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Direct Recognition of Foreign MHC Determinants by Naive T Cells Mobilizes Specific Vβ Families Without Skewing of the Complementarity-Determining Region 3 Length Distribution[1]

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The capacity of T cells to interact with nonself-APC, also referred to as direct allorecognition, is an essential feature of the cellular response involved in graft rejection. However, there is no study on TCR repertoire biases associated with direct restricted T cell activation. In this paper, we have addressed the impact of direct recognition on the whole naive T cell repertoire, using a new approach that provides, for the first time, an integrated depiction of the quantitative and qualitative alterations in the TCR Vβ transcriptome. This method can differentiate resting patterns from polyclonally activated ones, as evidenced by superantigen usage. According to this new readout, we show that direct recognition of nonself-MHC molecules triggers mRNA accumulation of several TCR Vβ families, specific to the combination studied. Moreover, in marked contrast to the situation that prevails in indirect allorecognition, T cell activation through the direct presentation pathway was not associated with skewing of the complementarity determining region (CDR) 3 length distribution. Altogether, these data argue for the significance of TCR contacts with the MHC framework in direct allorecognition. In addition, the TCR diversity mobilized by this interaction and the massive TCRβ mRNA accumulation observed after a few days of culture suggest that a significant proportion of naive T cells receive a signal leading to TCRβ transcriptional activation even though only a few of them engage in mitosis. ***The Journal of Immunology,*** **2001, 167: 3082–3088.**

Direct recognition of foreign MHC molecules has been proposed to describe the capacity of naive T cells to proliferate when cocultured with foreign APC (1, 2), as opposed to the recognition of a given peptide presented by self-MHC molecules, which is referred to as indirect alloreactivity (3). Because an unusually high fraction (from 1 to 5%) of naive T cells can be mobilized by this interaction to undergo mitosis after short-term coculture (4–8), direct recognition is believed to play a major role in allograft rejection, particularly in acute rejection crises in the early phase following transplantation. The fact that cardiac allografts from MHC-deficient mice are not acutely rejected is consistent with this concept (9, 10). Several studies, including crystallographic analyses of TCR/MHC complexes (11, 12), have established that molecular interactions of the TCR with allogeneic MHC molecules can involve TCR engagement with the MHC framework as well as with the peptide (13–15). The relative importance of the recognition of allopeptides as opposed to that of framework determinants is not precisely known. A succession of theories addressing this question has been proposed. First, Bevan and Matzinger (16) described a model of molecular mimicry, in which the alloMHC molecule, complexed with a given peptide, reproduced the conformation of a self-MHC/peptide complex. More recently, the availability of genetically modified MHC molecules and of rationally designed peptides has made more accurate studies of such interactions possible. Indeed, it has been demonstrated that TCR recognition of alloligands involves prominent molecular interactions with MHC framework determinants (17, 18). Such interactions with foreign MHC framework moieties would account for the high frequency of alloreactive T cells. Interestingly, crystallographic studies have provided some support for this theory (11, 19). Finally, as suggested by M. Bevan (20), foreign MHC molecules may be recognized by alloreactive CTL, even in the absence of peptide (14, 21). This cross-reactivity of the TCR for allo-MHC determinants may be related to the fact that T cell precursors are selected in the thymus on the basis of their intermediate affinity for self-MHC molecules (22), and it is likely that TCRs with a high affinity for allogeneic MHC have not been censured during thymic education.

T cell activation (including alloreactivity) can be investigated by studying TCR Vβ chain usage. Analysis of qualitative alterations in the T cell repertoire has made an essential contribution to the understanding of several biological situations including alloimmunity (23), tumor immunity (24), and autoimmune diseases (25). We have previously shown in vivo that T cells infiltrating allografts long after transplantation exhibit a strongly altered TCR Vβ repertoire in both rats and humans (23, 26). However, no equivalent data under conditions of direct restricted recognition of foreign APCs are available. In the absence of any quantification of the T cells concerned, the biological relevance of these analyses is questionable. Furthermore, these qualitative approaches can detect neither polyclonal

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activation nor T cells not selected on the basis of their complementarity determining region (CDR)[3] 3 length.

In this paper, we used a new approach, referred to as TcLand (for T cell Landscape; Institut National de la Santé et de la Recherche Médicale, Paris, France), which combines analysis of alterations to the Vβ TCR at the CDR3 level with quantification of each Vβ family mRNA within a T cell pool, to study TCR repertoire alterations induced by the direct presentation pathway. Our data show that T cell interaction with foreign APCs triggers the activation of specific TCR Vβ families regardless of their CDR3 region and affects a wide diversity of TCR. The relevance of these observations is discussed in the context of understanding the strength of direct recognition and its role in the mechanisms of acute graft rejection.

