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The imidazoquinoline R-848, originally identified as a highly effective antiviral agent, has recently been shown to be capable of potent B lymphocyte activation. The B cell-activating properties of R-848 are strikingly similar to the effects of the CD40 ligand CD154. The present study demonstrates that this similarity extends to the intracellular signaling pathways triggered by the compound, although both overlapping and distinct mechanisms of signaling were seen. Like CD40 ligation, R-848 stimulated activation of the stress-activated protein kinases c-Jun kinase and p38 and activated the NF-κB family of transcription factors. Both R-848- and CD40-mediated B cell differentiation were dependent upon NF-κB activation, although the relative importance of individual NF-κB family members appeared to differ between R-848- and CD40-mediated signals. Both signals were partially dependent upon induction of TNF-α and II-6, and the cytoplasmic adaptor molecule TNF receptor-associated factor 2 is involved in both R-848- and CD40-mediated differentiation. The Journal of Immunology, 2000, 165: 5552–5557.

The immunomodulators R-848 (S-28463) and R-837 (imi- quimod) and the inactive analogue S-26424 belong to the imidazoquinoline class of compounds. These compounds have been previously shown to have potent antiviral and antitumor properties in animals, and imiquimod has recently been indicated for treatment of genital warts caused by papilloma virus infection in humans (1–6). In addition to their previously demonstrated effectiveness in inducing cytokine secretion by monocytes and dendritic cells, the imidazoquinolines have been recently shown to possess potent activating properties for B lymphocytes (7–11). We have found that the properties of R-848 show remarkable similarity to physiologic effector functions induced in the B cell via its membrane CD40 molecules. In both normal B cells (11) and B cell lines (G. A. Bishop and M. A. Tomai, manuscript in preparation), R-848 stimulates Ab secretion and surface molecule up-regulation. Additional B lymphocyte-activating signals recently have been observed following treatment with R-848, including lymphokine secretion and enhanced resistance to apoptosis (G. A. Bishop and M. A. Tomai, manuscript in preparation). Interestingly, each of these activating signals can also be induced by various other B cell receptors in addition to CD40. For example, these downstream events can be induced in B cells by the binding of TI-2 Ags to the B cell Ag receptor (reviewed in Ref. 12), by the binding of LPS to the LPS receptor (G. A. Bishop and M. A. Tomai, manuscript in preparation), by engagement of CD40 with CD154 (reviewed in Ref. 13), and by various cytokines binding to their respective receptors (reviewed in Ref. 14). However, each of these receptors uses distinct initial signaling pathways. Thus, although R-848 shows marked similarity to CD40 signaling in its effects on the B cell, the signaling pathways used to achieve these results may differ.

Understanding the mechanism of action of low molecular weight organic compounds such as R-848 in B lymphocyte activation is of considerable interest, as they are structurally quite different from the receptors whose signaling effects they mimic. Experiments in progress have revealed that R-848 does not exert its effects by engaging a cell surface receptor on B cells, but it is not yet clear to which intracellular structures or receptors it binds (M. A. Tomai, unpublished observations). We thus undertook this study to determine which signaling pathways are exploited by the drug to result in B cell activation. Information such as this can contribute to both increased understanding of B cell activation pathways as well as improved small molecule design for enhanced activation of immune responses.

Materials and Methods

Cells

The mouse B cell lines CH12.LX and CHB3 have been previously described (15, 16). Production and characterization of CH12.LX cells expressing the Lac repressor (CH12.LAC) (17) as well as those inducibly expressing a mutant form of IκBα (18) or truncated, dominant negative, mutant TNFR-associated factor 2 (TRAF2) and TRAF3 (19) were described previously. The human EBV− B cell line Ramos was obtained from American Type Culture Collection (Manassas, VA). Cell lines were grown in RPMI 1640 containing 10% heat-inactivated FCS, 10−5 M 2-ME, and antibiotics (B cell medium (BCM)). Resting splenic B cells from normal 7- to 10-wk-old female (B6 × 129/J)F1 hybrid mice or p50−/− knockout mice (provided by Arthur Krieg, University of Iowa, Iowa City, IA) were purified by a discontinuous Percoll density gradient as previously described (20). Small dense B cells were recovered from the 70 to 75% interface for use in experiments.

Abbreviations used in this paper: TRAF, TNFR-associated factor; BCM, B cell medium; TNF-R, TNF receptor, DNTRAF, dominant negative TRAF; IPTG, isopropyl-thio-ß-D-galactoside; LacR, bacterial repressor of the Lac operon.

