Immunology 1996 **89** 245–249

Costimulation regulates the kinetics of interleukin-2 response to bacterial superantigens

E. MURAILLE,* S. DEVOS,† K. THIELEMANS,† J. URBAIN,* M. MOSER* & O. LEO* *Laboratoire de Physiologie Animale, Département de Biologie Moléculaire, Université Libre de Bruxelles, Rhode-St-Genèse and †Division of Haematology–Immunology, Medical School of the Vrije Universiteit Brussel, Brussels, Belgium*

SUMMARY

The aim of this study was to investigate the mechanisms by which B7-related costimulatory molecules (CD80, CD86) increase T-cell responsiveness to extracellular ligands. As a model study, the *in vitro* response of purified splenic $CD4^+$ T cells to a bacterial superantigen, SEB, was characterized. Previous analysis of this experimental model led us to conclude that expression of B7-related molecules is strictly required in order to activate $CD4^+$ T cells in the presence of bacterial superantigens. In the present report, we demonstrate that antigen-presenting cell-derived costimulatory signals regulate the kinetics of interleukin-2 (IL-2) production by SEB-activated splenic $CD4^+$ T cells. Indeed, experiments performed with purified subpopulations of antigen-presenting cells and using B7-transfected cell lines indicated that increased levels of CD80 and/or CD86 cell surface expression is associated with a faster kinetics of IL-2 production in response to SEB. Accordingly, blocking of CD80 or CD86-derived signals by specific monoclonal antibodies led to a slower kinetics of IL-2 production in response to SEB. Thus these data demonstrate that similar strength of signal through the T-cell receptor can lead to immune responses displaying distinct kinetics depending on the level of costimulatory ligands on APC.

INTRODUCTION

Optimal stimulation of T lymphocytes requires both T-cell receptor (TCR) engagement and an additional, contact-dependent, costimulatory signal delivered by an antigen-presenting cell (APC) (for review see ref. 1). The available experimental evidence suggests that while the specificity of a given T-cell response is dictated by the interaction of the TCR (including the CD4 and CD8 coreceptors) with an antigenic peptide–major histocompatibility complex (MHC), the magnitude of the response is strongly influenced by the APC-derived costimulus. Costimulation is neither antigen-specific nor MHC-restricted, and is potentially mediated by a large set of non polymorphic cell surface molecules interacting with counter-receptors expressed by T cells. The CD80 (B7, BB1, B7.1)[2] and CD86 (B70, B7.2)[3] cell surface proteins, both interacting with the T-cell-specific CD28 and/or cytotoxic T-lymphocyte antigen-4 (CTLA-4) counter-receptors, represent the most potent and best characterized costimulatory molecules expressed by APC.

The functional consequences of CD28/CTLA-4 ligation by their natural ligands CD80 and/or CD86 have been extensively studied *in vitro* using both naive and cloned T cells. These experiments have demonstrated that following TCR ligation, signals provided by the B7-related molecules increase the plateau levels of interleukin-2 (IL-2) protein secretion[4,5] and expression of cytokine cell surface receptors.[6] These biological effects appear to reflect both an increase in gene transcription and the stabilization of the relevant mRNA.[4–6] Although the proximal events of signal transduction mediated by CD28 are still the matter of investigation, the published evidence suggests that TCR and CD28-mediated signals follow two separate intracellular pathways which are finally integrated at specific stages critical for cytokine-gene transcription. In agreement with this hypothesis, it has been shown that simultaneous signalling through TCR and CD28 results in the synergistic activation of two mitogen-activated protein kinases, JNK1 and JNK2.[7] Recent findings suggesting that requirement for costimulation is strongly dependent on the intensity of TCR signalling are also in agreement with the idea that TCR and CD28 signals co-operate synergistically in T-cell activation. In fact, by stimulating T cells in the presence of graded doses of TCR ligands, we[8] and others[9] have recently observed that costimulation (provided by natural APC, CD80-transfectants or antibodies to CD28) increases T-cell responsiveness by lowering the threshold of TCR-dependent activation. Accordingly, it has been demonstrated that dendritic cells (DC), an APC population expressing high levels of CD80 and CD86, are able to stimulate T cells in the presence of small amounts of

Received 23 April 1996; revised 9 June 1996; accepted 9 June 1996.