Materials and Methods

MLR

Purified T cells were harvested from naive adult inbred LEW.1A (RT1^a) rats purchased from Janvier (Savigny/Orge, France) or from healthy unrelated human volunteers. T cell purity was systematically assessed using flow cytometry (>97% of TCR$^+$ (R7.3; European Collection of Animal Cell Cultures, Salisbury, U.K.) and no detectable MHC class II$^+$ cells) before use. Purified T cells were seeded at the final concentration of 1×10^5 cells/well in culture medium containing 10% heat-inactivated autologous serum (LEW.1A rat) and cultured for 5 days with allogeneic (LEW.1W (RT1^u) rats) or xenogeneic (inbred adult Syrian hamsters) enriched dendritic cell suspensions, obtained as described in detail elsewhere (27). For human proliferation assays, autologous PBMC were used as feeder. When indicated, 10 μg/ml Con A (Sigma-Aldrich, St. Quentin Fallavier, France), 50 ng/ml toxic shock syndrome toxin-1 (TSST1; Sigma-Aldrich), or 10 μg of OX3 hybridoma supernatant (anti-rat RT1-B^u MHC class II molecule; European Collection of Animal Cell Cultures) was added to the cultures on day 0. In parallel with the proliferation assays, cells from 48 replicates were prepared for mRNA extraction and subsequent repertoire analyses (at least 5×10^6 cells/informative sample).

Immunization and self-restricted response

LEW.1A rats were immunized in the footpad with 50 μg of xenoantigens (supernatants of sonicated hearts from inbred Syrian hamsters) in CFA. Draining lymph nodes were harvested 10 days after injection. Lymph node cells (3×10^5) were then cultured as described above for 3 days in complete medium with or without the addition of the same antigenic preparation at a final concentration of 12.5 μg/ml.

CFSE labeling and flow cytometry analyses

Aliquots of responder cell populations were resuspended at 5×10^7cells/ml in protein-free RPMI 1640, and CFSE (Molecular Probes, Eugene, OR) was added to a final concentration of 5 μM. Cells were then incubated for 20 min at 37°C. Labeled suspensions were immediately washed three times in cold RPMI 1640/10% serum. On day 5, CFSE-associated responder T cell fluorescence was analyzed by means of flow cytometry conducted on a FACScan (BD Biosciences, Mountain View, CA) running CellQuest software.

CDR3 fragment size determination

Total RNA was isolated as described (28), and 10 μg was reverse-transcribed using the Boehringer cDNA synthesis kit (Boehringer Mannheim, Meylan, France). Aliquots of the cDNA synthesis reaction were amplified with 1 of the 24 human (the pseudo-genes Vβ 10, 20, 21, and 24 were not included in the study) or 1 of the 20 rat Vβ family-specific primers and the corresponding Cβ primer (29, 30). The PCR amplification conditions were as previously described (23, 26). Immunoscope software (31) was used to obtain a semiquantitative analysis of the Vβ chain of the TCR repertoire at the CDR3. Reperturb software (32) was used to further quantify the alterations in CDR3 length distribution for each Vβ family in each sample.

[3] Abbreviations used in this paper: CDR, complementarity determining region; HPRT, hypoxanthine phosphoribosyltransferase; TSST1, toxic shock syndrome toxin-1.

Relative quantification of Vβ transcripts

Oligonucleotides and standard construction. To prepare each Vβ standard curve specific to the Vβ families in the rat and human, the Vβ primer was used in the forward position together with the Cβr primer in the reverse position (23). To prepare the Cβ standard curve, the Cβf and Cβr primers (specific for human or rat) were used in the forward and reverse positions, respectively. The target sequence was amplified in cDNA derived either from PBMCs from healthy human volunteers or from naive LEW.1A splenocytes, using forward (one Vβ or Cβf) and reverse (Cβr) oligonucleotides. For each Vβ family and Cβ, the standard concentration was derived from its absorbency value at 260 nm, and the number of copies per milliliter was then calculated using the m.w. of the cDNA. Subsequent dilutions of each Vβ, Cβ, and hypoxanthine phosphoribosyltransferase (HPRT) standard DNA were performed to establish a range of concentration similar to that of the target in the biological samples.

PCR amplification and analysis. A constant amount of cDNA for each dilution of each Vβ, Cβ, or HPRT standard was amplified in 25 μl of SYBR Green PCR Core Reagent (Applied Biosystems, Foster City, CA) with 0.6 U of AmpliTaq Gold polymerase, 0.25 U of AmpErase UNG, 200 μM each dNTP, 300 nM each primer, and 3 mM $MgCl_2$ in 1× SYBR Green PCR buffer. Amplifications were performed using an ABI Prism 7700 Sequence Detection System PerkinElmer machine (Applied Biosystems). The exact number of copies of the cDNA target sequence was deduced from the comparison of measured fluorescence with the standard curve and were standardized against the level of HPRT transcripts.

