Available online at www.sciencedirect.com
SCIENCE DIRECT®
Molecular Immunology 40 (2004) 1089–1094
Molecular Immunology
www.elsevier.com/locate/molimm

Degenerate recognition and response of human CD4+ Th cell clones: implications for basic and applied immunology

Yasuharu Nishimura[a,*], Yu-Zhen Chen[a], Yasushi Uemura[a,b], Yoshihiko Tanaka[a], Hirotake Tsukamoto[a], Takayuki Kanai[a], Hiroshi Yokomizo[a], Chyuns Yun[a], Takako Matsuoka[a,b], Atsushi Irie[a], Sho Matsushita[a,b]

[a] *Department of Immunogenetics, Graduate School of Medical Sciences, Kumamoto University, Honjo 1-1-1, Kumamoto 860-8556, Japan*
[b] *Department of Allergy and Immunology, Saitama Medical School, Moroyama 350-0495, Japan*

Abstract

It was once considered that the T cell response is an all or nothing type event, but recent studies have clearly indicated that T cells show many different types of activation in recognition of altered ligands for T cell receptors (TCR). In this review, we summarize our recent findings on the response of human CD4+ helper T (Th) cell clones to altered peptide ligands (APL); peptides carrying single or multiple residue substitutions in antigenic peptides. The extensive analyses revealed that TCR-antagonism and partial agonism are frequently observed by the stimulation with APLs substituted at particular amino acid residues of antigenic peptides. We observed unique partially agonistic APLs inducing prolongation of T cell survival without cell proliferation. Superagonistic APLs stimulated enhanced proliferation and production of cytokines in Th cell clones reactive to tumor-associated antigens. The other APL induced enhanced production of interleukin-12 by antigen presenting cells and subsequent enhancement of IFN-γ production by T cells reactive to allergens. By utilizing an HLA-DR-restricted T cell epitope library generated by mutated invariant chain genes, it was revealed that human Th cell clones recognize a more diverse array of peptides with multiple and simultaneous amino acid substitutions in an antigenic peptide. APLs also induced altered intracellular signaling events including intracellular calcium increase and phosphorylation of signaling molecules. This information provides basic knowledge regarding the characteristics of antigen recognition by human Th cells and the subsequent activation, and a novel method for manipulation of human Th cell responses by APLs, as a possible candidate for antigen-specific immuno-potentiating or immunosuppressive therapy.

Keywords: HLA class II molecule; Human CD4+ Th-cells; Antigenic peptides; Altered peptide ligands; T cell recognition; TCR antagonism; Peptide partial agonism

1. Introduction

The human histocompatibility leukocyte antigen class-II (HLA-II) molecule has a peptide binding groove on top of the molecule and binds antigenic peptides processed by antigen presenting cell (APC) to present them to CD4+ helper T (Th) cells (Germain and Margulies, 1993). Three to five amino acid residues were separated by one to two intervening residue(s) and acted as anchor residue(s) for peptide binding to HLA-II molecules (Sette et al., 1993; Hammer et al., 1993; Matsushita et al., 1994). On the other hand, side chains of amino acid residues flanking anchor residues proved to be the main recognition sites by T cell receptors (TCR); this was clearly established in crystallographic analyses of the DR molecule bound by either self (Brown et al., 1993) or non-self peptides (Stern et al., 1994).

CD4+ Th cells usually recognize non-self peptides in the context of self HLA-DR molecules. Recognition and responses of T cells were once considered to be an on/off phenomenon, however recent findings obtained using altered peptide ligands (APLs) carrying single residue substitutions in antigenic peptides presented by one major histocompatibility complex (MHC) class II molecule or one specific peptide presented by different MHC class II molecules showing a limited polymorphism revealed that

Abbreviations: HLA-II, human histocompatibility class II; APC, antigen presenting cell; Th cell, helper T cell; TCR, T cell receptor; APL, altered peptide ligand; MHC, major histocompatibility complex; IFN-γ, gamma interferon; IL, interleukin; CLIP, class II-associated invariant chain peptide; ZAP-70, zeta-associated protein-70

* Corresponding author. Tel.: +81-96-373-5310; fax: +81-96-373-5314.
E-mail address: mxnishim@gpo.kumamoto-u.ac.jp (Y. Nishimura).

0161-5890/$ – see front matter © 2003 Elsevier Ltd. All rights reserved.
doi:10.1016/j.molimm.2003.11.009

altered TCR ligands induce altered T cell responses in both mice and humans, including (1) T cell non-responsiveness, through TCR antagonism and (2) partial agonism inducing partial activation of T cells without cell proliferation (Sloan-Lancaster and Allen, 1996).

Previous analyses also revealed that the interactions of TCRs with MHC-wild-type peptides had stronger affinities and/or smaller off-rates than did those of TCRs with MHC-APL complexes (Lyons et al., 1996). These differences in characteristics of molecular interactions may induce insufficient engagements of TCR with MHC-APL complexes such that intracellular signals mediated by TCR through recognition of APLs are inadequate for full activation of T cells to induce cell proliferation. In some cases, inadequate signals induce unique altered T cell responses.

In this review, we will summarize our recent analyses on recognition by human $CD4^+$ Th cell clones of diverse peptides, and the heterogeneity of subsequent T cell responses and T cell activation signals induced, as summarized in Table 1.

2. Frequencies of agonistic and antagonistic single residue substituted APLs depend on position of substituted amino acid residues of the peptide

If there is a general rule for structures of APLs which stimulate or inhibit T cell responses to wild type antigenic peptides, it would be easier to generate peptides which augment or inhibit responses of human Th cells. We used a human Th1-cell like clone YN5-32 reactive to a streptococcal M12p54-68 peptide (^{54}NRDLEQAYNELSGEA^{68}) in the context of HLA-DR4 (DRB1*0406), and analyzed responses of YN5-32 to 156 independent APLs carrying single residue substitutions at residues 57 (P1)-65 (P9) of the peptide where P1 (position 1) means the putative most N-terminal DR anchor residue (Chen et al., 1996). As shown in Fig. 1, residues Leu-57 (P1), Ala-60 (P4) and Asn-62 (P6) were the most likely to be DR-anchor residues, and 30% (17/57) of APLs substituted at these residues exhibited full agonism to stimulate various magnitudes of proliferative responses in the T cell clone, whereas only 7.5% (3/40) of non-fully agonistic peptides exhibited TCR antagonism. On the other hand,

Table 1
Summary of our observations on responses of human $CD4^+$ Th-cell clones to APLs

Th-cell clone	Specificity	Observed immune responses to APLs	Reference
YN5-32	Streptococcal M12p54-68/DR4	TCR antagonism	
		Partial agonism; increases in cell size and expression levels of CD4, 11a, 28, 49d, 95 without anergy induction	Chen et al. (1996)
		Polymorphism at DRβ37 affected T cell recognition	Chen et al. (1997)
		Quantitative and qualitative alteration of intracellular calcium increase	Chen et al. (1998)
		Overexpression of partially agonistic TCR-ligand induced proliferation without phosphorylation of ZAP-70 and LAT	Irie et al. (2003)
SK2.11	AChRα p75-87/DQ6	TCR antagonism	Kanai et al. (1997).
BC20.7, BC33.5, BC42.1	BCGa p84-100/DR14	Partial agonism in recognition of artificial or natural self APLs; increased survival without antigenic stimuli or production of IL-4 and IFN-γ without cell proliferation	Matsushita et al. (1997)
C27	p21Ras p3-17/DR1	Superagonism; increased proliferation and production of IFN-γ and GM-CSF in recognition of cancer-associated mutated peptides and its APL	Yokomizo et al. (1997)
Y41.2	TEL/AML1 fusion peptide/DP17	Superagonism; increased proliferation and production of IFN-γ and GM-CSF in recodnition of APLs derived from leukemia-associated TEL/AML1 fusion peptide	Yun et al. (1999)
29.15.2	p21Ras p3-20/DR51	Superagonism; increased proliferation in recognition of APL identified by using a combinatorial peptide library and mass spectrometry	Tanaka et al. (1999)
ST1.9	*Cry j*1p335-346/DR52	Superagonism; increased production of IFN-γ	Ikagawa et al. (1996)
DT13.2	*Der f*1p18-31/DQ6	Superagonism; increased production of IFN-γ stimulated by increased production of IL-12 from antigen presenting cells	Matsuoka et al. (1996)
SA32.5, MK20.2	GAD65 p115-127/DR53	Generation of a multiple residue substituted epitope expression library by using CLIP-substituted invariant chain genes to identify agonistic APLs and mimicry microbial peptides	Uemura et al. (2003)

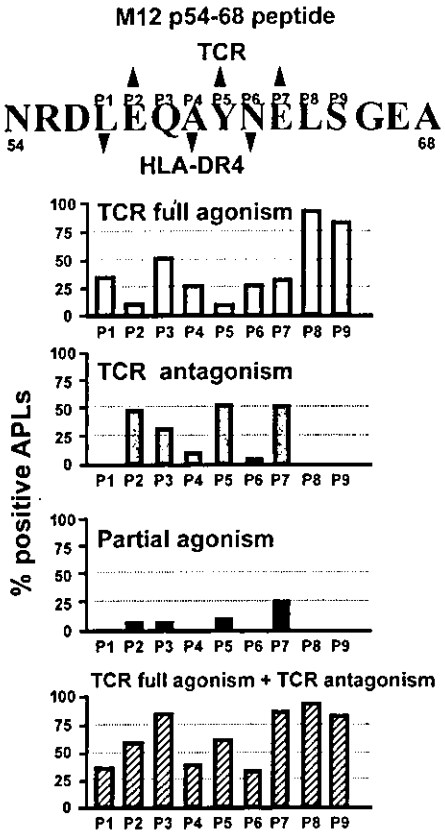


Fig. 1. Summary of responses of the human Th cell clone YN5-32 to 156 APLs carrying single residue substitutions in a streptococcal antigenic peptide M12p54-68. From P1 to P7 residues, residues were replaced with 19 other amino acids. The P8 and P9 residues were replaced with 10 and 11 other amino acids, respectively. Percentages of APLs exhibiting either full agonism (open bars), TCR antagonism (shedded bars) or partial agonism (closed bars) are indicated for each residue. APLs carrying substitutions at putative TCR contact residues, P2, P5 and P7, frequently exhibited TCR antagonism. Some of them, especially APLs substituted at P7, exhibited partial agonism. Because APLs with full agonism or TCR antagonism have to bind to MHC molecules, the frequencies of those peptides indicated by cross hatched bars represent the frequency of peptides with MHC-binding capacity.

residues Glu-58 (P2), Tyr-61 (P5) and Glu-63 (P7) were the most likely to be TCR-recognition sites and only 15.8% (9/57) of APLs stimulated proliferative responses in YN5-32 thereby indicating that substitutions at these residues frequently abrogate T cell recognition. Interestingly, as many as 60.4% (29/48) of non-fully agonistic APLs exhibited TCR antagonism to inhibit the proliferative response of YN5-32 to the wild-type peptide.

Eight (27.6%) of these antagonistic APLs carrying relatively conservative amino acid substitutions exhibited partial agonism to induce large increases in cell size and expression levels of CD4, CD11a (LFA-1α), CD28, CD49d (VLA-4α) and CD95 (Fas), on the T cell surface, as compared with responses to the wild-type peptide. This was the most prominent at Glu-63 (p7) where 5 of 10 antagonistic APLs exhibited partial agonism. These observations indicate that many APLs carrying substitutions at TCR recognition sites in the T cell epitope induce a partial agonism as well as TCR antagonism in YN5-32, as noted by other studies of mouse T cell clones. Differences, such as the absence of anergy induction or little increase in CD25 expression by partially agonistic APLs have been noted in human Th cells. The polymorphism (Ser-Tyr) at the $DR\beta^{37}$ residue induced conformational changes of peptides, which can be distinguished by YN5-32 TCR in some but not all peptides, providing further evidence for altered human T cell responses induced by minor modifications of TCR ligands (Chen et al., 1997).

Based on this knowledge of Th cell responses to APLs, we identified many antagonistic APLs which can inhibit proliferation of Th-cell clones auto-reactive to the acetylcholine receptor α chain derived self peptide in the context of the disease-susceptible HLA-DQ6 molecule and established from a patient with infant-onset myasthenia gravis unique to Asian populations (Kanai et al., 1997).

3. Unique partially agonistic APLs inducing prolonged survival of Th cells in the absence of antigenic stimulus

By utilizing three other human Th cell clones with distinct TCR-Vβ recognizing the same non-self mycobacterial *Bacillus Calmette-Guérin* a (BCGa) peptide/HLA-DR14 complex, we found another type of unique partial agonism, as follows (Matsushita et al., 1997). Stimulation of T cells with a one-residue-substituted APL or a minimally homologous self-peptide fragment can prolong the in vitro survival of T cells in the absence of antigenic stimuli, in a clone specific-manner. This prolongation is associated with the up-regulation of Bcl-x_L, without proliferation and these peptide-clone combinations are capable of inducing lymphokine secretion. Thus, peptide partial agonism may play a role in the survival of not only thymocytes but also mature Th cells, in the absence of non-self peptide ligands.

4. Augmentation of T cell responses (superagonism) stimulated by APLs: implication to peptide-based cancer immuno-therapy

A T cell response to a tumor requires a tumor antigen processed into peptides which can be presented to $CD8^+$ cytotoxic T cells by MHC class I molecules, and to $CD4^+$ Th cells by MHC class II molecules. While cytotoxic T cells can kill tumor cells directly, some Th1 cells can mediate cytotoxicity to tumors, amplify responses of cytotoxic T cells, and activate APC, through secretion of lymphokines to augment anti-cancer immunity. We established a Th cell clone reactive to oncogenic and mutated p21 Ras proteins as well as mutated peptides, in an HLA-DR1-restricted manner. We provided evidence for augmentation of proliferation and production of gamma interferon (IFN-γ) and granulocyte-macrophage colony-stimulating factor (GM-

CSF) by this T cell clone in recognition of APLs carrying a single residue substitution in the mutated P21 Ras peptide (Yokomizo et al., 1997).

We also identified superagonistic and single residue substituted APLs derived from leukemia-associated TEL/AML1 fusion peptide (IGRIA/ECILGMNPSR) (Yun et al., 1999). The APLs having Val or Leu substitutions at putative P8(Gly) or P9(Met) of the peptide respectively stimulated much stronger proliferation and production of Th1-type cytokines in a Th clone reactive to TEL/AML1 fusion peptide in the context of HLA-DP17. These superagonistic APLs can be given consideration for anti-leukemic immunotherapy.

To identify peptide superagonists in a systematic and sophisticated manner, we used a combinatorial peptide library and mass-spectrometry (Tanaka et al., 1999). The proliferative responses of a human $CD4^+$ T cell clone reactive to a self-K-Ras-derived peptide, Ras p3-20 (^{3}EYKLVVVGAGGVGKSALT^{20}), were tested using a set of X9 combinatorial peptide libraries containing the flanking residues (EYKLVXXXXXXXXXSALT, where X indicates random amino acids). Certain peptide libraries, such as EYKLVXXXXXXXMXXSALT and EYKLVXXXXXXXHXSALT, stimulated a marked proliferation of T cells. However, no combinations of substitutions tested, such as EYKLVXXXXXXXMHXSALT, exhibited additive effects. We subsequently synthesized peptides with degenerate sequences (a mixture of 480 species), where each position is composed of the wild-type residue or of amino acids that induced the proliferation of T cells, in positional scanning. Interestingly, one fraction of degenerate peptides, separated by reverse-phase HPLC, stimulated a much stronger proliferation than did the Ras p3-20; in addition, the retention time of this fraction was distinct from that of Ras p3-20. Mass spectrometry analysis of this fraction and flanking fractions identified five peptide species that exhibit strong signals in a manner that parallels the antigenic activity. Finally, 17 candidate peptide sequences were deduced from mass spectrometry and hydrophobicity scoring results, of which two peptides (EYKLVVVGAGGMLKSALT and EYKLVVVGAGGMIKSALT) did induce 52- and 61-fold stronger proliferation, respectively, compared with the Ras p3-20. These findings indicate that: (1) synthetic peptides that carry "the best" residue substitution at each position of combinatorial peptide libraries do not always exhibit superagonism, and (2) such a drawback can be overcome with the use of mass spectrometry. This approach provides new perspectives for accurate and efficient identification of peptide superagonists.

5. APL affects not only T cell responses but also APC responses to increase IL-12 production: Implication to peptide therapy inducing Th1-dominance

Human Th0 clone DT13.2 reactive to the group I allergen in *Dermatophagoides farinae* extracts (*Der f* I) p18-31 (^{18}RSLRTVTPIRMQGG^{31}) in the context of HLA-DQ6 (DQA1*0102/DQB1*0602) molecules was generated from a patient with bronchial asthma and DT13.2 produced both interleukin (IL)-4 and IFN-γ. Analysis of changes in DT13.2 responses to *Der f* I p18-31-derived APLs revealed that the substitution of ^{27}Arg to Lys resulted in a significant increase in IEN-γ production, with no remarkable changes either in proliferative response or in IL-4 production (Matsuoka et al., 1996). Interestingly, the selective enhancement of IFN-γ by the APL was accompanied by an increased production of IL-12 and this event was suppressed by an anti-IL-12 antibody down to the level of IFN-γ production induced by the wild-type peptide. The superagonistic APL derived from another Japanese Cedar pollen allergen (*Cry J*I) also augmented production of IFN-γ in a human Th0 clone reactive to *Cry J*I peptide/HLA-DR52 complex (Ikagawa et al., 1996).