Correspondence: Dr O. Leo, Laboratoire de Physiologie Animale, Université Libre de Bruxelles, 67 rue des Chevaux, B-1640 Rhode-Saint-Genèse, Belgium.

antigen.[10] The present study was undertaken in order to characterize further the physiological mechanisms by which costimulation increases the efficiency of a T-cell-dependent immune response. A detailed kinetics analysis of IL-2 production in response to saturating amounts of a well-defined TCR-specific ligand, the bacterial superantigen (SAg) SEB, has been performed. Our observations indicate that in addition to increased T-cell responsiveness to limiting concentration of SAg,[8] costimulatory functions also provoke a faster *in vitro* T-cell response to saturating doses of SAg.

MATERIALS AND METHODS

Mice

Female BALB/c mice, 6–8 weeks old, were purchased from Charles River Wiga (Sulzfeld, Germany) and maintained in a pathogen-free environment in our own animal facility.

Reagents and antibodies

The following antibodies to murine determinants were used in this study: 16–10A1 (anti-CD80, hamster IgG);[11] GL1 (anti-CD86, rat IgG2a).[3] IR418 (rat IgG2a) and PARSI-19 (hamster IgG) were used as isotype-matched controls. Purified SEB was purchased from Toxin Technology, Inc., (Sarasota, FL). Calcium ionophore A 23187 (dissolved in dimethyl sulphoxide (DMSO) at the concentration of 1 mg/ml) and phorbol myristate acetate (PMA, dissolved in anhydrous ethanol at a concentration of 10 μg/ml) were obtained from Sigma Chemical Co (St Louis, MO). These reagents had no effect on IL-2 determination at the concentrations used in this study (not shown).

Cell lines

The murine CD80 gene was cloned and introduced into the A20 B-cell lymphoma line (available through the American Type Culture Collection (ATCC), Rockville, MD) as previously described.[8] The murine CD86 gene was similarly cloned from Poly A + RNA extracted from purified dendritic cells of DBA/2 origin. The primers for the CD86 gene were the sense primer: 5′-ACCCACGATGGACCCCAGATG-3′ and the antisense primer 5′-CACTGCCTTCACTCTGCATTTG-3′. The CD86 polymerase chain reaction (PCR) product of 927 base pairs (bp) was cloned into the pcDNA3 expression vector (Invitrogen BV, Leek, the Netherlands) and sequenced. A clone without PCR mistakes was transfected into A20 cells and CD86-expressing clones selected following culture in G-418-supplemented medium.

In vitro *responses*

All *in vitro* immune responses were performed in serum-free media containing RPMI-1640 (Seromed; Biochem KG, Berlin Germany) supplemented with 2% HY ultroser (Gibco BRL, Merelbeke, Belgium), penicillin, streptomycin, non-essential amino acids, sodium pyruvate, 2-mercaptoethanol (2-ME) and L-glutamine (Flow ICN Biomedicals Bucks, UK) as previously described.[8] Responder $CD4^+$ T cells were purified following incubation of spleen cell suspensions with anti-CD8 (clone 83-12-5, murine anti-CD8·2 IgM) containing supernatant and subsequent incubation with mouse immunoglobulin-specific, biotin-coupled, rabbit immunoglobulin (produced in our laboratory). SIg^+ and $CD8^+$ cells were negatively selected using a Magnetic Cell Sorter (Miltenyi Biotec, Bergisch-Gladbach, Germany). The resulting population comprised over 85% of $CD4^+$ cells and no detectable $CD8^+$ T cells nor B cells. Splenic B cells and dendritic cells were purified as previously described.[8]