Integrated graphic representation of qualitative and quantitative repertoire alterations: TcLand

The profiles obtained from healthy human volunteers or naive rats used as controls in human or rat MLR, respectively, were measured, and the mean profiles for the 20 Vβ families were used as reference data. For each CDR3 length profile experiment, the experimental profiles were compared with the reference and the difference plotted as a landscape plot using Reperturb software. The percentage of alteration in CDR3 length distribution was represented as a color code in the landscape plots. This first analytical step yielded qualitative information only, because there was no indication of the amount of altered Vβ. To combine the qualitative alterations with the level of Vβ mRNA involved, the values obtained from the quantitative real-time PCR analysis were added to landscape plot graphics where the height of peaks on the landscape represents the amount of a given CDR3 size within each Vβ family. MatLab software was used to compute and display the data. This new means of representing the global TCR transcriptome, integrating qualitative and quantitative alterations in the whole T cell repertoire, is referred to as TcLand (for T cell landscape).

Results

Proliferative responses induced by direct recognition

MLR involving the direct recognition pathway alone were performed in either closely (LEW.1A/LEW.1W) or more distantly (LEW.1A/hamster) related combinations, using pure rat T cells as responders. In this experimental setting, both combinations produced significant proliferative responses on day 5, which was determined as the peak of the proliferative response (Fig. 1*A*, proliferation indexes = 73.7 and 29.5, respectively). Furthermore, the 87% inhibition obtained by adding an Ab directed against the MHC class II molecules of the stimulating APCs confirmed that the direct presentation pathway alone was operating in this experimental setting (Fig. 1*A*). As a first approach, we assessed the precursor frequency of allo- and xenoreactive T cells, using proliferation as a readout for T cell activation. To do this, we used a method based on the halving of CFSE labeling after each mitosis. In this way, in terms of the number of cells in each successive cellular generation (33), we estimated the proliferative precursor frequencies as being 1.3 and 0.7% in allogeneic and xenogeneic MLR, respectively (Fig. 1, *B* and *C*).

Absence of CDR3 length distribution alteration after direct allorecognition

According to these results, we assumed that T cell activation through the direct presentation pathway should have a significant

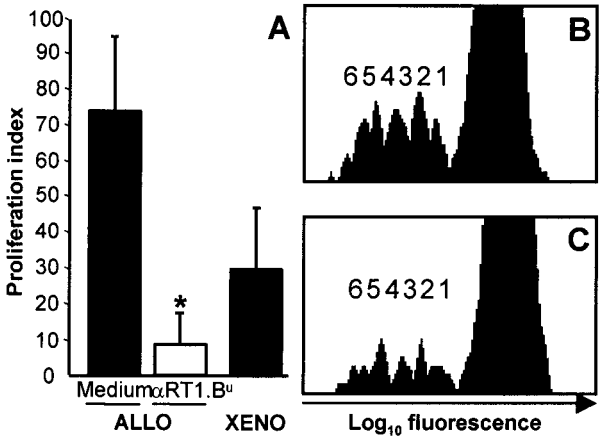

FIGURE 1. Direct proliferative allo- and xenoresponses. *A*, Purified T cells (10^5) from naive LEW.1A rats were cultured with 2×10^4 irradiated LEW.1W (allo) or hamster (xeno) enriched dendritic cells. When indicated, mAbs against donor MHC class II molecules were added to the culture. Proliferative responses were assessed on day 5. Results are means of proliferation indexes ± SD from at least four individual experiments (*, $p < 0.01$). *B* and *C*, FACS analysis of CFSE-labeled LEW.1A T cells harvested from 5-day MLR conducted in direct conditions. The numbered peaks represent successive division cycles with halving of the CFSE concentration in allo- (*B*) or xenocombination (*C*). The number of cells in each peak allows precursor frequencies to be calculated.

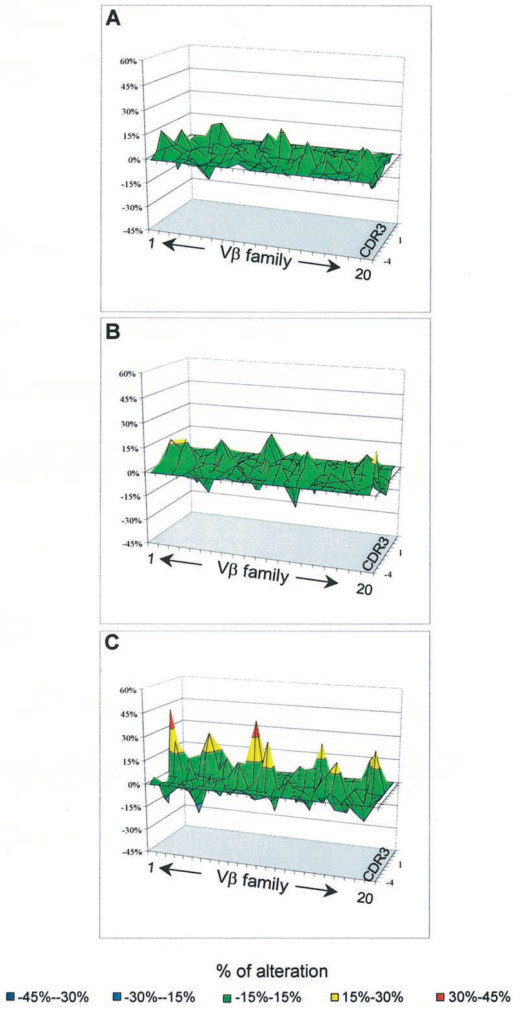

FIGURE 2. Qualitative alterations in CDR3 length distribution. The percentage of alteration (*z*-axis, color scale) for the 20 Vβ families (*x*-axis) with a given CDR3 length (*y*-axis) was calculated by comparing the area between the distribution for each MLR reaction and for resting T cells. LEW.1A T cell mRNA was harvested on day 5 after direct restricted alloresponse (*A*), direct restricted xenoresponse (*B*), or self-restricted xenogeneic response (*C*).