Our observations suggest that the mode of interaction between TCR and MHC/peptide complex may determine the Th1-predisposing condition by controlling the IL-12 production by APC. Furthermore, this kind of Th1-response inducing APLs may provide peptide therapy for diseases caused by Th2 responses such as allergy.

6. Generation of a Th cell epitope expression library for extensive analysis of degeneracy in peptides recognized by human Th cell clones

Because we found that the systematic detection of cross-recognized epitopes considering the combinatorial effect of amino acids within the epitope is impossible in approaches using positional scanning synthetic combinatorial peptide libraries, we established an alternative method by utilizing molecular genetic approaches. A DNA-based randomized epitope library using class II-associated invariant chain peptide (CLIP)-substituted invariant chains was generated (Fujii et al., 1998; Fujii et al., 2001; Uemura et al., 2003). This approach, by which multiple residues of an antigenic peptide were simultaneously randomized, has the great advantage of producing several conformations of the peptide/HLA-II complexes, and increasing the possibility to identify degenerate sequences with agonistic properties. GAD65-autoreactive T cell clones restricted by disease-susceptible HLA-DR53 and established from patients with type I diabetes were utilized as models. Analysis of agonistic epitopes indicate that recognition by each TCR was significantly affected by combinations of amino acids in the antigenic peptide, although the degree of combinatorial effect differed between each TCR. Protein database searching based on the TCR recognition profile proved successful in identifying several microbial and self-protein-derived mimicry epitopes with limited sequence homology to the original GAD65 epitope. Some of the identified mimicry epitopes were actually produced from recombinant microbial proteins by APCs to stimulate T cell clones. Our data

demonstrate the importance of the combinatorial nature of amino acid residues of epitopes to investigate diversity of T cell recognition and molecular mimicry, and the Th cell epitope display library we established provides a useful tool for these objectives.

7. Altered intracellular signalings induced in a Th-cell clone by APLs

In mouse T cell clones, TCR antagonistic or partially agonistic APLs induce partial phosphorylation of CD3ζ chains leading to the absence of phosphorylation and activation of ZAP-70 (Sloan-Lancaster et al., 1994; Madrenas et al., 1995). Studies of calcium signaling activity in mouse T cells stimulated with APLs indicated that the Ca^{2+} response induced by antagonistic APLs was smaller in amplitude and shorter in duration than that induced by fully agonistic ligands (Sloan-Lancaster et al., 1996; Wülfing et al., 1997).

To determine if APLs affect intracellular activation signals in human Th cells, we investigated changes in intracellular calcium concentrations ($[Ca^{2+}]_i$) in the Th cell clone YN5-32 stimulated with either fully agonistic peptide M12p54-68 or partially agonistic APL E63V (standing for APL having Val-substitution at amino acid residue 63 Glu), or simply antagonistic APL E58M as described in the Section 1 (Chen et al., 1998). Both E63V and E58M stimulated a Ca^{2+} response in ~40% of the T cells, whereas M12p54-68 did so in ~70% of T cells. The most predominant pattern of a Ca^{2+} increase induced by M12p54-68 was a small sinusoidal peak followed by a sustained high response. The most frequent pattern of calcium response induced by E63V was a continuous high response without a preceding sinusoidal peak, whereas that induced by E58M was large with frequent oscillations. Furthermore, our results suggest that the Ca^{2+} response induced by the fully agonistic peptide depends on activation of the genistein-sensitive signaling pathway, including protein tyrosine kinases, whereas the Ca^{2+} response to a simple antagonistic APL completely depends on activation of the GF109203X-sensitive signaling pathway, including protein kinase Cs and extracellular Ca^{2+}. These differences in the $[Ca^{2+}]_i$ response in recognition of different APLs may parallel the unique T cell activation patterns induced by APLs in human T cells.

We then asked whether forced overexpression of partially agonistic TCR-ligands on APCs provides high-avidity TCR-ligands to stimulate T cell proliferation, we generated L cell transfectants expressing various numbers of HLA-DR4 covalently linked with APLs derived from M12p54-68 peptide and observed responses of the cognate T cell clone YN5-32. Some overexpressed HLA-DR4/partially agonistic APL complexes induced T-cell proliferation in a density-dependent manner, however tyrosine-phosphorylation of ZAP-70 and linker for activated T cells (LAT) and kinase activity of ZAP-70 were not detectable (Irie et al., 2003). Our data suggest the presence of an unique signaling pathway coupling TCR-ligation with T cell proliferation in a ZAP-70 less dependent manner, and this activation pathway is observed when TCRs are engaged with relatively low affinity TCR ligands expressed in high density on the surface of APC. This suggests that T cell activation signals are not uniform and they can be alternatively activated depending on binding characteristics between TCRs and their ligands.

8. Conclusions

In conclusion, we observed various kinds of responses to APLs in human Th cell clones, as summarized in Table 1, and the implications of our findings are as follows. (1) It is so far difficult to predict degeneracy of Th cell recognition in a given TCR by analyzing the past literature, and our Th cell epitope expression library using CLIP-substituted invariant chain genes will provide a breakthrough in this field. (2) Our findings may support the following ideas, (1) maintenance of Th cell survival (memory ?) by self APLs in the absence of stimuli with non-self peptides, (2) triggering of autoreactive Th cells by non-self agonistic APLs (molecular mimicry), and (3) a possible application of APLs to augmentation of desirable anti-microbial or anti-tumor immunity, or to inhibition of pathological immune responses such as allergy and autoimmunity. Our analyses of human Th cell responses to APLs have provided pertinent information on the basic immunology of human Th cell biology and also on the strategy for new methods for manipulation of antigen-specific responses of human Th cells.

Acknowledgements

We are grateful to M. Ohara (Fukuoka) for helpful comments. This work was supported in part by Grants-in-Aid 11694294, 11557027 and 14370115 from the Ministry of Education, Science, Technology, Sports, and Culture, Japan, and a Research Grant for Intractable Diseases from Ministry of Health and Welfare, Japan.

References

Brown, J.H., Jardetzky, T.S., Gorga, J.C., Stern, L.J., Urban, R.G., Strominger, J.L., Wiley, D.C., 1993. Three-dimensional structure of the human class II histocompatibility antigen HLA-DR1. Nature 364, 33–39.

Chen, Y.Z., Matsushita, S., Nishimura, Y., 1996. Response of a human T cell clone to a large panel of altered peptide ligands carrying single residue substitutions in an antigenic peptide: characterization and frequencies of TCR agonism and TCR antagonism with or without partial activation. J. Immunol. 157, 3783–3790.

Chen, Y.-Z., Matsushita, S., Nishimura, Y., 1997. A single residue polymorphism at $DR\beta^{37}$ affects recognition of peptides by T cells. Human Immunol. 54, 30–39.

Chen, Y.-Z., Lai, Z.-F., Nishi, K., Nishimura, Y., 1998. Modulation of calcium response by altered peptide ligands in a human T cell clone. Eur. J. Immunol. 28, 3929–3939.

Fujii, S., Senju, S., Chen, Y.-Z., Ando, M., Matsushita, S., Nishimura, Y., 1998. The CLIP-substituted invariant chain efficiently targets an antigenic peptide to HLA class II pathway in L cell. Human Immunol. 59, 607–614.

Fujii, S., Uemura, Y., Iwai, L.K., Ando, M., Senju, S., Nishimura, Y., 2001. Establishment of an expression cloning system for $CD4^+$ T cell epitopes. Biochem. Biophys. Res. Comm. 284, 1140–1147.

Germain, R.N., Margulies, D.H., 1993. The biochemistry and cell biology of antigen processing and presentation. In: Paul, W.E., Fathman, C.G., Metzger, H. (Eds.), Annual Review of Immunology 11. Annual Reviews, Inc., Palo Alto, CA, pp. 403–450.

Hammer, J., Valsasnini, P., Tolba, K., Bolin, D., Higelin, J., Takacs, B., Sinigaglia, F., 1993. Promiscuous and allele-specific anchors in HLA-DR-binding peptides. Cell 74, 197–203.

Ikagawa, S., Matsushita, S., Chen, Y.Z., Ishikawa, T., Nishimura, Y., 1996. Single amino acid substitutions on a Japanese cedar pollen allergen (*Cry j*1)-derived peptide induced alterations in human T cell responses and T cell receptor antagonism. J. Allergy Clin. immunol. 97, 53–64.

Irie, A., Chen, Y.-Z., Tsukamoto, H., Jotsuka, T., Masuda, M., Nishimura, Y., 2003. Unique T cell proliferation associated with PKCμ activation and impaired Zap-70 phosphorylation in recognition of overexpressed HLA/partially agonistic peptide complexes. Eur. J. Immunol. 33, 1497–1507.

Kanai, T., Nomura, Y., Segawa, M., Takagi, K., Senju, S., Matsushita, S., Nishimura, Y., 1997. Immuno-suppressive peptides for a human T cell clone autoreactive to a unique acetylcholine receptor α subunit peptide presented by the disease susceptible HLA-DQ6 in infant-onset myasthenia gravis. Human Immunol. 56, 28–38.

Lyons, D.S., Lieberman, S.A., Hampl, J., Boniface, J.J., Chien, Y., Berg, L.J., Davis, M.M., 1996. A TCR binds to antagonist ligands with lower affinities and faster dissociation rates than to agonists. Immunity 5, 53–61.

Madrenas, J., Wange, R.L., Wang, J.L., Isakov, N., Samelson, L.E., Germain, R.N., 1995. ζ Phosphorylation without ZAP-70 activation induced by TCR antagonists or partial agonists. Science 267, 515–518.

Matsuoka, T., Kohrogi, H., Ando, M., Nishimura, Y., Matsushita, S., 1996. Altered TCR ligands affect APC responses: upregulation of IL-12 by an analogue peptide. J. Immunol. 157, 4837–4843.

Matsushita, S., Takahashi, K., Motoki, M., Komoriya, K., Ikagawa, S., Nishimura, Y., 1994. Allele specificity of structural requirement for peptides bound to HLA-DRB1*0405 and-DRB1*0406 complexes:Implication for the HLA-associated susceptibility to methimazole-induced insulin autoimmune syndrome. J. Exp. Med. 180, 873–883.

Matsushita, S., Kohsaka, H., Nishimura, Y., 1997. Evidence for self-and non-self-peptide partial agonists that prolong clonal survival of mature T cells in vitro. J. Immunol. 158, 5685–5691.

Sette, A., Sidney, J., Oseroff, C., del Guercio, M.F., Southwood, S., Arrhenius, T., Powell, M.F., Colon, S.M., Gaeta, F.C., Grey, H.M., 1993. HLA DR4w4-binding motifs illustrate the biochemical basis of degeneracy and specificity in peptide-DR interactions. J. Immunol. 151, 3163–3170.

Sloan-Lancaster, J., Shaw, A.S., Rothbard, J.B., Allen, P.M., 1994. Partial T cell signaling: altered phospho-ζ and lack of zap70 recruitment in APL-induced T cell anergy. Cell 79, 913–922.

Sloan-Lancaster, J., Allen, P.M., 1996. Altered peptide ligand-induced partial T cell activation: molecular mechanisms and roles in T cell biology. In: Paul, W.E., Fathman, C.G., Metzger, H. (Eds.), Annual Review of Immunology, 14, Annual Reviews, Inc., Palo Alto, CA, pp. 1–27.

Sloan-Lancaster, J., Steinberg, T.H., Allen, P.M., 1996. Selective activation of the calcium signaling pathway by altered peptide ligands. J. Exp. Med. 184, 1525–1530.

Stern, L.J., Brown, J.H., Jardetzky, T.S., Gorga, J.C., Urban, R.G., Strominger, J.L., Wiley, D.C., 1994. Crystal structure of the human class II MHC protein HLA-DR1 complexed with an influenza virus peptide. Nature 368, 215–221.

Tanaka, Y., Ohyama, H., Ogawa, M., Nishimura, Y., Matsushita, S., 1999. Identification of peptide superagonists for a self-K-ras-reactive $CD4^+$ T cell clone. J. Immunol. 162, 7155–7161.

Uemura, Y., Senju, S., Maenaka, K., Iwai, L.K., Fujii, S., Tabata, H., Tsukamoto, H., Hirata, S., Chen, Y.-Z., Nishimura, Y., 2003. Systematic analysis of the combinatorial nature of epitopes recognized by TCR leads to identification of mimicry epitopes for GAD65 specific TCRs. J. Immunol. 170, 947–960.

Wülfing, C., Rabinowitz, J.D., Beeson, C., Sjaastad, M.D., McConnell, H.M., Davis, M.M., 1997. Kinetics and extent of T cell activation as measured with the calcium signal. J. Exp. Med. 185, 1815–1825.

Yokomizo, H., Matsushita, S., Murakami, S., Fujita, H., Shirouzu, M., Yokoyama, S., Ogawa, M., Nishimura, Y., 1997. Augmentation of immune response by an analog of the antigenic peptide in a human T cell clone recognizing mutated Ras-derived peptides. Human Immunol. 52, 22–32.

Yun, C., Senju, S., Fujita, H., Tsuji, Y., Irie, A., Matsushita, S., Nishimura, Y., 1999. Augmentation of immune response by altered peptide ligands of the antigenic peptide in human $CD4^+$ T cell clone reacting to TEL/AML1 fusion protein. Tissue Antigens 54, 153–161.

Generation and genetic modification of dendritic cells derived from mouse embryonic stem cells

Satoru Senju, Shinya Hirata, Hidetake Matsuyoshi, Masako Masuda, Yasushi Uemura, Kimi Araki, Ken-ichi Yamamura, and Yasuharu Nishimura

We developed a method to generate dendritic cells (DCs) from mouse embryonic stem (ES) cells. We cultured ES cells for 10 days on feeder cell layers of OP9, in the presence of granulocyte-macrophage colony-stimulating factor in the latter 5 days. The resultant ES cell–derived cells were transferred to bacteriologic Petri dishes without feeder cells and further cultured. In about 7 days, irregularly shaped floating cells with protrusions appeared and these expressed major histocompatibility complex class II, CD11c, CD80, and CD86, with the capacity to stimulate primary mixed lymphocyte reaction (MLR) and to process and present protein antigen to T cells. We designated them ES-DCs (ES cell–derived dendritic cells), and the functions of ES-DCs were comparable with those of DCs generated from bone marrow cells. Upon transfer to new dishes and stimulation with interleukin-4 plus tumor necrosis factor α, combined with anti-CD40 monoclonal antibody or lipopolysaccharide, ES-DCs completely became mature DCs, characterized by a typical morphology and higher capacity to stimulate MLR. Using an expression vector containing the internal ribosomal entry site–puromycin *N*-acetyltransferase gene or a Cre-lox–mediated exchangeable gene-trap system, we could efficiently generate ES cell transfectants expressing the products of introduced genes after their differentiation to DCs. ES-DCs expressing invariant chain fused to a pigeon cytochrome C epitope presented the epitope efficiently in the context of E^k. We primed ovalbumin (OVA)–specific cytotoxic T lymphocytes in vivo by injecting mice with ES-DCs expressing OVA, thus demonstrating immunization with ES-DCs genetically engineered to express antigenic protein. The methods may be applicable to immunomodulation therapy and gene-trap investigations of DCs. (Blood. 2003;101: 3501-3508)

Introduction

Dendritic cells (DCs) are the most potent antigen-presenting cells (APCs) responsible for priming of naive T cells in the immune response. DCs are involved in the maintenance of immunologic self-tolerance in the periphery, inducing regulatory T cells or anergy of autoreactive T cells.[1-3] It has been reported that distinct subpopulations of DCs preferentially induce differentiation of either T helper 1 (Th1) or Th2 cells.[4-6] Therefore, DCs physiologically play a central role in immunoregulation. Manipulation of functions of DCs by genetic modification and in vivo transfer of DCs with modified property is considered a promising means to control immune responses in an antigen-specific manner.[7] As for the methods for gene transfer to DCs, electroporation, lipofection, and virus vector–mediated transfection have been developed. However, there are several problems related to presently used means (ie, efficiency of gene transfer, stability of gene expression, potential risk accompanying the use of virus vectors, and the immunogenicity of virus vectors). Although improvements have been made in these methods, development of more efficient and safer means is desirable.[8,9]

Embryonic stem (ES) cells are characterized by pluripotency and infinite propagation capacity. Non–virus-mediated methods for gene transfer, including targeted gene integration and procedures for isolation of appropriate recombinant cell clones, have been established for ES cells. Recently, a novel Cre-lox–mediated exchangeable gene-trap system has been developed using TT2 ES cells.[10] The method enables efficient gene-trap, plasmid rescue for the analysis of trapped genes, and targeted integration of replacement vectors to the gene-trapped sites. Genetic modification of ES cells and their subsequent in vitro differentiation to DCs would be an attractive strategy for genetic manipulation of DCs and for analysis of gene functions in DCs.