An adequate number of responder cells was stimulated by SEB in a total volume of 0·2 ml in 96-well V-bottom plates. The plates were centrifuged at 50 *g* for 5 min at the onset of culture in order to pellet responder and antigen-presenting cells and synchronize cell cultures. Cultures were maintained at 37° in a humidified incubator (7% CO_2). Supernatants were collected at different time-points, frozen and equivalent dilutions assayed for IL-2 content by a bioassay using a subclone of the CTL.L cell line insensitive to murine IL-4.[12]

RESULTS

Experimental strategy

Bacterial SAg, such as the SEB, behave as bifunctional agents able to simultaneously interact with selected Vβ regions of the TCR and with a conserved domain of the MHC class II molecule, causing TCR aggregation of a large proportion of peripheral T cells (reviewed in ref. 13). By binding to an unoccupied site on the MHC molecule, SAg bypass physiological requirements for antigen capture and processing, and do not compete with processed self-peptides for the generation of a high-affinity TCR ligand. The role of costimulatory functions on the kinetics of T-cell activation was therefore studied by incubating splenic murine T cells with selected APC populations and saturating amounts of SEB as a TCR ligand.

Expression of B7.1 or B7.2 molecules enhances the kinetics of SEB-induced IL-2 production *in vitro*

In a first series of experiments, the role of B7-related molecules on the kinetics of IL-2 production was studied using cell transfectants. Two stable lines expressing high levels of the murine CD80 (A20-$CD80^{hi}$) or CD86 molecules (A20-$CD86^{hi}$) were produced following transfection of the corresponding gene into a class II^+ B-cell line constitutively expressing low levels of B7-related molecules (clone A20). As shown in Fig. 1, the parental (A20-wt) and the transfected line displayed equivalent amounts of cell surface I-E molecules while expressing different levels of the CD80 and CD86 molecules. Purified $CD4^+$ T cells were stimulated *in vitro* by SEB in the presence of the parental and transfected A20 sublines, and by pharmacological agents (calcium ionophore and PMA) known to bypass the early steps of signal transduction. These agents were therefore assumed to induce the fastest kinetics of IL-2 production. Supernatants were collected at different time-points and tested for IL-2 content by a sensitive bioassay, as shown in Fig. 2. This experiment demonstrated that increased levels of CD80 or CD86 cell surface expression are associated with faster kinetics of IL-2 production in response to SEB. The slower kinetics of IL-2 production induced by the CD86-transfectant as compared to CD80 transfectant is probably related to the weaker expression of cell surface-associated CD86 (see Fig 1). In spite of very different kinetics, A20-wt and B7-transfected A20 cell line induced similar levels of IL-2 following 20 hr of *in vitro* culture in the presence of saturating