impact on the TCRβ repertoire. CDR3 length distributions were analyzed after short-term MLR under such conditions, using the classic qualitative method (Immunoscope). Fig. 2 shows that, despite a strong proliferative response (see Fig. 1*A*), purified rat T cells stimulated by the direct presentation pathway had a Gaussian distribution of CDR3 lengths in both allo- (Fig. 2*A*) and xenocombinations (Fig. 2*B*). Analysis of qualitative alterations in the global TCRβ repertoire using Reperturb software showed that variations in CDR3 length distribution profile almost never exceeded 15% (a low level of alteration) of that observed in resting cells in almost all Vβ families. To avoid any possibility of the existence of subtle alterations restricted to T cell subtypes, Immunoscope analyses were also performed on distinct $CD4^+$ and $CD8^+$ T cell subtypes. In agreement with the results obtained with the global T cell population, neither pattern exhibited any significant alterations in their CDR3 length distribution (data not shown). In contrast, and given only as an example of a significantly altered pattern (Fig. 2*C*), purified T cells from LEW.1A draining lymph nodes sensitized in vivo (indirect pathway) and stimulated with soluble acellular cardiac xenoantigens, had a strongly biased distribution profile of CDR3 length. Indeed, on day 5, several oligoclonal expansions (Vβ1, 5, 11, and 13) appeared after recognition of xenoantigens presented on self-APCs. Therefore, whereas the presentation of allo- or xenoantigens in the context of autologous MHC molecules gives rise to the expected oligoclonal expansions, probably specific to a limited set of peptides presented on self-MHC, the direct recognition of foreign MHC/peptide complexes mobilizes naive T cells without significant selection in terms of the CDR3 length of the TCR β-chain.

Integrated depiction of qualitative and quantitative alterations of the TCRβ repertoire: TcLand

Because of the vigorous proliferative response observed in our direct type MLR, we hypothesized that such qualitative analyses at the level of the CDR3 region were not sufficient to account for the complexity of T cell activation in this model. For example, polyclonal expansions of specific Vβ families would not be detected by these methods. To overcome this limitation, we took advantage of the transcriptional activation of the TCR β-chain in activated T cells (34, 35). For each Vβ family, mRNA accumulation was quantified by real-time RT-PCR, and these values were displayed according to the distribution of CDR3 lengths, as determined by the Immunoscope method (31). The new approach we propose here (TcLand) permits the accumulation of polyclonal or restricted Vβ mRNA to be depicted globally. To evaluate the efficiency and the accuracy of this new method, we studied the influence of polyclonal stimuli on the TCRβ repertoire. In addition, to further illustrate the potential of this new approach, these experiments were performed using human and rat T cells. As shown in the Fig. 3, the TcLand profiles obtained from resting (Fig. 3*A*), Con A-activated (Fig. 3*B*), and TSST1-stimulated (Fig. 3*C*) human T cell populations appeared clearly different despite the absence of any alteration in the distribution of the CDR3 length (color scale). Indeed, the activation induced by Con A affected all Vβ families (increase in the Vβ/HPRT ratio), whereas, as previously described (36), the TSST1 effect was restricted to $V\beta2^+$ T cell. Similar patterns were

