Lack of *ex vivo* peripheral and intrahepatic α-fetoprotein-specific CD4+ responses in hepatocellular carcinoma

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Hepatocellular carcinoma (HCC) is one of the most common malignancies with a poor prognosis and limited therapeutic options that is often characterized by the expression of the tumor-associated antigen α-fetoprotein (AFP). CD4+ helper T cells are important in generating potent anticancer immunity as they prime and expand CD8+ T-cell memory and may also have direct antitumor activity. However, very little information is currently available about the relative frequency, immunodominance and peripheral versus intratumoral distribution of AFP-specific CD4+ T-cell responses in patients with HCC.

We, therefore, analyzed AFP-specific CD4+ responses in blood and tumor tissue of patients with HCC by using overlapping peptides spanning the entire AFP protein and novel sensitive approaches such as antigen-specific upregulation of CD154. We found that AFP-specific CD4+ T-cell responses were not detectable in the peripheral blood *ex vivo*. However, after *in vitro* stimulation, AFP-specific CD4+ T-cell responses were detectable in a large fraction of patients targeting different previously unreported epitopes with no clear immunodominance. These results indicate that AFP-specific CD4+ T-cell responses are not completely deleted but only present at very low frequencies. Importantly, AFP-specific CD4+ T-cell responses were also rarely detectable in tumor tissue, suggesting that the relative absence of these cells in the circulation *ex vivo* is not due to a rapid accumulation to the tumor side. Taken together, these results suggest that the lack of sufficient CD4+ T-cell help, especially within the tumor tissue, may be one central mechanism responsible for the failure of AFP-specific immune responses to control HCC progression.

Key words: α-fetoprotein, hepatocellular carcinoma, CD4+, T-cell responses

Abbreviations: AFP: α-fetoprotein; APC: antigen-presenting cell; CMV: cytomegalovirus; DC: dendritic cell; EDTA: ethylenediaminetetraacetic acid; FACS: fluorescence-activated cell sorting; HBV: hepatitis B virus; HCC: hepatocellular carcinoma; HCV: hepatitis C virus; ICS: intracellular cytokine staining; IL-2: interleukin-2; ITL: intratumoral lymphocytes; PBMC: peripheral blood mononuclear cells; PBS: phosphate-buffered saline; SER: Staphylococcus enterotoxin B; TNF-α: tumor necrosis factor alpha; TT: tetanus toxoid

Additional Supporting Information may be found in the online version of this article

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Hepatocellular carcinoma (HCC) is the fifth most common malignancy worldwide with a poor prognosis and limited therapeutic options. Therefore, the development of novel therapeutic strategies is of high priority. Immunotherapy represents a promising potential option for several reasons: First, a correlation has been reported between high numbers of tumor-infiltrating T cells in HCC tissue and the prognosis of disease.1,2 Second, adoptive immunotherapy with anti-CD3- and interleukin-2-stimulated autologous lymphocytes lowers postsurgical HCC recurrence rates in humans.3 Finally, the induction of anti-α-fetoprotein (AFP) cell-mediated immune responses can control tumor growth in the mouse model.4

AFP is a serum marker for HCC that is elevated in 50–80% of patients with HCC. Physiologically, AFP is highly expressed in fetal liver, gastrointestinal tract and yolk sac but is transcriptionally downregulated after birth. Importantly, after birth, AFP can be elevated in patients with HCC or testicular cancers.5 Of note, this can be associated with the emergence of AFP-specific immune responses. For example, several human leukocyte antigen (HLA) class I-restricted AFP-specific epitopes have been identified by different approaches and shown to be present in HCC patients.6–9