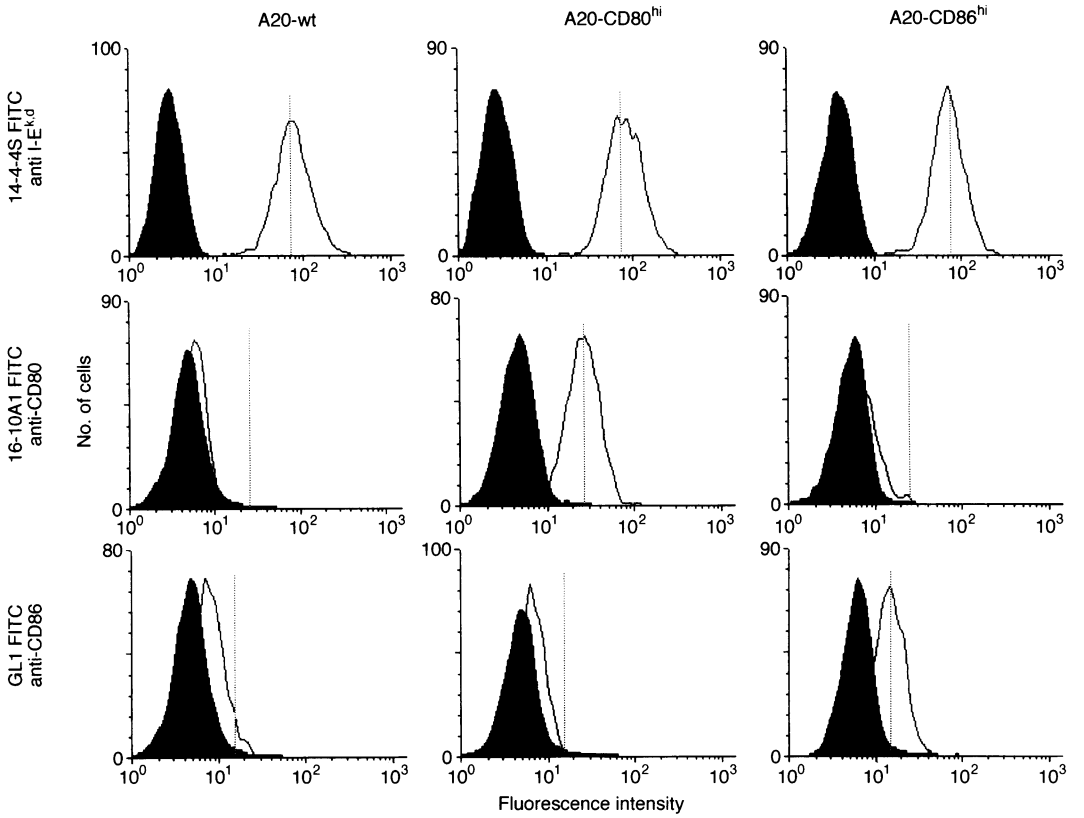

Figure 1. Flow cytometry analysis of wild-type and CD80/CD86-transfected A20 B-cell lymphoma lines. CD80- and CD86-transfected cell lines were derived as described in the Material and Methods. Cell surface antigen expression was assessed by incubating cells with fluorescein isothiocyanol-labelled mAb to I-$E^{k.d}$, CD80 or CD86 (open histograms), or the corresponding isotype-matched control mAb (closed histograms). All staining was performed in the presence of saturating doses of 24G2, a rat mAb to mouse FcRII/III , in order to inhibit FcR binding of staining reagents.

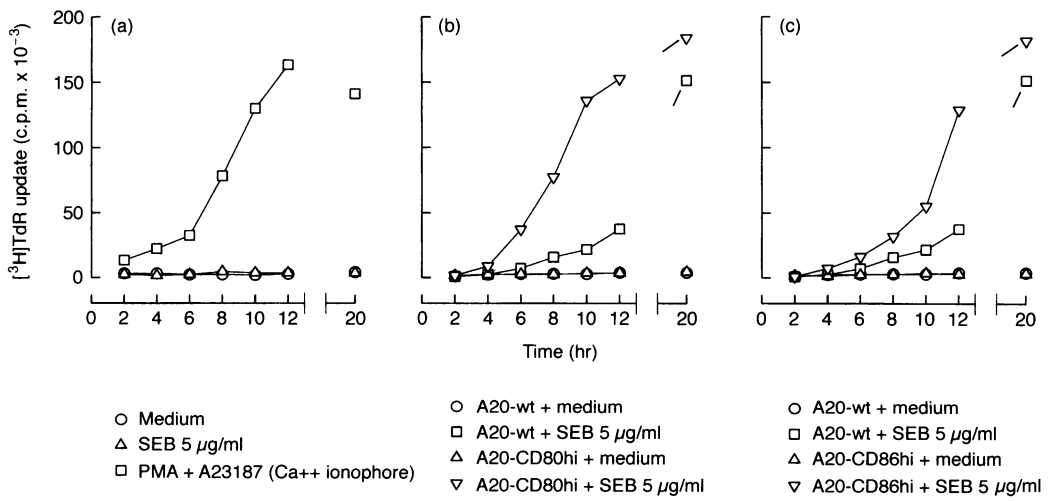

Figure 2. Costimulatory activity of the A20-CD80 and A20-CD86 transfected cell line. (a) Splenic CD4 T cells (10^5 cells/well) were stimulated in the absence of accessory cells by media, SEB (5 μg/ml) or a combination of PMA (10 ng/ml) and calcium ionophore (60 ng/ml). (b, c) The same responder cell population was stimulated by media or SEB (5 μg/ml) in the presence of control and transfected A20 cell lines (5×10^4 cells/well). The figure represents data collected from a single experiment representative of four. Culture supernatants were assayed for IL-2 content by bioassay. Results are expressed as c.p.m of [^{3}H]thymidine incorporation by the IL-2-dependent cell line CTLL.

doses of SEB (Fig. 2). Note that the IL-2 response induced by A20-wt in the presence of SEB has also been found to be B7-costimulation dependent, as it was completely inhibited by a combination of anti-CD80 and anti-CD86 mAb (data not shown).

