

Exacerbated inflammatory responses in transgenic mice expressing an inhibitor of apoptosis protein (OplAP)

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Abstract

Members of the inhibitor of apoptosis protein family are involved not only in suppressing apoptosis, but also in signal transduction, cell division, and are associated with some types of cancers. Here we show that transgenic expression of the inhibitor of apoptosis protein OplAP in murine T lymphocytes leads to a significant increase in T-cell receptor-induced cell activation, proliferation and cytokine production. Transgenic T lymphocytes expressing OplAP have a lower proliferation threshold in response to T-cell receptor stimulation. Unstimulated OplAP transgenic T lymphocytes show elevated nuclear levels of NF- κ B transcription factor that increase after *in vivo* antigen peptide treatment. OplAP transgenic animals present an exacerbated inflammatory response in an experimental contact hypersensitivity model, suggesting increased T-cell activation *in vivo*. These data indicate a new role for the inhibitor of apoptosis proteins in T-lymphocyte activation and proliferation.

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Keywords: inflammation; T lymphocyte; proliferation; transcription factors; inhibitor of apoptosis

Abbreviations: BIR, baculovirus IAP repeat; BRUCE, BIR repeat containing ubiquitin-conjugating enzyme; CARD, caspase-recruitment domain; CHS, contact hypersensitivity; DNBS, dinitrobenzene sulfonic acid; DNFB, 2,4-dinitrofluorobenzene; IAP, inhibitor of apoptosis proteins; IKK, I κ B kinase complex; MEKK, mitogen-activated protein/ERK kinase kinase; OplAP, IAP generated from the *Orgyia pseudotsugata* nuclear polyhedrosis virus; tg, transgenic; wt, wild type

Introduction

The inhibitor of apoptosis proteins (IAPs) constitute a family of cell death suppressors, conserved throughout evolution, which are found in many species from virus to mammals.¹

The IAPs are characterized by the existence of at least one copy of a common baculovirus IAP repeat (BIR) domain, which is essential for their antiapoptotic properties.² Most IAPs also contain a characteristic zinc finger motif, called RING, located at the carboxy-terminus.² The RING domain of many IAPs is described to have ubiquitin-ligase activity and to interact with ubiquitin complex components.^{3,4} Furthermore, the IAP BIR repeat containing ubiquitin-conjugating enzyme (BRUCE) has an additional domain with ubiquitin-conjugating enzyme (E2) activity.⁵ Although IAPs were initially discovered due to their ability to suppress apoptosis, other cellular functions have been demonstrated for certain members of this family. Some IAPs induce activation of the transcription factor NF- κ B by increasing its nuclear levels.^{6–10}

The baculoviral OplAP was one of the first IAP family members identified; it shares the characteristic structural composition with cellular counterparts such as XIAP, c-IAP1 and c-IAP2: two BIR motifs located in the amino-terminal region and a RING zinc finger in the carboxy-terminus of the protein.² Although OplAP was originally described as a caspase inhibitor in insect cells,¹¹ it also inhibits apoptosis when expressed in mammalian cells.^{8,12} We recently reported that, in addition to blocking apoptosis, OplAP expression in developing thymocytes also increases NF- κ B nuclear levels, which seems to be coupled to the enhanced activation and proliferative response of transgenic (tg) thymocytes.⁸

T lymphocytes are necessary for generation and regulation of the immune response. T-cell stimulation through the T-cell receptor (TCR) triggers several signaling pathways that ultimately lead to the activation of certain transcription factors, including members of the NF- κ B family. NF- κ B is a key regulatory element of immune and inflammatory responses^{13,14} and activates transcription of genes that encode proteins required for the immune response, such as immune receptors, inflammatory cytokines and chemokines.¹⁵

In resting T cells, NF- κ B is maintained inactive, bound to I κ B proteins including I κ B α , a major NF- κ B inhibitor in lymphocytes.¹⁶ Many biological factors, such as TCR triggering, induce I κ B α phosphorylation by the I κ B kinase (IKK) complex, followed by polyubiquitination and proteasome-dependent degradation. NF- κ B is then free to bind to DNA and activate transcription of its target genes.¹³