Indeed, in a recent comprehensive analysis using overlapping AFP peptides, we have shown that the majority of patients with HCC showed AFP-specific CD8+ T-cell responses.
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specific CD8 T-cell response are not well understood and may include the action of regulatory T cells, inhibitory receptors or immunosuppressive cytokines.10–13 It is also possible that the CD8+ T-cell failure may be due to the lack of AFP-specific CD4+ T-cell help. Indeed, although CD4+ helper T cells are important in generating potent anticancer immunity as they prime and expand CD8+ T-cell memory14 and may also have direct antitumor activity,15–17 very little information is currently available about the AFP-specific CD4+ T-cell response. Studies have shown that CD4+ T-cell responses to immunodominant AFP-derived peptides are mainly detectable in HCC patients with low serum AFP concentrations and at an early stage of disease.18,19 However, in these studies, only a limited set of peptides was studied; the analyses were based on prediction programs, were limited to the peripheral blood and only used the measurements of functional effector functions as a readout. Thus, the important question as to whether AFP-specific CD4+ T-cell responses are present ex vivo but exhausted and functionally impaired in their effector functions could not be addressed. To overcome these limitations, we used the novel CD154 assay that is robust, independent from the HLA type as well as from the knowledge of previously identified epitopes.20 Of note, by using this approach, we could recently show that antigen-specific CD4+ T cells are not deleted but fully exhausted in chronically HCV-infected patients.21 In our study, we set out to analyze the actual frequency, function and immunodominance of AFP-specific CD4+ T-cell responses as well as their peripheral versus intrahepatic distribution by using these novel assays.

Material and Methods

Subjects and study samples

Thirty patients with HCC and ten healthy subjects were enrolled in the study (Table 1). Fifty milliliters of EDTA-anticoagulated blood was obtained after informed consent and in agreement with federal guidelines and after approval by the local ethics committee. Peripheral blood mononuclear cells (PBMCs) were isolated by Pan-coll density gradient (Pan Laboratories, Eidenbach, Germany) and washed three times with phosphate-buffered saline (PBS) (Gibco, Karlsruhe, Germany). Twelve of 30 patients with HCC underwent diagnostic liver biopsy or potentially curative liver resection. Part of the liver biopsy or resection was put into RPMI 1640 medium (Gibco) containing 10% fetal bovine serum and processed as described below.

Synthetic peptides and recombinant proteins

Overlapping peptides spanning the entire AFP protein (18 amino acids each, overlapping by ten amino acids) were obtained from JPT Peptide Technologies GmbH and used in a final concentration of 10 μg/mL. AFP protein (BioProcessing, Portland, ME) was used in a final concentration of 10 μg/mL. A lysate of CMV-infected cells was obtained by Virusys (North Berwick, ME; final concentration 4 μg/mL). Staphylococcus enterotoxin B (SEB final concentration 0.1 μg/mL) and phorbol 12-myristate 13-acetate (PMA, 10 ng/mL; Sigma-Aldrich, Taufkirchen, Germany) were used as positive controls as indicated. Tetanus toxoid (TT; Sanofi pasteur MSD, Leimen, Germany; final concentration 4 μg/mL) was used only in selected cases as additional control antigen.

Antibodies

All antibodies used were obtained from BD Pharmingen (San Jose, CA) and were used according to the manufacturer’s instructions. The following antibodies were used: anti-CD4-APC (Clone RPA-T4), Via-Probe-PerCP, anti-IFN-γ-FITC (Clone 25723.11), anti-CD107a-PE (Clone H4A3), anti-IL-2-FITC (Clone 5344.111), anti-TNF-α-FITC (Clone 6401.1111) and anti-CD154-PE (Clone TRAP1).

