

EASL meeting report

Immunological techniques in viral hepatitis

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The need to quantitate and monitor immune responses of large patient cohorts with standardized techniques is increasing due to the growing range of treatment options for hepatitis B and hepatitis C, the development of combination therapies, and candidate experimental vaccines for HCV. In addition, advances in immunological techniques have provided new tools for detailed phenotypic and functional analysis of cellular immune responses. At present, there is substantial variation in laboratory protocols, reagents, controls and analysis and presentation of results. Standardization of immunological assays would therefore allow better comparison of results amongst individual laboratories and patient cohorts. The EASL-sponsored and AASLD-endorsed Monothematic Conference on Clinical Immunology in Viral Hepatitis was held at the University College London, United Kingdom, Oct 7–8, 2006 to bring together investigators with research experience in clinical immunology of hepatitis B virus (HBV) and hepatitis C virus (HCV) infections for in-depth discussion, critical evaluation and standardization of immunological assays. This report summarizes the information presented and discussed at the conference, but is not intended to represent a consensus statement. Our aim is to highlight topics and issues that were supported by general agreement and those that were controversial, as well as to provide suggestions for future work.

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1. Introduction

In preparation of the conference, speakers and moderators completed a questionnaire on immunological research practices instituted in their laboratories. The presentations at the conference reviewed the advantages, disadvantages and pitfalls of individual techniques

according to specific topics (Table 1). The conference participants consisted of senior investigators (59% MDs, 32% PhDs), the majority with hands-on experience in the discussed techniques during the past 5 years in either academic (63%) or industry (18%) settings (Table 2). Eighty-one percent of the conference participants were actively studying immune responses either

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Abbreviations: ACD, acid citrate dextrose; CFSE, carboxyfluorescein diacetate succinimidyl ester; CPDA, Citrate phosphate dextrose; cpm, counts per minute; CTL, cytotoxic T-cell; DC, dendritic cells; DMSO, dimethylsulfoxid; EDTA, ethylenediaminetetraacetic acid; ELISpot, enzyme-linked immunospot; FBS, fetal bovine serum; FCC, flow cytometry cytotoxic T-cell assay; GM-CSF, granulocyte macrophage colony-stimulating factor; HBV, hepatitis B virus; HCV, hepatitis C virus; HCVpp, retroviral HCV pseudoparticle; IC70, 70% inhibitory capacity; mDC, myeloid dendritic cell; MHC, major histocompatibility complex; MIP-1 α , macrophage inhibitory protein 1 α ; MIP-1 β , macrophage inhibitory protein 1 β ; NK, natural killer cell; NKT, natural killer T-cell; PBMC, peripheral blood mononuclear cells; PBS, phosphate-buffered saline; pDC, plasmacytoid dendritic cell; RANTES, regulated upon activation, normal T-cell expressed, and secreted; RPMI, Roswell Park Memorial Institute cell culture medium; TLR, toll-like receptor; TNF- α , tumor necrosis factor α .

Table 1
Faculty participants

Faculty member	Institution	Topic
Barbara Rehermann	National Institutes of Health, USA	Immunological Assays in Hepatitis: The Problem of Standardization
<i>Session on Innate Immune Responses (Moderator: David Adams)</i>		
Pablo Sarobe	University of Navarra, Spain	Dendritic Cells
Ulrich Spengler	University of Bonn, Germany	NK Cell Assays
<i>Ex vivo Quantitation of T Cell Responses (Moderator: Nikolai Naoumov)</i>		
Heiner Wedemeyer	Hannover Medical School, Germany	ELISpot Assays
Paul Klenerman	University of Oxford, UK	HLA class I and II Tetramers and Intracellular Cytokine Staining
Micheline Nascimbeni	Institute Cochin, France	FACS-based Assays for Cytokine Production
Ian McInnes	University of Glasgow, UK	Lessons from Analysing T Cell Responses in Rheumatology
<i>Characterizing T Cell Specificity (Moderator: Barbara Rehermann)</i>		
Georg Lauer	Massachusetts General Hospital, USA	T Cell Epitope Mapping
Gabriele Missale	University of Parma, Italy	Generation and Application of T Cell Lines and Clones
David Bowen	Columbus Children's Research Institute, USA	Analysis of Liver-Infiltrating Lymphocytes
<i>Specific CD4, CD8 and B Cell Effector Functions (Moderator: Vincenzo Barnaba)</i>		
Helmuth Diepolder	University of Munich, Germany	Assays for CD4 T Cell Functions
Kyong-Mi Chang	University of Pennsylvania & VA Medical Center, USA	Assays for Cytolytic Effector Functions
Mario Mondelli	University of Pavia, Italy	Characterization of B Cells
<i>The Virus and Modeling of Virus Host Interaction (Moderator: Jean-Michel Pawlotsky)</i>		
Jane McKeating	University of Birmingham, UK	Analysis and Quantitation of Neutralizing Antibodies
Stuart Ray	Johns Hopkins University, USA	Analysis of Viral Sequence Evolution in Relation to Immune Selection Pressure
Alan Perelson	Los Alamos National Laboratory, USA	Mathematical Modeling

during the natural course of viral hepatitis (35%), during therapy with interferon/ribavirin and/or antivirals (25%) or during vaccine and/or immunotherapy trials (21%). Two-thirds of the audience had coauthored publications on immune responses in viral hepatitis and/or other diseases (Table 2). During the conference, the audience was polled on the same questions on laboratory practices as the speakers, which served as a starting point for discussions.