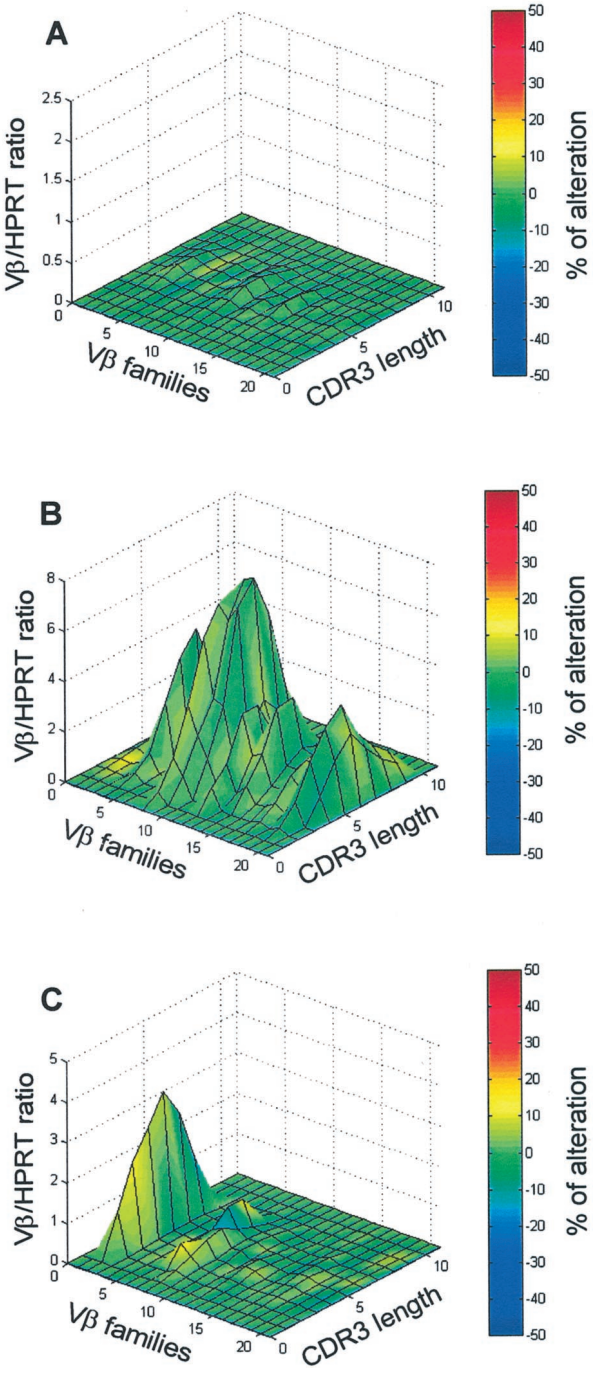

FIGURE 3. TcLand representation of integrated analysis of qualitative and quantitative alterations in the TCRβ repertoire. Human T cells were reacted against autologous stimulator cells in the presence of medium (*A*), Con A (*B*), or superantigen TSST1 (*C*). Total mRNA was extracted on day 3 and used for qualitative and quantitative analyses of Vβ CDR3 length distribution. These two data were combined and expressed as a TcLand graphic representation. Briefly, the *x*-axis shows Vβ families, the *z*-axis the Vβ:HPRT transcript ratio, and the *y*-axis the distribution of CDR3 lengths. The color scale represents the percentage of alteration. The results presented here are representative of three individual experiments.

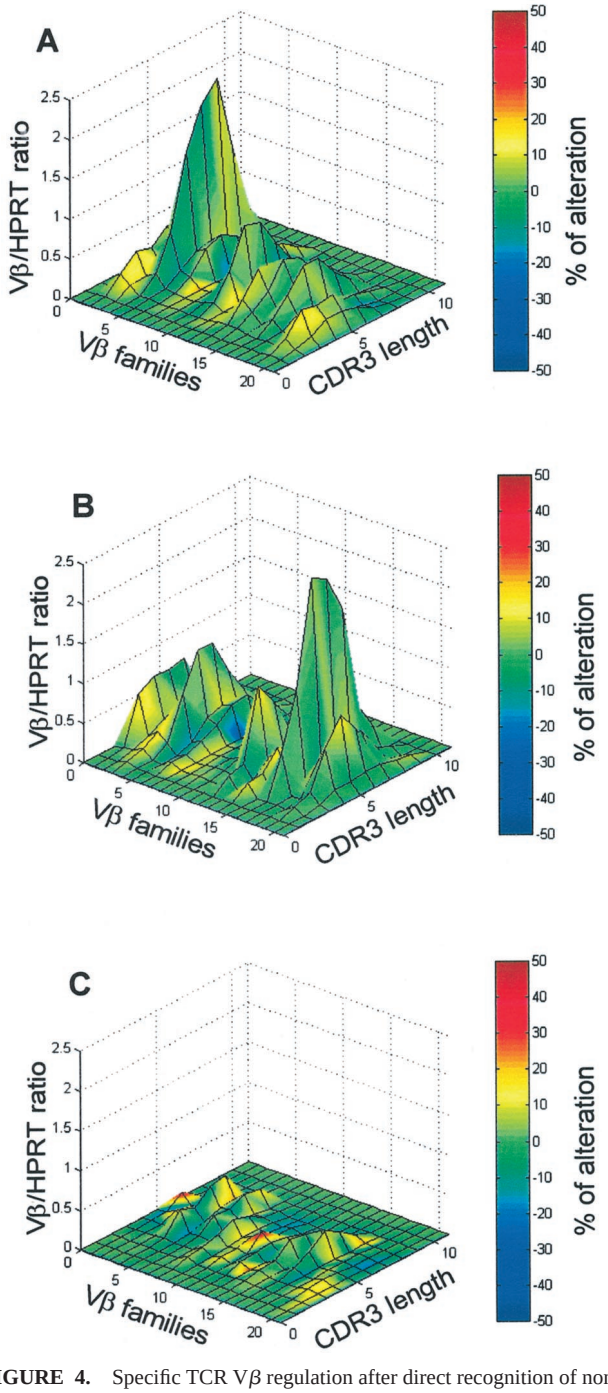

FIGURE 4. Specific TCR Vβ regulation after direct recognition of non-self-MHC molecules. Representative TcLand profiles derived from LEW.1A T cells harvested from 5-day MLR conducted in direct restricted allogeneic combination (*A*), xenogeneic combination (*B*), or self-restricted xenoresponses (*C*).

generated using rat T cells as the responder population (data not shown). Thus as expected, TcLand allows unaltered T cell populations to be discriminated, whereas they would appear highly similar after CDR3 qualitative analyses.