Antigen-specific expansion of PBMC

A total of 10 × 10⁶ PBMC were resuspended in 10 mL RPMI 1640 medium containing 10% fetal calf serum, 1% streptomycin/penicillin, 1.5% 1 M Hepes and 100 U/mL IL-2 (Hoffmann-La Roche) and were stimulated with AFP protein. No additional antigen-presenting cells (APCs) were added as PBMCs already contain a sufficient amount of the latter. Recombinant IL-2 was added on Day 5 of culture. After a total of 10–12 days of culture, the cells were assayed for IFN-γ secretion as described below in response to a 5-hr stimulation with pools of overlapping AFP peptides. If responses were detectable, peptide pools were deconvoluted, and PBMCs were then retested and stimulated with individual peptides. Background levels (no peptide or irrelevant peptide) were between 0.01 and 0.08%. The average of all AFP-pool responses in healthy donors was <0.015% after subtraction of the background, and none of these responses was >0.03% after subtraction of the background. Positive responses were, therefore, defined as >0.03% peptide-specific IFN-γ secretion after subtraction of the background. This sensitive threshold was validated by the successful deconvolution of peptide pool-specific responses to the single peptide level (representative stainings are shown in Supporting Information Fig. S2) and is in line with previous observations of our group for both, HCV-specific CD4+ and CD8+ T-cell responses.21,22 Assays were performed on fresh PBMC and for some selected samples on cryopreserved cells.
CD4 selection and polyclonal expansion of PBMC
A total of $4 \times 10^6$ PBMCs were resuspended in 5 mL PBS and incubated with magnetic beads coupled to anti-CD4 antibodies (Dynabeads; Dynla, Oslo, Norway) for 20 min at 4°C. Bound CD4+ T cells were isolated by a particle magnetic concentrator. The purity of CD4+ T cells was confirmed to be $>95\%$ by fluorescence-activated cell sorting (FACS) analysis. FACS analysis was performed on a BD FACSCanto II flow cytometer. CD4+ T cells were plated into one well of a 24-well plate (Corning) in 1 mL complete medium containing 100 U/mL IL-2 (Hoffmann-La Roche), 0.04 μg/mL anti-human CD3 monoclonal antibody (Immuno-tect, Marseilles, France) and $2 \times 10^6$ irradiated autologous PBMC as feeder cells. Twice a week, 1 mL of medium was exchanged and 100 U/mL IL-2 was added. After 2 weeks, the expanded CD4+ PBMC and intratumoral lymphocytes were tested for AFP-specific T-cell responses by intracellular IFN-γ staining, first on pool level and, if tested positively, on a single peptide level.

Isolation and expansion of intratumoral CD4+ T cells
Isolation of tumor-infiltrating T cells was performed as described. Briefly, liver biopsy specimens were homogenized using a 70-μm Dounce tissue grinder (BD Biosciences, Heidelberg, Germany). Cell suspensions were incubated with magnetic beads coupled to anti-CD4 antibodies (Dynabeads)
for 20 min at 4°C. Bound CD4+ T cells were isolated using a particle magnetic concentrator. The purity of CD4+ T cells was >95% by FACS analysis. The intratumoral CD4+ T cells were then expanded and stained for IFN-γ production as described for PBMC (see above). Background levels of nonspecific IFN-γ production (no peptide or irrelevant peptide) for intratumoral lymphocytes and PBMC after nonspecific expansion were between 0 and 0.05%. Importantly, the expansion of peripheral and intratumoral CD4+ T cells from a given patient was always performed in parallel and for the same time before analysis to obtain comparable results. The expansion is needed to obtain a sufficient number of intratumoral T cells for analysis, and the same approach has been successfully used in HCV immunobiology. All nonspecifically expanded PBMC and intratumoral lymphocytes were used fresh.

**Intracellular IFN-γ staining and cytokine assays**
The intracellular IFN-γ staining was performed essentially as described. Briefly, PBMC ex vivo or after antigen-specific expansion and nonspecifically expanded PBMC and intratumoral T cells were stimulated with peptides (10 μg/mL), 1 μL/mL Brefeldin A (BD Pharmingen) and IL-2 (50 U/mL). After incubation for 5 hr (37°C, 5% CO2), cells from each well were blocked with immunoglobulin G1 antibodies and stained with antibodies against CD4 and with Via-Probe to exclude dead cells. After permeabilization with Cytofix/Cytoperm (BD Pharmingen), cells were stained with antibodies against IFN-γ (BD Pharmingen) and fixed in 100 μL 2% paraformaldehyde/PBS per well before FACS analysis. The frequency of cytokine-positive T cells was defined as the difference between the frequency detected in peptide-stimulated and unstimulated cells with a minimum of 0.03%. CD107a staining of PBMC was performed as described previously.26 Briefly, PBMC ex vivo or after antigen-specific expansion and nonspecifically expanded PBMC and intratumoral T cells were stimulated with peptides (10 μg/mL), 1 μL/mL Brefeldin A (BD Pharmingen) and IL-2 (50 U/mL). After incubation for 5 hr (37°C, 5% CO2), cells from each well were blocked with immunoglobulin G1 antibodies and stained with antibodies against CD4 and with Via-Probe to exclude dead cells. After permeabilization with Cytofix/Cytoperm (BD Pharmingen), cells were stained with antibodies against IFN-γ (BD Pharmingen) and fixed in 100 μL 2% paraformaldehyde/PBS per well before FACS analysis. As a control group, ten healthy subjects were also tested for AFP-specific CD4+ T-cell responses.