2. History of immunological techniques employed to study immune responses in viral hepatitis

Historically, the first assays to study HBV- and HCV-specific T-cell responses were based on in vitro expansion of virus-specific T-cells. Proliferation assays were the first approach to assess CD4 T-cell responses to HBV antigens. Peripheral blood mononuclear cells (PBMCs) were stimulated with recombinant viral proteins. CD4+ T-cell lines and clones were established from blood or liver of infected patients [1–4] and chimpanzees [5,6]. For assessment of CD8+ T-cell responses, PBMCs were stimulated with short synthetic peptides for 2–3 weeks and then assessed for their ability to kill patient-derived autologous EBV-B cell lines loaded with the same peptide or infected with recombinant vaccinia viruses [7–9]. The advantage of this technique was its

high sensitivity, because it combined the in vitro expansion of low-frequency HBV-specific CD8 T-cell populations with an assessment of their effector function. Disadvantages were the preselection of peptides based on HLA-binding motifs and the possible loss of low-avidity T-cells during in vitro expansion.

While in vitro expansion techniques were very useful for the identification of CD4+ and CD8+ T-cell epitopes, it soon became necessary to quantitate the number of epitope-specific T-cells. Using an in vitro expansion technique, this was first performed with limiting dilution cultures to estimate the frequency of cytotoxic T-cell precursors [10,11]. When ex vivo techniques such as ELISpot and tetramer-technology became available, however, it became evident that the number of virus-specific T-cells in the blood was much higher than previously estimated [12]. Both the ELISpot and tetramer assays allowed direct ex vivo quantitation of virus-specific T-cells without in vitro expansion. In addition, the tetramer technology allowed the detection of virus-specific T-cells independent of their function. Further developments concerned the synthesis and use of large panels of overlapping peptides spanning entire viral proteins. This facilitated a comprehensive assessment of all T-cell specificities, quantitation of both CD4+ and CD8+ T-cell responses in a single assay in the context of all given HLA-alleles and, when peptide pools were set up in a matrix format, simultaneous identification of candidate epitopes [13,14].

Table 2
Demographics of conference participants (*n* = 170)

Occupation	
Academia	63%
Clinical patient care and laboratory	34%
Laboratory research or diagnostics	29%
Hospital	
Clinical patient care	17%
Industry	
Research and development	18%
Clinical Trials	10%
Marketing	7%
None of the above	1%
None of the above	2%
Primary qualification	
Degree in medicine	59%
PhD in biomedical science	25%
PhD in fields other than biomedical science	7%
Student	4%
Time since graduation	
<5 years	17%
5–10 years	32%
10–20 years	38%
>20 years	13%
Level of experience with immunological assays discussed at this conference	
Hands-on experience at present or in the last 5 years	65%
Hands-on experience only during training	14%
No hands-on experience but theoretical knowledge from publications	14%
No experience	7%
Where was training acquired	
In dedicated research laboratory for >12 months	50%
In dedicated research laboratory for <12 months	13%
General training in immunology	17%
No training in immunology	20%
Co-author of publications on the immune response in viral hepatitis or other diseases	
Yes	63%
Less than 5 publications	36%
10–20 publications	16%
More than 20 publications	11%
No	37%
Involved in projects investigating the immune response in viral hepatitis	
Natural history/outcome of infection	35%
Interferon and/or antiviral agents	25%
Vaccine trials and/or immunotherapy	21%
Animal models	2%
None of the above	13%

3. Shipment, freezing and thawing of blood samples for immunological assays

One of the most important and often overlooked aspect of immunological studies concerns the handling of blood samples prior to the actual experiments (Fig. 1). The first consideration is the choice of the anticoagulant (Fig. 1a). Whereas sodium heparin was the most commonly used anticoagulant by conference

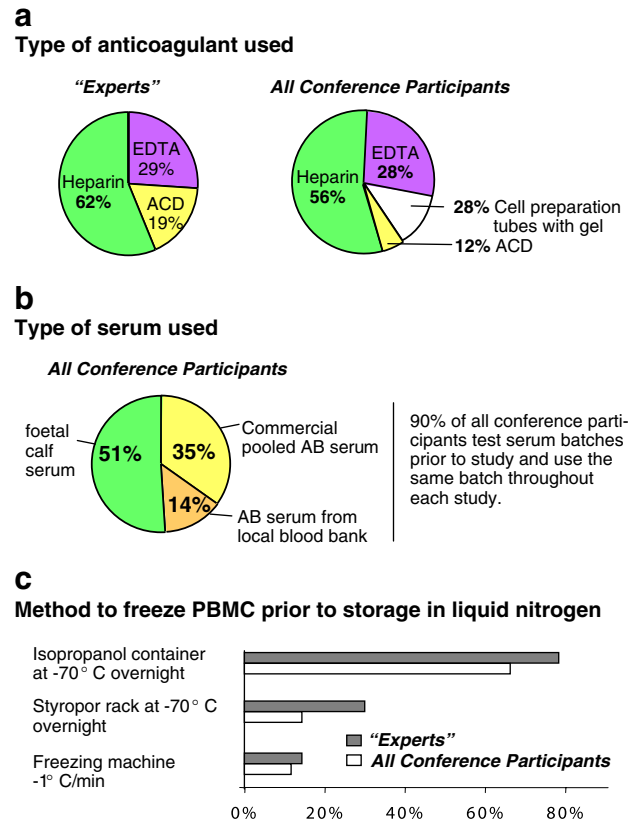


Fig. 1. General considerations for immunological assays. The answers to the indicated questions reflect laboratory practices of “experts” (21 international laboratories including those of the speakers and moderators polled prior to the conference) and those of 170 conference participants. The data in (c) reflect multiple answers per person.

participants, one needs to be aware that heparin may activate cells, especially macrophages and monocytes, and therefore affect expression of sensitive activation markers such as CD69 and CD62L. In contrast, calcium chelators (EDTA, ACD or CPDA) render activation of PBMC more difficult, especially when subsequent washing steps are conducted in Ca- and Mg-free PBS.