For hematopoietic differentiation of ES cells in vitro, embryoid body–mediated methods and the OP9-coculture method have been established.[11,12] OP9 is a bone marrow stromal cell line that originated from macrophage colony-stimulating factor (M-CSF)–defective op/op mouse,[13] and generation of various hematopoietic cells from ES cells using OP9 cells as feeder cells has been reported, including granulocytes, erythrocytes, B lymphocytes, and osteoclasts.[11-15] The method has been applied to several molecular and cellular analyses for investigations of hematopoiesis.[16-19]

In the current study, we attempted to establish a method to develop DCs from ES cells in vitro. We adapted the OP9-coculture

From the Division of Immunogenetics, Department of Neuroscience and Immunology, Kumamoto University Graduate School of Medical Sciences, Kumamoto, Japan; and the Division of Developmental Genetics, Department of Organogenesis, Institute of Molecular Embryology and Genetics, Kumamoto University, Japan.

Submitted July 26, 2002; accepted October 1, 2002. Prepublished online as *Blood* First Edition Paper, October 24, 2002; DOI 10.1182/blood-2002-07-2254.

Supported by Grants-in-Aid 11557027, 12213111, 14370115, 14657082, and 14570421 from the Ministry of Education, Science, Technology, Sports, and Culture of Japan.

Reprints: Yasuharu Nishimura, Division of Immunogenetics, Department of Neuroscience and Immunology, Kumamoto University Graduate School of Medical Sciences, 2-2-1 Honjo, Kumamoto 860-0811, Japan; e-mail: mxnishim@gpo.kumamoto-u.ac.jp.

The publication costs of this article were defrayed in part by page charge payment. Therefore, and solely to indicate this fact, this article is hereby marked "advertisement" in accordance with 18 U.S.C. section 1734.

method for hematopoietic differentiation of ES cells. For induction of differentiation to DCs, we used granulocyte-macrophage colony-stimulating factor (GM-CSF), the cytokine essential for in vitro generation of DCs from hematopoietic cells.[20-22] The generated ES cell–derived DCs were morphologically and functionally comparable with those differentiated in vitro from bone marrow cells. For gene transfer to ES cells to generate genetically modified DCs, we adopted 2 means: an expression vector containing β-actin promoter and internal ribosomal entry site (IRES)–puromycin *N-acetyltransferase* gene and Cre-lox–mediated targeted gene integration into gene-trapped ES cell clones.

Materials and methods

Mice

Balb/c, CBA, and C57BL/6 mice were obtained from Clea Animal (Tokyo, Japan) or Charles River (Hamamatsu, Japan) and kept in specific pathogen-free conditions. Male CBA and female C57BL/6 mice were mated to generate (CBA × C57BL/6) F1 mice.

Peptides, cell lines, and cytokines

The E^k-binding peptide pigeon cytochrome C $(PCC)_{88\text{-}104}$, KAERADLIAY-LKQATAK, and K^b-binding peptide ovalbumin $(OVA)_{257\text{-}264}$, SIINFEKL, were synthesized with 9-fluorenylmethyloxycarbonyl (F-MOC) method on an automatic peptide synthesizer (PSSM8; Shimadzu, Kyoto, Japan) and purified using high-performance liquid chromatography. The ES cell line TT2, derived from (CBA × C57BL/6) F1 blastocysts,[23] was maintained as described.[24] The T-cell hybridomas, RF33.70, recognizing $OVA_{257\text{-}264}$ in the context of K^b, and 2B4, recognizing $PCC_{88\text{-}104}$ in the context of E^k, have been described elsewhere.[25,26] The M-CSF–defective bone marrow–derived stromal cell line, OP9,[12] was maintained in α–minimum essential medium (α-MEM) supplemented with 20% fetal calf serum (FCS); and to form feeder cell layers, OP9 cells were seeded onto culture plates precoated with gelatin. Recombinant mouse GM-CSF was provided by Kirin Brewery (Tokyo, Japan). Recombinant mouse interleukin-4 (IL-4), tumor necrosis factor α (TNF-α), and IL-1β were purchased from Peprotec (London, United Kingdom).

Hematopoietic differentiation of TT2 ES cells

The induction of hematopoietic differentiation of TT2 ES cells was done as described.[11] After 15 days of culture on feeder cell layers of OP9 without exogenous cytokines, ES cell–derived cells were harvested by pipetting and were then subjected to cytospin preparation and May-Giemsa staining.

Induction of differentiation of ES cells into DCs

The procedure for induction of differentiation of ES cells into DCs is shown in Figure 1. ES cells were suspended in α-MEM supplemented with 20% FCS and seeded (1.5×10^4/2 mL medium/well) onto OP9 cell layers in 6-well plates. On day 3, half of the medium was removed and 2 mL fresh medium was added to each well. On day 5, cells were harvested using phosphate-buffered saline (PBS)/0.25% trypsin/1 mM EDTA (ethylenediaminetetraacetic acid), reseeded onto fresh OP9 cell layers, and cultured in α-MEM supplemented with 20% FCS and GM-CSF (1000 U/mL). At this step, cells recovered from 3 wells of 6-well culture plates were suspended in 20 mL medium and seeded into one 150-mm dish. On day 10 (5 days after the transfer), floating cells were recovered by pipetting. On average, 4 to 8×10^6 cells were recovered from one 150-mm dish, thus indicating 100 to 200 times increase in cell number from undifferentiated ES cells. The recovered cells were transferred to bacteriologic Petri dishes (2.5×10^5 cells/90-mm dish) without feeder cells, and cultured in RPMI-1640 medium supplemented with 10% FCS, GM-CSF (500 U/mL), and 2-mercaptoethanol. After days 17 to 19, 1.5 to 2×10^5 floating or loosely adherent cells were recovered per dish (ES cell–derived dendritic cells [ES-DCs]), the number of cells increasing about 100 times over the number of undifferentiated ES cells. When over half the number of cells became adherent after day 12, the transfer of floating cells to fresh dishes on around day 15 improved the purity and yield of ES-DCs. To induce a complete maturation of ES-DCs, cells cultured for longer than 10 days in Petri dishes were transferred to fresh Petri dishes and cultured in RPMI/10% FCS without GM-CSF. The next day, IL-4 (10 ng/mL), TNF-α (5 ng/mL), plus anti-CD40 mAb (10 μg/mL, clone 3/23), or IL-4, TNF-α, plus lipopolysaccharide (LPS; 1 μg/mL) were added. After 2 or 3 days, cells were harvested by pipetting and used for functional and flow cytometric analysis. In most experiments, some cells harvested on days 5 and 10 were freeze-stocked for future use.

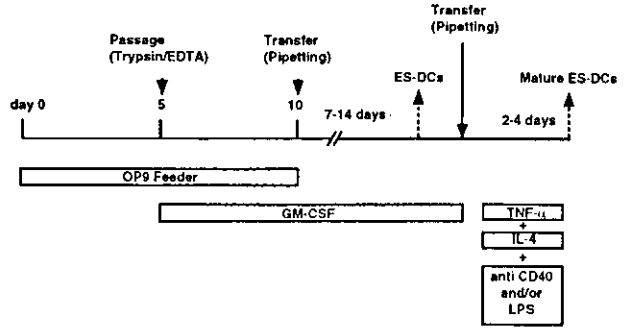


Figure 1. Overview of the culture protocol for generation of DCs from ES cells.

Generation of DCs from mouse bone marrow cells

Generation of dendritic cells from mouse bone marrow cells was done according to the reported procedures[20,27] with some modifications. In brief, bone marrow cells were isolated from (C57BL/6 × CBA) F1 mice and cultured in bacteriologic Petri dishes (1.5×10^6/90-mm dish) in RPMI-1640 medium supplemented with 10% FCS, GM-CSF (500 U/mL), and 2-ME (50 μM). Culture medium was changed by half on days 5 and 10, and floating cells harvested by pipetting between 9 to 12 days of the culture were used as bone marrow–derived DCs (BM-DCs) in functional experiments. For the purpose of maturation, TNF-α (5 ng/mL) was added on the day before analysis.

Flow cytometric analysis

Staining of cells and analysis on a flow cytometer (FACScan, Becton Dickinson, San Jose, CA) was done as described previously.[28] The procedure for intracellular staining with anti-human CD74 mAb was also described previously.[29] Antibodies used for staining were as follows: fluorescein isothiocyanate (FITC)–conjugated anti-H-$2K^b$ (clone CTKb, mouse IgG2a; Caltag, Burlingame, CA), anti-I-A^b (clone 3JP, mouse IgG2a), anti-I-E^k (clone 8705-A, mouse IgG2a; Cedarlane, Hornby, Canada), anti-mouse CD11c (clone N148, hamster IgG; Chemicon, Temecula, CA), R-PE–conjugated anti-mouse CD80 (clone RMMP-1, rat IgG2a; Caltag), R-PE–conjugated anti-mouse CD86 (clone RMMP-2, rat IgG2a; Caltag), anti-mouse CD40 (clone 3/23, rat IgG2a; Serotec, Oxford, United Kingdom), R-PE–conjugated anti-F4/80 (A3-1, rat IgG2b; Serotec), anti-mouse CD205 (clone NLDC-145, rat IgG2a; Serotec), FITC-conjugated anti-mouse CD8α (clone 53-6.7, rat IgG2a; Pharmingen, San Diego, CA), FITC-conjugated anti-human CD74 (clone M-B741, mouse IgG2a; Pharmingen), FITC-conjugated goat anti–mouse Ig (Pharmingen), FITC-conjugated goat anti–hamster IgG (Caltag), FITC-conjugated goat anti–rat Ig (Pharmingen), mouse IgG2a control (clone G155-178; Pharmingen), FITC-conjugated mouse IgG2a control (clone G155-178; Pharmingen), R-PE–conjugated rat IgG2a control (clone LO-DNP-16; Caltag), FITC-conjugated rat IgG2a control (clone LODNP-57; Beckman-Coulter, Tokyo, Japan), hamster IgG control (clone 530-6; Caltag), and rat IgG2a control (clone LO-DNP-16; Caltag).

Mixed lymphocyte reaction (MLR)

Splenic mononuclear cells were prepared from unprimed female Balb/c mice, and T cells were isolated from the splenic mononuclear cells by

magnetic cell sorting using anti-CD90 (Thy1.2) supermagnetic MicroBeads (Miltenyi Biotec, Bergisch-Gladbach, Germany), and then used as responders. Graded numbers of stimulator cells were x-ray irradiated (35 Gy) and cocultured with responder cells (1.5×10^5) in wells of 96-well round-bottomed culture plates and cultured for 4 days. [^{3}H]-thymidine (6.7 Ci/mmol [247.9 GBq/mmol]) was added to the culture (1 μCi/well [0.037 MBq/well]) in the last 16 hours. At the end of the culture, cells were harvested onto glass fiber filters (Wallac, Turku, Finland), and the incorporation of [^{3}H]-thymidine was measured by scintillation counting.

Antigen presentation assay

DCs were seeded onto 96-well flat-bottomed culture plates with or without PCC protein (50 μg/mL; Sigma, St Louis, MO) or peptide (10 μM). After 6 hours, 2B4 hybridoma cells were added (5×10^4/well). After 24 hours of culture, the supernatant (50 μL/well) was collected and added to cultures of the IL-2–dependent cell line, CTLL-20 (5×10^3/100 μL/well), in 96-well flat-bottomed culture plates. After 16 hours, [^{3}H]-thymidine was added and cells were incubated for a further 8 hours. The incorporation of [^{3}H]-thymidine by CTLL-20 was measured by scintillation counting. In some experiments, ES-DCs were fixed as follows. Cells were washed with PBS and suspended in PBS at 10^6 cells/mL, and diluted glutaraldehyde was added to the final concentration of 0.002%. After incubation for 30 seconds at room temperature, equal volume of 0.2 M L-lysine/PBS was added and mixed gently, and cells were incubated for 1 minute at room temperature. Cells were sequentially washed with PBS and with culture medium and used in the experiments. In experiments using BM-DCs pulsed with peptide, OVA or PCC peptide was added to the final concentration of 1 or 10 μM, incubated for 4 hours, washed twice, and used as stimulator cells.

Plasmid construction

To obtain pCI-PCC, the expression vector presenting PCC epitope on major histocompatibility complex (MHC) class II molecules and driven by the SRα promoter, double-stranded oligo DNA encoding the PCC epitope, 5′-AAGGCAGAAAGGGCAGACCTAATAGCTTATCTTAAACAAGCT-ACTGCCAAG-3′, was inserted into the previously reported human invariant chain–based epitope-presenting vector, pCI.[30] The coding region of this construct was transferred to pCAG-IP,[31] a mammalian expression vector containing the chicken β-actin promoter and IRES-puromycin *N*-acetyltransferase gene cassette, to generate pCAG-PCC-IP. The replacement vector, p6SEFPPF, and the Cre expression vector, pCAGGS-Cre, have been described elsewhere.[10,32-35] A cDNA fragment coding for OVA protein was inserted into a mammalian expression vector pCAGGS to make pCAGGS-OV. The DNA fragment containing β-actin promoter–cDNA for OVA-rabbit β-globin poly (A) signal was excised from this construct and inserted into p6SEFPPF replacing the enhanced green fluorescent protein–coding sequence to obtain p6AOVP.

Quantitative analysis of β-galactosidase activity of ES-DCs

Generation and characterization of gene-trapped TT2 ES cell clones, in which lox-*β-geo* (*β-galactosidase/neomycin resistance* fusion gene)–lox cassette was introduced as a reporter gene, have been reported elsewhere.[10] ES cell clones were differentiated to DCs according to the procedure described above. After differentiation, ES-DCs were suspended in lysis buffer (150 mM NaCl/20 mM tris(hydroxymethyl)aminomethane [Tris-HCl], pH 8.0), and cell lysates were prepared by 2 cycles of freezing and thawing. For each cell lysate sample, the protein concentration was measured using a protein assay kit (MIRCO BCA assay kit; Pierce, Rockford, IL) and the β-galactosidase (β-gal) activity was quantified using a β-gal assay kit (Gene Therapy Systems, San Diego, CA) using chlorophenol red-β-D-galactopyranoside as substrate. Relative β-gal activity (β-gal activity/protein concentration) was calculated for each sample, and the gene-trapped ES cell clone showing the highest β-gal activity after DC differentiation was selected for transfection with the replacement vector.

Transfection of ES cells

TT2 ES cells maintained on layers of primary embryonic fibroblasts (PEFs) were harvested and suspended in Dulbecco modified Eagle medium at a concentration of 2.5×10^7/mL, and 1×10^7 cells were electroporated in a 4-mm gap cuvette under the condition of 200 V and 950 μF. For transfection with pCI-PCC or pCAG-PCC-IP, 40 μg linearized plasmid DNA was used. For Cre-mediated targeted integration of the replacement vector into a gene-trap ES clone, 20 μg each of p6AOVP and pCAGGS-Cre in circular form were used for transfection. Site-specific integration into ES cells using a pair of mutant lox, lox71 and lox66, has been described previously.[33] Transfected ES cells were cultured on PEF feeder layers in 90-mm culture dishes and selected with puromycin (2 μg/mL) on days 3, 5, and 7 for 24 hours each, and drug-resistant colonies were picked up on day 9 into 24-well culture plates with PEFs.

Transfer of ES-DCs into mice and cytotoxity assay

ES-DCs were stimulated with IL-4, TNF-α, and anti-CD40 mAb and injected intraperitoneally into mice (5×10^5 cells/mouse). Injections were given twice at a 7-day interval, and 7 days after the second injection, mice were killed and spleen cells isolated. Spleen cells were treated with hemolysis buffer (140 mM NH_4Cl, 10 mM HEPES [*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid], pH 7.4) for one minute, washed, and cultured in 24-well culture plates (2.5×10^6/well) in 45% RPMI/45% AIMV/10% horse serum supplemented with recombinant human IL-2 (100 U/mL) and $OVA_{257-264}$ peptide (0.1 μM). After 5 days, cells were recovered, and viable cells isolated with lympholyte-M (Cedarlane) were used as effector cells. As target cells, EL-4, a thymoma cell line which originated from a C57BL/6 mouse, was labeled with sodium [^{51}Cr]-chromate for one hour at 37°C and washed twice with RPMI/10% FCS. Subsequently, target cells were incubated in 24-well culture plates (1×10^6 cells/well) with or without 10 μM OVA peptide for 3 hours, harvested, washed with RPMI/10% FCS, and seeded onto 96-well round-bottomed culture plates (5×10^3 cells/well). Effector cells were added (5×10^4cells/well) to the target cells and incubated for 4 hours at 37°C. At the end of the incubation, the plates were centrifuged, and supernatants (50 μL/well) were harvested and counted on a gamma counter. The percentage of specific lysis was calculated as: $100 \times$ [(experimental release − spontaneous release)/(maximal release − spontaneous release)]. Spontaneous release and maximal release were determined in the presence of either medium or 1% Triton X-100, respectively.

Results

Application of OP9-coculture method to generate hematopoietic cells from TT2 ES cells

For induction of hematopoietic differentiation of ES cells, we used OP9 cells, a bone marrow stromal cell line defective in the M-CSF gene,[13] as feeder cells. Successful hematopoietic differentiation by the OP9-coculture method of ES cell lines derived from 129 strain of mice, such as D3 and E14, has been reported.[11,12,14,17] In the current study, we used another line of ES cell, TT2, established from (CBA × C57BL/6) F1 blastocysts,[23] which has been used in gene targeting to generate many lines of mutant mice and also for a large-scale gene-trap project.[10,24]

To determine if the OP9-coculture method can be applied to TT2, we cultured TT2 ES cells following the reported culture procedure.[11] TT2 ES cells, maintained on PEFs in the presence of leukemia inhibitory factor (LIF), were transferred onto the OP9 cell layer and cultured without exogenous cytokines (Figure 2A). Most of the ES cell colonies showed a differentiated morphology in 4 to 5 days (Figure 2B). After 5 days of culture on OP9 feeder layers, ES cell–derived cells were transferred onto freshly prepared OP9 feeder layers and cultured for another 10 days. Cells of various morphologies floating or loosely adherent to the feeder cells appeared. We harvested the differentiated cells and examined them after May-Giemsa staining. As shown in Figure 2C-E, we observed hematopoietic cells of at least erythroid, myeloid, and megakaryocytic lineage. Therefore, this method is applicable also to TT2 ES cells.

