation. *Nat Immunol.* 4: 835–842.
24. Sallusto, F., E. Kremmer, B. Palermo, A. Hoy, P. Ponath, S. Qin, R. Forster, M. Lipp, and A. Lanzavecchia. 1999. Switch in chemokine receptor expression upon TCR stimulation reveals novel homing potential for recently activated T cells. *Eur. J. Immunol.* 29: 2037–2045.
25. Manz, R. A., A. Thiel, and A. Radbruch. 1997. Lifetime of plasma cells in the bone marrow. *Nature* 388: 133–134.
26. Slifka, M. K., R. Antia, J. K. Whitmire, and R. Ahmed. 1998. Humoral immunity due to long-lived plasma cells. *Immunity* 8: 363–372.
27. Bernasconi, N. L., E. Traggiai, and A. Lanzavecchia. 2002. Maintenance of serological memory by polyclonal activation of human memory B cells. *Science* 298: 2199–2202.
28. Di Genova, G., J. Roddick, F. McNicholl, and F. K. Stevenson. 2006. Vaccination of human subjects expands both specific and bystander memory T cells but antibody production remains vaccine specific. *Blood* 107: 2806–2813.
29. Donnenberg, A. D., G. J. Elfenbein, and G. W. Santos. 1984. Secondary immunization with a protein antigen (tetanus toxoid) in man. Characterization of humoral and cell-mediated regulatory events. *Scand. J. Immunol.* 20: 279–289.
30. Fernandez, V., J. Andersson, U. Andersson, and M. Troye-Blomberg. 1994. Cytokine synthesis analyzed at the single-cell level before and after revaccination with tetanus toxoid. *Eur. J. Immunol.* 24: 1808–1815.
31. Kaech, S. M., E. J. Wherry, and R. Ahmed. 2002. Effector and memory T-cell differentiation: implications for vaccine development. *Nat. Rev. Immunol.* 2: 251–262.
32. Lanzavecchia, A., and F. Sallusto. 2000. Dynamics of T lymphocyte responses: intermediates, effectors, and memory cells. *Science* 290: 92–97.
33. Barbey, C., E. Pradervand, N. Barbier, and F. Spertini. 2007. Ex vivo monitoring of antigen-specific CD4+ T cells after recall immunization with tetanus toxoid. *Clin. Vaccine Immunol.* 14: 1108–1116.
34. Nakanishi, K., D. I. Cohen, M. Blackman, E. Nielsen, J. Ohara, T. Hamaoka, M. E. Koshland, and W. E. Paul. 1984. Ig RNA expression in normal B cells stimulated with anti-IgM antibody and T cell-derived growth and differentiation factors. *J. Exp. Med.* 160: 1736–1751.
35. Rinkenberg, J. L., J. J. Wallin, K. W. Johnson, and M. E. Koshland. 1996. An interleukin-2 signal relieves BSAP (Pax5)-mediated repression of the immunoglobulin J chain gene. *Immunity* 5: 377–386.
36. Turner, C. A., Jr., D. H. Mack, and M. M. Davis. 1994. Blimp-1, a novel zinc finger-containing protein that can drive the maturation of B lymphocytes into immunoglobulin-secreting cells. *Cell* 77: 297–306.
37. Kallies, A., and S. L. Nutt. 2007. Terminal differentiation of lymphocytes depends on Blimp-1. *Curr. Opin. Immunol.* 19: 156–162.
38. Amu, S., A. Tarkowski, T. Dorner, M. Bokarewa, and M. Brisslert. 2007. The human immunomodulatory CD25+ B cell population belongs to the memory B cell pool. *Scand. J. Immunol.* 66: 77–86.
39. Bergamin, F., I. E. Vincent, A. Summerfield, and K. C. McCullough. 2007. Essential role of antigen-presenting cell-derived BAFF for antibody responses. *Eur. J. Immunol.* 37: 3122–3130.