A second consideration is that large, multicenter clinical studies often use a central research laboratory for the immunological assays. This necessitates the shipment of whole blood or isolated PBMC either in RPMI medium with 10% serum at controlled temperature or cryopreserved and shipped on dry ice [15]. For shipment of whole blood, ACD and CPDA are the preferred anticoagulants because dextrose increases cell survival and maintains cell functionality. On the other hand, shipment of cryopreserved cells has the advantage that PBMC from different time points can be thawed later and tested simultaneously, thus reducing inter-assay variability. Conference participants generally agreed that cryopreservation does not adversely affect immunological readouts such as tetramer and intracellular cytokine staining and ELISpot

analyses, but it may significantly reduce cell numbers, proliferative capacity and expression of activation markers and chemokine receptors.

The third consideration is the technique for cryopreservation and thawing cells, which was discussed in detail. Almost all (95%) of the conference participants use cryopreservation medium containing 80–90% FBS, 10% DMSO and 0–10% RPMI medium. The vast majority (67%) of participants froze cryovials overnight in an isopropanol container at -70°C prior to long-term storage in the vapor phase of liquid nitrogen (Fig. 1c). Whereas there was agreement that at least 80% cell viability is required for subsequent immunological assays, the importance of the thawing protocol and the preparation of the cells prior to the assay appeared underestimated. Recommendations to increase cell viability included step-wise dilution of the thawed cell suspension in PBS over a period of up to 10 min, in order to decrease osmotic stress, and use of DNase to prevent clumping of thawed PBMC [16]. It was also recommended to allow cells to “rest” for several hours or overnight in complete cell culture medium at 37°C , 5% CO_2 prior to cell counting and start of the immunological assay, in order to overcome functional variability between samples. This is critical for assays such as cytokine ELISpots which are based on defined “input” cell numbers.

4. Types of immunological and virological analyses

T-cell assays with PBMC from patients were the most commonly used assays and performed by 85% of the conference participants (Table 3). B-cell or antibody assays were performed by 39% and viral sequence evolution was investigated by 31% of the conference participants. About a third of the participants also analysed dendritic cell functions and about the same percentage studied NK/NKT-cells (Table 3). The following paragraphs therefore describe the advantages, disadvantages

and pitfalls of individual assays that are most commonly used for these purposes. Recommendations for assay details that should be included in published manuscripts are also provided (Table 4).

5. Analysis of T-cell responses

5.1. Cytokine ELISpot assay

ELISpot assays were first established to quantitate antibody-secreting B-cells [17] and were then modified to detect cytokine-secreting T-cells [18]. ELISpot assays were the method of choice to monitor HBV- and HCV-specific immune responses, followed by proliferation assays, tetramer staining and intracellular cytokine staining (Fig. 2). ELISpot assays can be performed in a semiautomated manner, allowing rapid screening of many individual, antigen-specific responses in complete PBMC. Using overlapping peptides, the breadth of CD4 and CD8 T-cell responses in the context of all autologous HLA alleles can be analysed and multiple cytokines can be tracked simultaneously [19]. The ELISpot assay was considered superior to the proliferation assay because it yields good responses even with cryopreserved PBMC [20]. It was considered superior to the intracellular cytokine staining/flow cytometry assay because of its 10- to 100-fold higher sensitivity [21].

Several critical ELISpot parameters were discussed in detail. First, it was emphasized that each batch of primary and secondary antibody should be titrated alone and in combination to determine the optimal concentration. The primary antibody is usually a monoclonal antibody with high affinity for the lymphokine of interest, whereas the detection antibody often binds to several epitopes to better amplify the signal (for a list of antibody combinations, see [22]).

Although 15- to 20-mer peptides detect both CD4 and CD8 T-cell responses, 20-mer peptides were considered more useful than 15-mer peptides for detection of CD4+ T-cell responses, whereas much shorter peptides (ideally shorter than 12 amino acids) are optimal for detecting CD8+ T-cell responses [23,24]. It is important to recognize that even by using overlapping peptides one may never detect all T-cell responses because (i) peptides are usually longer than the optimal epitopes, (ii) epitopes may be located in the overlap between peptides or in hydrophobic regions and (iii) peptide sequences commonly do not match the sequence of the infecting virus.

Cultures should be set up in at least duplicate, but ideally triplicate wells with at least 200,000 cells/well to allow calculation of standard deviations (Fig. 3a and b). Controls must be included on each plate and consist of a mitogen and a recall antigen, such as tetanus toxoid or CMV lysate as positive controls; medium only

Table 3
Immunological parameters studied

Parameter	% of conference participants studying this parameter
T cells	85
Antibodies and/or B cells	39
Antibodies, but not B cells	21
B cells	18
Viral Sequence evolution	31
Dendritic cells	30
Direct ex vivo functions of plasmacytoid and myeloid DC	54
In vitro matured, monocyte-derived DC	46
NK cells	21
NKT cells	14

Table 4
Recommendations on experimental details to be included in the methods section of publications

Issue	Required information
Sample preparation	Report use of fresh or frozen cells, type of anticoagulant; cell viability; length of resting period between thawing and assay
ELISpot analysis	Report concentrations and clones of the used antibodies; use of fresh or frozen; rested or not rested cells; assay duration; number of replicates per condition; type of positive and negative controls and criteria for cut-off (ideally both internal comparison to negative control wells and external comparison to uninfected control subjects)
Intracellular cytokine staining	Report minimal number of positive events acquired; exclude dead cells by flow cytometry; include representative dot plots; establish threshold of positivity with unstimulated cells from patients with hepatitis and with stimulated cells from seronegative control subjects
Tetramer staining	Evaluate background staining with PBMC from HLA-matched, uninfected subjects and/or from HLA-mismatched, infected subjects; report number of tetramer+ events in cases where frequencies are very low or populations are enriched by using magnetic beads; exclude dead cells by flow cytometry; include representative dot plots
Proliferation assay	Indicate maximum cpm of incorporated [³ H]-thymidine and range of cpm in negative controls, in addition to how the stimulation index was calculated.
Cytotoxicity assay (⁵¹ Cr release)	Indicate spontaneous and maximum lysis of target cells; antigen concentration; effector to target ratio

and wells with irrelevant proteins or peptides as negative controls. Ideally, each assay should also include PBMC from high responders and nonresponders as external standards [25].