Previous work showed that the expression of some IAPs is associated with increased nuclear levels of NF- κ B via an unknown pathway.^{7–9} Since NF- κ B is an essential regulator of the T-cell response, we used T-lineage-specific tg expression in mice to study the effects of the IAP protein OplAP on T-lymphocyte function and physiology. The data illustrate a new role for IAPs in T-cell responses; OplAP enhances T-lymphocyte activation, proliferation and cytokine production probably by increasing nuclear NF- κ B levels. Increased *in vivo* T-cell activation is also demonstrated by an exacerbated inflammatory response in OplAP tg mice.

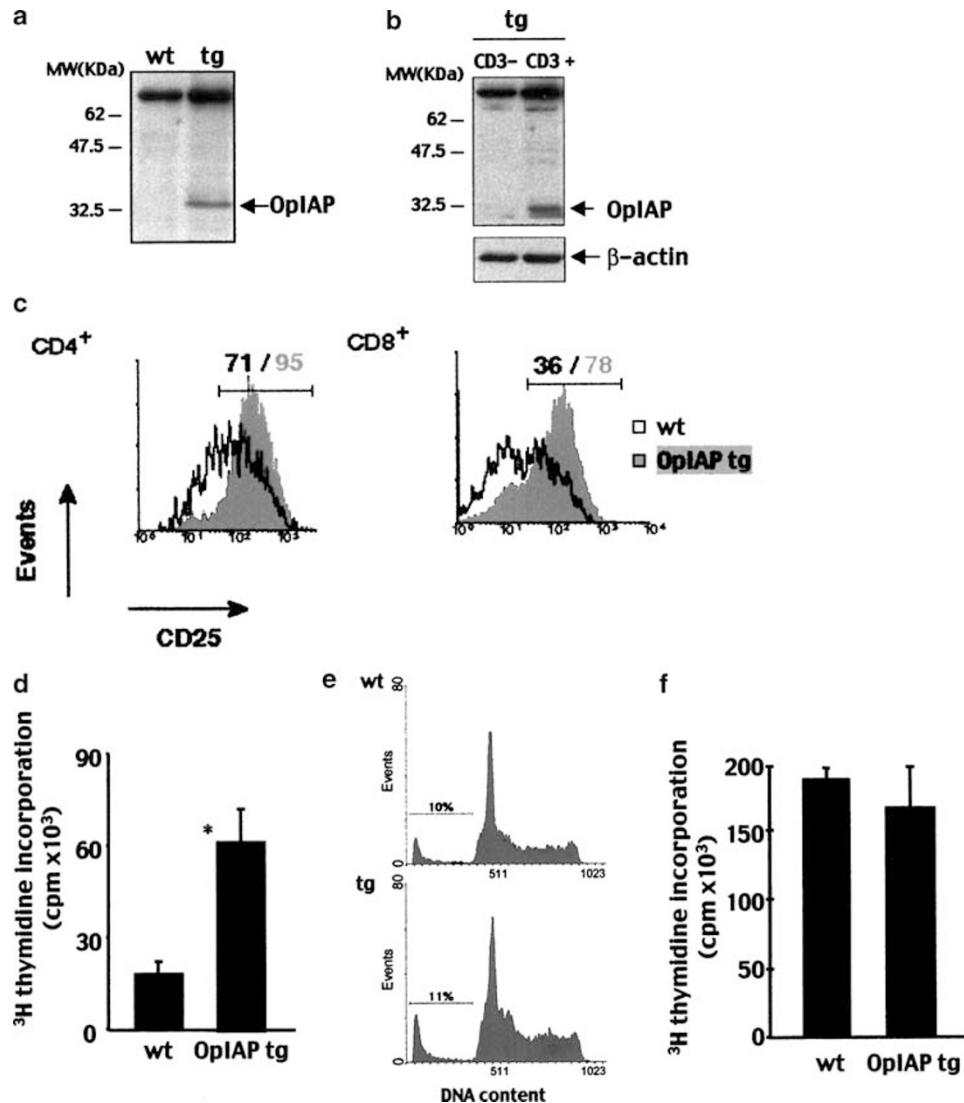


Figure 1 Increased CD3-mediated activation and proliferative responses of OplAP tg T lymphocytes. **(a)** Analysis of OplAP protein levels in lymph node extracts from OplAP tg and control wt mice, detected by Western blot using polyclonal anti-OplAP antiserum. **(b)** OplAP levels in CD3-negative and -positive cells purified by FACS from the lymph nodes of OplAP tg mice. **(c)** Percentage of CD25-positive cells in CD3-triggered mature T lymphocytes (gated on live CD8⁺ or CD4⁺) from wt (white histograms) and OplAP tg (gray histograms) mice 24 h after stimulation. Basal expression was undetectable in unstimulated cells ($\leq 5\%$) and is not shown. **(d)** T-lymphocyte proliferation measured as ³H thymidine incorporation after 48 h cell culture in the presence of plastic-bound anti-CD3 antibody (5 μ g/ml). The asterisk indicates statistical significance between bars ($P=0.0032$; Student's two-tailed *t*-test). **(e)** The graphs show the DNA content of CD3-positive cells after PI staining from wt (top) and OplAP tg (bottom) total lymph node cultures after 48 h anti-CD3 stimulation. Bars and the numbers indicate the percentage of dead cells (sub-G₀/G₁ DNA content) in the proliferation cell cultures shown in **d**. **(f)** Bars show ³H thymidine incorporation of wt and OplAP tg T-lymphocyte cultures 48 h after PMA plus ionomycin stimulation. The data in **(d, f)** represent the mean \pm S.D. of values for wt ($n=3$) and OplAP tg mice ($n=3$). No specific proliferation (≤ 500 cpm) was detected in unstimulated cultures