Cells constitutively expressing high levels of costimulatory molecules induce a rapid IL-2 response *in vitro*

In order to confirm that costimulatory functions allow APC to induce a faster *in vitro* response to SEB, we have examined the kinetics of IL-2 secretion induced by SEB in the presence of splenic APC populations known to express different levels of costimulatory functions. Figure 3 shows that dendritic cells, (DC), an APC population expressing high levels of CD80 and CD86 molecules, induced a very fast *in vitro* IL-2 response, similar to the response induced by a combination of calcium ionophore and PMA. In marked contrast, B lymphocytes, a cell population expressing low levels of CD80 and CD86 molecules, only stimulate a weak IL-2 production, with a slower kinetics.

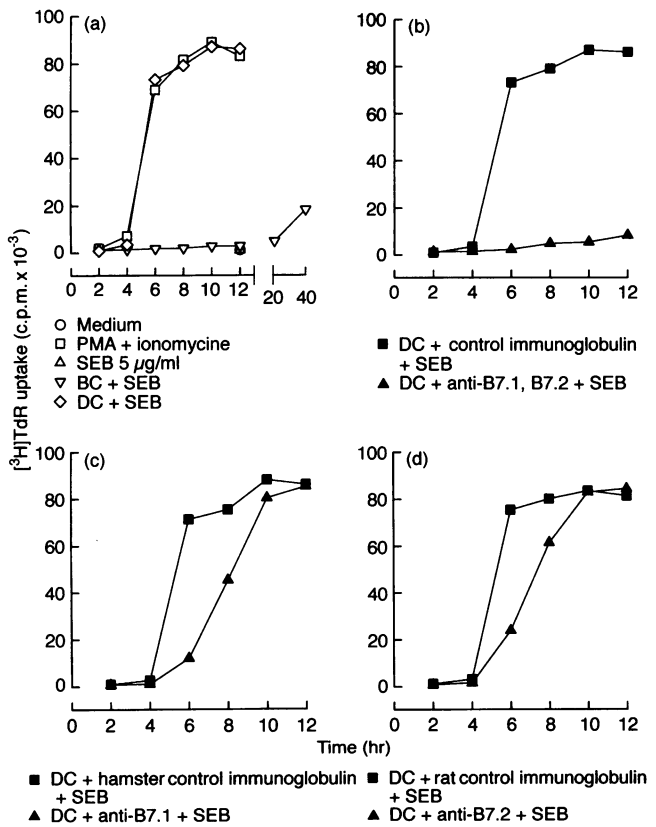

Figure 3. Costimulatory activity of splenic antigen-presenting cell populations. Splenic CD4 T cells (3×10^5 cells/well) were stimulated by pharmacological agents, SEB (5 μg/ml), or purified APC in the presence of SEB. Dendritic cells (DC, 5×10^3 cells/well) and splenic B cells (BC, 5×10^5 cells/well) were purified as described in the Materials and Methods. Antibodies to B7-related molecules or isogenic control mAb were added at a final concentration of 0·2 μg/ml. A 1:1 mixture of mAbs to CD80 and CD86, each present at 0·1 μg/ml was used. The figure represents data collected from a single experiment representative of four. Culture supernatants were assayed for IL-2 content by bioassay and results are expressed as c.p.m.