If an automated ELISpot reader is used, the spot size, mean and maximum spot intensity, circularity, mean well intensity and contrast that defines a positive spot should be described and consistently used [26]. These parameters need to be set specifically for the target cell population and the cytokine of interest. For example,

IL-10 spots by T-cells are several times larger than IL-10 spots by monocytes [27].

To allow comparison of published results, publications should include information on concentrations and clones of the antibodies, the use of fresh or frozen, rested or not-rested cells, culture time, number of replicates per condition, type of positive and negative controls and the criteria for definition of a positive result (Table 4). There was active debate on the definition of the cut-off of positivity. As indicated in Table 5, the absolute number of cytokine spots does not necessarily indicate a vigorous response, because the background (spots in the absence of antigen) might be high. Whereas 63% of 21 polled international immunological laboratories used an internal cut-off with a positive response defined as being at least 2-fold higher than the background in the cultures without antigen, only 42% used an external control group with positive responses defined as being higher than the mean plus 2 or 3 standard deviations of the response of uninfected subjects and less than 25% applied both an internal and external control. Ideally, both internal and external controls should be used to calculate the cut-off.

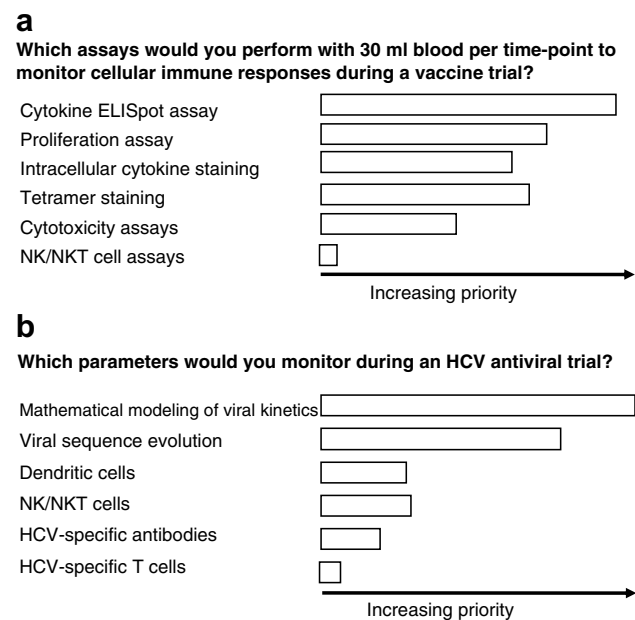


Fig. 2. Assays of choice to monitor immune and viral parameters during vaccine and antiviral trials. Conference participants were asked to rank the respective assays (a) and studied parameters (b) from highest to lowest priority.

5.2. Intracellular cytokine staining and flow cytometry

There was general consensus among conference participants that detection of intracellular cytokines by flow cytometry is not sensitive enough to detect low frequency HCV-specific T-cells ex vivo in HCV infection, except in some cases with acute hepatitis C. This technique is more useful if applied on antigen-specific T-cell lines to “deconvolute” recognized peptide mixtures [13] and to analyse whether responses are mediated by CD4 or CD8 T-cells. The related cytokine secretion assays, in which a specific reagent fixes the secreted cytokines to the surface of the cell that secretes them, have the addi-

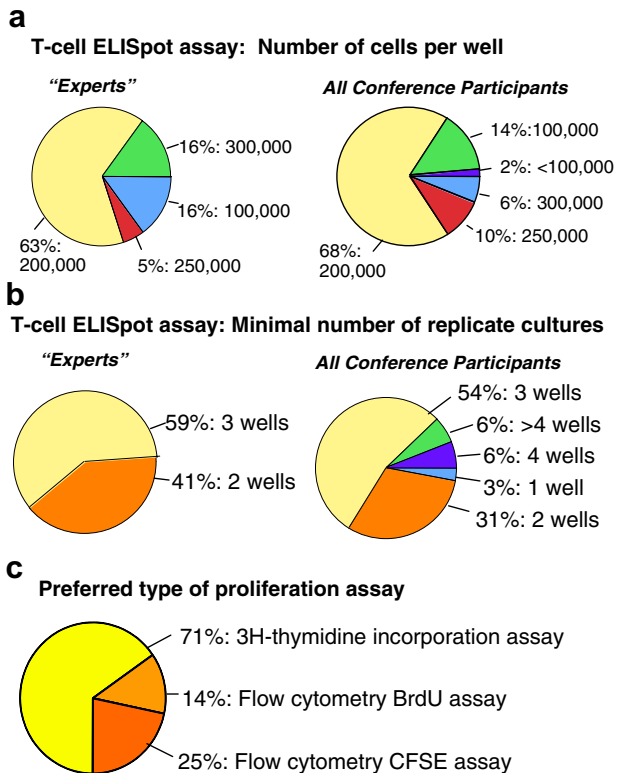


Fig. 3. Conditions for ELISpot and proliferation assays. The answers to the indicated questions reflect laboratory practices of “experts” (21 international laboratories including those of the speakers and moderators polled prior to the conference) and those of 170 conference participants.

tional advantage that cytokine-secreting cells can be positively selected with magnetic beads and cultured further or cloned.

It was noted that the addition of antibodies against costimulatory molecules CD28 and CD49d increases the cytokine response 3- to 4-fold and that antibodies against CD8 β are preferred for simultaneous phenotyping, because antibodies against CD8 α do also stain NK and NKT-cells. It should also be considered that activated cells downregulate CD3 and may be difficult to distinguish from CD3-negative cells. In this context, intracellular CD69 may be a useful marker to identify activated cells and also serve as a positive control for intracellular staining and inhibition of protein secretion [28]. When intracellular TNF- α is to be detected, brefeldin A should be included in addition to monensin

because monensin does not sufficiently block TNF- α secretion.