Results

OplAP expression in peripheral T lymphocytes enhances TCR-induced activation and proliferation

Previously we reported that OplAP expression in developing thymocytes enhances TCR-triggered proliferation and activation of these cells. To study whether OplAP also affects TCR-mediated responses in mature T cells, we used tg mice expressing OplAP under the control of the p56lck proximal promoter.⁸ Western blot analysis of cytosolic extracts

revealed the presence of OplAP in peripheral T cells (Figure 1a and b). OplAP tg mice showed no alteration in total cell number or T-lymphocyte repertoire in lymph nodes compared to their wild-type (wt) counterparts. Similarly, no differences were found in the total cell number or T-lymphocyte percentages in lymph nodes from OplAP/F5TCR double tg animals, in which the majority of T lymphocytes are CD8⁺ (Table 1). We studied the effect of OplAP expression on T-lymphocyte activation and proliferative responses following anti-CD3/TCR triggering. At 24 h after anti-CD3 stimulation, OplAP tg T lymphocytes expressed much higher surface levels of the CD25 activation marker compared to wt T cells

Table 1 Total cell numbers and percentages of CD8⁺ and CD4⁺ T lymphocytes in lymph nodes from wt and OplAP tg mice on a wt (left) and on a F5TCR (right) background

	Cell number ($\times 10^6$)	% CD8 ⁺	% CD4 ⁺	F5TCR		
				Cell number ($\times 10^6$)	% CD8 ⁺	% CD4 ⁺
Wt	8.3 \pm 3.6	35.3 \pm 9.4	30.4 \pm 6.3	7.9 \pm 2.8	68.3 \pm 5.2	6.1 \pm 1.1
OplAP tg	8.4 \pm 2.8	34.5 \pm 6.4	33.3 \pm 6.4	10.1 \pm 0.8	74.3 \pm 6.3	7.0 \pm 1.2

Numbers correspond to the average \pm S.D. for values of wt ($n=11$), OplAP ($n=12$), F5TCR ($n=3$) and F5TCR/OplAP ($n=4$) mice

(Figure 1c). Although anti-CD3-induced CD25 expression was higher in both CD4⁺ and CD8⁺ tg T-lymphocyte subpopulations, more notable differences were found between tg and wt mice in CD8⁺ T cells (Figure 1c). The percentage of viable cells was similar in cultures from wt and OplAP tg total lymph node cells 24 h after anti-CD3 stimulation (not shown).