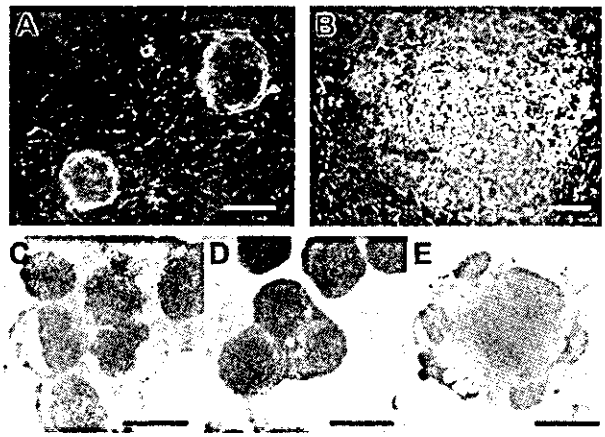


Figure 2. Hematopoietic differentiation of TT2 ES cells on feeder cell layers of OP9. (A-B) Phase-contrast micrographs of TT2 ES cell colonies on OP9 feeder cell layers on day 3 (A) and day 5 (B) are shown. (C-E) May-Giemsa staining of cytospin specimens of hematopoietic cells derived from TT2 ES cells. TT2 cells were cultured on OP9 feeder cell layer for 15 days in total, without addition of exogenous cytokines. Floating cells were applied to cytospin preparations and stained with May-Giemsa. Cells of myeloid (C), erythroid (D), and megakaryocytic (E) lineages are shown. Scale bars represent 50 μm (A-B); and 20 μm, (C-E).

Development of DCs from ES cells

To induce differentiation to DCs, the above-described mesodermally differentiated ES cell–derived cells harvested from a 5-day culture on OP9 feeder layers were cultured on fresh OP9 cell layers in the presence of exogenous GM-CSF (Figure 1). In comparison with the culture without exogenous GM-CSF, addition of this cytokine resulted in appearance of a larger number of floating cells. On day 8 (3 days after the transfer), we observed many round and relatively homogenous floating cells (Figure 3A) and most expressed CD11b, suggesting their commitment to myeloid cell lineage. On day 10, cells floating or loosely adherent to feeder cells were recovered by pipetting and transferred to bacteriologic Petri dishes without feeder cells (Figure 3B-C). After this passage, some (< 30%) of the transferred cells adhered to the dish surface and resembled macrophages. On days 15 to 18, floating cells could be divided roughly into 2 types, 1 with a round shape and of a larger size, the other smaller and irregularly shaped with protrusions (Figure 3D). In addition, clusters of floating cells (Figure 3E) of the latter type were observed after days 17 to 19, and the cell clusters gradually increased. Floating cells were positive for MHC class I, MHC class II, CD80, CD86, DEC205, and CD11c (Figure 4A-B). Based on the morphology, surface phenotype, and function

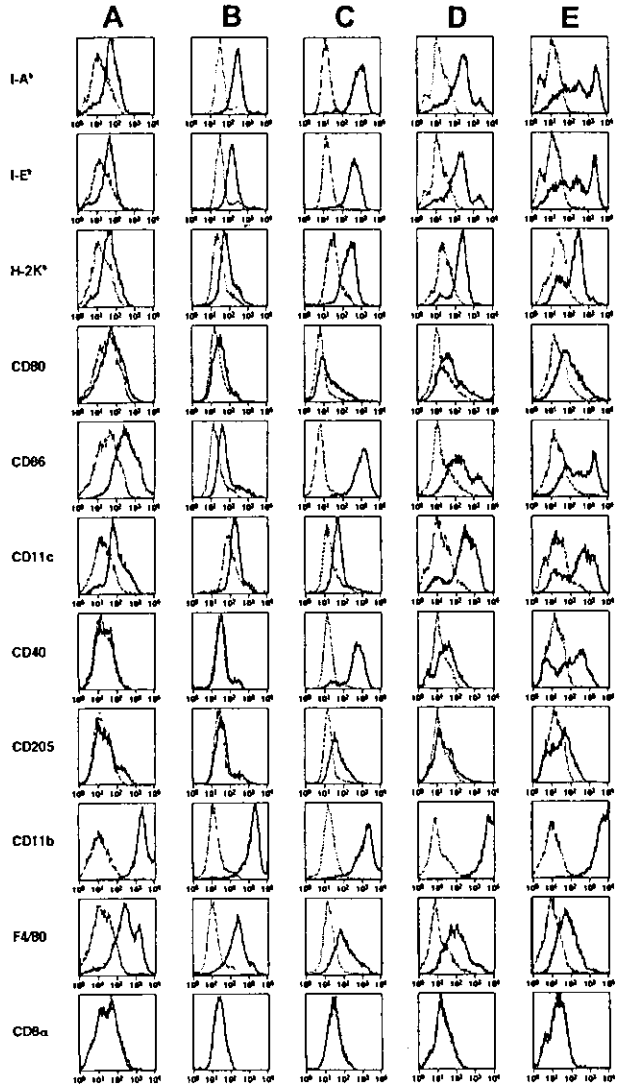


Figure 4. Surface phenotypes of DCs differentiated from ES cells and bone marrow cells. ES-DCs on day 17 (A), day 26 (B), and when given maturation stimuli (C) were analyzed using flow cytometry on the surface expression of indicated molecules. (C) For analysis on expression of CD11c, CD40, and CD205, cells were stimulated with IL-4, TNF-α, plus LPS, and for the analysis on expression of other molecules, cells stimulated with IL-4, TNF-α, plus anti-CD40 mAb were used. For comparison, DCs generated from bone marrow cells by 10-day culture in the presence of GM-CSF (D) and those stimulated with TNF-α for 20 hours (E) were also analyzed. Staining patterns with specific antibodies (thick lines) and isotype-matched controls (thin dotted lines) are shown.

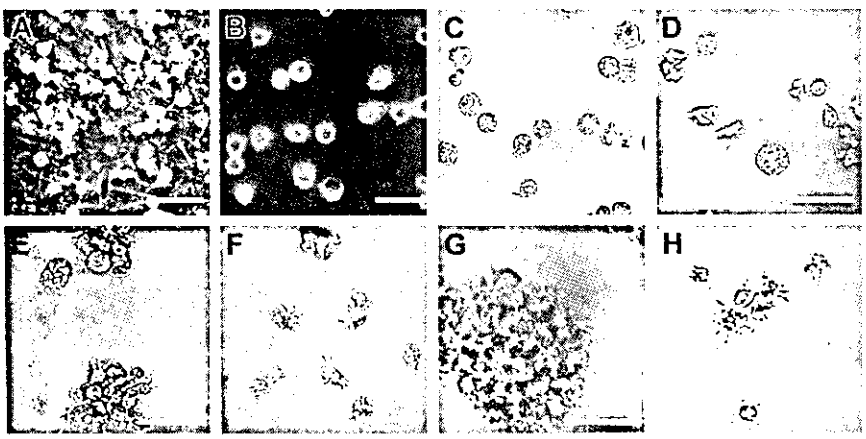


Figure 3. Morphology of ES cell–derived DCs. ES cell–derived cells on day 8 (A), day 12 (B-C), day 17 (D-E), and day 27 (F) of differentiation culture are shown. Cells on day 24 were recovered and stimulated for 2 days with IL-4, TNF-α, plus agonistic anti-CD40 mAb (G), or with IL-4, TNF-α, plus LPS (H). Panels A and B are phase-contrast micrographs. Scale bars represent 20 μm.

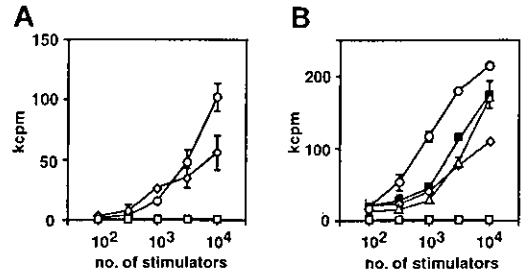


Figure 5. Allogeneic MLR-stimulating capacity of ES-DCs. (A) Graded numbers of ES-DCs harvested on day 17 (◇), ES-derived cells on day 10 (□), and (CBA × C57BL/6) F1 BM-DCs cultured for 11 days (○) were irradiated and cocultured with splenic T cells (1.5×10^5) isolated from Balb/c mice for 4 days, and the proliferative response of T cells in the last 16 hours of the culture was measured by [^{3}H]-thymidine uptake. (B) ES-DCs harvested on day 24 and stimulated with IL-4/TNF-α/anti-CD40 (◇), IL-4/TNF-α/LPS (△), or IL-4/TNF-α/anti-CD40/LPS (■) were used as stimulators. BM-DCs cultured for 10 days and stimulated with TNF-α (○) for 20 hours were also used as stimulators. For control, ES-derived cells cultured on OP9 feeder cell layers for 10 days were also used as stimulators (□). Mean values in kilo cpm (kcpm) ± standard deviation for triplicate cultures are shown.

(described below), we referred to cells with protrusions as ES-DCs. ES-DCs were positive for F4/80 and CD11b and negative for CD8α, suggesting that they were of myeloid lineage. However, further analysis may be necessary to definitely determine the lineage of ES-DCs. Further culture in Petri dishes in the presence of GM-CSF resulted in appearance of cells with longer protrusions showing morphology of mature DCs (Figure 3F).

Induction of maturation of ES-DCs

The induction of maturation by separating floating cells from adherent cells was noted in culture of bone marrow– and skin-derived DCs.[20,27,36,37] To induce further maturation of ES-DCs, we recovered floating cells from 14- to 16-day cultures in Petri dishes (on days 24-26 in Figure 1) and transferred these to new Petri dishes. Some transferred cells became typical mature DCs in 2 to 3 days, bearing large veils or long dendritic protrusions, along with dead cells and adherent cells. We tested several factors reported to have effects on maturation of DCs (IL-4, TNF-α, IL-1β, anti-CD40 mAb, and LPS) in single use or in combinations. As a result, we found that the combination of IL-4, TNF-α, plus anti-CD40, or IL-4, TNF-α, plus LPS drastically increased the number of typical mature DCs. Most of the mature ES-DCs formed clusters if stimulated with IL-4, TNF-α, plus anti-CD40 (Figure 3G). On the other hand, most floating cells did not cluster if stimulated with IL-4, TNF-α, plus LPS (Figure 3H). The stimulated ES-DCs expressed higher levels of surface MHC class I, MHC class II, CD80, and CD86 than did cells before the treatment (Figure 4A-C). In addition, cells stimulated with IL-4, TNF-α, plus LPS expressed CD40, for which ES-DCs before stimulation were negative. On the other hand, expression of CD11b and F4/80 decreased slightly and that of CD11c did not change significantly. We compared the surface phenotype of ES-DCs with that of BM-DCs generated by culture in the presence of GM-CSF for 10 days (Figure 4D) and those further stimulated with TNF-α (Figure 4E). Levels of expression of MHC class II, CD40, CD80, and CD86 were almost comparable between mature ES-DCs and BM-DCs.

Stimulation of primary MLR by ES-DCs

To test ES-DCs for the capacity to activate naive T cells, we did primary MLR assays using ES-DCs as stimulators. Because H-2 of TT2 cells is of the k/b haplotype, allogenic splenic T cells purified from unprimed Balb/c mice ($H\text{-}2^d$) were used as responders. ES-DCs prepared from 7-day culture in Petri dishes (day 17 in Figure 1) had a capacity to stimulate MLR comparable with that of BM-DCs cultured for 10 days[27] (Figure 5A), although it is possible that BM-DCs generated by different culture protocols have stronger stimulation capacity. In contrast, ES cell–derived cells harvested from a 10-day culture on OP9 feeder cell layers (day 10 in Figure 1) did not stimulate T cells. We also tested DCs given maturation stimuli (Figure 5B). We tested the T-cell stimulatory capacity of ES-DCs treated with 3 different stimulation cocktails: IL-4/TNF-α/anti-CD40 mAb, IL-4/TNF-α/LPS, or IL-4/TNF-α/anti-CD40 mAb/LPS. Consistent with the elevation of surface MHC class II and costimulatory molecules, the maturation stimuli enhanced the capacity to stimulate T cells.

Antigen processing and presentation by ES-DCs

To examine the APC function of ES-DCs, presentation of PCC epitope to a T-cell hybridoma, 2B4, recognizing $PCC_{88\text{-}104}$ in the context of $I\text{-}E^k$, was tested. ES-DCs cultured for 17 days in Petri dishes (day 27 in Figure 1) and bone marrow–derived DCs cultured for 10 days were harvested and incubated with PCC protein, to allow for capture of the antigen. After 6 hours, 2B4 cells were added and presentation of the PCC epitope by DCs was measured by quantifying the IL-2 produced by 2B4. We compared functions of these 2 kinds of DCs under the conditions of different numbers of APCs and concentrations of antigenic protein. As shown in Figure 6A-B, APC activity of ES-DCs was even stronger than BM-DCs. To rule out the possibility that the response of T cells was induced by direct binding of peptide fragments contaminated in the PCC protein to I-E molecules, we fixed ES-DCs with glutaraldehyde. As shown in Figure 6C, fixation of ES-DCs before addition of the protein abrogated the response of T cells, whereas ES-DCs fixed under the same condition and added with $PCC_{88\text{-}104}$ synthetic peptide induced the response of 2B4. These results indicate that ES-DCs can capture and process soluble protein antigens and present the resultant peptides in the context of MHC class II molecules.

Genetic modification of ES-DCs using an expression vector containing IRES-puromycin *N*-acetyltransferase cassette

For the genetic modification of ES-DCs, we planned to introduce expression vectors into ES cells and develop DCs from the ES cell transfectants. For this purpose, we first used the MHC class II–restricted epitope presentation vector, pCI,[29,30] in which the

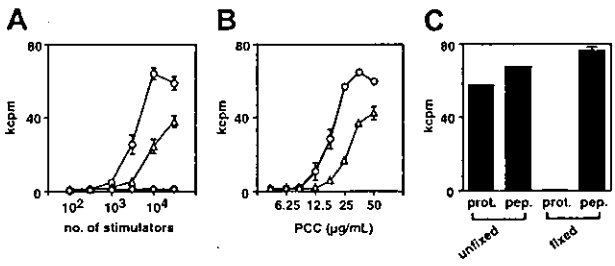


Figure 6. Antigen-presenting capacity of ES-DCs. (A) Graded numbers of ES-DCs (○) and BM-DCs (△) were incubated with PCC protein (50 μg/mL) for 6 hours, and subsequently 2B4 hybridoma cells were added (5×10^4/well). ES-DCs (●) and BM-DCs (◇) were also tested in the absence of the protein. After 24 hours, culture supernatant was collected and IL-2 produced by the hybridoma was measured by proliferation of CTLL-20 cells. (B) ES-DCs (○) and BM-DCs (△) were plated (3×10^4/well) and incubated with graded doses of PCC protein for 6 hours and subsequently added with 2B4 cells. IL-2 production by 2B4 was measured as in panel A. (C) ES-DCs fixed with glutaraldehyde or left unfixed were plated (3×10^4/well) and incubated with PCC protein (50 μg/mL, prot.) or $PCC_{88\text{-}104}$ peptide (10 μM, pep.) for 6 hours, and subsequently 2B4 hybridoma cells were added. Results were expressed as mean cpm of triplicate (A-B) or duplicate (C) cultures ± standard deviation.

class II–associated invariant chain peptide (CLIP) region of the human invariant chain (Ii) is substitutive with antigenic peptides and the expression in mammalian cells is driven by the SRα promoter. We inserted a DNA fragment coding $PCC_{88\text{-}104}$ into pCI to make pCI-PCC and transfected ES cells with this vector. We differentiated 48 transfected ES cell clones to DCs, and examined their expression of human Ii (CD74), using flow cytometry. To our disappointment, only 1 of 48 clones expressed human Ii and the level of expression of the clone was very low.

To improve the efficiency of expression of the introduced genes, we used a vector containing a β-actin promoter and an IRES-*puromycin N-acetyltransferase* cassette, pCAG-IP.[31] We transferred the DNA fragment coding for Ii-$PCC_{88\text{-}104}$ fusion protein from pCI-PCC to pCAG-IP to obtain pCAG-PCC-IP (Figure 7A) and transfected ES cells with this construct. We picked up puromycin-resistant ES cell clones and differentiated them to DCs. We observed that 24 (85%) of 28 clones analyzed expressed human Ii at the level readily detectable by flow cytometric analysis (Figure 7B-C). We examined ES-DCs presenting the PCC epitope generated by this procedure for the potential to stimulate the PCC-specific T-cell hybridoma, 2B4. As shown in Figure 7D, the ES-DCs introduced with the Ii-$PCC_{88\text{-}104}$ expression vector stimulated the T-cell hybridoma more efficiently than that of BM-DCs prepulsed with 10 μM synthetic PCC peptide for 4 hours.