As reported for the ELISpot assay, there is a good correlation between using fresh and frozen/thawed cells [15]. Overnight resting of thawed PBMC at 37 °C increases their functionality and increases the mean fluorescence intensity of positive cells [29].

Another crucial point is the exclusion of dead cells by flow cytometry, which can be achieved using different dyes. Ethidium bromide enters live cells slowly, thus a longer incubation period is required. Ethidium monoazide covalently binds to nucleic acid by illumination with visible light, but results in a relatively low mean fluorescence intensity [30]. Propidium iodide results in intense staining, which may be difficult to compensate for. 7-aminoactinomycin, whose emission spectra overlap less with those of phycoerythrin, is less bright and therefore easier to compensate [31]. It should be considered, however, that the fluorescence intensity of 7-aminoactinomycin staining is affected by fixation and permeabilisation. The addition of actinomycin-D in the steps following fixation reduces transfer of 7-AAD to viable cells that occurs following fixation, and thus allows live/dead cell discrimination post-fixation [32].

It was recommended that dot plots rather than bar graphs or tables should be shown to demonstrate how clearly separated cell populations are. The threshold of positivity should be established with unstimulated cells from patients with viral hepatitis and with stimulated cells from seronegative control subjects.

5.3. Tetramer staining and flow cytometry

If intracellular cytokine staining is to be combined with MHC class I or II tetramer staining, tetramer staining should ideally be performed prior to T-cell stimulation because the latter is associated with T-cell receptor downregulation. The staining pattern of tetramers should be confirmed by analysis of T-cell lines or clones whenever possible [33] and background staining should be evaluated by staining PBMC from HLA-matched, uninfected subjects and from HLA-mismatched, infected subjects. It should be noted that not all tetramer+ cells degranulate or secrete IFN- γ after stimulation. Although MHC class I tetramers have been used to analyse HBV- [34] and HCV-specific [35] T-cells for several years, the frequency of peripheral blood T-cells that can be stained *ex vivo* is usually below the detection limit in most patients with persistent infection [26,34]. It is about 10-fold higher in the liver, but the small number of lymphocytes that can be isolated from a biopsy typically allows only a small selection of tetramers to be tested [34–36]. Because the frequency of MHC class II tetramer-positive cells in viral hepatitis is even lower than that of MHC class I tetramer-positive cells enrichment of tetramer-positive cells with magnetic beads is

Table 5
Impact of control readings on the result of a hypothetical IFN- γ ELISpot assay

Assay	No antigen [IFN- γ spots]	Antigen [IFN- γ spots]	Delta	Ratio
A	50	150	100	3
B	5	50	45	10
C	1	25	24	25

recommended for phenotypic analyses of these cells [37]. Exclusion gating of dead cells, B cells, monocytes, macrophages and irrelevant T-cell populations is recommended to reduce background staining. Dot plots rather than bar graphs should be included to demonstrate the frequency of tetramer-positive events in the CD4- or CD8-positive, as well as in the CD4- or CD8-negative T-cell populations. When frequencies of tetramer+ cells are very low or populations are enriched with magnetic beads, the number of tetramer-positive events should be indicated in published reports.

5.4. Proliferation assays

A vigorous proliferative response to viral antigens was one of the first immunological parameters associated with recovery from acute hepatitis B [38,39] and C [40]. Due to its sensitivity and capacity for semiautomation, conference participants ranked the proliferation assay second after the ELISpot assay for monitoring immune responses during vaccine and antiviral drug trials (Fig. 2). The ^3H -thymidine incorporation assay was preferred (Fig. 3c) due to extensive published data available for comparison, the small number of required cells (as low as 5×10^4 cells/well) and the assay's high-throughput. It was noted that variations in the negative control (proliferation in the absence of antigen) may greatly affect the assay result (stimulation index). Thus, a lower than usual negative control, e.g. 50 cpm rather than 400 cpm ^3H -thymidine incorporated, results in a false 8-fold increase of the stimulation index. Conversely, a higher than usual negative control, e.g. 800 cpm rather than 400 cpm ^3H -thymidine incorporated, which can be due to a large percentage of dying or dead cells in the culture, may falsely decrease the stimulation index twofold. For these reasons, it is recommended to indicate not only the stimulation index but the total cpm of incorporated ^3H -thymidine and/or the range of cpm of the negative controls in publications. The conference participants also noted that PBMC proliferation cannot be equated with CD4+ T-cell proliferation because B-cells and CD8+ T-cells have also been shown to proliferate in response to recombinant viral proteins and/or their breakdown products. Flow cytometry-based assays, such as the carboxyfluorescein diacetate succinimidyl ester (CFSE)- and bromodesoxyuridine-based proliferation assays, or depletion experiments should therefore be used to identify the predominant responding cell populations. Overall, T-cell proliferative responses and cytokine production provide complementary information and may be differentially affected in patients with chronic viral hepatitis or during antiviral treatment [41]. Therefore, these assays should be applied in parallel to characterize the functional profiles of T-cells in chronic viral infections [42].

5.5. Cytotoxicity assays

Cytotoxicity is an essential CD8+ T-cell function and implicated in the killing of virus-infected hepatocytes, that display viral peptides within cell surface MHC class I molecules. Cytotoxic effector function can be examined from either the target or the effector cell perspective. In vitro assays are therefore either a direct measure of cytotoxicity, i.e. based on target cell lysis (^{51}Cr release assay, flow cytometry CTL (FCC) or caspase assay), or an indirect measure, based on the detection of specific molecules that CD8+ T-cells use to lyse target cells (flow cytometric CD107a/b degranulation assay, perforin/granzyme ELISpot assay). The ^{51}Cr -release assay is the historical assay in which cytotoxic T-cell (CTLs) lines are co-cultured for 4–6 h with HLA-matched ^{51}Cr labeled target cells, which do or do not express the cognate antigen. Cytotoxicity is indicated by the amount of ^{51}Cr released into the supernatant from lysed target cells. The advantages of this assay are its high sensitivity (especially when performed with T-cell lines and/or clones) and the direct detection of target cell lysis.