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**Results**

R-848 stimulates activation of c-Jun kinase and p38 in B cells

One of the earliest events induced by CD40 signaling in B cells is the activation of the stress-activated protein kinases c-Jun kinase and p38 (24–26). R-848 also rapidly induced both kinases in the representative B cell line CH12.LX, as well as in purified splenic B cells (Fig. 1). Similar results were found by cross-linking either mouse or transfected human CD40. The inactive analogue of R-848 (S-26424), however, had no effect on the activity of these kinases in either cell type.

R-848 induces NF-κB activation in both mouse and human B cells

CD40 signaling to B cells induces strong activation of the NF-κB family of transcription factors, and this event has been shown to be necessary for CD40-mediated B cell differentiation and up-regulation of the B7 costimulatory molecules (18). As it has been recently shown that R-848, like CD40, induces B cell differentiation to Ab secretion and can enhance CD40-mediated B7 up-regulation (11), it was of interest to determine whether R-848 also induces NF-κB activation in B cells. Fig. 2A shows that within 5 min R-848 (but not its inactive analogue) induced nuclear translocation of NF-κB in the mouse B cell lines CH12.LX and CHB3, both of which have been previously shown to display an activated phenotype following R-848 treatment (G. A. Bishop and M. A. Tomai, manuscript in preparation). Peak increases were seen between 15 and 60 min, with levels decreasing in CHB3 cells by 4 h. In addition, R-848 induced NF-κB activation in human B cell lines, as shown in Fig. 2A in the EBV–

**Ab secretion assays**

To induce Ab secretion, CH12.LX cells (1500 cells/well in 150 μl) inductively expressing IgBaaAA or dominant negative TRAF2 (DNTRAF2) or DNTRAF3 were incubated for 24 h in 96-well flat-bottom microtiter plates in the presence of 100 μM IPTG to induce production of the IgBaaAA or DNTRAF proteins. Various stimuli were then added in a volume of 50 μl, and cells were incubated for an additional 48 h. Stimuli included various combinations of the following, all determined in preliminary experiments to be saturating final concentrations: R-848 (200 ng/ml), the anti-CD40 mAb 1C10 (1 μg/ml), IL-6 (10 ng/ml), anti-IL-6 mAbs 32C11 and 20F311 (3 μg/ml), TNF-α (25 ng/ml), or the anti-TNF mAbs MP6-XT22 and MP6-XT5 (10 μg/ml). CH12.LX cells inducibly produce IgM specific for an Ag, phosphatidylcholine, which is present on the mem-
FIGURE 1. R-848 stimulates the activation of c-Jun N-terminal kinase and p38 kinase in B lymphocytes. A, c-Jun N-terminal kinase activity. Left panel, CH12.LX mouse B cells stimulated for 5 min with BCM alone (lane 1). 1 μg/ml control mAb (lane 2), 1 μg/ml anti-CD40 mAb (lane 3), 1 μg/ml analogue S-26424 (lane 4), or 1 μg/ml R-848 (lane 5). Stimulation conditions and assay for phosphorylation of the c-Jun substrate are described in Materials and Methods. Right panel, Resting splenic B cells from (B6 x 129/J)F2 mice stimulated for 10 min with 1 μg/ml analogue S-26424 (lane 1), 1 μg/ml R-848 (lane 2), or 0.6 M sorbitol (lane 3). B, p38 activity. Left panel, CH12.LX cells expressing transfected human CD40 stimulated for 5 min with 1 μg/ml R-848 (lane 1), 1 μg/ml analogue S-26424 (lane 2), 1 μg/ml anti-hCD40 mAb (lane 3), 1 μg/ml anti-mCD40 mAb (lane 4), or PMA plus ionomycin (lane 5). Right panel, Resting splenic B cells from (B6 x 129/J)F2 mice stimulated for 10 min with BCM alone (lane 1), 1 μg/ml analogue S-26424 (lane 2), or 1 μg/ml R-848 (lane 3). Stimulation conditions and kinase activity assays are described in Materials and Methods. Results are representative of three similar experiments.