**Ex vivo CD154 staining of PBMC**
The procedure was performed as described previously. A total of 1 × 10⁶ fresh PBMCs were stimulated with pools of 11 overlapping 18-mer peptides for 5 hr in the presence of IL-2 (50 U/mL). Corresponding samples were stimulated with CMV-lysate or TT as control antigens for 16 hr and cocultured with a CD154-PE antibody as described previously. The cells were blocked with immunoglobulin G1 and then stained with Via-Probe (to exclude dead cells, which may bind nonspecifically to the PE magnetic beads) and anti-CD4-APC. Cells were then fixed in 100 μL 2% paraformaldehyde/PBS per well and analyzed for CD154 expression by FACS analysis. FACS analysis was performed on a BD FACSCanto II flow cytometer. Ex vivo assays for CD154 expression or IFN-γ secretion were performed on either fresh or cryopreserved PBMC, and experiments performed side by side revealed comparable results.

**Results**

**Patient cohort**
The AFP-specific CD4+ T-cell response was tested in 30 patients with HCC. The characteristics of the patients are summarized in Table 1. The diagnosis of HCC was based on the American Association for the Study of Liver Diseases guidelines. The most frequent causes for HCC development in our cohort were chronic viral infection (n = 8) and alcohol (n = 11) or both of these two risk factors (n = 3). One patient had hemochromatosis, whereas in seven patients the cause of HCC development remained unknown. AFP was elevated (AFP > 7 ng/mL) in 18 patients, ranging from 7.4 to >60,500 ng/mL. Nineteen of all patients enrolled in the study were therapy naive. The most frequent therapy applied was transarterial chemoembolization (n = 9). As a control group, ten healthy subjects were also tested for AFP-specific CD4+ T-cell responses.

**Absence of AFP-specific IFN-γ-producing CD4+ T cells in the peripheral blood**
In a first set of experiments, we analyzed the AFP-specific CD4+ T-cell response in 22 patients with HCC and compared it to the CMV-specific CD4+ T-cell response in the same cohort of patients. For these experiments, PBMCs from HCC patients were stimulated with overlapping peptides spanning the entire AFP protein for 5 hr before IFN-γ staining. As shown in Figure 1a, we did not detect any AFP-specific IFN-γ production ex vivo. In contrast, 13 of these patients (59%) displayed a CMV-specific CD4+ T-cell response that is in a similar range as previously described by us and other groups.21,28,29 Original dot blots from these data are shown in Figure 1c. Results were similar in healthy donors: three of the eight healthy donors (38%) displayed a CD4+ T-cell response against CMV, whereas we were unable to detect specific CD4+ T-cell responses directed against AFP in these subjects (Fig. 1b). Thus, the results clearly indicate that HCC patients do not have a general impairment of their CD4+ T-cell response but rather a specific lack of AFP-specific CD4+ T-cell responses.

**Detection of AFP-specific CD4+ T-cell responses after antigen-specific expansion**
Next, we set out to determine whether AFP-specific CD4+ T-cell responses can be detected after antigen-specific expansion. For these experiments, PBMCs derived from HCC patients and healthy donors were stimulated with the whole AFP protein for 10–12 days and subsequently tested for AFP-specific responses to overlapping peptides covering the entire AFP protein. Importantly, by using this approach, we were unable to detect AFP-specific CD4+ T-cell responses in healthy donors (Fig. 2a). In contrast, AFP-specific CD4+
T-cell responses were readily detectable in 15 of 29 (52%) HCC patients tested (Fig. 2a). In these 15 patients, a median of 2 [range: 1–8] epitopes was targeted, and these responses were found in a frequency between 0.035 and 3.43%. Original dot blots from these results are shown in Figure 2b. All positive peptide-specific CD4⁺ T-cell responses are listed in Table 2. It is important to note that the AFP-specific CD4⁺ T-cell responses were heterogeneous and spread over the entire AFP protein with no consistently recognizable immunodominant epitopes (Fig. 2c).

