Introduction
Activation of resting T lymphocytes requires at least two signals: one provided by engagement of the T-cell antigen receptor (TCR)–CD3 complex by foreign antigen associated with self-major histocompatibility complex and the second by co-stimulatory molecule(s) present on antigen presenting cells (reviewed in [1]). These signals trigger the G0–G1 transition of the cell cycle as well as the induction of interleukin (IL) 2 secretion and IL-2 receptor expression [1]. TCR signals alone are insufficient to allow T-cell proliferation [1] and thus an understanding of the molecular interactions and signals generated by co-stimulatory molecules is of considerable importance. The major co-stimulatory signal involved in T-cell activation involves the interaction of B7.1 (CD80), a molecule found on antigen-presenting cells, with the T-cell molecule CD28, which is believed to provide a potential ‘second signal’ required for T-cell activation (reviewed in [2]). Additional family members have subsequently been identified [1,2], which has given the potential for at least four interactions in T-cell co-stimulation involving B7.1, B7.2 (CD86), CD28 and CTLA-4 (Table 1).

Ligation of CD28 alone has little, if any, effect on resting T-cell proliferation but this interaction has been shown to control proliferation and IL-2 production from TCR-stimulated CD28' T cells [1,2]. CD28 also mediates strong up-regulation of other cytokines including IL-4, IL-8, IL-13, γ-interferon and granulocyte/macrophage colony-stimulating factor as well as components of the IL-2 receptor [1]. T cells that are deprived of co-stimulatory signals enter into a state of proliferative hyporesponsiveness (anergy) or may undergo apoptosis [1,2]. These data along with transgenic experiments in vivo [3] provide ample evidence for a critical role of CD28-mediated signals in controlling T-cell responsiveness. Manipulation of CD28 interactions has already provided exciting results in both transplantation and tumour therapy settings and is likely to be equally valuable in studying autoimmune diseases [1]. It is therefore of considerable interest to determine and understand the biochemical signals by which CD28 can specify these functional outcomes.

There are several ways in which CD28 may mediate co-stimulation: (i) CD28 may activate distinct signal transduction pathways, complementing those activated by TCR and thus allowing full activation of T cells, which would fit well with the idea of co-stimulation; (ii) CD28 may enhance the amplitude or duration of a TCR-triggered signal, thereby crossing a threshold to activate downstream signalling cascades; (iii) CD28 may provide signals similar to those provided by the TCR, but at different time points or in different cellular compartments. Although CD28 can clearly trigger signals in common with the TCR, certain CD28-mediated signal transduction events occur independently of TCR stimulation. For instance, CD28 can trigger cyclosporin A (CsA)-resistant cytokine production, whereas TCR-induced cytokine production is generally inhibited by CsA [4] and CD28 ligation increases tyrosine phosphorylation on cellular substrates distinct from those induced by the TCR [5,6]. This paper will summarize the evidence that a likely candidate for the unidentified second signal provided by CD28 is the signalling pathway initiated by phosphoinositide (PI) 3-kinase activation [7].

Phosphoinositide 3-kinase
PI 3-kinase activity is generated by a growing family of biochemically distinct PI 3-kinase enzymes (Table 2). The use of the term PI 3-kinase should be taken to mean the phosphotyrosine/SH2-coupled form unless otherwise stated, a heterodimer that has a unique dual specificity as both a lipid and protein serine kinase [7] and consists of an 85 kDa regulatory subunit containing two SH2 domains and an SH3 domain, tightly associated with a catalytic 110 kDa subunit [7]. PI 3-kinase phosphorylates the membrane phosphatidylinositol lipids at the 3 position on the inositol ring, resulting in the generation of PtdIns(3)P, PtdIns(3,4)P2 and...
Pharmacological Targets in the Immune Response

Table I

Members of the B7 and CD28 families

<table>
<thead>
<tr>
<th></th>
<th>B7.1</th>
<th>B7.2/B70</th>
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<td>20</td>
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<td>—</td>
<td>90</td>
<td>40</td>
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<td>5</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>CTLA-4</td>
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<td>B7.1</td>
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<td>+</td>
</tr>
<tr>
<td>B7.2</td>
<td></td>
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</table>