Non-radioactive alternatives include several flow cytometry-based assays. In the CFSE/PKH or FATAL assay, target cells are peptide-loaded, labeled with a fluorochrome such as CFSE and/or PKH-26, co-cultured with effector T-cells and monitored for cell death by disappearance or changes in forward/side scatter characteristics (i.e. fluorescent targets move to the left of the standard flow cytometry live lymphoid gate). In the flow cytometry CTL (FCC) or caspase assays [43], target cells are labeled with two fluorophores covalently linked to peptides with caspase cleavage sites. Activation of caspases in target cells results in cleavage of the peptide and in loss of fluorophore–fluorophore interaction, thereby enabling detection of caspase-activated target cells by flow cytometry or fluorescence microscopy. Since caspase activation occurs shortly after the CTL target cell encounter, this assay can provide an early measure of CTL-mediated apoptosis [43].

Indirect measures of cytotoxicity monitor activation of cytotoxic T-cells rather than lysis of target cells. These include the detection of CD107a/b on the surface of CD8+ T-cells by flow cytometry [44] and the release of granzyme [45] or perforin [46] by ELISpot analysis. As cytotoxic CD8+ T-cells are activated, CD107a/b-positive lytic granules move to the immunological synapse between the CTL and target and release granzymes and perforin. Crucial aspects of the degranulation assay are optimization of the staining conditions (which is best achieved by serial dilution of CD107-specific antibody into stimulated and unstimulated cell suspensions), addition of CD107-specific antibodies prior to T-cell stimulation, addition of monensin to the cell suspension to neutralize the endogenous pH, and rigorous exclusion of dead cells, monocytes and B cells because dead cells

with increased cell permeability will stain positive for CD107 and both monocytes and B cells stain CD107a/b-dull. Although degranulation is a pre-requisite for perforin/granzyme-mediated target lysis, it should also be considered that the granule content depends on the maturation status of the CD8+ T-cell [47] and that granules of central memory cells may not contain perforin or granzymes. Likewise, it should be considered that the 16–24 h ELISpot assay will not distinguish between CD8+ T-cells that express perforin/granzyme *ex vivo* and those that rapidly upregulate them upon *in vitro* stimulation. Thus, it might be advisable to combine these assays with a full phenotypic characterization of the responding cell population.

5.6. Use of T-cell lines and clones

Within the last 10 years, the above-described *ex vivo* assays have largely replaced *in vitro* T-cell expansion techniques. T-cell expansion may introduce a bias for selection of clonal T-cell populations because it is strictly dependent on T-cell differentiation and functional state. T-cell expansion may also change phenotype and precursor frequency of the cell population of interest. Nevertheless, the use of T-cell lines and clones remains important for several purposes. First, T-cell lines and clones are valuable reagents for epitope mapping [4,13], especially for the identification of minimal–optimal epitopes with serial dilutions of truncated peptides, for the definition of HLA-restriction and for the demonstration that the epitope is endogenously processed. Notably, many epitopes have been mapped with T-cell lines generated from liver biopsies, which typically yield too few (3×10^4 – 1×10^5) T-cells for *ex vivo* analysis [5,8,48]. Second, T-cell lines and clones are also useful to analyse the effect of viral sequence mutations on T-cell recognition and activation. In fact, most viral T-cell escape mutations and/or antagonists have been fully characterized with T-cell clones [6,49,50]. Third, the clonality of T-cell response directed to specific epitopes (V-beta analysis, T-cell receptor sequencing) [51] and the crossreactivity of epitope-specific T-cells [52] can be studied with T-cell clones, if confirmed that these responses exist *ex vivo*.

6. Analysis of B-cells and antibodies

6.1. B-cell phenotype and function

B-cell subsets can be characterized phenotypically by flow cytometry using monoclonal antibodies and functionally by proliferation assays and immunoglobulin secretion assays. Phenotypic analysis of B cells requires fresh PBMC, because expression of the activation markers CD69, CD86, and the chemokine receptor CXCR3 is

downregulated on cryopreserved and thawed PBMC. Furthermore, it should be noted that positive selection of CD19/CD20-positive B-cells with magnetic beads results in downregulation of CD19 and CD20 [53].

Naïve B-cells require three signals for optimal activation: antigen or complexing of the B-cell receptor with anti-human immunoglobulin, cognate T-cell help, and innate immunity-derived signals such as unmethylated single-stranded DNA motifs (CpG oligodeoxynucleotides) which stimulate B cells via Toll-like receptor (TLR) 9 [54]. In contrast, memory B cells require only a single signal [54], which does not need to be antigen-specific and may consist of either bystander T-cell help (CD40L plus cytokines such as IL-10 or IL-2) or innate immunity-derived signals (CpG). CpG 2006 (CpG B) should be preferred to other oligodeoxynucleotides because of its exclusive activation of B cells. In contrast, CpG 2216 (CpG A) does stimulate plasmacytoid dendritic cells (pDC) and CpG 2395 (CpG C) stimulates both B cells and pDC [55].

The B-cell ELISpot assay is most useful to determine antigen-specific B-cell frequency. For B cells, the automated ELISpot reader needs to be specifically set to count spots of a more rounded morphology and a greater size. B cell spots typically display a size of 65–162 U as compared to the typical T-cell spot size of 25–30 U (1 U equals 0.01 mm²). Secretion of antigen-specific and non-specific immunoglobulins can also be detected by ELISA in the culture supernatant of stimulated B cells and usually correlates well to the ELISpot results. B cell proliferation can be determined either by the classical [³H]-thymidine incorporation assay or by labeling with CFSE. Assessing proliferation by CFSE staining is superior to ³H-thymidine incorporation because it allows direct identification of the dividing cells.