R-848-mediated Ab secretion is dependent upon NF-κB activation

Previous studies showed that CD40-mediated differentiation of B cells to Ab secretion is highly dependent upon CD40-induced NF-κB activation (18). We have found that R-848, like CD40, induces Ab secretion and synergizes with signals delivered through the B cell Ag receptor. It was thus of interest to determine whether the R-848-mediated activation of NF-κB is required for the ability of the drug to induce Ab production. To do so, we used a subclone of CH12.LX that stably and inductively expresses a mutant form of the NF-κB inhibitory molecule, IkBα. In this mutant molecule (IkBoAA), the two serine residues that are phosphorylated as a consequence of activation signals, leading ultimately to the degradation of IkBα, have been changed to alanines. Expression of the mutant protein is normally repressed by constitutive expression of the bacterial Lac operon repressor protein (LacR) in this cell line, but can be induced by inclusion of IPTG in the culture medium. We have previously shown that induced expression of IkBoAA in CH12.LX effectively blocks NF-κB activation (18). Fig. 5A shows that R-848-mediated activation of B cells expressing only LacR (CH12.LAC) was not inhibited by IPTG, but cells induced to express IkBoAA showed highly diminished Ab secretion in response to the drug. This dependence of the Ab response on NF-κB activation was quite similar to that seen for CD40 signaling, as shown in Fig. 5B, where the two stimuli are compared in the same experiment. Thus, NF-κB activity by R-848 is important for mediating the downstream effects of the drug on B lymphocytes.

R-848-induced differentiation involves both IL-6 and TNF

We have recently found that R-848 treatment of B cells increases both the gene expression and protein production of the cytokines IL-6 and TNF (G. A. Bishop and M. A. Tomai, manuscript in preparation), both of which have been shown to stimulate B cell Ab

p50−/− mice. Small, dense, resting splenic B cells were purified as described in Materials and Methods and stimulated for 20 min with BCM alone (lane 2), 1 μg/ml analogue plus 1 μg/ml isotype control mAb EM95 (lanes 3 and 6), 1 μg/ml 1C10 (lanes 4 and 7), or 1 μg/ml R-848 (lanes 5 and 8). Lane 1 contained labeled probe alone, which migrated to the bottom of the gel and is thus not visible here. EMSA was performed as described in Materials and Methods. Results are representative of two similar experiments. Wt, wild type.
production (27). To extend these findings, we wished to determine whether either cytokine was involved in R-848-mediated B cell differentiation. Fig. 6A shows that treatment of CH12.LX cells with a saturating amount of exogenous IL-6 (the maximal amount induced by R-848) induced Ab production, although not to the same extent as R-848 itself. R-848-induced differentiation could be completely blocked with a saturating concentration of the IL-6-blocking mAb, 20F3–11 (second set of bars), but was unaffected by a nonblocking IL-6-specific mAb (32C11). Fig. 6A also shows that R-848-induced Ab production was inhibited ~50% by treatment with 20F3–11. This suggests that IL-6 is an important component of the mechanism by which R-848 induces B cells to secrete Ab, but cannot completely account for the effect. Fig. 6B shows that, similarly, a saturating amount of exogenously added TNF-α stimulated Ab secretion by CH12.LX, but was less effective than R-848 itself. A mixture of two anti-TNF-blocking mAbs also reduced R-848-induced Ab secretion, by ~60%. Finally, the results shown in Fig. 6C demonstrate that the presence of blocking Abs to both IL-6 and TNF reduced CD40- and R-848-stimulated Ab production to a greater extent than blocking either cytokine alone. These results indicate that both IL-6 and TNF play important roles in R-848-induced B cell differentiation.

TRAF2 molecules play a role in R-848-mediated signaling

CD40 is a member of the TNF receptor (TNF-R) family of molecules, and the cytoplasmic proteins known as TRAFs have been strongly implicated as playing important roles in signaling through this family of molecules (28). The TRAFs are thought to exert their effects by direct binding to the cytoplasmic domains of the various TNF-R family molecules in whose functions they are involved. However, we have recently shown that TRAF2 can strongly influence CD40-mediated B cell differentiation even when it cannot directly associate with CD40 (19). An indirect way in which TRAF2 could influence Ab secretion is that CD40 signals are known to induce B cell TNF-α production, and TNF can induce Ab secretion (27, 29). In addition, we have recently found that R-848 induces CH12.LX cells to produce TNF-α (G. A. Bishop and M. A. Tomai, manuscript in preparation). TRAF2 binds to the cytoplasmic domains of both CD40 and the TNF-R (28), so it could indirectly influence CD40- and/or R-848-induced Ab secretion by affecting TNF-R signaling. We thus tested whether induced expression of a truncated, DNTRAF2 molecule in B cells affects R-848-induced differentiation, as Fig. 6B shows that this effect is
partially dependent upon TNF production. Fig. 7A shows that induced expression of DNTRAF2 inhibits both CD40- and R-848-mediated Ab secretion to approximately the same extent as does treatment with anti-TNF mAbs (see Fig. 6B). We have recently found that DNTRAF2 also inhibits TNF-induced Ab production by CH12.LX cells (G. A. Bishop and B. S. Hostager, manuscript in preparation). However, induced expression of DNTRAF3, which binds CD40 but not the TNF-R, inhibits only CD40-mediated differentiation and does not affect R-848-mediated activation (Fig. 7B).