PtdIns(3,4,5)P3 [12]. These D-3 phosphatidylinositol lipids represent only a minor fraction of cellular phosphatidylinositol lipids and are not substrates for phospholipase C [12]. However, considerable importance has been attached to this pathway, particularly concerning their putative role as regulatory molecules [7,12]. Kinetic evidence has suggested that PtdIns(3,4,5)P3, formed by a PtdIns(4,5)P2-specific 3-kinase, is the probable physiological intracellular mediator [12]. The association of PI 3-kinase with activated growth factor receptors and correlation of the production of D-3 phosphatidylinositol lipids with growth factor stimulation led to the suggestion that PI 3-kinase could be involved in signalling cell proliferation [12]. The advent of selective PI 3-kinase inhibitors such as wortmannin [13] has begun to clarify the role of this enzyme in cell signalling. Wortmannin inhibits a number of cellular responses such that PI 3-kinase is now implicated in the regulation of growth factor-induced membrane ruffling, histamine secretion, activation of neutrophils and translocation of glucose transporters [7,12]. However, the mechanisms by which the signal is transmitted downstream of the lipid metabolites remain obscure.

Coupling and activation of PI 3-kinase by CD28

The cytoplasmic domain of CD28 lacks any direct enzymic activity and is presumed to signal via the recruitment of cellular enzymes. Most notably, there is a consensus sequence motif (p)Y375MN, similar to the core phosphotyrosine-containing consensus motif, namely YXXM [14], which has specificity for the SH2 domains of the p85 subunit of PI 3-kinase and therefore suggested a direct interaction between PI 3-kinase and CD28. Moreover, CD28 also contains two proline-rich motifs (P178RRP and P190YAP), which conform to the PXXP SH3 binding consensus sequence [15] and these regions of the CD28 tail may also mediate interactions with PI 3-kinase and/or other signalling proteins.

The first indication that CD28 could indeed couple to PI 3-kinase was the demonstration that ligation of CD28 by B7.1 induced the accumulation of D-3 phosphatidylinositol lipids, the products of PI 3-kinase activation, in the leukaemic T cell line Jurkat [16]. Later studies revealed that B7.2 or monoclonal antibody ligation of CD28 also induced formation of D-3 phosphatidylinositol lipids in Jurkat cells [17]. Moreover, the p85 subunit of PI 3-kinase can
align with the cytosolic domain of CD28 via the YMNM motif [18]. The interaction of the Y<sup>173</sup>YMNM motif with the SH2 domains of p85 depends on phosphorylation of the Y<sup>173</sup> within the motif and CD28 is indeed phosphorylated on tyrosine after ligation [18]. Site-directed mutagenesis within the (p)Y<sup>173</sup>YMNM motif showed that mutation of Y<sup>173</sup> results in the elimination of PI 3-kinase binding [18]. There is substantial evidence that CD28 couples to protein tyrosine kinase(s) (PTK) because CD28 ligation results in the tyrosine phosphorylation of a number of other substrates in addition to CD28 [5,6,18]. The identity of the CD28-coupled PTKs remains unclear, although studies have suggested an association with p56<sup>ak</sup> and p59<sup>erm</sup> [19]. However, the association of CD28 with PI 3-kinase, but not CD28-induced Ca<sup>2+</sup> mobilization or tyrosine phosphorylation of certain substrates, has been shown to occur in p56<sup>ak</sup> deficient cell lines [20]. Hence it has been suggested that there are at least two PTK pathways activated in response to CD28 ligation, one of which is dependent on p56<sup>ak</sup> activity [20]. The identity of the PTK(s) that regulate the other pathways is unknown.

**Effects of PI 3-kinase inhibition on CD28-mediated co-stimulation**

The functional significance of the CD28/PI 3-kinase association was highlighted by reports showing that murine T-cell hybridomas expressing point mutations of the (p)Y<sup>173</sup> residue within the CD28 cytoplasmic tail are no longer able to produce IL-2 after CD28 ligation [18]. The importance of PI 3-kinase to CD28-dependent IL-2 production from both leukemic and normal T cells has been demonstrated in studies using the specific inhibitors of PI 3-kinase wortmannin and the structurally unrelated LY294002 [17,21]. Whereas wortmannin inhibits the CD28-stimulated accumulation of D-3 phosphatidylinositol lipids in metabolically <sup>32</sup>P-labelled Jurkat cells (in the low nanomolar range with an IC<sub>50</sub> less than 10 nM), similar concentrations of wortmannin actually potentiate CD28-dependent IL-2 production [17]. In contrast, the effects of wortmannin on the accumulation of D-3 phosphatidylinositol lipids in normal primary T cells are unknown because it is exceedingly difficult to measure D-3 phosphatidylinositols in these cells. However, wortmannin inhibits CD28-mediated co-stimulation of IL-2 from primary T cells at nanomolar concentrations, and assay in vitro of immunoprecipitated PI 3-kinase from primary T cells confirms that PI 3-kinase is inhibited in these cells (S. Ward, unpublished work). The effects of wortmannin on IL-2 production are also reproduced by the unrelated inhibitor LY294002 in all the T-cell models [17] and strongly suggests that the actions of wortmannin are due to PI 3-kinase inhibition.