Cre-lox–mediated targeted gene introduction into gene-trapped ES cell clones

As an alternative strategy to efficiently obtain ES cell transfectants, which expressed the introduced genes after differentiation to DCs, we used targeted gene integration into gene-trapped ES cell clones. Cell lineage–specific gene-expression patterns are regulated not only by transcription factors that bind specific nucleotide sequences but also by epigenetic mechanisms; that is, activation and inactivation of specific chromatin domains are controlled by the status of histone acetylation and DNA methylation. We supposed that, by integrating expression constructs into certain chromosomal region replacing some gene, which is actively transcribed in DCs, we could efficiently obtain ES cell transfectant clones expressing the introduced genes after differentiation to DCs.

A library of gene-trapped TT2 ES cells has been recently generated, in which a lox-*β-geo*-lox cassette as reporter gene was inserted into various chromosomal regions, trapping various genes.[10] From the gene-trapped ES cell library, we first selected 20 ES cell clones that showed strong β-gal activity when differentiated into mesodermal cell lineage. These ES cell clones were subjected to the DC-differentiation culture, and resultant ES-DCs were analyzed for β-gal activity. From the 20 gene-trapped ES cell clones, we chose 1 clone that showed the highest β-gal activity after differentiation to DCs and that had a DC-differentiation capacity comparable with that of parental TT2 ES cells. We transfected the selected clone with a replacement vector, p6AOVP, to introduce OVA expression cassette (OVA cDNA driven by the rabbit β-actin promoter ligated with the puromycin *N*-acetyltransferase gene driven by the pgk promoter) replacing the *β-geo* sequence (Figure 8A). After cotransfection with p6AOVP and pCAGGS-Cre, which was for transient expression of Cre recombinase, and selection with puromycin, we picked up puromycin-resistant clones and induced their differentiation to DCs. We analyzed the expression of OVA protein after differentiation by detecting the capacity of each cell clone to stimulate RF33.70, a T-cell hybridoma specific to $OVA_{257\text{-}264}$. Of 10 clones examined, 9 (90%) strongly stimulated the hybridoma. The result of analysis using ES-DCs generated from one of the ES cell clones introduced with the replacement vector is shown in Figure 8B. The ES-DCs were superior to BM-DCs prepulsed with 1 or 10 μM $OVA_{257\text{-}264}$ peptide in stimulating the hybridoma. Taken together, this exchangeable gene-trap system allows one to efficiently generate ES cell transfectants, which highly express the introduced genes after differentiation to DCs.

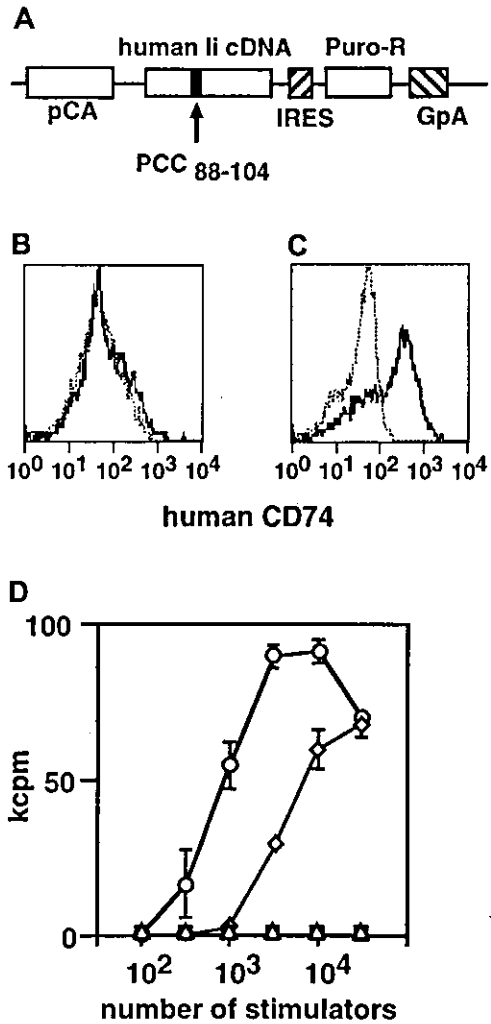


Figure 7. Introduction of an epitope-presenting vector to ES-DCs. (A) The structure of PCC epitope presentation vector, pCAG-PCC-PI, is shown. CLIP region of human Ii cDNA was replaced with an oligo DNA encoding $PCC_{88\text{-}104}$. The expression of this gene is driven by the chicken β-actin promoter (pCA). The mutant Ii coding sequence is followed by IRES-puromycin *N*-acetyltransferase gene (Puro-R) and polyadenylation signal sequence of the human growth hormone (GpA). (B-C) Flow cytometric analysis of ES-DCs on the expression of human Ii. ES-DCs without (B) or with (C) expression construct were stained intracellularly with antihuman Ii (CD74) (thick lines) or isotype-matched control mAb (thin dotted lines). (D) Stimulation of PCC-specific T-cell hybridoma, 2B4, by ES-DCs with (○) or without (■) PCC epitope-presenting vector. As a control, BM-DCs pulsed with PCC peptide (10 μM) for 4 hours (◇) or left unpulsed (△) were also used as stimulators. Stimulator cells and hybridomas were cocultured for 24 hours, and IL-2 produced by 2B4 was measured by proliferation of CTLL-20 cells. Results were expressed as mean cpm of triplicate cultures ± standard deviation.

Priming of antigen-specific cytotoxic T cells with genetically modified ES-DCs

The capacity of ES-DCs introduced with an OVA-expression vector as described above to prime OVA-specific T cells in vivo was analyzed. ES-DCs (5×10^5) with or without OVA expression vector were injected intraperitoneally into syngenic (CBA × C57BL/6)

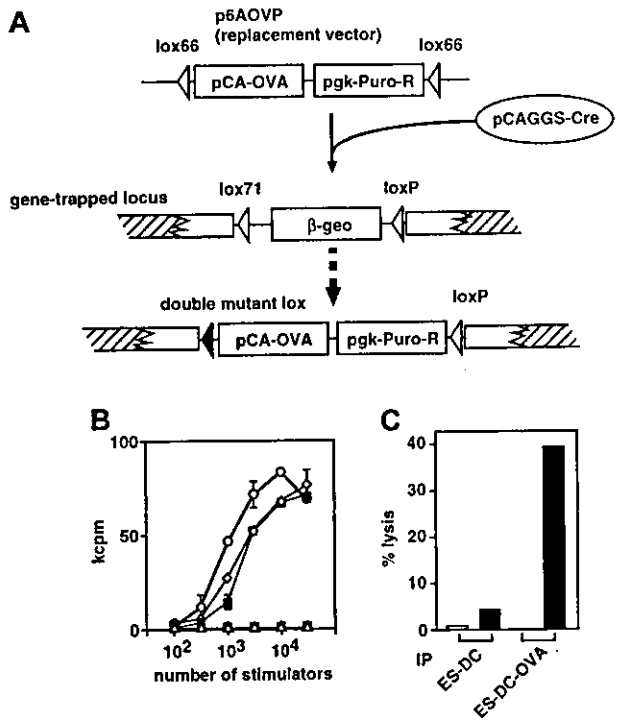


Figure 8. Generation of ES-DCs expressing OVA antigen and in vivo priming of OVA-specific cytotoxic lymphocytes. (A) Cre/mutant lox–mediated targeted integration of the expression cassette to a gene-trapped locus is schematically depicted. The expression cassette, the OVA cDNA driven by the chicken β-actin promoter (pCA-OVA) ligated with the puromycin *N*-acetyltransferase gene driven by the pgk promoter (pgk-Puro-R), was inserted to the gene-trapped site, replacing the β-geo sequence by the effect of Cre recombinase transiently expressed from pCAGGS-Cre. The combination of a lox66 of the replacement vector and a lox71 of a trapped locus resulted in a double-mutant lox, which inhibited further recombination of the replaced allele and increased the frequency of proper recombinant ES cell clones. (B) Stimulation of OVA-specific T-cell hybridoma, RF33.70, with ES-DCs with (○) or without (□) expression of OVA was analyzed. As a control, bone marrow–derived DCs pulsed with 10 μM (◇) or 1 μM (■) of $OVA_{257-264}$ for 4 hours or left unpulsed (△) are also used as stimulators. Stimulators and hybridomas were cocultured for 24 hours, and IL-2 produced by 2B4 was measured by proliferation of CTLL-20 cells. Results were expressed as mean cpm of triplicate cultures ± standard deviation. (C) ES-DCs with (ES-DC-OVA) or without (ES-DC) expression of OVA protein were injected intraperitoneally on days 0 and 7 into syngenic F1 mice. Splenocytes from the injected mice were harvested on day 14 and cultured in the presence of $OVA_{257-264}$ (0.1 μM) for 5 days. The resultant cells were assayed for the capacity to lyse EL-4 tumor cells either pulsed with 10 μM OVA peptide (■) or left unpulsed (□). Results were expressed as mean specific lysis of triplicate assays and the standard deviation of triplicates was less than 2.5%.

F1 mice twice with a 7-day interval. Splenocytes were isolated 7 days after the second injection and cultured in vitro in the presence of a suboptimal concentration (0.1 μM) of $OVA_{257-264}$ peptide, the major K^b-binding epitope of OVA protein. After 5 days, viable cells were recovered and assayed for their capacity to kill EL-4 thymoma cells ($H\text{-}2^b$) prepulsed with the OVA peptide. The results shown in Figure 8C indicated that cytotoxic T cells specific to the OVA epitope were primed in vivo with ES-DCs expressing OVA protein but not with ES-DC without OVA. These results demonstrate that ES-DCs genetically engineered to express an antigenic protein have the capacity to prime antigen-specific cytotoxic T cells in vivo.

Discussion

We developed a method to generate DCs from mouse ES cells in vitro. The ES-DCs were comparable with bone marrow cell–derived DCs in morphology, surface phenotypes, and function. Several improvements have been made in the procedure for differentiation culture. For feeder cells used in days 5 to 10 of culture, we compared OP9 with other bone marrow stromal cell lines, PA6 and ST2, both producing M-CSF. Although DCs could differentiate when PA6 or ST2 cells were used as feeder cells, the number of generated DCs was fewer and the phenotype of the generated DCs differed. With PA6 or ST2, generated DCs did not express CD80 and CD205, and their activity to stimulate MLR was weaker than that of DCs produced with OP9. The use of tissue culture–grade dishes in the culture after transfer from the OP9 feeder cell layer (after day 10 in Figure 1) gave rise to a fewer number and a lower purity of ES-DCs than did the use of bacterial-quality Petri dishes. If we used dishes of tissue-culture grade, many cells firmly adhered to the dish surface, resembling macrophages or fibroblasts, and inhibited the generation of ES-DCs. The beneficial effect of bacterial-quality Petri dishes to DC development has been noted also for culture of BM-DCs.[27] GM-CSF has been reported to be essential for in vitro generation of DCs from hematopoietic cells.[20-22] GM-CSF is also necessary for generation of ES-DCs. We applied the current culture protocol to ES cell lines other than TT2. We tested 3 lines of ES cells, D3, R1, and CCE, and observed that all of these lines also differentiated to DCs, thereby suggesting that the method is applicable to most lines of mouse ES cells.

Recently, another method to generate DCs in vitro from mouse ES cells has been reported.[38] In the procedure, embryoid bodies (EBs) made from ES cells were cultured for 14 days in the presence of GM-CSF and IL-3. Resultant DCs, referred to as esDCs, lack expression of CD8α and Dec-205 (CD205), suggesting myeloid origin. Upon stimulation with LPS, they were matured and became highly competent to stimulate T cells. Characteristics of the EB-derived esDCs seemed to be similar to those of our ES-DCs. Compared with our method, the EB-mediated method is simple in that feeder cells are not necessary. On the other hand, our method allows one to microscopically observe individual cells following differentiation from ES cells to mature DCs.

For investigation of the physiologic function of genes, generation of mutant mice by gene targeting in ES cells is a potent and widely used means. However, it takes a relatively long time to develop mutant mice by gene targeting, and if the disrupted gene is essential for embryogenesis, homozygous mutant mice cannot be obtained because of embryonic lethality. ES cell clones homozygous for mutated allele can be obtained from single-allele mutant cell clones by selection with a high dose of a selection drug[39] or sequential gene targeting with 2 targeting vectors bearing different selection markers.[28] The method for in vitro generation of DCs from ES cells may prove useful for analyzing genes essential for both embryogenesis and function of DCs. Using the TT2 ES cell line, the generation of a large-scale library of gene-trap ES clones is now in progress. Using the method established in the current study, it is possible to efficiently screen large numbers of gene-trap ES cell clones to search for genes expressed in DCs. After selection of ES cell clones in which genes expressed in DCs were trapped, one can generate homozygous mutant ES cell clones by high-dose drug selection, differentiate them to DCs, and evaluate the functional significance of the identified genes in DCs.

For genetic modification of ES-DCs, we found 2 means for gene transfer to ES cells useful: an expression vector containing IRES-*puromycin N-acetyltransferase* gene and Cre-lox–mediated targeted gene integration into gene-trapped ES cell clones. Both worked very efficiently to generate ES cell transfectant clones expressing the gene products after DC differentiation; the frequency of appropriate transfectant clones in picked-up drug-resistant clones was 85% and 90% when we used the vector with IRES-*puromycin N-acetyltransferase* gene and targeted gene integration, respectively. We are now preparing ES-DCs

expressing both antigenic protein and immunomodulating molecules, such as immunostimulatory or inhibitory molecules, cytokines, or chemokines. One of our goals is to manipulate immune responses in an antigen-specific manner by in vivo transfer of such engineered DCs. We have already succeeded in introducing 2 different plasmid vectors with different selection markers, the puromycin- and neomycin-resistance gene, by sequential transfection, and confirmed that generation of DCs from double-transfected ES cells is also feasible. Possible future applications of this method may be treatment of autoimmune and allergic diseases, inhibition of graft rejection in transplantation medicine, antitumor immunotherapy, and vaccination against intractable infectious diseases. Recently, human ES cells have been generated.[40,41] In the mouse system, a method was devised to generate ES cell lines with an appropriate genetic background by nuclear transfer from somatic cells to already established ES cells.[42,43] With the advances in ES cell–related technology, immunomodulation therapy using DCs generated from genetically engineered ES cells may be considered.

Acknowledgments

We thank Drs T. Nakano, K. Inaba, K. Matsushima, K. Iwabuchi, and K. Matsuno for valuable suggestions; Dr S. Aizawa for TT2; Drs N. Takakura and T. Suda for OP9 and PA6; Dr K. Lock for RF33.70; Drs T. Koda and M. Bevan for a cDNA clone for OVA; Dr H. Niwa for pCAG-IP; and Kirin Brewery for rGM-CSF. M. Ohara provided helpful comments on the manuscript.