Since activation precedes T-cell proliferation, we tested anti-CD3-induced proliferation in OplAP tg compared to wt T lymphocytes. At 48 h after anti-CD3 triggering, tg T lymphocytes expressing OplAP showed a three-fold greater proliferative response compared to wt T cells, as measured by ³H-thymidine incorporation (Figure 1d). Cell cycle analysis by PI staining showed that the percentage of cell death in the CD3-positive cell population (subG₀/G₁ DNA content) was similar in wt and OplAP cell cultures (Figure 1e). The increased proliferation exhibited by OplAP T-cell cultures after CD3 stimulation is thus probably due to a greater ability to proliferate rather than increased survival. To determine whether the OplAP effect on T-lymphocyte proliferation is specific to the TCR/CD3 signaling pathway, we analyzed T-lymphocyte proliferation induced by PMA plus ionomycin. Thymidine incorporation values at 48 h after treatment were similar in T-lymphocyte cultures from wt and OplAP tg mice (Figure 1f). These data indicate that OplAP increases anti-CD3-induced T-lymphocyte activation as well as proliferation specifically associated with TCR/CD3 triggering.

OplAP tg T lymphocytes have a lower proliferation threshold

We determined the proliferative response of wt and OplAP tg T lymphocytes to suboptimal concentrations of anti-CD3 antibody. OplAP tg T lymphocytes proliferated at an anti-CD3 dose (0.6 μ g/ml) that was insufficient to induce wt T-cell proliferation (Figure 2). At a higher anti-CD3 dose (1.25 μ g/ml), wt T cells showed marginal proliferation, whereas OplAP tg T-lymphocyte proliferation values were nearly 10-fold higher than those of wt T cells. No differences were found in anti-CD3-induced cell death between wt and OplAP tg T-lymphocyte cultures (not shown). OplAP tg T lymphocytes thus exhibit a lower proliferation threshold than wt T cells, as well as greatly increased proliferation at nonlimiting doses of anti-CD3 antibody.

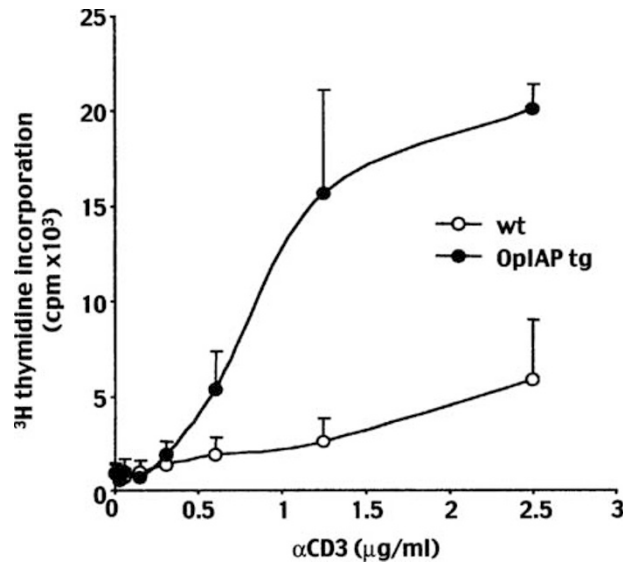


Figure 2 Reduced proliferation threshold of tg T lymphocytes. Proliferation values for wt (open symbols) and OplAP tg (closed symbols) T lymphocytes 48 h after stimulation with anti-CD3 antibody. Results are shown as mean and standard deviation (S.D.) of triplicate samples. The result is representative of three independent experiments