Partial blocking of costimulatory functions using a monoclonal antibody (mAb) to the CD80 or CD86 molecules expressed by DC, resulted in delayed IL-2 production *in vitro* with no effect on the magnitude of the response, further suggesting that CD28/B7 signalling plays a role in the kinetics of *in vitro* T-cell responses. As previously demonstrated for unfractionated spleen cells in a previous study,[14] simultaneous blocking of CD80 and CD86 signals in this model completely abrogated the IL-2 production induced by SEB in the presence of purified DC as APC (Fig. 3b).

DISCUSSION

The main conclusion from this work is that the kinetics of IL-2 production in response to SEB is influenced by the level of B7-related costimulatory molecules expressed by APC. The faster IL-2 response observed when T cells are stimulated by APC expressing high levels of costimulatory molecules could correspond to a higher signal delivered to each of the responding cells or to a higher number of T cells being activated at a given time. As our experimental design cannot distinguish between these two possibilities, further work will be required in order to determine the precise mode of action of B7-related costimulatory molecules.

Previous work performed in a murine T-cell clone has demonstrated a striking complexity of the kinetic regulation of IL-2 production by antibodies to CD28.[15] In this model, the most pronounced effect of anti-CD28 antibodies appeared to be an increase in IL-2 mRNA stability leading to a sustained secretion of the IL-2 protein. These studies also demonstrated that anti-CD28 mAb can augment IL-2 production by a T-cell clone 30- to 100-fold, with little effect on the early kinetics of the IL-2 response. In contrast, data presented herein suggest that at saturating amounts of TCR ligands, costimulatory functions induce a faster IL-2 response with little effect on the peak level of cytokine production. Differences in responding cell populations (T-cell clones versus naive populations), TCR (antibodies to TCR structure versus SEB) and CD28 ligands (anti-CD28 versus B7-related molecules) probably accounts for the observed discrepancies. Note that these observations are not mutually incompatible, as costimulatory factors may induce a faster response from naive T cells, while augmenting the peak level of cytokine production from activated T cells.

The early response to SAg induced by costimulatory-bearing APC may be of physiological relevance, as in contrast to most *in vitro* experimental models, Ag/MHC complexes may only be available to T cells *in vivo* for a limited amount of time. Kinetics studies performed in SEB-treated mice demonstrated that blood levels of exotoxin peaked 1 hr after administration and rapidly declined within another hour, with no detectable SEB in the spleen at 24 hr.[16] Of note, *in vivo* responses to SEB are characterized by a very rapid T-cell response, as several T-cell-derived cytokines can be detected in the serum as early as 90 min following intravenous injection of SEB.[17] This observation and the fact that antibodies to CD86 molecules inhibit *in vivo* responses to SEB[18] strongly suggest that bacterial SAg are presented *in vivo* by cells constitutively expressing CD86 molecules such as dendritic cells.[19]

In summary, we conclude that the delivery of APC-derived costimulatory signals represents a possible mechanism regulating both the kinetic (this study) and the amplitude of T-cell responses to extracellular ligands.[4,5]

ACKNOWLEDGMENTS

The authors wish to thank G. Dewasme, M. Swanepoel and F. Tielemans for expert technical assistance and H. Reiser, R. J. Hodes and H. Bazin for providing reagents. This work presents research results of the Belgian Programme in Interuniversity Poles of Attraction initiated by the Belgian State, Prime Minister's office, Science Policy Programming. The authors wish to thank the Fonds Emile Defay for providing additional financial support. The scientific responsibility is assumed by its authors. E.M. is supported by a grant from the FRIA (Belgium). S.D. is suported by the IWT (Belgium). O.L and M.M are research Associates from the FNRS (Belgium).