The reason for the conflicting results in Jurkat cells and normal cells is not known, but similar heterogeneity in the regulation of biochemical signals between normal and Jurkat T cells has also been observed with respect to the regulation of phospholipase C by phorbol esters [22]. The basis for the difference between Jurkat cells and primary T cells could reflect the varying ability of CD28 to couple to multiple signalling pathways such as phospholipase C, calcium mobilization, p21<sup>ras</sup> activation and tyrosine phosphorylation [23,24] depending on the nature of the CD28 stimulus (e.g. antibody or natural ligand) and/or the activation state of the cell, since CD28-induced IL-2 production is inhibited by CsA in tumour cells or pre-activated T cells [25] but is CsA-insensitive [4] in resting T cells (reviewed in [2]). These alternative signalling pathways could contribute to the regulation of IL-2 production to varying degrees at various stages of cell growth. In leukemic cells, it seems that CD28-activated PI 3-kinase mediates a negative feedback mechanism to limit the amount of IL-2 produced in response to CD28 ligation. The ability of PI 3-kinase to regulate either a negative or positive signal for IL-2 production implies that CD28 and/or PI 3-kinase function(s) may change during the course of cellular differentiation, and this may also be critical to understanding the proposed inhibition of T-cell activation [26] and/or T-cell apoptosis [27] by CTLA-4 in some models, since CTLA-4 has also been reported to couple to PI 3-kinase [28].

In addition to CD28 [16-18], other T-cell molecules such as the IL-2 receptor [29], the TcR/CD3 complex [30] and CD4 [30] are known to associate with PI 3-kinase via interactions with the src family kinase p59<sup>gr</sup> or p56<sup>ak</sup> [29,30]. Moreover, both the TCR [31] and CD7 [31] also induce D-3 phosphatidylinositol lipid accumulation after ligation, albeit at much reduced levels than those of CD28-mediated accumulation, which are generally 4-5-fold greater than either CD3- or CD7-induced increases [31]. It is therefore unclear why activation of this pathway, which is clearly coupled to and activated by multiple T-cell-surface receptors, should be a critical event
in T-cell co-stimulation when specifically activated by CD28. There are, however, several mechanisms by which PI 3-kinase could mediate multiple cellular processes. First, the lipid product PtdIns(3,4,5)P3 is restricted to the membrane compartment in which it is made, giving rise to a high degree of compartmentalization such that activation of PI 3-kinases in distinct locations may have profoundly different consequences. Secondly, the existence of multiple forms of PI 3-kinase (Table 2) allows for differential regulation of each distinct PI 3-kinase isoform. Indeed, in T cells the α and β forms of p85 are differentially regulated by the TCR [32]. Thirdly, PI 3-kinase may actually be a multifunctional enzyme because the p110 subunit exhibits dual specificity as both a lipid kinase and a protein serine kinase [7] and the p85 subunit can act as an ‘adaptor’ molecule as demonstrated for insulin-receptor signalling [33], by virtue of interactions with other signalling molecules mediated by the SH2 or SH3 domains or even the proline-rich regions of p85 [7]. This means that data relating to the use of wortmannin to inhibit PI 3-kinase function must be interpreted carefully, because even though the lipid kinase activity of the p110 subunit can be inhibited by wortmannin, the p85 subunit is still able to function as an adaptor molecule and mediate protein–protein interactions.

Identification of downstream targets for CD28-activated PI 3-kinase

The major outcome of CD28 activation is the production of cytokines, an effect mediated by the modulation of the transcription of cytokine genes. A major aim of future studies will be to identify the downstream targets of PI 3-kinase that allow this pathway to mediate transcriptional events: this is currently the subject of intense investigations. Of the putative downstream effector molecules reported, there are perhaps three candidate biochemical targets relevant to CD28 signalling. The first is p70/85 S6 kinase (α1 and α11 isoforms, referred to collectively as p70S6k), a rapamycin-sensitive, ubiquitous mitogen-activated Ser/Thr kinase that is necessary for cells to enter S-phase after mitogen stimulation [34] and is phosphorylated on Thr-252 in the catalytic domain by PI 3-kinase [35]. Moreover, wortmannin inhibits the activation of p70S6k by insulin and platelet-derived growth factor [36]. Indeed, CD28 ligation induces activation of p70S6k in resting purified T lymphocytes [37] and Jurkat cells (R. Parry and S. Ward, unpublished work). The immunosuppressant rapamycin inhibits the effects of CD28-induced p70S6k activation [37], IκBα down-regulation and c-Rel translocation [38]. In contrast, rapamycin has no effect on CD28-induced PI 3-kinase activation, implying that PI 3-kinase lies upstream of p70S6k (R. Parry and S. Ward, unpublished work). Thus there may be an upstream regulation of IκBα and/or c-Rel by a signalling cascade involving the metabolic products of PI 3-kinase and p70S6k. This mechanism provides a direct link between CD28 and the modulation of transcriptional factors involved in IL-2 gene regulation.