Increased cytokine production by OplAP tg T lymphocytes

To estimate the degree of T-cell activation, we analyzed cytokine production by wt and OplAP tg T lymphocytes following anti-CD3 stimulation. At 24 h poststimulation, four-fold higher IL-2 levels were detected in OplAP T-lymphocyte culture supernatants compared to those of wt cells (Figure 3a). This difference was reduced after 48 h, probably due to cytokine accumulation in the supernatants; IL-2 levels nonetheless remained higher in OplAP tg T-cell cultures (Figure 3a). IFN- γ , which is also produced after T-cell activation, was detected at significantly higher levels (nearly 100-fold) in OplAP tg T-lymphocyte supernatants than in wt T cell cultures at 24 h after anti-CD3 stimulation (Figure 3a). Similarly to IL-2, these differences were reduced after 48 h of culture, although IFN- γ levels in OplAP T-cell supernatants remained much higher than those observed in wt cultures (Figure 3a). At 48 h after CD3 triggering, TNF α , another cytokine involved in T-cell responses, was detected only slightly higher in tg T-cell culture supernatants than in those

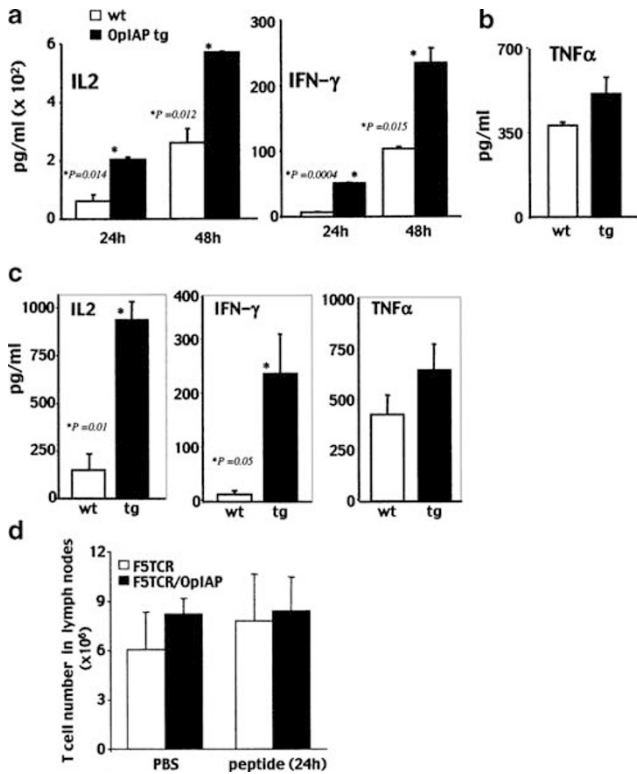


Figure 3 Enhanced *in vitro* and *in vivo* cytokine production by OplAP tg T lymphocytes after anti-CD3/TCR triggering. **(a)** IL-2 and IFN- γ levels in T-lymphocyte culture supernatants measured by ELISA at 24 and 48 h after incubation on anti-CD3-coated plates. **(b)** TNF- α supernatant levels in the same cultures measured 48 h after stimulation. The graphs represent cytokine concentration (mean \pm S.D.) for wt ($n=3$; open bars) and OplAP ($n=3$; closed bars) tg T-lymphocyte cultures. **(c)** Serum levels of IL-2, IFN- γ and TNF- α in F5TCR (wt; $n=7$; open bars) and F5TCR/OplAP tg mice (tg, $n=7$; closed bars), measured 90 min after antigenic peptide injection. Cytokine levels were below the detection limit (≤ 15 pg/ml) in unstimulated cultures and in untreated mice. **(d)** Number of viable T lymphocytes of F5TCR ($n=3$) and F5TCR/OplAP tg ($n=4$) mice injected with the antigenic peptide or with PBS. The asterisks indicate the P -value (Student's two-tailed t -test) for groups of data statistically significant between wt and OplAP tg mice

from wt T cells (Figure 3b). Since cell survival is not enhanced in OplAP cell cultures stimulated with anti-CD3 (see Figure 1f), the increased cytokine production in *ex vivo* anti-CD3-activated OplAP T lymphocytes appears to be due to enhanced T-cell activation and not to increased numbers of T cells producing cytokines.