References

1. Huang FP, Platt N, Wykes M, et al. A discrete subpopulation of dendritic cells transports apoptotic intestinal epithelial cells to T cell areas of mesenteric lymph nodes. J Exp Med. 2000;191:435-444.
2. Steinbrink K, Graulich E, Kubsch S, Knop J, Enk AH. CD4(+) and CD8(+) anergic T cells induced by interleukin-10-treated human dendritic cells display antigen-specific suppressor activity. Blood. 2002;99:2468-2476.
3. Steinman RM, Turley S, Mellman I, Inaba K. The induction of tolerance by dendritic cells that have captured apoptotic cells. J Exp Med. 2000;191:411-416.
4. Rissoan MC, Soumelis V, Kadowaki N, et al. Reciprocal control of T helper cell and dendritic cell differentiation. Science. 1999;283:1183-1186.
5. Pulendran B, Smith JL, Caspary G, et al. Distinct dendritic cell subsets differentially regulate the class of immune response in vivo. Proc Natl Acad Sci U S A. 1999;96:1036-1041.
6. Arpinati M, Green CL, Heimfeld S, Heuser JE, Anasetti C. Granulocyte-colony stimulating factor mobilizes T helper 2-inducing dendritic cells. Blood. 2000;95:2484-2490.
7. Klein C, Bueler H, Mulligan RC. Comparative analysis of genetically modified dendritic cells and tumor cells as therapeutic cancer vaccines. J Exp Med. 2000;191:1699-1708.
8. Van Tendeloo VF, Ponsaerts P, Lardon F, et al. Highly efficient gene delivery by mRNA electroporation in human hematopoietic cells: superiority to lipofection and passive pulsing of mRNA and to electroporation of plasmid cDNA for tumor antigen loading of dendritic cells. Blood. 2001;98:49-56.
9. Dyall J, Latouche JB, Schnell S, Sadelain M. Lentivirus-transduced human monocyte-derived dendritic cells efficiently stimulate antigen-specific cytotoxic T lymphocytes. Blood. 2001;97:114-121.
10. Araki K, Imaizumi T, Sekimoto T, et al. Exchangeable gene trap using the Cre/mutated lox system. Cell Mol Biol. 1999;45:737-750.
11. Nakano T, Kodama H, Honjo T. Generation of lymphohematopoietic cells from embryonic stem cells in culture. Science. 1994;265:1098-1101.
12. Nakano T, Kodama H, Honjo T. In vitro development of primitive and definitive erythrocytes from different precursors. Science. 1996;272:722-724.
13. Kodama H, Nose M, Niida S, Nishikawa S, Nishikawa S. Involvement of the c-kit receptor in the adhesion of hematopoietic stem cells to stromal cells. Exp Hematol. 1994;22:979-984.
14. Yamane T, Kunisada T, Yamazaki H, Era T, Nakano T, Hayashi SI. Development of osteoclasts from embryonic stem cells through a pathway that is c-fms but not c-kit dependent. Blood. 1997;90:3516-3523.
15. Nakayama N, Fang I, Elliott G. Natural killer and B-lymphoid potential in CD34+ cells derived from embryonic stem cells differentiated in the presence of vascular endothelial growth factor. Blood. 1998;91:2283-2295.
16. Stanford WL, Caruana G, Vallis KA, et al. Expression trapping: identification of novel genes expressed in hematopoietic and endothelial lineages by gene trapping in ES cells. Blood. 1998;92:4622-4631.
17. Motoyama N, Kimura T, Takahashi T, Watanabe T, Nakano T. bcl-x prevents apoptotic cell death of both primitive and definitive erythrocytes at the end of maturation. J Exp Med. 1999;189:1691-1698.
18. Hidaka M, Stanford WL, Bernstein A. Conditional requirement for the Flk-1 receptor in the in vitro generation of early hematopoietic cells. Proc Natl Acad Sci U S A. 1999;96:7370-7375.
19. Era T, Takagi T, Takahashi T, Bories JC, Nakano T. Characterization of hematopoietic lineage-specific gene expression by ES cell in vitro differentiation induction system. Blood. 2000;95:870-878.
20. Inaba K, Inaba M, Romani N, et al. Generation of large numbers of dendritic cells from mouse bone marrow cultures supplemented with granulocyte/macrophage colony-stimulating factor. J Exp Med. 1992;176:1693-1702.
21. Inaba K, Steinman RM, Pack MW, et al. Identification of proliferating dendritic cell precursors in mouse blood. J Exp Med. 1992;175:1157-1167.
22. Zhang Y, Wang Y, Ogata M, Hashimoto S, Onai N, Matsushima K. Development of dendritic cells in vitro from murine fetal liver-derived lineage phenotype-negative c-kit(+) hematopoietic progenitor cells. Blood. 2000;95:138-146.
23. Yagi T, Tokunaga T, Furuta Y, et al. A novel ES cell line, TT2, with high germline-differentiating potency. Anal Biochem. 1993;214:70-76.
24. Senju S, Iyama K, Kudo H, Aizawa S, Nishimura Y. Immunocytochemical analyses and targeted gene disruption of GTPBP1. Mol Cell Biol. 2000;20:6195-6200.
25. Hedrick SM, Matis LA, Hecht TT, et al. The fine specificity of antigen and Ia determinant recognition by T cell hybridoma clones specific for pigeon cytochrome c. Cell. 1982;30:141-152.
26. Rock KL, Rothstein L, Gamble S. Generation of class I MHC-restricted T-T hybridomas. J Immunol. 1990;145:804-811.
27. Lutz MB, Kukutsch N, Ogilvie AL, et al. An advanced culture method for generating large quantities of highly pure dendritic cells from mouse bone marrow. J Immunol Methods. 1999;223:77-92.
28. Senju S, Negishi I, Motoyama N, et al. Functional significance of the Fas molecule in naive lymphocytes. Int Immunol. 1996;8:423-431.
29. Fujii S, Senju S, Chen YZ, Ando M, Matsushita S, Nishimura Y. The CLIP-substituted invariant chain efficiently targets an antigenic peptide to HLA class II pathway in L cells. Hum Immunol. 1998;59:607-614.
30. Fujii S, Uemura Y, Iwai LK, Ando M, Senju S, Nishimura Y. Establishment of an expression cloning system for CD4+ T cell epitopes. Biochem Biophys Res Commun. 2001;284:1140-1147.
31. Niwa H, Masui S, Chambers I, Smith AG, Miyazaki J. Phenotypic complementation establishes requirements for specific POU domain and generic transactivation function of Oct-3/4 in embryonic stem cells. Mol Cell Biol. 2002;22:1526-1536.
32. Araki K, Araki M, Miyazaki J, Vassalli P. Site-specific recombination of a transgene in fertilized eggs by transient expression of Cre recombinase. Proc Natl Acad Sci U S A. 1995;92:160-164.
33. Araki K, Araki M, Yamamura K. Targeted integration of DNA using mutant lox sites in embryonic stem cells. Nucleic Acids Res. 1997;25:868-872.
34. Araki K, Imaizumi T, Okuyama K, Oike Y, Yamamura K. Efficiency of recombination by Cre transient expression in embryonic stem cells: comparison of various promoters. J Biochem. 1997;122:977-982.
35. Taniguchi M, Sanbo M, Watanabe S, Naruse I, Mishina M, Yagi T. Efficient production of Cre-mediated site-directed recombinants through the utilization of the puromycin resistance gene, pac: a transient gene-integration marker for ES cells. Nucleic Acids Res. 1998;26:679-680.
36. Yamaguchi Y, Tsumura H, Miwa M, Inaba K. Contrasting effects of TGF-beta 1 and TNF-alpha on the development of dendritic cells from progenitors in mouse bone marrow. Stem Cells. 1997;15:144-153.
37. Yamada N, Katz SI. Generation of mature dendritic cells from a CD14+ cell line (XS52) by IL-4, TNF-alpha, IL-1 beta, and agonistic anti-CD40 monoclonal antibody. J Immunol. 1999;163:5331-5337.
38. Fairchild PJ, Brook FA, Gardner RL, et al. Directed differentiation of dendritic cells from mouse embryonic stem cells. Current Biol. 2000;10:1515-1518.
39. Mortensen RM, Conner DA, Chao S, Geisterfer-Lowrance AA, Seidman JG. Production of homozygous mutant ES cells with a single targeting construct. Mol Cell Biol. 1992;12:2391-2395.
40. Thomson JA, Itskovitz-Eldor J, Shapiro SS, et al. Embryonic stem cell lines derived from human blastocysts. Science. 1998;282:1145-1147.
41. Reubinoff BE, Pera MF, Fong CY, Trounson A, Bongso A. Embryonic stem cell lines from human blastocysts: somatic differentiation in vitro. Nat Biotechnol. 2000;18:399-404.
42. Wakayama T, Tabar V, Rodriguez I, Perry AC, Studer L, Mombaerts P. Differentiation of embryonic stem cell lines generated from adult somatic cells by nuclear transfer. Science. 2001;292:740-743.
43. Hochedlinger K, Jaenisch R. Monoclonal mice generated by nuclear transfer from mature B and T donor cells. Nature. 2002;415:1035-1038.

Systematic Analysis of the Combinatorial Nature of Epitopes Recognized by TCR Leads to Identification of Mimicry Epitopes for Glutamic Acid Decarboxylase 65-Specific TCRs[1]

Yasushi Uemura,* Satoru Senju,* Katsumi Maenaka,[§¶] Leo Kei Iwai,*[‖] Shinji Fujii,*[†] Hiroki Tabata,*[‡] Hirotake Tsukamoto,* Shinya Hirata,* Yu-Zhen Chen,* and Yasuharu Nishimura[2]*

Accumulating evidence indicates that recognition by TCRs is far more degenerate than formerly presumed. Cross-recognition of microbial Ags by autoreactive T cells is implicated in the development of autoimmunity, and elucidating the recognition nature of TCRs has great significance for revelation of the disease process. A major drawback of currently used means, including positional scanning synthetic combinatorial peptide libraries, to analyze diversity of epitopes recognized by certain TCRs is that the systematic detection of cross-recognized epitopes considering the combinatorial effect of amino acids within the epitope is difficult. We devised a novel method to resolve this issue and used it to analyze cross-recognition profiles of two glutamic acid decarboxylase 65-autoreactive CD4$^+$ T cell clones, established from type I diabetes patients. We generated a DNA-based randomized epitope library based on the original glutamic acid decarboxylase epitope using class II-associated invariant chain peptide-substituted invariant chains. The epitope library was composed of seven sublibraries, in which three successive residues within the epitope were randomized simultaneously. Analysis of agonistic epitopes indicates that recognition by both TCRs was significantly affected by combinations of amino acids in the antigenic peptide, although the degree of combinatorial effect differed between the two TCRs. Protein database searching based on the TCR recognition profile proved successful in identifying several microbial and self-protein-derived mimicry epitopes. Some of the identified mimicry epitopes were actually produced from recombinant microbial proteins by APCs to stimulate T cell clones. Our data demonstrate the importance of the combinatorial nature of amino acid residues of epitopes in molecular mimicry. *The Journal of Immunology,* **2003, 170: 947–960.**

Activation of autoreactive CD4$^+$ T cells is a crucial step in the development of T cell-mediated autoimmunity, and cross-reactivity between microbial and self Ags, a phenomenon known as molecular mimicry, is one of the mechanisms that account for the link between infection and autoimmunity (1–3). Thereby, identification of microbial Ags mimicking self Ags would provide insights into the disease process and new therapeutic or preventative strategies for autoimmune diseases. In the last decade, many studies have demonstrated degeneracy in Ag recognition by TCR, thus showing its high flexibility (2, 4–8). Degeneracy of TCR recognition can appear itself in various functional outcomes, depending on the affinity of the MHC/peptide ligand to TCR, which includes T cell responses ranging from full activation to strong antagonism (9–11).

In functional analysis using altered peptide ligands, it was reported that TCRs can discriminate not only between two peptides differing at a TCR contact residue but also between those differing at only a single MHC anchor residue, which does not significantly alter the binding affinity of a peptide to MHC (12, 13). In addition, it has been reported that certain TCR recognition is affected by amino acids adjacent or not adjacent to TCR contact residues or by amino acid combinations in antigenic peptides (14–17). These observations indicate that a substitution at a certain residue would induce conformational changes of peptides and may affect other residues. In contrast, a quantitative strategy using combinatorial peptide libraries with a positional scanning format (PS-SCLs)[3] and biometric score matrices dissected and predicted peptide mimicry ligands of a given cognate T cells (18, 19). However, the precise effect of successive combinations of residues in the antigenic peptide on recognition of certain TCR has heretofore not been clarified. In addition, there is no available technology that allows for systematic separation and identification of diverse T cell epitopes from a mixture of randomized peptide ligands.

Several groups, including ours, reported invariant chain (Ii)-based epitope-presenting vectors, in which class II-associated invariant chain peptide (CLIP, Ii_{89-101}) was replaced by antigenic

*Division of Immunogenetics, Department of Neuroscience and Immunology, Kumamoto University Graduate School of Medical Sciences and [†]First Department of Internal Medicine and [‡]Department of Pediatrics, Kumamoto University School of Medicine, Kumamoto, Japan; [§]Structural Biology Center, National Institute of Genetics, Mishima, Shizuoka, Japan; [¶]Biosystem Science, School of Advanced Science, Graduate University for Advanced Studies, Hayama, Kanagawa, Japan; and [‖]Laboratory of Immunology, Heart Institute (Incor), University of Sao Paulo Medical School, Sao Paulo, Brazil

Received for publication June 20, 2002. Accepted for publication November 13, 2002.

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

[1] This work was supported in part by Grants-in-Aid 11694294, 11557027, and 14370115 from the Ministry of Education, Science, Technology, Sports, and Culture, Japan, and a Research Grant for Intractable Diseases from Ministry of Health and Welfare, Japan. K.M. was supported in part by the year; 2000 Joint Research Project (Soken/K01-4) of Sokendai (The Graduate University for Advanced Studies) and a research grant from the Nakajima Foundation.

[2] Address correspondence and reprint requests to Dr. Yasuharu Nishimura, Division of Immunogenetics, Department of Neuroscience and Immunology, Kumamoto University Graduate School of Medical Sciences, Honjo 2-2-1, Kumamoto 860-0811, Japan. E-mail address: mxnishim@gpo.kumamoto-u.ac.jp

[3] Abbreviations used in this paper: PS-SCLs, positional scanning synthetic combinatorial peptide libraries; CLIP, class II-associated invariant chain peptide; GAD, glutamic acid decarboxylase; Ii, invariant chain; DC, dendritic cell; CDR, complementarity-determining region.

0022-1767/03/$02.00

peptides (20–23). Using this vector system, we reported a method to identify epitopes cross-recognized by autoreactive T cell clones from a library of randomized peptides (24).

In the current study, we used this vector to make libraries of the glutamic acid decarboxylase 65 epitope ($GAD65_{115-127}$)-based degenerate peptides, where three successive residues within the epitope were randomized. GAD65 is one of the important islet Ags implicated in autoimmunity of the NOD mouse and Type I diabetes in humans. We used two T cell clones established from Japanese patients with Type I diabetes and restricted by disease susceptible HLA-DR53 and they responded to GAD65 protein (25). The epitope ($GAD65_{115-127}$) used in this study was also reported to be immunodominant in studies using HLA-DR4 transgenic mice (26, 27). Epitopes stimulating GAD65-reactive $CD4^+$ T cell clones were isolated from the series of epitope libraries. Recognition properties of these TCRs were intensively analyzed, and combinatorial effects of amino acid residues within antigenic peptide on recognition by TCRs were investigated. The information obtained by screening of this epitope expression library included the combinations of amino acid residues with TCR agonism that could not be predicted using panels of single amino acid substituted peptide analogs. Data acquisition of stimulatory TCR ligands combined with a pattern match search allowed for identification of self or microbe-derived peptides cross-recognized by $CD4^+$ T cell clones autoreactive to GAD65.

Materials and Methods

T cell clones and T cell proliferation assay

Dodecamer peptide analogs with single-amino acid substitutions derived from $GAD65_{116-127}$ were purchased from Chiron Mimotopes (Clayton South, Victoria, Australia), and 13-mer peptides were synthesized as described (28). Two human $CD4^+$ T cell clones, SA32.5 and MK20.2, recognizing $GAD65_{116-127}$ (NILLQYVVKSFD) in the context of HLA-DR53 molecules (DRA*0101 + DRB4*0103) susceptible to type I diabetes were used throughout the study (25). T cells were fed weekly with 50 U/ml human rIL-2 and the irradiated DR53-matched allogenic PBMCs prepulsed with the $GAD65_{111-131}$ (LQDVMNILLQYVVKSFDRSTK) in RPMI 1640 supplemented with 10% heat-inactivated human plasma, 100 U/ml penicillin, 100 μg/ml streptomycin, and 2 mM L-glutamine. T cell proliferation assays were set up in 96-well flat-bottom culture plates (Falcon; BD Biosciences, San Jose, CA) with 3×10^4 T cells and irradiated (6000 cGy) DR53-positive 1.5×10^5 PBMCs or 1.0×10^4 dendritic cells (DCs) per well in the presence of peptides or recombinant proteins at various concentrations. After 48 h of culture, [^{3}H]thymidine was added (1 μCi/well); and after an additional 16 h, cells were harvested onto glass fiber filters, and radioactivity was counted on a beta scintillation counter (Wallac, Gaithersburg, MD).

In vitro generation of DCs

DCs were generated from $CD14^+$ monocytes purified by positive immunoselection from HLA-DR53-positive allogeneic PBMCs, using an anti-CD14 mAb coupled onto magnetic microbeads (CD14 microbeads; Miltenyi Biotec, Auburn, CA). The $CD14^+$ monocytes were cultured at 1×10^6 cells/ml in the presence of 100 ng/ml GM-CSF and 100 U/ml IL-4 (Ono Pharmaceutical, Osaka, Japan) in RPMI 1640 supplemented with 10% human plasma, 2 mM L-glutamine, and 100 μg/ml streptomycin. Cultures were fed on days 3 and 5 with fresh medium containing GM-CSF and IL-4. On day 5, DCs were treated with TNF-α (20 ng/ml). On day 7, the nonadherent cells were harvested and served as mature DCs. For the assessment of HLA class II-restricted presentation of recombinant microbial proteins, mature DCs were cultured with recombinant proteins for 14 h before use as APCs for proliferation assay.

Epitope expression library

The procedure for construction of an epitope-presenting library is described in our previous report (24). Briefly, oligonucleotide fragments encoding degenerate $GAD65_{115-127}$ were synthesized and purified using polyacrylamide gel (Genemed Synthesis, South San Francisco, CA). These oligonucleotide fragments were amplified by PCR with 5′-biotinylated primers, 5′-TCC CTC CTG GTG ACT CTG CTC CTC-3′ and 5′-ATT GTT ATC TGC TGT TCC GAC TTG-3′. The purified PCR products were digested with *Dra*I and *Sac*I, purified with streptavidin-agarose, and ligated to *Sma*I-*Sac*I-digested pCI, the CLIP-substituted epitope expression vector. The construct encodes Ii protein inserted with partially degenerate fusion peptides based on $GAD65_{115-127}$ (MNILLQYVVKSFD) instead of Ii_{89-101} (SKMRMATPLLMQA) within the CLIP sequence. *Escherichia coli* (DH5α) was transformed with the ligation mixture, and the transformants were divided into 96-well culture blocks (Qiagen, Studio City, CA) to generate transformant pools of 30–50 clones and grown overnight in Luria-Bertani medium containing ampicillin (100 μg/ml). The amplified plasmid DNA was purified using a QIAprep 96 Turbo Miniprep system (Qiagen). The complexity of each degenerate-$GAD65_{115-127}$ expression sublibraries was $\sim 1.5 \times 10^4$–2.0×10^4.