In 13 of 18 patients (72%) with elevated AFP level (>7 ng/mL) AFP-specific CD4⁺ T-cell responses were detectable. In two remaining patients showing T-cell responses, serum AFP level was not determined. Patients with normal serum AFP level (\(n = 10\)) did not show responses. Statistical analysis revealed that an elevated serum AFP level correlated significantly with the presence of AFP-specific CD4⁺ T-cell responses in the peripheral blood in our study (\(p = 0.0003\), obtained by Fisher’s exact test). The serum AFP level of responding patients was predominantly mildly elevated and significantly higher compared to that of nonresponding patients (\(p = 0.024\), Fig. 2d). This is in agreement with the study by Behboudi et al., who could detect CD4⁺ T-cell responses to AFP mainly in patients with mildly elevated serum AFP level.¹⁹ However, in contrast to our findings, responding patients in that study showed significantly lower serum AFP levels compared to nonresponding patients.

Further analysis of the effector functions of 12 AFP-specific IFN-γ-producing CD4⁺ T-cell responses revealed that some of these cells were also able to produce TNF-α, whereas only one response was able to degranulate (CD107a⁺/1/12) and two produced IL-2 (2/12) (Supporting Information Fig. S1a). Interestingly, CMV-specific CD4⁺ T cells showed a similar functional profile, although with a
higher tendency to degranulate (3/5) (Supporting Information Fig. S1a). Original dot blots from these data are shown in Supporting Information Figure S1b.

**Analysis of de novo AFP-specific CD154 (CD40 ligand) expression**

The fact that IFN-γ-producing AFP-specific CD4+ T-cell responses are readily detectable after antigen-specific expansion but not ex vivo raises the important question of whether AFP-specific CD4+ T cells might indeed be present ex vivo but primarily impaired in specific effector functions, such as IFN-γ production. To address this issue, we determined de novo CD154 expression in response to AFP antigens in selected patients who showed AFP-specific IFN-γ production after antigen-specific expansion. De novo CD154 expression is a sensitive ex vivo assay that has the advantage of being
| ID  | Peptide no. | Amino acid | AFP-spec. expansion PBMC %IFNγ+/CD4+ | Unspecific expansion PBMC %IFNγ+/CD4+ | ITL %IFNγ+/CD4+ |
|-----|-------------|------------|-------------------------------------|----------------------------------------|----------------|
| HCC 2 | 23 | 177–194 | 0.04                               | --                                    | --             |
| HCC 3 | 23 | 177–194 | 0.09                               | --                                    | --             |
| HCC 4 | 24 | 185–202 | 0.08                               | --                                    | --             |
| HCC 5 | 25 | 193–210 | 0.05                               | --                                    | --             |
| HCC 6 | 30 | 233–250 | 0.04                               | --                                    | --             |
| HCC 7 | 25 | 185–202 | 0.04                               | --                                    | --             |
| HCC 8 | 28 | 217–234 | 0.08                               | --                                    | --             |
| HCC 9 | 42 | 329–346 | --                                 | 0.05                                  | --             |
| HCC 10 | 43 | 337–354 | --                                 | 0.04                                  | --             |
| HCC 11 | 44 | 345–362 | --                                 | 0.06                                  | --             |
| HCC 12 | 47 | 369–386 | --                                 | 0.04                                  | --             |
| HCC 13 | 52 | 409–426 | --                                 | 0.07                                  | --             |
| HCC 14 | 53 | 417–434 | --                                 | 0.07                                  | --             |
| HCC 15 | 54 | 425–442 | --                                 | 0.16                                  | --             |
| HCC 16 | 55 | 433–450 | --                                 | 0.35                                  | --             |
| HCC 17 | 56 | 441–458 | 0.07                               | --                                    | --             |
| HCC 18 | 61 | 481–498 | 0.06                               | --                                    | --             |
| HCC 19 | 65 | 513–530 | 0.07                               | --                                    | --             |
| HCC 20 | 7 | 49–66 | 0.07                               | --                                    | --             |
| HCC 21 | 64 | 505–522 | 0.04                               | --                                    | --             |
| HCC 22 | 66 | 521–538 | 0.05                               | --                                    | --             |
| HCC 23 | 45 | 353–370 | 0.11                               | --                                    | --             |
| HCC 24 | 52 | 409–426 | 0.14                               | --                                    | --             |
| HCC 25 | 75 | 593–610 | --                                 | --                                    | 0.07           |
| HCC 26 | 36 | 281–298 | 0.17                               | --                                    | --             |
| HCC 27 | 42 | 329–346 | 0.05                               | --                                    | --             |
| HCC 28 | 1 | 1–18 | --                                 | --                                    | 0.79           |
| HCC 29 | 32 | 249–266 | 3.43                               | nd                                    | nd             |
| HCC 30 | 33 | 257–274 | 0.67                               | nd                                    | nd             |
| HCC 31 | 13 | 97–114 | 0.12                               | nd                                    | nd             |
| HCC 32 | 14 | 105–122 | 0.04                               | nd                                    | nd             |
| HCC 33 | 15 | 113–130 | 0.11                               | nd                                    | nd             |
| HCC 34 | 17 | 129–146 | 0.13                               | nd                                    | nd             |
| HCC 35 | 18 | 137–154 | 0.07                               | nd                                    | nd             |
| HCC 36 | 19 | 145–162 | 0.15                               | nd                                    | nd             |
| HCC 37 | 20 | 153–170 | 0.04                               | nd                                    | nd             |
| HCC 38 | 21 | 161–178 | 0.04                               | nd                                    | nd             |
| HCC 39 | 52 | 409–426 | 0.09                               | nd                                    | nd             |
| HCC 40 | 45 | 353–370 | 0.04                               | nd                                    | nd             |
| HCC 41 | 24 | 185–202 | 0.26                               | nd                                    | nd             |
| HCC 42 | 26 | 201–218 | 0.21                               | nd                                    | nd             |
robust and independent from the HLA type and previous knowledge of AFP epitopes. We have recently used this approach to show that HCV-specific CD4⁺ T cells are indeed present *ex vivo* but are unable to perform effector functions, such as IFN-γ production.²¹ In case of HCC, however, as shown in Figure 3a, even by using this sensitive approach, we were unable to detect AFP-specific T-cell responses *ex vivo*, whereas CMV- and TT-specific responses were readily detectable. These results clearly indicate that AFP-specific CD4⁺ T-cell responses are not detectable in the peripheral blood of patients with HCC, at least when using the most sensitive methods currently available. However, they can be expanded by antigen-specific stimulation indicating that they are not completely deleted from the peripheral pool albeit present at a very low frequency.