6.2. Assessment of antibodies that neutralize HCV infection *in vitro*

Whereas antibodies to structural and nonstructural HBV and HCV antigens have been used for more than two decades in diagnostic assays of past or current infection, the role of recently described antibodies that neutralize HCV infection of hepatoma cells *in vitro* is still less clear. Presentations and discussions at the conference focused on assays to detect these latter antibodies, which inhibit HCV glycoprotein-dependent entry of retrovirus-HCV pseudoparticles (HCVpp) [56,57] and/or infection by *in vitro* produced infectious particles of the JFH-1 strain of HCV [58–60]. Using this system, HCV infection can be assessed by either quantifying virus-encoded reporter genes, measuring intra- and extracellular HCV RNA or by determining the frequency of viral antigen expressing cells. Determination of the frequency of HCV-antigen (e.g. NS5A) expressing cells by flow cytometry 48 h after infection was considered

the most precise approach to quantitate the percentage of infected cells. For quantitation, end-point titrations of sera and determination of the antibody concentration with a 70% inhibitory capacity (IC70) were recommended. Other considerations that are yet to be incorporated into the model include the observations (i) that plasma-derived HCV but not in vitro generated virus is associated with lipoproteins, (ii) that the presence of high density lipoproteins modulates the efficacy of in vitro neutralizing antibodies [61,62], and (ii) that in vivo clearance of HCV may also be influenced by Fab and Fc functions of antibodies.

7. Analysis of viral kinetics and sequence evolution

The 3.2 kb HBV and the 9.6 kb HCV genome sequences reflect both ancient and recent selections, from geographic separations (resulting in the major genotypes) to selection driven by humoral and cellular immune responses of previous or current hosts. Presentations and discussions at the conference focused on goal-directed methods to model viral replication and infection kinetics during antiviral therapy and to analyse viral sequence data in the context of host immune responses.

As regards to mathematical modeling, the usefulness of simultaneously studying viral kinetics and immune responses was previously shown [63] and the need for further application was emphasized at the conference.

As regards to viral sequence analysis, possible pitfalls during amplification of the viral genome such as selective and error-prone amplification due to the use of low-fidelity polymerase [64,65], contamination [66], PCR-mediated recombination [67] or template resampling [68] were discussed. According to the recent consensus proposal for HCV nomenclature [69], primer positions should be based on H77 (AF009606), regardless of genotype. Direct sequencing of PCR products can reduce the impact of error-prone amplification, and generate a single sequence from each specimen, thereby reducing cost and simplifying analysis. However, this approach may be challenging for genomic regions that have few conserved sites for primer binding, may fail if there are length polymorphisms, may generate ambiguous results in variable regions, and generally performs poorly when divergent mixtures of viral strains are present. If cloning rather than direct sequencing of PCR products is performed, 10–20 clones from several independent PCR products should be sequenced, increasing the expense and complexity of the analysis. Digital PCR addresses many of the limitations of both cloning and direct sequencing, but is laborious and expensive [70].

For analysis of sequence data a number of critical factors need to be considered, which include but are

Table 6

Useful Websites for analysis of sequence data

Standardization of numbering for primers, oligonucleotides, and oligopeptides
HCV sequence databases
Europe: http://euhcvdb.ibcp.fr/euHCVdb/
US/LANL: http://hcv.lanl.gov
Comparison to reference sequence (database, cohort, individual).
HBV sequence databases
UK: http://www.hpa-bioinfodatabases.org.uk/hepatitis/main.php
Japan: http://s2as02.genes.nig.ac.jp/
HCV sequence databases
Europe: http://euhcvdb.ibcp.fr/euHCVdb/
US/LANL: http://hcv.lanl.gov
Japan: http://s2as02.genes.nig.ac.jp/
Identifying evidence of immune selection pressure
Datamonkey: http://www.datamonkey.org
PAML: http://abacus.gene.ucl.ac.uk/software/paml.html
SNAP: http://hcv.lanl.gov/content/hcv-db/SNAP/SNAP.html
Mega: http://www.megasoftware.net

not limited to comparison to reference sequences (databases, cohort sequences, individual sequences), and identification of evidence of immune selection pressure. For sequence analysis, several databases are provided in Table 6. For evaluation of sequence mutations, comparison to the sequence of the infecting virus or an earlier sequence isolated from the same patient was considered ideal [71,72]. If this is not possible, it was proposed to compare to a consensus sequence rather than to a specific prototype sequence, e.g. to a consensus genotype 1a sequence rather than to a specific genotype 1a prototype such as H77. Finally, all new nucleotide sequences should be submitted to a public database such as EMBL, GenBank, DDBJ databases upon publication of the data.

8. Analysis of NK and NKT-cells and dendritic cells

Natural killer (NK) cells comprise approximately 15% of PBMC and are found at a higher percentage in the liver and some other organs. At least two distinct subsets of NK cells have been described, which are characterized by their relative surface expression of the cellular markers CD16 (also known as FcγRIII) and CD56. CD16^{hi}/CD56^{low} NK cells constitute the major subpopulation in the blood, whereas CD16^{low}/CD56^{hi} NK cells comprise the major fraction of NK cells in the liver. CD16^{hi}/CD56^{low} NK cells are predominantly responsible for natural cytotoxicity and antibody-dependent cellular cytotoxicity and produce only small amounts of inflammatory cytokines. In contrast, CD16^{low}/CD56^{hi} NK cells, which express the high-affinity IL-2 receptor, are a potent source of inflammatory cytokines and chemokines but exert only poor cytolytic activity. NK cell