Screening of epitope expression library by detecting IFN-γ production

Library DNA pools and expression vectors for HLA-DRA*0101 and DRB4*0103 were mixed with Transfectam reagent (Promega, Madison, WI) in serum-free DMEM. The DNA-Transfectam mixtures were then added to the COS-7 cells (1×10^4 cells/well) in 96-well flat-bottom culture plates and incubated for 90 min at 37°C. After removal of the transfection medium, the COS-7 cells were incubated overnight in DMEM supplemented with 10% FCS. After 24 h, cells were washed twice with DMEM, and T cells were added at a concentration of 3×10^4/well in RPMI 1640 supplemented with 10% heat-inactivated human plasma, 100 U/ml penicillin, 100 μg/ml streptomycin, and 2 mM L-glutamine. After 48 h of incubation, the supernatant was collected, and IFN-γ concentration was measured using a standard ELISA (Endogen, Woburn, MA). The library of DNA pools, for which a significant production of IFN-γ was detected was used to transform the bacteria to prepare a sublibrary of DNA pools consisting of ~10 clones. Secondary screening was done as described above, using sublibraries. Single plasmid clones were obtained after three rounds of screening. DNA sequences of the purified plasmid clones were analyzed using BigDye Terminator Cycle Sequencing Ready Reaction Kits and the ABI PRISM 310 Genetic Analyzer (Applied Biosystems, Foster City, CA). For construction of expression vectors encoding mimicry peptides or analogs of GAD65 epitope, both strands of oligonucleotide fragments encoding these peptides were synthesized (Espec Oligo Service, Tsukuba, Japan), annealed, and ligated to *Sma*I-*Sac*I-digested pCI. The amplified plasmid DNAs were purified for transfection. The agonistic activity was assessed in an IFN-γ secretion assay at various dilutions with pCI (wild Ii: irrelevant DNA).

Analysis of TCR V-(D)-J junctional regions of GAD65-autoreactive T cell clones

Total RNA was extracted from T cells using the TRIzol reagent (Life Technologies, Gaithersburg, MD), and first-strand cDNA was synthesized using Superscript RNase H$^-$ reverse transcriptase (Life Technologies) and random hexamers. cDNA was subjected to PCR amplification for rearranged TCR-α with 29 5′-TCRAV family-specific oligonucleotides (Vα1–32) and a 3′-TCRAC (Cα) constant primer, and for rearranged TCR-β with 27 5′-TCRBV family-specific oligonucleotides (Vβ1–25) and a 3′-TCRBC (Cβ) constant primer (29). The amplified PCR products of the α-chain and of the β-chain were cloned into a plasmid vector, pGEM-T (Promega), and sequenced. The resulting sequences were analyzed using IMGT, the international ImMunoGeneTics database (http://imgt.cnusc.fr: 8104/).

Northern blot analysis

Northern blot analysis was conducted as described (30). To prepare the probes, the MK20.2 cDNA was subjected to PCR amplification for 5′-TCRVα15- or 5′-TCRVα16-specific oligonucleotide and 3′-TCRJα region oligonucleotide primers, and each PCR product was TA cloned, digested, and gel purified. The two cDNA fragments of the *TCRAV* gene were labeled with [α-^{32}P]dCTP. After hybridization and quantitative analysis of signal intensities, probes were stripped, and a second hybridization was conducted using an β-actin probe. To assess cross-reactivity of the Vα-specific probes, TCRAV cDNA fragments were arrayed onto two copies of nylon membrane filters and hybridized using TCRAV cDNA probes.

Generation of recombinant proteins

Genomic DNA of *Legionella pneumophila* (strain Philadelphia-1) was kindly provided by Drs. T. Akaike and T. Akuta (Kumamoto University School of Medicine, Kumamoto, Japan). *Lactococcus lactis* (subsp. *lactis*) was provided by the Institute of Physical and Chemical Research (Wako, Japan). Bacterial genomic DNA were purified using DNeasy Tissue Kits (Qiagen). The *O*-succinylbenzoic acid-CoA ligase gene (coding for aa 101–201)/*L. lactis*, putative PTS system, lactose-specific component IIBC

gene (coding for aa 426–546)/*Streptococcus pyogenes* (ATCC 19615), putative dihydrolipoamide dehydrogenase gene (coding for aa 101–205)/*Neisseria meningitidis*, glutamine amidotransferase, class I gene (coding for aa 1–104)/*Streptococcus pneumoniae* (ATCC 49619), and the pilus assembly protein *PilB* gene (coding for aa 28–131)/*Legionella pneumophila* were PCR amplified and cloned into the plasmid vector (pGEM-T-easy vector system). Fusion proteins containing relatively small fragment (100–120 aa) of microbial proteins were generated, because larger recombinant proteins tend to become insoluble in bacteria and are difficult to purify. The inserted fragments were digested and ligated directionally into the prokaryotic expression vector pGEX-4T (Pharmacia, Peapack, NJ) to produce GST-fusion protein. The integrity of the constructs was confirmed by DNA sequencing. The procedure for protein induction and purification were described in our previous report (31). The purity and integrity of the fusion protein were confirmed by SDS-PAGE. The recombinant proteins were concentrated and separated from small peptide fragments with Centricon-30 (Amicon, MA), and the buffer was replaced with culture medium.

Results

A novel strategy to analyze combinatorial effects of amino acids on the antigenic peptide in exhibition of molecular mimicry

The goal of this study was to develop new and comprehensive methods for analysis of the combinatorial effects of residues in antigenic peptides on recognition by HLA class II-restricted TCRs. We also tried to clarify the significance of the effect of amino acid combination within antigenic peptide in recognition by TCR. By adopting the strategy, we sought to identify candidate mimicry epitopes for GAD65-autoreactive T cell clones established from type I diabetes patients.

Specificity of GAD65-reactive T cell clones SA32.5 and MK20.2 analyzed using single-amino acid residue-substituted peptide analogs

To investigate structural features of peptides cross-recognized by autoantigen-specific TCRs, we analyzed HLA-DR53-restricted SA32.5 and MK20.2 $CD4^+$ T cell clones reactive to GAD65 (peptide 116–127). These T cell clones were established from two independent type I diabetes patients in our previous study (25). At the beginning of this study, we verified that the two T cell clones expressed single TCRs, because it has been reported that a significant fraction of T cells in human peripheral blood expresses dual TCRs (32). The complementarity-determining region (CDR) sequences of the Ag contact sites were defined. As shown in Table I, TCR-α and TCR-β-chains of SA32.5 T cell clone revealed functional *TCRAV1S2* and *TCRBV9S1* gene rearrangements as well as an out of frame rearrangement of *AV27S1*. TCR α- and TCR β-chains of MK20.2 TCR revealed an in frame dual Vα rearrangement (*TCRAV15S1* and *TCRAV16S1* transcripts) and TCRBV3S1 rearrangement. In Northern blot analysis (Fig. 1*A*), T cell clone MK20.2 expressed the *TCRAV16S1* but not the *TCRAV15S1* gene transcript. Therefore, *TCRAV16S1* combined with the *TCRBV3S1*

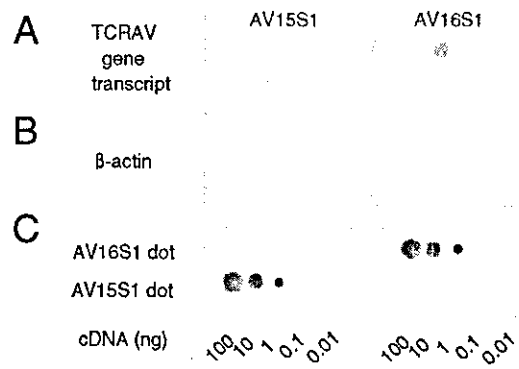


FIGURE 1. Identification of the TCR α-chain expressed in T cell clone MK20.2 by Northern blot analysis. *A*, Probes of TCRAV15S1 (*left lane*) and TCRAV16S1 (*right lane*) were hybridized in parallel with replica filters. *B*, Filters were probed with β-actin probe for control of amounts of RNA. *C*, Cross-hybridization analysis of TCRAV16S1 and TCRAV15S1 cDNA probes. To assess the cross-hybridization of each probe, cDNA dot hybridization was done using replica filters. Replicate dot blots on which the indicated amounts of TCRAV16S1 (*upper dots*) and TCRAV15S1 (*lower dots*) were manually spotted and hybridized with cDNA probes of TCRAV15S1 (*left*) or TCRAV16S1 (*right*). Cross-hybridization of each probe was not observed.

gene product mediates Ag recognition in MK20.2 TCR. These data indicated that reactivities of these T cell clones were determined by each single TCR. These T cell clones use distinct CDR3α and CDR3β sequences at the amino acid level and a distinct CDR3 length, whereas both T cell clones use the same Jβ rearrangement.

Next we analyzed these T cell clones to understand the scheme of Ag recognition properties, using conventional means, in which we examined proliferative responses of these T cell clones to the 35 peptide analogs carrying chemically conservative or nonconservative single-amino acid substitution in the native 12-mer epitope ($GAD65_{116-127}$). As shown in Fig. 2, several amino acids with different chemical properties (lysine, aspartic acid, asparagine, serine, and valine) were tested for position 117 (isoleucine in the native ligand). MK20.2 tolerated all of these substitutions. In contrast, SA32.5 did not tolerate the negatively charged aspartic acid. In the analysis using the same substitutions as position 117, these clones showed distinct specificity at position 118 (leucine in the native ligand) and at position 119 (leucine in the native ligand). With substitution at position 123 (valine in the native ligand), MK20.2 responded to hydrophobic residues (leucine, isoleucine, alanine, and methionine), in several orders of magnitude. In contrast, SA32.5 showed a significant response to only isoleucine among the tested substitutions at this position. SA32.5 tolerated

Table I. *TCR gene usage and TCR V-(D)-J junctional region sequences of α- and β-chains expressed in $GAD65_{116-127}$-autoreactive T cell clones SA32.5 and MK20.2*[a]

	TCRV	*FW*	*CDR3*	*FW*	*TCRJ*	CDR3 Length
SA32.5	***TCRAV1S2***	**CAV**	**SGQGAQKL**	**VFG**	***AJ 54*01***	8
	TCRAV27S1 (out of frame)	CAV	DSRVRNWSQZZADIWKRNNSECZT			
MK20.2	*TCRAV15S1*	CAD	SLLSPNSGSARQL	TFG	*AJ 22*01*	13
	TCRAV16S1	**CAA**	**WNNFNKF**	**YFG**	***AJ 21*01***	7
SA32.5 (*TCRBC2*)	***TCRBV9S1***	**CAS**	**SPTGQGAHTGEL**	**FFG**	***BJ 2-2*01***	12
MK20.2 (*TCRBC2*)	***TCRBV3S1***	CAS	SSTGVSPGEL	FFG	***BJ 2-2*01***	10

[a] Functional TCRs for each T cell clone are represented in boldface. The deduced amino acid sequence of the CDR3 loop is shown putatively supported by two framework branches (*FW*). Amino acids preceding "CA" of V region and those following the highly conserved "FG" of J region are not shown. Two T cell clones used distinct CDR3α and CDR3β sequences at the amino acid level and distinct CDR3 length. Both T cell clones used the same Jβ rearrangement.

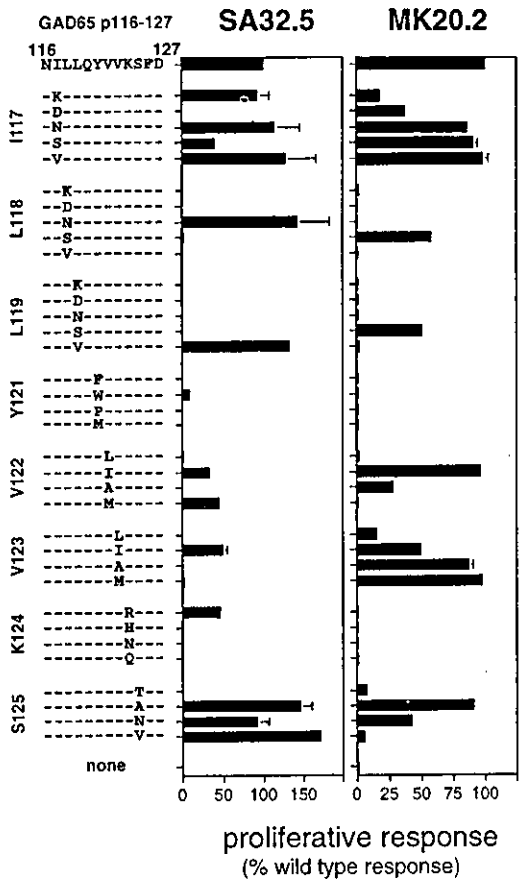


FIGURE 2. Proliferative response of $GAD65_{116-127}$-autoreactive human T cell clones to peptide analogs carrying a single-amino acid substitution in the $GAD65_{116-127}$ peptide. Data are given as percentages of wild-type response which were standardized by calculating the percentage to the response to wild-type peptide. The T cell responses to wild-type $GAD65_{116-127}$ were 27,021 cpm for SA32.5 and 23,146 cpm for MK20.2. Medium control response without peptide was <200 cpm. All data are expressed as the mean value of duplicate determinations ± SD.

tyrosine to tryptophan substitution at position 121 and lysine to arginine substitution at position 124, albeit with a significantly weaker response. In contrast, MK20.2 did not respond to these conservative substitutions at these positions. These data indicated that these T cell clones differ in responses against peptide analogs with a single-amino acid substitution. In particular, peptide analogs carrying replacement of Y121 and K124 with even conservative amino acids completely abrogated reactivity of MK20.2, suggesting that these residues are directly contacted by MK20.2 TCR.

Construction of the T cell epitope expression library using CLIP-substituted Ii genes

We constructed a set of T cell epitope expression libraries by using an Ii-based epitope presenting plasmid vector, pCI, in which the CLIP region of Ii was substituted with MHC class II-restricted epitopes (Fig. 3*A*). In the libraries, sequences of peptides are derived from the $GAD65_{115-127}$, and three successive residues within the sequence were totally randomized, the theoretical maximum complexity of each library being 20^3 (Fig. 3*B*). We defined the I117 of $GAD65_{115-127}$ as the relative position 1 in this study. As shown in Fig. 3*B*, the library set (series CIR) is composed of seven sublibraries: CIR−1–2; CIR2–4; CIR3–5; CIR4–6; CIR5–7; CIR6–8; and CIR7–9. (CIR−1–2 represents a library in which three successive residues from a relative position −1 to 2 in the T

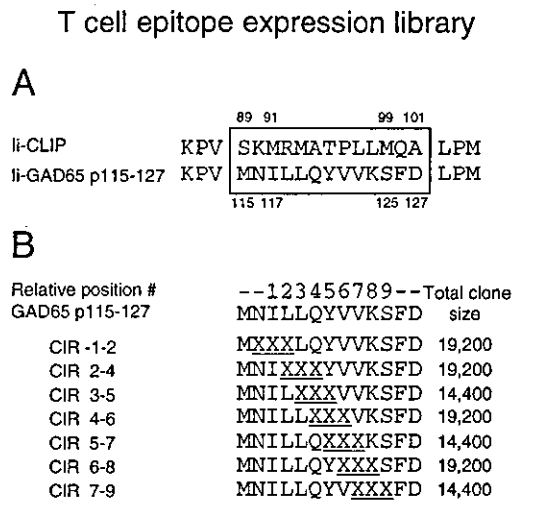


FIGURE 3. Design of a T cell epitope expression library using CLIP-substituted Ii genes. *A*, For construction of this library, the CLIP-encoding region (Ii_{89-101}) was genetically replaced with $GAD65_{115-127}$ to align I117 of $GAD65_{115-127}$ to M91 of Ii_{89-101}, the first DR-anchoring residue of the CLIP. *B*, The series of T cell epitope expression library was composed of seven sublibraries: CIR−1–2; CIR2–4; CIR3–5; CIR4–6; CIR5–7; CIR6–8; and CIR7–9. CIR−1–2 represents a sublibrary in which three successive residues from relative positions −1 to 2 on the $GAD65_{115-127}$ were replaced by randomized amino acids. Each sublibrary was aligned and is indicated in single-letter amino acid code. X indicates a random amino acid encoded by nucleotide triplets NNK, where N stands for any nucleotide and K stands for G or T. In each of three randomized codons, the third position was limited to G or T to minimize the appearance of stop codons.

cell epitope $GAD65_{115-127}$ are replaced by randomized amino acids.) The positions of inserted randomized amino acids are serially overlapped between individual sublibraries covering the core epitope $GAD65_{116-125}$. Individual libraries contained ~14,400–19,200 DNA clones and were divided into subpools composed of 30–50 clones. Summation of all the complexity of peptides is estimated to be at least 120,000 species.

Two $GAD65_{116-127}$-autoreactive and HLA-DR53-restricted TCRs responded differently to T cell epitope expression libraries: cross-reactivity scanning

The epitope recognition by the two $GAD65_{116-127}$-reactive TCRs was further investigated using the T cell epitope expression system to identify the diverse peptide ligands of these T cell clones. The two T cell clones were examined in parallel with their production of IFN-γ in response to a set of T cell epitope expression libraries. Fig. 4 shows the frequency of pools stimulating T cell clones in the epitope libraries. The vector DNA encoding native $GAD65_{115-127}$ mixed with a 100× excess amount of the wild-type Ii gene was assigned an arbitrary activity of 1 U, leading to determination of the relative activity of each separated pool. Thus, these TCRs responded to pools of each epitope library at different frequencies. MK20.2 responded to many of the pools of CIR−1–2 (99.7%) and CIR2–4 (66.0%) libraries with >0.5 U of response. In contrast, this TCR responded to a few of the pools in relatively C terminus-randomized libraries (CIR3–5 to CIR7–9 libraries) (<7%). These results suggest that the highest specificity of MK20.2 TCR exists at the relatively C-terminal side of $GAD65_{115-127}$. In contrast, SA32.5 showed a broader response profile against several libraries spanning the epitope functional core. SA32.5 TCR showed a number of strong responses with library CIR6–8 (73.4%). These results collectively indicate that the spectrum of fine specificity in TCR recognition was clearly different between these two TCRs.