**Analysis of intratumoral AFP-specific CD4⁺ T-cell responses**

The relative rarity of circulating AFP-specific CD4⁺ T cells in the peripheral blood could be explained by a rapid accumulation of these cells within the tumor tissue. To address this question, the intratumoral AFP-specific CD4⁺ T-cell response was analyzed in a total of 12 patients. Liver-derived lymphocytes were either isolated from diagnostic liver biopsies (n = 7) or from potentially curative liver resections (n = 5) and expanded nonspecifically as described in the Material and Methods section. The antigen nonspecific expansion is necessary to obtain a sufficient amount of lymphocytes required for this comprehensive analysis. Lymphocytes derived from the peripheral blood were expanded in the exact same manner to allow a strict comparison of the strength and hierarchy of the immune response between the two compartments. In addition, the AFP-specific CD4⁺ T-cell response was also analyzed after AFP-specific expansion in 11 of these patients (Figs. 4a and 4b). Importantly, as shown in Figure 4, AFP-specific CD4⁺ T-cell responses, each targeting a single epitope, were detectable in the tumor-derived lymphocytes of only 2 of 12 (17%) patients. Similarly, AFP-specific CD4⁺ T-cell responses were detectable in the blood of only 1 of 12 (8%) patients analyzed, although this patient (HCC3) did mount a response to eight different epitopes (Figs. 4a and 4b, Table 2). This patient had a mildly elevated serum AFP level and was therapy naive. Figure 4c shows original dot blots of an intratumoral CD4⁺ T-cell response to AFP. As expected, antigen-specific expansion of PBMC led to the detection of AFP-specific responses in 7 of 11 (64%) patients, again suggesting that these cells are present at least at low frequencies (Figs. 4a and 4b).