TcLand analysis after allo- and xenogeneic MLR

We then applied this analysis to mRNA harvested from MLR performed in direct restricted allo- or xenogeneic combinations, which showed unaffected CDR3 length profiles despite significant proliferative responses. Fig. 4 confirms the Gaussian distribution of CDR3 lengths, but also demonstrates the specific regulation of certain Vβ families that accumulated strongly after direct recognition of foreign APCs. Indeed, using this method, some differences in Vβ family usage were observed when allo- or xenogeneic APCs were cocultured with the same T cell population, indicating

that Vβ families, despite their Gaussian CDR3 profile, were not mobilized at random when stimulated through the direct presentation pathway. For instance, as compared with the resting situation (Fig. 3*A*), in the allocombination (Fig. 4*A*) preferential expansions of Vβ2, 4, 6, 10, 14, and 16 were observed, whereas in the xenocombination (Fig. 4*B*) the Vβ families 1, 4, 12, 16, and 19 were the most profoundly regulated. Moreover, as shown in Fig. 4*C*, T cells stimulated by self-APCs were not associated with a significant increase in the Vβ:HPRT ratio, illustrating the specificity of TCR interactions with nonself-MHC determinants. These characteristic landscapes, observed in the different species combinations, indicate that despite an unaltered distribution of CDR3 length, the TCRβ repertoire mobilized by the direct Ag presentation pathway does not occur randomly but is polarized toward specific Vβ families. In addition, the observation that some Vβ families were not regulated, as well as the results of superantigen stimulation, excludes the possibility of T cell activation mediated by the release of a nonspecific growth factor.

Direct allorecognition mobilizes a wide repertoire diversity

To estimate the extent of TCRβ diversity mobilized by direct allorecognition, relative CDR3 length distributions obtained under various conditions of stimulation were compared with the profile of resting T cells. Each mRNA species significantly regulated was plotted as a filled square on a grid encompassing the entire TCRβ transcriptome (Fig. 5). The TCRβ diversity of affected T cells was represented by the percentage of grid squares exhibiting a significant accumulation of mRNA as compared with naive T cells. As expected, this representation accurately reflects both the global activation induced by Con A stimulation (>80% of the possible mRNA species were regulated) (Fig. 5*A*) and the Vβ2$^+$-restricted activation induced by TSST1 (5% of mRNA species were regulated, Fig. 5*B*). The patterns obtained after allogeneic and xenogeneic direct MLR are shown in Fig. 5, *C* and *D*. In both situations, the diversity of mRNA species regulated appeared surprisingly high. Indeed, 34% of the possible mRNAs were significantly regulated by the direct allorecognition pathway, and a roughly similar value (39%) was obtained in the xenogeneic combination. Taken together, these results indicate that more than a third of the possible mRNA species underwent significant changes when activated by the direct recognition pathway, and that T cells activated by this pathway span a large part of the TCRβ transcriptome.

Estimation of the proportion of alloreactive T cells using TCRβ regulation as a readout

The wide diversity of mRNA species involved in direct allo- or xenorecognition does not correspond to an actual proportion of alloreactive cells, because each of the grid squares only represents a pool of mRNA species sharing the same CDR3 length but with different sequences. Thus, a square with a significant accumulation of TCRβ mRNA may arise either from a very strong up-regulation of a few T cell clones or from a homogenous Vβ mRNA up-regulation involving most of the T cells as exemplified in Fig. 6. Therefore, to evaluate the extent of naive T cell activation through the direct presentation pathway, we compared TCRβ mRNA accumulation obtained after short-term direct MLR with that observed in conditions of maximum activation arbitrarily represented by Con A stimulation (Fig. 7). An estimation of the minimal number of T cells that would have to be maximally activated to account for the observed variation in the Cβ:HPRT ratio was obtained by the following formula: minimum estimation of alloreactive T cells = 100 × (experimental ratio/resting ratio) − 1/(maximum ratio/resting ratio) − 1. Using this readout, the minimum number of T cells reactive to foreign APCs in the allo- and xenogeneic combinations were estimated to be 33 and 50%, respectively. Interestingly, this estimate of the minimum number of cells involved in direct allorecognition on day 5 was roughly similar to the values of the diversity mobilization (Fig. 5), favoring option *A* of Fig. 6.