Table 7

Areas for further development identified by conference participants

1. Need for bench-marking of cellular assays
 - Use of common antigen and peptide panels
 - Standardized definition of positive and negative responses in all assays
 - Establishment of study groups to develop consensus statements for assay standardization
2. Continued search for correlates of cellular immunity and protection
 - Widening the spectrum of cytokines and activation markers studied
 - Development of TGF- β ELISpot, development of more robust multicolor ELISpots
 - Identification of better phenotypic markers to define memory T cell subpopulations
 - Development of assays to assess T cell-dependent and -independent B cells
 - Genotype-specific assessment of neutralizing antibodies
3. Consideration for multicenter trials
 - Development of better methods for cryopreservation and for obtaining higher viability of thawed PBMC
 - Standardization of transport, sample handling and storage conditions according to FDA-Good Laboratory Practices (GLP)-guidelines
 - Development of consensus for statistical analysis of results and assay validation
 - Assessment of standardized samples and protocols in several laboratories to compare results, variability/precision
4. Flow cytometry
 - Update and evolve guidelines for multicolour flow cytometry, new labeling techniques, Q-dots
 - Development of guidelines on adequate statistical analysis of multicolour flow cytometry
 - Optimization of class II tetramer technology
 - Generation of tetramers/pentamers spanning a wider range of MHC alleles
5. Need for additional assay and model development
 - Development of sensitive nonradioactive methodology/assays to measure cytolytic function
 - Improvement of NK cell and dendritic cell functional assays
 - Development of techniques for in situ analysis of virus-specific T cells in liver biopsies
 - Development of new animal models

function is controlled by integration of signals from both inhibitory and stimulatory receptors. The assays available to study NK cell cytotoxicity and cytokine production are similar to those described for T-cells with the exception that the MHC-negative cell line K562 is used as a target in cytotoxicity assays and that chemokines such as CC chemokine ligand 3 (CCL3, also known as MIP1- α), CCL4 (also known as MIP-1 β) and CCL5 (also known as RANTES) can be detected in addition to cytokines such as IFN- γ , tumor necrosis factor α (TNF- α) and granulocyte/macrophage colony-stimulating factor (GM-CSF).

NKT-cells can be defined using a number of different criteria in man – this includes expression of both the NK cell marker CD56 and the T-cell marker CD3 [73], or alternatively expression of a very restricted T-cell receptor repertoire (typically consisting of TCR V α 24 and V β 11 chains in humans) and recognition of glycolipid antigens in the context of the MHC class I molecule CD1d [74]. Classical NKT-cells can be either CD4+ or CD4/CD8-double negative. By contrast, nonclassical NKT-cells encompass TCR $\alpha\beta$ and TCR $\gamma\delta$ T-cells, do not use the T-cell receptor V α 24 chain and do not express the CD8 β -chain [75]. Classical and nonclassical NKT-cells are more abundant in the liver than in other organs and constitute up to 30% of the intrahepatic lymphocyte population [76]. While their natural antigen is not known, the marine sponge antigen α -galactosyl ceramide is used as a reliable experimental tool to activate all classical NKT-cells. CD1d-tetramers can be used to

identify NKT-cells in flow cytometry-based IL-4 and/or IFN- γ assays and CD1d-expressing targets should be used in cytotoxicity assays.

The analysis of dendritic cells (DCs) in viral hepatitis is of interest because both chronic hepatitis B and C are characterized by the lack of T-cell responses against viral antigens and DCs play an important role in the priming of T-cell immunity. The role of DCs, myeloid DC (mDC), and plasmacytoid DC (pDC) is best analysed directly from circulating cells, rather than using monocytes that are differentiated into DC during in vitro culture. Enumeration of DCs is usually carried out by flow cytometry using a combination of antibodies. DCs are defined as lineage (CD3, CD14, CD16, CD19, CD20 and CD56) negative, HLA-DR+ and either CD11c+ (mDC) or CD123+ (pDC). mDC can be cultured in the presence of GM-CSF and pDC can be cultured in the presence of IL-3. In both cases, DCs are subsequently stimulated with different reagents (TLR ligands, pro-inflammatory cytokines, CD40L, etc.) to induce their maturation or used to stimulate T-cells. Mature DCs express high levels of MHC class I and class II molecules, costimulatory molecules (CD80, CD86), adhesion markers (CD54), chemokine receptors (CCR7) and other molecules such as CD40 and CD83. However, expression of these molecules does not predict whether these activated DC are immunogenic or tolerogenic, which requires functional assays. These include analysis of their endocytosis capacity (analysed by flow cytometry using fluorochrome-labeled particles) and

cytokine production (IFN- α for pDC and IL-12, TNF- α and IL-10 for mDC). The latter can be analysed by flow cytometry of PBMC because each DC subset expresses different TLRs, thereby responding to different stimuli (influenza and herpes simplex virus-mediated activation of TLR7 and -9 on pDC and peptidoglycan, lipopeptide, poly(I:C), flagellin-mediated activation of TLR1, 2, 3, 5 and 6 on mDC, respectively). Alternatively, simultaneous labeling with population-specific antibodies and anti-cytokine antibodies allows precise characterization of the responding cell population. Finally, the T-cell stimulatory ability of DCs is usually evaluated in co-culture experiments with allogeneic or autologous T-cells.

9. Conclusions and future recommendations

The Monothematic conference on Clinical Immunology in Viral Hepatitis provided the first opportunity for a focused discussion and critical evaluation of immunological techniques by clinical investigators and translational immunologists. Future efforts should aim at standardization of methods amongst laboratories involved in immune monitoring as part of clinical trials. One initiative in this direction is the use of common reagents. For example, several sets of 18-mer peptides with an 11-amino acid overlap (>80% purity) spanning the 1a (H77), the HCV 1b (J4) and the HCV 3a sequences can be provided by the NIH NIAID Biodefense and Emerging Infections Research Resources Repository (BEI, <http://www.beiresources.org>). Other initiatives may aim at the comparison of laboratory performances with standardized test samples as it has been performed in HIV vaccine trials [77]. Finally, the conference participants identified a number of areas for further development (Table 7) and it is hoped that technological progress in these areas can be achieved and discussed at future conferences.

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