**Discussion**

Our study was performed to analyze the peripheral and intratumoral AFP-specific CD4⁺ T-cell responses in patients with HCC. The first important finding of our study is that these responses are almost completely absent *ex vivo* in this patient cohort. Indeed, by using sensitive approaches, such as ICS, we failed to detect AFP-specific CD4⁺ T-cell responses *ex vivo* in the peripheral blood. Importantly, however, by using control antigens such as CMV or TT, we were easily able to detect antigen-specific immune responses, clearly indicating the specific absence of AFP-specific CD4⁺ T-cell responses *ex vivo* and not a general state of tumor-induced T-cell

**Table 2. AFP-specific CD4⁺ T-cell responses (Continued)**

| ID  | Peptide no. | Amino acid | AFP-spec. expansion PBMC %IFN-γ⁺/CD4⁺ | Unspecific expansion PBMC %IFN-γ⁺/CD4⁺ | ITL %IFN-γ⁺/CD4⁺ |
|-----|-------------|------------|---------------------------------------|----------------------------------------|------------------|
| 29  | 225–242     | 0.15       |                                       |                                        |                  |
| 32  | 249–266     | 0.62       |                                       |                                        |                  |
| 33  | 257–274     | 0.25       |                                       |                                        |                  |
| HCC 22 | 37     | 289–306   | 0.08                                   | nd                                     | nd               |
| 42  | 329–346     | 0.06       |                                       |                                        |                  |
| 58  | 457–474     | 0.04       |                                       |                                        |                  |
| 59  | 465–482     | 0.05       |                                       |                                        |                  |
| 60  | 473–490     | 0.09       |                                       |                                        |                  |
| 61  | 481–498     | 0.07       |                                       |                                        |                  |
| 64  | 505–522     | 0.09       |                                       |                                        |                  |
| 66  | 521–538     | 0.23       |                                       |                                        |                  |
| HCC 26 | 21     | 161–178   | 0.05                                   | nd                                     | nd               |
| HCC 27 | 28     | 217–234   | 0.08                                   | nd                                     | nd               |
| 29  | 225–242     | 0.05       |                                       |                                        |                  |

AFP-specific responses are displayed as percentage of IFN-γ⁺/CD4⁺ cells of total CD4⁺ T cells. Abbreviations: PBMC: peripheral blood mononuclear cell; ITL: intratumoral lymphocytes; nd: not done.
suppression. Moreover, in agreement with a previous study, we were able to detect AFP-specific immune responses after antigen-specific expansion, strongly suggesting that these cells are not completely deleted from the T-cell pool but probably just present at very low frequencies. This is also supported by a study by Alisa et al. who have shown that AFP-specific CD4+ T-cell responses are detectable, but primarily in patients with an early stage of HCC (Okuda I or II) or with AFP levels <1,000 ng/mL, and only after antigen-specific expansion, like in our study. In addition, Evdokimova et al. reported the absence of AFP-specific CD4+ T-cell responses ex vivo but could detect such responses by ELISpot after activation with AFP protein-fed and AdVhAFP-engineered dendritic cells. Thus, these combined results clearly indicate the absence of AFP-specific CD4+ T-cell responses ex vivo in the peripheral blood of patients with HCC.

Here, by using the highly sensitive and specific CD154 assay, we further extend these findings by showing that AFP-specific CD4+ T cells are indeed almost completely absent from the circulation and not just present in a dysfunctional state. In contrast, by using the same approach, we could recently show that HCV-specific CD4+ T cells are readily detectable in chronic HCV infection although they were also undetectable by functional assays and thus in a dysfunctional state. These results indicate that two different major mechanisms contribute to CD4+ T-cell failure in HCC and HCV: almost complete absence of T cells in HCC and T-cell dysfunction in HCV. This is further supported by our finding that AFP-specific CD4+ T-cell responses were also virtually absent in the tumor tissue. These results clearly indicate that the relative absence of these cells in the blood is not due to a rapid accumulation to the tumor site. This is an important finding because it suggests that the absence of sufficient CD4+ help may be one central mechanism responsible for the failure of AFP-specific immune responses to HCC. Indeed, CD4+ T cells have been shown to be key regulators of the adaptive immune responses in tumor models and chronic viral infections, e.g., HBV and HCV, where the absence of CD4+ T-cell help causes tumor progression or viral persistence.