In vivo cytokine production in response to T-cell activation was also tested in OplAP-expressing mice. We used the F5TCR tg mouse model, in which a tg TCR that recognizes a specific influenza virus nucleoprotein peptide is expressed in most T cells.¹⁸ Injection of the antigenic peptide into F5TCR mice leads to T-lymphocyte activation, as well as cytokine production that can be detected in the serum.¹⁸ On measuring *in vivo* cytokine production in F5TCR and F5TCR/OplAP tg mice 90 min after antigenic peptide injection, we detected marked differences in IL-2 and IFN- γ production. Levels of both cytokines were approximately 10-fold higher in F5TCR/OplAP tg mouse serum compared to F5TCR littermates (Figure 3c); TNF- α concentrations were slightly higher in F5TCR/OplAP serum compared to those of F5TCR mice (Figure 3c). At 24 h after antigen peptide injection, we

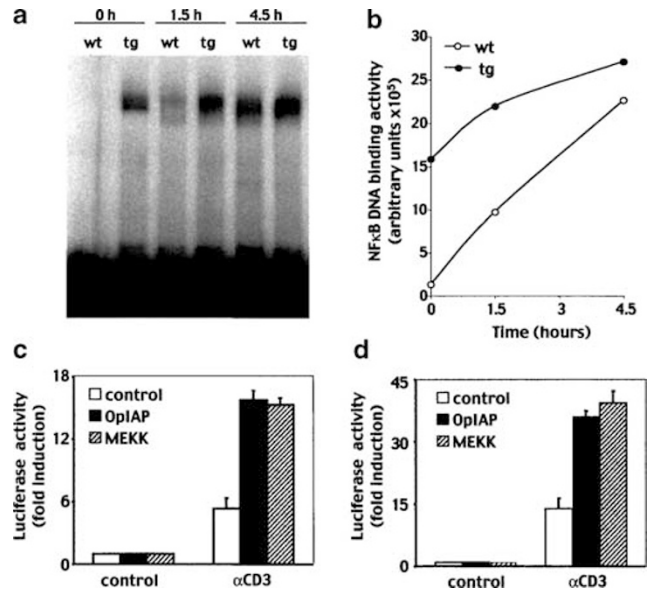


Figure 4 OplAP induces increased NF- κ B nuclear translocation and activity. **(a)** EMSA analysis using lymph node nuclear extracts from F5TCR (wt) and F5TCR/OplAP (tg) mice treated for different times with peptide antigen. **(b)** NF- κ B DNA-binding activity quantified in the lymph node nuclear extracts in panel (a) from F5TCR (wt, open symbols) and F5TCR/OplAP (closed symbols) mice. Activity is expressed as arbitrary units following phosphorimager quantification. The data show a representative result of three independent experiments. **(c)** Anti-CD3-induced NF- κ B activation in Jurkat cells transiently transfected with an empty vector (empty bars) or vectors encoding a MEKK active domain (hatched bars) or OplAP (filled bars). The bars represent the x -fold induction of NF- κ B-regulated firefly luciferase activity 14 h poststimulation, after normalization using a reporter with constitutive Renilla luciferase activity (TK-RL). **(d)** IL-2 promoter transcription activity in Jurkat cells transfected with an empty vector (empty bars) or vectors encoding a MEKK active motif (hatched bars) or OplAP (filled bars), after anti-CD3 stimulation. Transcriptional activation is represented as the x -fold induction of firefly luciferase activity normalized to Renilla luciferase activity. **(c, d)** Show results of one experiment, representative of three performed

detected similar T-cell numbers in lymph nodes of F5TCR/OplAP compared to F5TCR (Figure 3d); the increased cytokine production detected at early times is thus not associated with increased tg T-cell survival.