A second target for PI 3-kinase may be the signalling cascades involved in the regulation of AP-1. AP-1 transcriptional activity, which requires both TCR and CD28-mediated signals

Table 2
Biochemically distinct PI 3-kinases

<table>
<thead>
<tr>
<th>PI 3-kinase</th>
<th>Substrate</th>
<th>Subunits</th>
<th>IC50 for wortmannin</th>
<th>References</th>
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<td>PTK/SH2 coupled</td>
<td>PtdIns</td>
<td>Regulatory p85 (α, β, γ)</td>
<td>&lt; 10 nM</td>
<td>[7]</td>
</tr>
<tr>
<td></td>
<td>PtdIns(4)P</td>
<td>Regulatory p55(κ)</td>
<td></td>
<td>[8]</td>
</tr>
<tr>
<td></td>
<td>PtdIns(4,5)P2</td>
<td>Catalytic p110 (α and β)</td>
<td>43 nM</td>
<td>[9,10]</td>
</tr>
<tr>
<td>G protein coupled</td>
<td>PtdIns</td>
<td>(PI 3-kinase γ)</td>
<td>2.5 nM</td>
<td>[10]</td>
</tr>
<tr>
<td></td>
<td>PtdIns(4)P</td>
<td>p110 (γ)</td>
<td></td>
<td>[9,10]</td>
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<td></td>
<td>PtdIns(4,5)P2</td>
<td>p110 (γ)</td>
<td></td>
<td>[10]</td>
</tr>
<tr>
<td>Vps34</td>
<td>PtdIns</td>
<td>p110, p150</td>
<td></td>
<td>[10,11]</td>
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</table>
is regulated at the level of c-jun and c-fos gene transcription and by post-translational modification of their products [40]. Induction and regulation of both c-fos and c-jun seem to be under the regulation of separate members of the mitogen-activated protein (MAP) kinase family, namely extracellular-signal regulated kinases (ERK) and c-Jun N-terminal kinase(s) (JNK, also referred to as stress-activated protein kinases) respectively [41]. There is accumulating evidence that PI 3-kinase may be involved in the regulation of either or both of the signalling cascades involving ERKs and JNK and hence in the regulation of c-fos and c-jun. First, fos transcription can be activated in a ras-dependent manner by a constitutively active p110 subunit mutant [42]. Secondly, the ras-related small GTP-binding protein rac, which has been implicated as an upstream regulator of the kinase cascades involved in the activation of JNK [43,44], seems to be a downstream effector for PI 3-kinase [45]. Interestingly, JNK has been reported to be synergistically activated by CD28 and TCR ligation, suggesting a pivotal role in T-cell activation for this kinase [46].

Studies in vitro have shown that the protein kinase C (PKC) isozymes δ, ε, η [47] and ζ [7] are activated by D-3 phosphatidylinositol lipids, and studies with phorbol esters have previously shown PKC to be involved in the control of many signalling pathways including p21ras and the MAP kinases ERK and JNK. Indeed, the PKC-responsive element in the IL-2 gene enhancer contains sites for the transcription factors NFkB, AP-1 and the nuclear factor of activated T cells (NFAT-1) [48]. The role of specific PKC isozymes, however, is poorly determined but PKCζ has been implicated in the induction of AP-1 and NFAT-1 induction [49], whereas PKCζ has been suggested to play a role in NFkB induction in fibroblasts [50], although not so far in T cells [49].

Conclusions
PI 3-kinase activation is clearly a pivotal signalling event in CD28 co-stimulation. The ultimate goal and biggest challenge in this field is to identify all the members of the PI 3-kinase family and the downstream components of the PI 3-kinase signalling pathway and ultimately to regulate these events. The growing number of events linked to PI 3-kinase activation as well as the increasingly diverse nature of the PI 3-kinase family means that it may be possible to design selective drugs to target distinct PI 3-kinase enzymes. Given the important role of PI 3-kinase in CD28-mediated T-cell co-stimulation, these drugs would be expected to have a large therapeutic potential in this area.

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Pharmacological Targets in the Immune Response

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