**Figure 3.** Absence of detectable AFP-specific CD4+ T-cell responses ex vivo by de novo CD154 expression. (a) PBMCs of nine patients showing AFP peptide-specific IFN-γ secretion after antigen-specific expansion were stained ex vivo for de novo CD154 (CD40L) expression on CD4+ T cells by using pools of AFP peptides. Analysis is based on percentage of CD154+/CD4+ cells of total CD4+ T cells. CMV lysate and tetanus toxoid were used as controls. (b) Representative blots of de novo CD154 expression ex vivo. The lymphocyte population was gated on the basis of forward scatter and side scatter. Gating strategy was then used to identify Via-probe-negative lymphocytes. SEB was used as positive control (pos. control). Unstimulated samples (unstim.) served as a negative control. Pool 1 contains AFP peptides 1–11.
The mechanisms responsible for the weak or absent AFP-specific CD4+ T-cell responses in HCC are currently unclear. Possible mechanisms include an AFP-induced impairment of APCs,34 dysfunctional dendritic cells,35 the induction of AFP-specific TGF-β-producing CD4+ T cells36 or the action of CD4+ CD25+ regulatory T cells.10–12 Indeed, high serum AFP levels >2,500 ng/mL have been shown to impair APC function.34 However, in our study, patients with a serum level up to >5,000 ng/mL showed AFP-specific responses; thus, no evidence of a marked immune-suppressive effect of serum AFP up to this level could be observed although only a few patients with high serum levels were enrolled in our study. It is also possible that AFP-specific CD4+ T cells undergo deletion during thymic migration, as AFP is a tumor-associated, but not completely tumor-specific antigen. The readily detection of AFP-specific CD8+ T-cell responses, however, argues against the thymic deletion of AFP-specific T cells as a major mechanism.5

Our results may have important implications for vaccine design in HCC. Indeed, to develop a successful vaccine, it will be important to induce both arms of the adaptive immune responses, CD8+ and CD4+ T cells. Studies in other tumors have clearly demonstrated that the combination of CD4+ and CD8+ T-cell epitopes provides a more powerful and long-lasting immunity than CD8+ epitopes alone.37 In addition, autologous DC vaccination using a tumor cell line lysate that allows presentation of CD4+ and CD8+ T-cell epitopes has been shown to be safe and to have antitumor

Figure 4. Lack of AFP-specific CD4+ T-cell responses in the tumor and the peripheral blood after unspecific expansion. (a) CD4+ T cells from PBMC and intratumoral lymphocytes (ITL) derived from 12 HCC patients were analyzed by using overlapping peptides spanning the entire AFP followed by intracellular IFN-γ secretion assay. In addition, PBMCs of 11 of these patients were expanded specifically as described in Figure 2a and analyzed for intracellular IFN-γ secretion by CD4+ T cells (AFP-spec. stim.). Each dot displays the sum of all peptide-specific responses for a respective patient. Analysis is based on percentage of CD4+/IFN-γ+ cells of total CD4+ population. (b) Percentages of patients showing AFP-specific CD4+ T-cell responses after nonspecific expansion and after AFP-specific expansion are displayed as bars. (c) Representative plots of AFP-specific CD4+ T-cell response directed against peptide 1 (AFP1–18) in the intratumoral compartment after nonspecific expansion. The lymphocyte population was gated on the basis of forward scatter and side scatter. It was then gated on Via-probe-negative lymphocyte population. As a negative control, unstimulated samples (unstim.) were used. PMA served as positive control (pos. control).
efficacy even with generation of antigen-specific immune responses in the serum of patients with HCC. However, for the successful development of peptide-based immune therapies, it will be important to identify dominant AFP-specific CD4+ and CD8+ T-cell epitopes, as until now only a few such epitopes have been identified. In our study, we were unable to detect a clear immunodominance of the CD4+ and CD8+ T-cell responses that were spread across the entire AFP protein. This is similar to our findings regarding AFP-specific CD8+ T-cell responses in patients with HCC. These combined results suggest that AFP-specific immunotherapeutic strategies should include the whole AFP, allowing the endogenous processing and presentation of several different AFP-specific CD4+ and CD8+ T-cell epitopes, which may have better clinical efficacy than vaccination with a limited set of AFP peptides.

In sum, the results of our study demonstrate the almost complete absence of AFP-specific CD4+ and CD8+ T-cell help in the peripheral blood ex vivo and in tumor tissues. This is in contrast to the easy detection of AFP-specific CD8+ T-cell responses in the same compartments, as previously described. These results clearly suggest that AFP-specific CD4+ and CD8+ T-cell responses are differentially regulated and that the absence of sufficient help is a hallmark of HCC-specific immune responses, probably explaining the failure of these responses to control HCC progression.

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