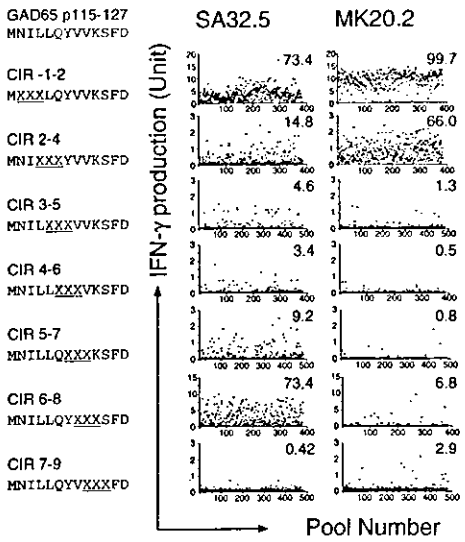


FIGURE 4. Response of $GAD65_{116-127}$-autoreactive TCRs to pools of a peptide expression library. Each pool was expected to contain ~30–50 distinct peptide ligands. IFN-γ production for each pool is expressed in terms of units. The response to pCIG (relevant DNA; $GAD65_{115-127}$) 100-fold diluted with pCI (irrelevant DNA; CLIP) was defined to be a relative value 1 U IFN-γ production ranging from 232 to 506 pg/ml. Percentages of positive pools for individual sublibraries are indicated. Positive pools that stimulated a significant IFN-γ production (relative value >0.5) were assigned. The negative control culture was stimulated by pCI, and IFN-γ production stimulated by pCI ranged between 5.75 and 36.3 pg/ml. The experiment was repeated twice with reproducible results. Randomized residues are underlined.

Isolation of diverse stimulatory peptides recognized by TCRs

The T cell epitope expression library was used to identify a series of agonistic peptide ligands for these TCRs. We screened 2,796 pools (total clone size was estimated to be 120,000); and after 3 rounds of screening, possible stimulatory peptides with higher antigenicity for SA32.5 (85 ligands) and MK20.2 (63 ligands) TCRs, respectively, were identified. The wild-type $GAD65_{115-127}$ sequence was isolated from several libraries but not from library CIR−1–2, CIR3–5, and CIR6–8. For some peptide sequences identified, the same sequences were isolated more than twice; and in ~70% of such cases, peptides were encoded by distinct nucleotide sequences. The sequences of stimulatory peptides and their stimulatory activity are shown in Fig. 5. MK20.2 TCR responded to most of the pools (99.7%) of library CIR−1–2 in the first round of screening (Fig. 4). Therefore, agonistic peptide ligands for MK20.2 TCR were not isolated from this library, and sequences isolated from library CIR−1–2 as stimulatory ligands for SA32.5 TCR were tested for their capacity to stimulate MK20.2 TCR (Fig. 5*A*). With regard to library CIR2–4 for SA32.5 TCR (14.8%) and MK20.2 TCR (66.0%) and the library CIR6–8 for SA32.5 TCR (73.4%), stimulatory ligands were not exhaustively isolated because of the high frequency of positive pools.

Analysis of stimulatory ligands from library CIR−1–2 (MXXXLQYVVKSFD library)

The panel of stimulatory ligands isolated from library CIR−1–2 (Fig. 5*A*) shows that both SA32.5 and MK20.2 tolerated phenylalanine, methionine, leucine, isoleucine, valine, and cysteine at relative position 1 (isoleucine in the native ligand). These amino acids were compatible with the HLA-DR primary anchor residue that was restricted by the Val/Gly dimorphism at DRβ86 where valine is used in the HLA-DR53 molecule (33, 34). For SA32.5 TCR, relative position 2 showed a strong preference for asparagine and leucine. MK20.2 TCR tolerated most of the agonistic sequences identified with SA32.5 TCR with different stimulatory capacities. However, MK20.2 TCR did not respond to peptides that incorporated asparagine at relative position 2.

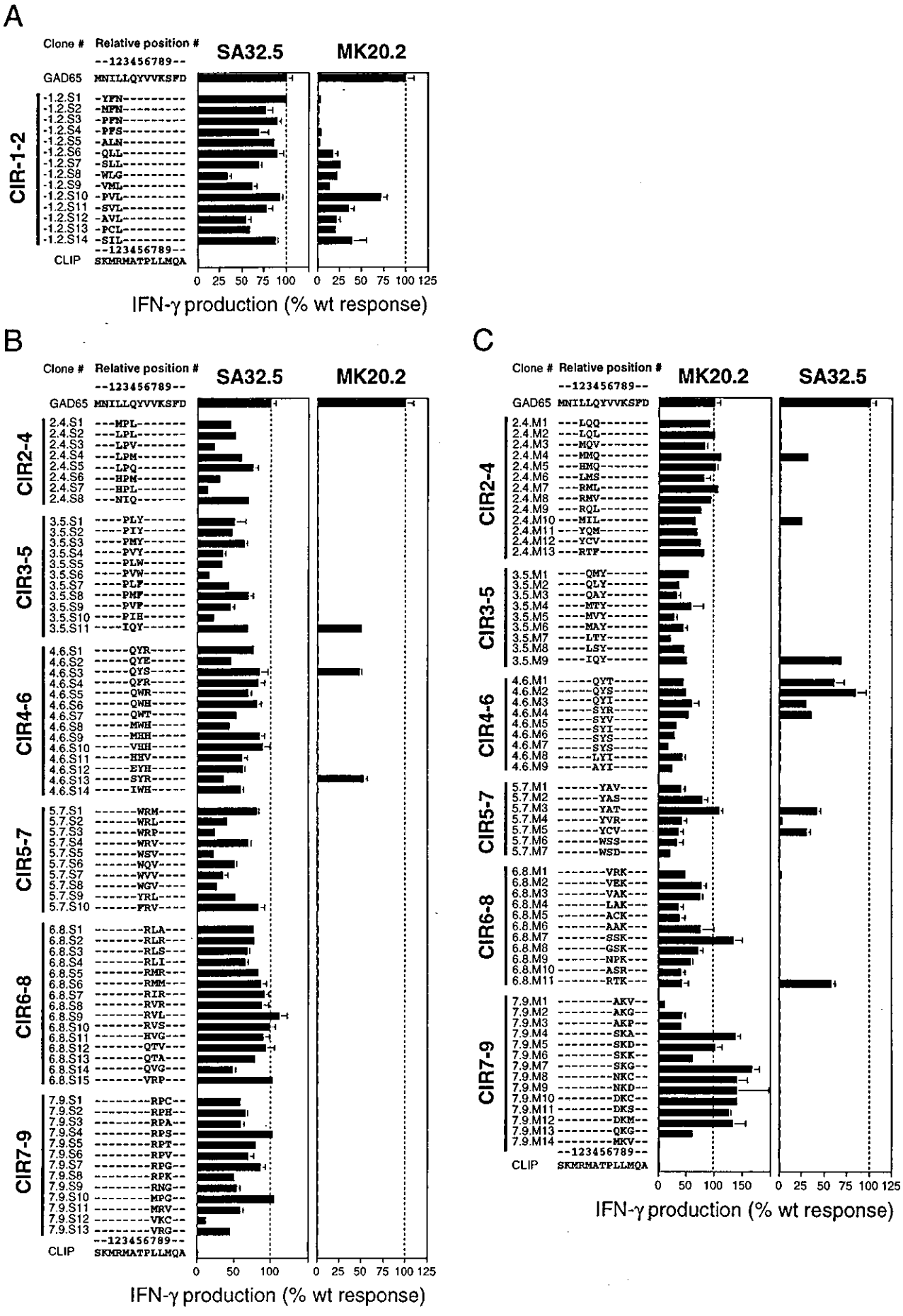
Analysis of stimulatory ligands for SA32.5 TCR: CIR2–4 to CIR7–9 libraries

SA32.5 TCR had a preference for leucine and asparagine at relative position 2 in screening of CIR−1–2 (Fig. 5*A*) and CIR2–4 (Fig. 5*B*), and methionine and histidine were also tolerated in CIR2–4. This TCR showed a strong preference for proline with an imino ring at relative position 3 in the screening of two separate libraries, CIR2–4 and CIR3–5. At this position, isoleucine sharing a chemically conservative side chain with native leucine was also tolerated. At relative position 4, leucine, methionine, valine, and glutamine were tolerated almost equally in CIR2–4 and CIR3–5 libraries. In CIR3–5, isoleucine was also tolerated; however, glutamine at relative position 4, the same amino acid as in the native ligand, was the most frequently observed (7 of 14) in CIR4–6. At this position, histidine, glutamic acid, and serine were also tolerated in CIR4–6. At relative position 5, located at the center of the epitope, this TCR did not tolerate chemically conservative single amino acid substitutions except for tryptophan in the analysis peptide analogs with single-amino acid residue substitution (Fig. 2). However, ligands containing tyrosine, phenylalanine, and tryptophan, which share an aromatic ring, and histidine with an imidazole ring at this position were isolated from the two libraries, CIR3–5 and CIR4–6. Neighboring residues may compensate for conformational mimicking.

Tryptophan at relative position 5 was the most frequently observed amino acid (8 of 10) in stimulatory ligands isolated from CIR5–7. At relative position 6, this TCR has a preference for positively charged arginine and histidine in CIR4–6, CIR5–7, and CIR6–8. However, glutamic acid, serine, threonine, and valine were also tolerated in CIR4–6. In addition, serine, glutamine, valine, and glycine were also tolerated in CIR5–7. Similarly, glutamine and valine were also tolerated in CIR6–8. At relative position 7, this TCR showed a preference for hydrophobic residues (methionine, leucine, valine, and proline) in CIR5–7 and (leucine, methionine, valine, and isoleucine) in CIR6–8. This preference in chemical character is not significantly changed in these two libraries. However, there is a drastic change in the preference in CIR7–9 at this position in which the 3-mer randomized portion was moved to the C-terminal side only by one amino acid from CIR6–8. This TCR has a preference for positively charged arginine at relative position 7. In addition, threonine and arginine in CIR6–8 and methionine and valine in CIR7–9 were also permitted at this position. At relative position 8, several amino acids with different chemical properties (arginine, proline, serine, isoleucine, methionine, leucine, valine, alanine, and glycine) were tolerated almost equally in CIR6–8. On the contrary, similar drastic changes in preference observed at position 7 were also observed at position 8 in CIR7–9, and this TCR has a strong preference for proline at this position. Several amino acids with different chemical properties were tolerated at relative position 9.

Epitope expression library revealed the importance of linear combinations of residues in recognition by SA32.5 TCR

By observing the preference of residues in peptides in recognition by SA32.5 TCR, preferable residues at each position in the N-terminal side were relatively similar in each separated library. However, in the peptide C-terminal side (Fig. 5*B*, CIR5–7,


A
Clone # Relative position #
--123456789--
SA32.5
MK20.2
GAD65 MNILLQYVVKSFD
CIR-1-2
-1.2.S1 -YFN---------
-1.2.S2 -MFN---------
-1.2.S3 -PFN---------
-1.2.S4 -PFS---------
-1.2.S5 -ALN---------
-1.2.S6 -QLL---------
-1.2.S7 -SLL---------
-1.2.S8 -WLG---------
-1.2.S9 -VML---------
-1.2.S10 -PVL---------
-1.2.S11 -SVL---------
-1.2.S12 -AVL---------
-1.2.S13 -PCL---------
-1.2.S14 -SIL---------
--123456789--
CLIP SKMRMATPLLMQA
0 25 50 75 100
0 25 50 75 100 125
IFN-γ production (% wt response)
B
Clone # Relative position #
--123456789--
SA32.5
MK20.2
GAD65 MNILLQYVVKSFD
CIR2-4
2.4.S1 ---MPL-------
2.4.S2 ---LPL-------
2.4.S3 ---LPV-------
2.4.S4 ---LPM-------
2.4.S5 ---LPQ-------
2.4.S6 ---HPM-------
2.4.S7 ---HPL-------
2.4.S8 ---NIQ-------
CIR3-5
3.5.S1 ----PLY------
3.5.S2 ----PIY------
3.5.S3 ----PMY------
3.5.S4 ----PVY------
3.5.S5 ----PLW------
3.5.S6 ----PVW------
3.5.S7 ----PLF------
3.5.S8 ----PMF------
3.5.S9 ----PVF------
3.5.S10 ----PIH------
3.5.S11 ----IQY------
CIR4-6
4.6.S1 -----QYR-----
4.6.S2 -----QYE-----
4.6.S3 -----QYS-----
4.6.S4 -----QFR-----
4.6.S5 -----QWR-----
4.6.S6 -----QWH-----
4.6.S7 -----QWT-----
4.6.S8 -----MWH-----
4.6.S9 -----MHH-----
4.6.S10 -----VHH-----
4.6.S11 -----HHV-----
4.6.S12 -----EYH-----
4.6.S13 -----SYR-----
4.6.S14 -----IWH-----
CIR5-7
5.7.S1 ------WRM----
5.7.S2 ------WRL----
5.7.S3 ------WRP----
5.7.S4 ------WRV----
5.7.S5 ------WSV----
5.7.S6 ------WQV----
5.7.S7 ------WVV----
5.7.S8 ------WGV----
5.7.S9 ------YRL----
5.7.S10 ------FRV----
CIR6-8
6.8.S1 -------RLA---
6.8.S2 -------RLR---
6.8.S3 -------RLS---
6.8.S4 -------RLI---
6.8.S5 -------RMR---
6.8.S6 -------RMM---
6.8.S7 -------RIR---
6.8.S8 -------RVR---
6.8.S9 -------RVL---
6.8.S10 -------RVS---
6.8.S11 -------HVG---
6.8.S12 -------QTV---
6.8.S13 -------QTA---
6.8.S14 -------QVG---
6.8.S15 -------VRP---
CIR7-9
7.9.S1 --------RPC--
7.9.S2 --------RPH--
7.9.S3 --------RPA--
7.9.S4 --------RPS--
7.9.S5 --------RPT--
7.9.S6 --------RPV--
7.9.S7 --------RPG--
7.9.S8 --------RPK--
7.9.S9 --------RNG--
7.9.S10 --------MPG--
7.9.S11 --------MRV--
7.9.S12 --------VKC--
7.9.S13 --------VRG--
--123456789--
CLIP SKMRMATPLLMQA
0 50 100
0 25 50 75 100 125
IFN-γ production (% wt response)
C
Clone # Relative position #
--123456789--
MK20.2
SA32.5
GAD65 MNILLQYVVKSFD
CIR2-4
2.4.M1 ---LQQ-------
2.4.M2 ---LQL-------
2.4.M3 ---MQV-------
2.4.M4 ---MMQ-------
2.4.M5 ---HMQ-------
2.4.M6 ---LMS-------
2.4.M7 ---RML-------
2.4.M8 ---RMV-------
2.4.M9 ---RQL-------
2.4.M10 ---MIL-------
2.4.M11 ---YQM-------
2.4.M12 ---YCV-------
2.4.M13 ---RTF-------
CIR3-5
3.5.M1 ----QMY------
3.5.M2 ----QLY------
3.5.M3 ----QAY------
3.5.M4 ----MTY------
3.5.M5 ----MVY------
3.5.M6 ----MAY------
3.5.M7 ----LTY------
3.5.M8 ----LSY------
3.5.M9 ----IQY------
CIR4-6
4.6.M1 -----QYT-----
4.6.M2 -----QYS-----
4.6.M3 -----QYI-----
4.6.M4 -----SYR-----
4.6.M5 -----SYV-----
4.6.M6 -----SYI-----
4.6.M7 -----SYS-----
4.6.M8 -----LYI-----
4.6.M9 -----AYI-----
CIR5-7
5.7.M1 ------YAV----
5.7.M2 ------YAS----
5.7.M3 ------YAT----
5.7.M4 ------YVR----
5.7.M5 ------YCV----
5.7.M6 ------WSS----
5.7.M7 ------WSD----
CIR6-8
6.8.M1 -------VRK---
6.8.M2 -------VEK---
6.8.M3 -------VAK---
6.8.M4 -------LAK---
6.8.M5 -------ACK---
6.8.M6 -------AAK---
6.8.M7 -------SSK---
6.8.M8 -------GSK---
6.8.M9 -------NPK---
6.8.M10 -------ASR---
6.8.M11 -------RTK---
CIR7-9
7.9.M1 --------AKV--
7.9.M2 --------AKG--
7.9.M3 --------AKP--
7.9.M4 --------SKA--
7.9.M5 --------SKD--
7.9.M6 --------SKK--
7.9.M7 --------SKG--
7.9.M8 --------NKC--
7.9.M9 --------NKD--
7.9.M10 --------DKC--
7.9.M11 --------DKS--
7.9.M12 --------DKM--
7.9.M13 --------QKG--
7.9.M14 --------MKV--
--123456789--
CLIP SKMRMATPLLMQA
0 50 100 150
0 25 50 75 100 125
IFN-γ production (% wt response)