Taken together, these data indicate that OplAP enhances cytokine production in activated T lymphocytes following anti-CD3 stimulation in culture and, more importantly, after *in vivo* antigen-triggered activation in the F5TCR model.

OplAP tg T lymphocytes show higher nuclear NF- κ B levels

NF- κ B transcription factors play an essential role in TCR-mediated T-lymphocyte proliferation and activation.^{19,20} Previously, we showed that developing thymocytes from tg mice expressing OplAP under the Ick promoter have elevated nuclear NF- κ B levels.⁸ We examined NF- κ B levels in the nuclei of mature OplAP tg T lymphocytes, which may explain the increased activation and proliferation responses observed in tg T lymphocytes. We performed electrophoretic mobility shift assays (EMSA) using nuclear extracts of *in vivo* antigenic peptide-triggered lymphocytes from F5TCR and F5TCR/OplAP tg mice. We detected increased NF- κ B DNA binding activity in OplAP tg lymphocytes compared to F5TCR littermates (Figure 4a). This increase was time-dependent, peaking at 4.5 h after antigenic peptide injection (Figure 4b). To further confirm that the increased NF- κ B activity was due to OplAP expression, we performed luciferase reporter assays in Jurkat cells. Jurkat cells transfected with OplAP or MEKK active domain showed increased luciferase activity following anti-CD3 stimulation (Figure 4c). Similarly, Jurkat cells transfected with OplAP or MEKK active domain showed increased IL-2 promoter transcription activity following anti-CD3 stimulation (Figure 4d).

OplAP mice. Unstimulated F5TCR/OplAP lymphocytes had high nuclear NF- κ B levels, compared to near absence in the nuclei of wt lymphocytes (Figure 4a, b). Antigenic peptide injection induced an increase in nuclear NF- κ B levels in both wt and tg lymphocytes, with a greater increase in wt cells. At 4.5 h after *in vivo* peptide challenge, nuclear NF- κ B levels remained higher in tg compared to wt lymphocytes (Figure 4a, b). Taken together, the data indicate that OplAP expression is associated with high NF- κ B DNA-binding activity in resting lymphocytes, which increases after *in vivo* antigenic peptide challenge.

OplAP directly induces NF- κ B activation in TCR/CD3-triggered T cells

To further test the effect of OplAP expression on TCR-induced NF- κ B activation, we examined NF- κ B reporter activation in the JHM12 Jurkat T-cell line. These cells were cotransfected with a vector containing OplAP and another carrying the luciferase gene driven by a promoter element downstream of NF- κ B-binding site enhancer elements. As reported,²¹ CD3 triggering induced slight NF- κ B activation, which was higher in cells cotransfected with a constitutively active MEKK kinase domain (positive control). MEKK acts as a mediator in the costimulatory pathway in T cells, leading to optimal NF- κ B activation.²² OplAP-transfected JHM12 cells showed NF- κ B reporter activation levels similar to those observed in MEKK-expressing cells (Figure 4c). We also

tested NF- κ B transcriptional activity by examining luciferase expression driven by a hIL-2 promoter containing NF- κ B-binding sites. Similar to the data obtained with NF- κ B-binding site enhancers, the IL-2 promoter-regulated luciferase expression induced after CD3 triggering was higher in OplAP-transfected JHM12 cells than in control (empty vector-transfected) cells (Figure 4d). Comparable elevated luciferase activity was observed in cells transfected with OplAP or with MEKK. In contrast, expression of OplAP or MEKK alone in JHM12 cells did not induce NF- κ B activation, which appeared to require CD3/TCR triggering. These results would explain the phenotype of TCR-stimulated OplAP tg T lymphocytes, since increased proliferation, CD25 activation marker expression and cytokine production may be directly related to elevated NF- κ B activity.