Discussion

In this paper, we show that TCRβ mRNA expression is exquisitely regulated following stimulation through the direct recognition pathway, and we present a new approach for the study of global TCR repertoire alterations. Using a method combining qualitative

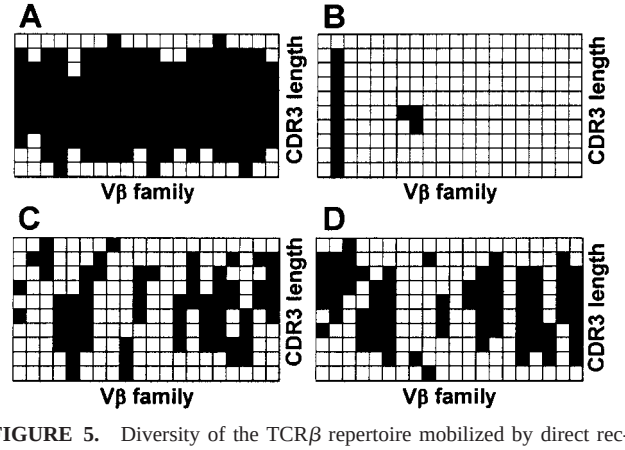

FIGURE 5. Diversity of the TCRβ repertoire mobilized by direct recognition. Total mRNA was harvested from 5-day MLR conducted in the presence of Con A (*A*) or TSST1 (*B*), specific to human Vβ2$^+$ T cells and used for quantitative analysis of Vβ mRNA accumulation. These values were displayed on a grid encompassing the entire TCRβ transcriptome, according to the CDR3 length distribution. To illustrate the diversity of the mRNA species mobilized, statistical analysis (Student's *t* test) of the variation from the resting level was performed for each value in the respective experimental groups ($n = 3$ in each group). Each mRNA species significantly regulated was plotted as a filled square. mRNA harvested from either allogeneic (*C*) or xenogeneic (*D*) direct MLR were analyzed in the same way.

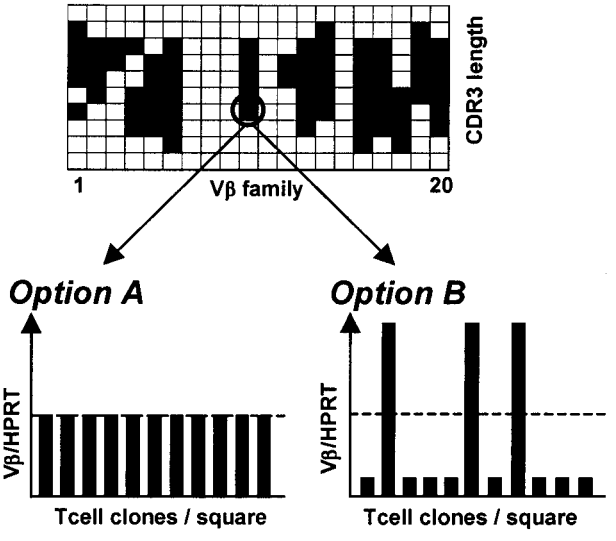

FIGURE 6. Two hypotheses for significant mRNA accumulation in a grid square. Each square of the grid represents a pool of mRNA sharing the same CDR3 length with a different sequence. Each mRNA species (1 CDR3 length in a given Vβ family) can appear regulated as a result of either homogenous up-regulation in almost all of the T cell clones (*A*) or strong up-regulation of TCRβ expression in a few clones (*B*).

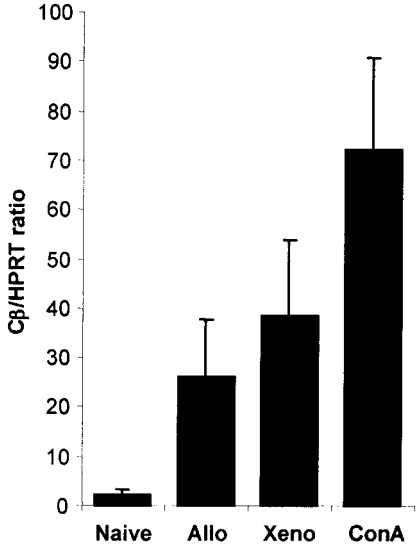

FIGURE 7. TCR β-chain regulation as a readout for T cell activation. The transcriptional regulation of the TCR β-chain was quantified in resting T cells and compared with that measured after stimulation by TSST1, allogeneic APCs, xenogeneic APCs, or Con A. For each situation, the level of Cβ expression was assessed using real-time quantitative RT-PCR and standardized according to the level of HPRT expression. The results are expressed as the mean Cβ:HPRT ratio $\pm$ SD.

alterations in CDR3 length distribution with quantitative measurements of mRNA accumulation for each Vβ family of the global TCRβ transcriptome, we show that, in contrast to what happens in T cells stimulated by self-APCs, T cells activated through the direct recognition pathway are selected on the basis of their Vβ segment rather than on the basis of their CDR3 region. This type of interaction mobilizes a wide diversity of T cell clones and strongly suggests that a significant proportion of naive T cells could be prone to interact with foreign MHC molecules, even though only few of them engage in mitosis. These findings may help to understand how naive T cells can lead to vigorous allo- and xenograft rejection within a few days.