Discussion

The present study was designed to investigate the molecular mechanisms of B lymphocyte activation by the immunomodulator R-848. The ability of this agent to stimulate B cell function has important implications for its potential as a vaccine adjuvant in stimulating B cell responses, particularly as it clearly mimics in many ways the signals received by B cells through their CD40 molecules. Indeed, imiquimod has been shown to act as a vaccine adjuvant when given together with herpes simplex virus glycoprotein D in guinea pigs (30, 31). Understanding how R-848 mediates its effects can lead to development of even more effective immunomodulatory agents as well as allow optimization of its use.

Similar to the natural signal provided by CD40 ligation (18), R-848 stimulates activation of stress-activated protein kinases (Fig. 1). There is no evidence, however, that R-848 directly activates protein tyrosine kinases (M. A. Tomai, unpublished observations). Similarly, although there is indirect evidence that CD40 signaling may ultimately involve tyrosine kinases, convincing in vivo evidence that CD40 associates with tyrosine kinases has been difficult to obtain (reviewed in Ref. 32). R-848, like CD40 (21) and another potential new adjuvant, Cpg oligonucleotides (33) also activates the NF-κB family of transcription factors (Figs. 2 and 3), and R-848-induced IgM secretion depends upon NF-κB activation (Fig. 4). NF-κB activation by the imidazoquinolines has also been demonstrated in human monocytes (10). The activation of members of the NF-κB family has been shown to make important contributions to many immune activation processes (34) and provides an important proviability signal to cells (35–39). Of particular interest was the finding that although CD40-induced NF-κB activation was dependent upon the p50 subunit, R-848-induced NF-κB activation, although inducing nuclear translocation of p50, was able to proceed in the absence of this subunit (Fig. 3). This result suggests that although the two types of signal are each able to induce nuclear translocation of the same Rel family subunits, CD40 preferentially stimulates the movement of hetero- and homodimers containing p50, while R-848 can also stimulate translocation of dimers lacking p50.

Our previous studies showed that R-848 induces production of a variety of cytokines in cells of the immune system, including B lymphocytes (7, 40). The present study determined that R-848-induced B cell production of both TNF-α and IL-6 play important roles in R-848-induced Ab secretion (Fig. 6). IL-6 has been shown to potently preserve B cell viability (27, 41, 42) and stimulate B cell differentiation (27, 43–46). Additionally, IL-6 production has been demonstrated to play a significant role in CD40-mediated B cell differentiation (47–49). Thus, R-848 again shows its ability to mimic signals delivered to B cells during normal T-dependent Ab responses. Although TNF can induce apoptosis in a variety of cells via the type I TNF-R (50), it has been shown that TNF induces human B cells to secrete Ab (27, 51), a finding we have recently reproduced in mouse B cells (B. S. Hostager and G. A. Bishop, manuscript in preparation). Thus, TNF production induced by R-848 has a positive effect in stimulating an Ab response. We also show that this effect requires participation of the cytoplasmic adapter molecule TRAF2, which has been shown to participate in signal transduction via the TNF-R (52). The result that DNTRAF3, which binds CD40 but not the TNF-R, does not affect R-848 signaling (Fig. 7) supports the hypothesis that TRAF2 affects R-848 signaling indirectly via affects on TNF-R signaling.

IL-6 and TNF are known mediators of inflammation in a variety of clinical situations (53, 54), and a number of therapeutic strategies have sought to block the actions of these cytokines. However, in designing more effective vaccines and adjuvants, it may clearly prove desirable to stimulate B lymphocyte production of IL-6 and TNF to obtain a more effective initial Ab response. The present study shows that R-848 can achieve this response, using molecular mechanisms remarkably similar to those used by CD40 signaling to B lymphocytes. The ability of R-848 to stimulate IL-12 production may also contribute to the potential of this compound as an adjuvant (7, 9). Mimicking the normal T cell-dependent pathways of B cell activation may prove a promising strategy to effective manipulation of the adaptive Ab response.

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