REFERENCES

1. SCHWARTZ R.H. (1990) A cell culture model for T lymphocytes clonal anergy. *Science* **248,** 1349.
2. LINSLEY P.S., CLARK E.A. & LEDBETTER J.A. (1990) T-cell antigen CD28 mediates adhesion with B cells by interacting with activation antigen B7/BB1. *Proc Natl Acad Sci USA* **87,** 5031.
3. HATHCOCK K.S., LASZLO G., DICKLER H.B., BRADSHAW, J., LINSLEY P. & HODES R.J. (1993) Identification of an alternative CTLA-4 ligand costimulatory for T cell activation. *Science* **262,** 905.
4. LINDSTEN T., JUNE C.H., LEDBETTER J.A., STELLA G. & THOMPSON C.B. (1989) Regulation of lymphokine messager RNA stability by a surface-mediated T cell activation pathway. *Science* **244,** 339.
5. FRASER J.D., IRVING B.A., CRABTREE G.R. & WEISS A. (1991) Regulation of interleukine-2 gene enhancer activity by the T cell accessory molecule CD28. *Science* **251,** 313.
6. CERDAN C., MARTIN Y., COURCOUL M., MAWAS C., BIRG F. & OLIVE D.J. (1995) CD28 costimulation up-regulates long-term IL-2R beta expression in human T cells through combined transcriptional and post-transcriptional regulation. *Immunology* **154,** 1007.
7. SU B., JACINTO E., HIBI M., KALLUNKI T., KARIN M. & BEN-NERIAH Y. (1994) JNK is involved in signal integration during costimulation of T lymphocytes. *Cell* **77,** 727.
8. MURAILLE E., DE BECKER G., BAKKUS M. *et al.* (1995) Co-stimulation lowers the threshold for activation of naive T cells by bacterial superantigens. *Int Immunol* **7,** 295.
9. DUBEY C., CROFT M. & SWAIN S.L. (1995) Costimulatory requirements of naive CD4+ T cells. ICAM-1 or B7-1 can costimulate naive CD4 T cell activation but both are required for optimum response. *J Immunol* **155,** 45.
10. BHARDWAJ N., YOUNG J.W., NISANIAN A.J., BAGGERS J. & STEINMAN R.M. (1993) Small amounts of superantigen, when presented on dendritic cells, are sufficient to initiate T cell responses. *J Exp Med* **178,** 633.
11. RAZI-WOLF Z., GALVIN F., GRAY G. & REISER H. (1993) Evidence for an additional ligand, distinct from B7, for the CTLA-4 receptor. *Proc Natl Acad Sci USA* **90,** 11182.
12. MULLER W. & VANDERNABEELE P. (1987) A T cell clone which responds to interleukine 2 but not to interleukine 4. *Eur J Immunol* **17,** 579.
13. KOTZIN B.L., LEUNG D.Y.M., KAPPLER J. & MARRACK P. (1993) Superantigens and their potential role in human disease. *Adv Immunol* **54,** 100.
14. MURAILLE E., DE SMEDT T., THIELEMANS K., URBAIN J., MOSER M. & LEO O. (1995) Activation of murine T cells by bacterial superantigens requires B7-mediated costimulation. *Cell Immunol* **162,** 315.
15. UMLAUF S.W., BEVERLY B., LANTZ O. & SCHWARTZ R.H. (1995) Regulation of interleukine 2 gene expression by CD28 costimulation in mouse T-cell clones: both nuclear and cytoplasmic RNAs are regulated with complex kinetics. *Mol Cell Biol* **15,** 3197.
16. OCHI A., YUH K., MIGITA K. & KAWABE Y. (1992) Effects of staphylococcal toxins on T-cell activity in vivo. In: *Biological Significiance of Superantigens. Chem Immunol* (ed. B. Fleischer), vol. 55, p. 115. Karger, Basel.
17. GONZALO J.A., BAIXERAS E., GONZALEZ-GARCIA A. *et al.* (1994) Differential in vivo effects of a superantigen and an antibody targeted to the same T cell receptor. Activation induced cell death vs passive macrophage-dependent deletion. *J Immunol* **152,** 1597.
18. MURAILLE E., DE SMEDT T., THIELEMANS K., URBAIN J., MOSER M. & LEO O. (1995) B7.2 provides co-stimulatory functions in vivo in responses to staphylococcal enterotoxin B. *Eur J Immunol* **25,** 2111.
19. INABA K., WITMER-PACK M., INABA M. *et al.* (1994) The tissue distribution of the B7–2 costimulator in mice: abundant expression on dendritic cells in situ and during maturation in vitro. *J Exp Med* **180,** 1849.