Qualitative analysis of repertoire biases has been extremely useful in coupling constraint CDR3 sequences and recognition of a specific peptide/MHC complex. However, Immunoscope/Spectrotyping analyses have major limitations. First, a constraint CDR3 sequence, only assessed through qualitative methods, may not necessarily indicate biologically relevant events. In contrast, the same Gaussian distribution pattern of CDR3 length may reflect either an exhaustive and polyclonal expansion, or a state of unresponsiveness in a T cell population, with little apparent difference. Therefore, quantitative assessment of mRNA of each Vβ family, and its relationship to qualitative alterations in CDR3 lengths, yields crucial additional information about the in vivo relevance of private TCR alterations, as well as about the possible immunological relevance of a given Gaussian pattern. For instance, in view of the large number of cells concerned, the strongly expanded but unaltered Vβ families, as observed in direct MLR on day 5, may be extremely important biologically (such as in early acute rejection).

Interestingly, this new representation of global TCR landscapes showed that the direct recognition pathway actually results in a vigorous regulation of TCRβ mRNA. However, this regulation does not occur randomly but is restricted to certain Vβ families, in a pattern dependent on the genetic combination studied. This specific pattern of activated Vβ families, as well as the profile obtained after superantigen stimulation, excludes the possibility of a nonspecific activation by soluble factors released during the culture. The reproducibility of profiles in the rat model is essentially due to the fact that we used inbred strains. Although this is not the case in humans, this method should nevertheless prove very useful for the long-term follow-up of the individual evolution of the TCRβ transcriptome during various disease processes, including autoimmune or viral disease and to monitor the effect of anti tumor vaccination.

An important finding revealed by our study concerns the diversity of TCRβ mRNA species regulated and the number of alloreactive T cells involved in direct allo- or xenorecognition. Indeed, $\sim$35% of the mRNA species (Vβ families/CDR3 length) underwent significant accumulation. This suggests that a high proportion of T cells are involved in the direct recognition of nonself-MHC determinants, and that T cells activated by this pathway span a large part of the TCRβ transcriptome. However, as assessed on day 5, these results do not correspond to an actual frequency calculation in a naive T cell population. Indeed, the alloreactive T cell precursor frequency ranges from 0.1 to 5%, according to the readout chosen (6, 7, 37, 38). Interestingly, as demonstrated by the CFSE measurement, proliferation and TCRβ mRNA accumulation do not correlate, as it has been recently demonstrated for other activation markers (39). This difference may be related to the affinity required in the TCR/MHC interaction. Low affinities could be sufficient to trigger Vβ mRNA regulation, whereas a higher threshold is required to stabilize the numerous ligand-receptor interactions and to allow T cells to proliferate and achieve a complete activation state. Interestingly, it has recently been shown that mitosis is not absolutely required for T cell to acquire effector functions such as cytotoxic activity or IFN-γ production (40–42), suggesting the biological relevance of these findings.

Finally, we show here that T cell activation through the direct presentation pathway occurs in the absence of skewing in the distribution of the CDR3 lengths, even when repertoire analyses are performed on purified $CD4^+$ and $CD8^+$ T cells. Because prominent recognition of the peptide by the CDR3 region of the TCR would have resulted in altered usage of CDR3 lengths, these results are consistent with the model derived from structural analyses of the molecular interactions involved in direct allorecognition. Indeed, the large number of contacts made by the TCR with the MHC framework (11, 12) and the relatively low sensitivity to peptide sequence variations (17, 18, 43) suggest that direct recognition of nonself-MHC molecules by T cells may be more dependent on interactions of the TCR with the allo-MHC framework determinants than with the peptide itself (20). This low peptide specificity has also been recently suggested by the low contact number between the CDR3 regions of the BM3.3 TCR and an allogeneic MHC-peptide ligand (44). In this way, as demonstrated for superantigens (45), prominent interactions of allogeneic MHC/peptide complexes with the CDR1 and CDR2 region encoded by the Vβ segment should trigger expansions of specific Vβ families rather than selecting unique CDR3 lengths. However, our estimation of the pool size of alloreactive T cells after 5 days of culture (about one-third of the responding cells) may give rise to an alternative hypothesis. Indeed, xenogeneic and allogeneic peptides presented in the context of foreign MHC molecules might represent enough diversity to be recognized by one-third of the repertoire in a CDR3-dependent manner. However, the similar proportion of the TCRβ repertoire reacting against allogeneic (32%) and xenogeneic (38%) APCs, despite large variations in protein sequences between species (46), which potentially feed APCs with a high diversity of peptides, rather argue for the first hypothesis. Interestingly, preliminary evidence suggests that the patterns observed in vivo during acute rejection of a MHC mismatched cardiac allograft show the same vigorous TCRβ mRNA accumulation associated with

unaltered CDR3 length distribution.[4] Altogether, these observations could be instrumental in explaining the strength of the alloimmune response, leading to organs of a large size being acutely rejected within a few days in unprimed recipients.

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