Increased contact hypersensitivity (CHS) response in OplAP tg mice

To assess the *in vivo* effect of the elevated T-lymphocyte activation and proliferative response, we analyzed the CHS reaction in mice. CHS is a hapten (2,4-dinitrofluorobenzene (DNFB))-induced T-cell response that results in allergic contact dermatitis and inflammation. Epicutaneous application of hapten leads to an oligoclonal T-cell response mediated mainly by CD8⁺ cells, whereas CD4⁺ cells have downregulatory effects at later phases.²³ We tested whether OplAP expression in T lymphocytes affects the CHS reaction to DNFB in tg animals. Mice were first sensitized, and the inflammatory response to hapten challenge was then evaluated by measuring ear swelling. At 48 h postchallenge, OplAP tg mice showed a clear increase in DNFB-mediated allergic response, as approximately two-fold higher ear swelling values were observed in OplAP tg mice compared to wt littermates (Figure 5a).

To confirm that the increased CHS response was due to the enhanced T-cell response in the tg mice, we analyzed the antigen-specific proliferative response of lymphocytes from DNFB-sensitized mice. Lymph node cells were cultured alone or in the presence of 2,4-dinitrobenzene sulfonic acid (DNBS), a water-soluble form of the hapten; after 48 h, cell proliferation was determined by thymidine incorporation. This analysis showed a clear increase in the proliferative response of OplAP tg compared to that of wt cells (Figure 5b). Lymphocytes from nonsensitized wt and OplAP tg mice did not proliferate in response to DNBS stimulation (not shown). The antigen-independent proliferative response to PMA and ionomycin showed no differences between wt and OplAP tg lymphocytes from DNFB-sensitized mice (not shown). Ear lesions were characterized histopathologically, confirming that the elevated inflammation in OplAP tg animals was associated with greater alteration in dermal tissue (Figure 5c). These data indicate that the increased inflammatory response in OplAP tg mice to CHS is due to the elevated antigen-specific T-lymphocyte response.

Taken together, the results indicate that OplAP enhances tg T-lymphocyte proliferation and activation, reflected as an increased inflammatory response in the CHS model.

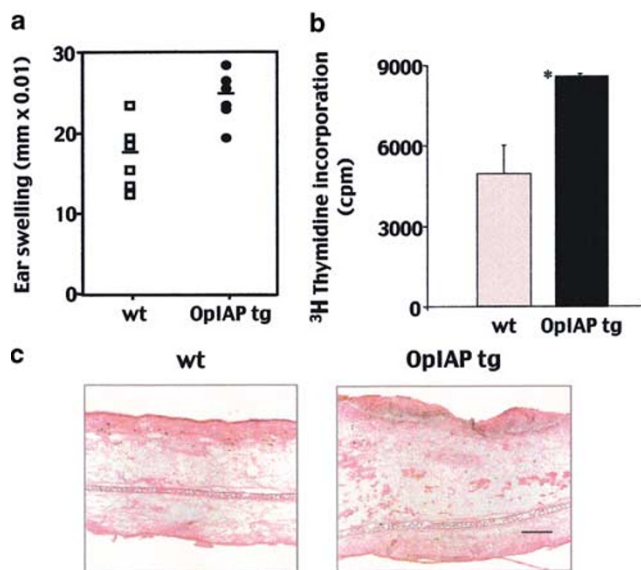


Figure 5 Elevated response of OplAP tg mice in CHS inflammation. **(a)** Ear swelling values of wt ($n=10$, open squares) and OplAP tg mice ($n=10$, closed circles), 48 h after DNFB challenge of ears ($P=3.5 \times 10^{-5}$, Student's two-tailed *t*-test). **(b)** ³H thymidine incorporation of wt ($n=3$, open symbols) and OplAP tg lymphocytes ($n=3$, closed symbols), 48 h after *in vitro* DNBS stimulation using 300×10^3 cells per well. The asterisk indicates statistical significance between bars ($P=0.0035$, Student's two-tailed *t*-test). Lymphocytes were obtained from mice 5 days after abdominal DNFB sensitization. **(d)** Hematoxylin/eosin-stained cryosections of haptens-challenged ears from wt (left panel) and OplAP tg (right panel) mice at 24 h postchallenge (magnification, $\times 10$)

Discussion

We analyzed how the expression of an IAP, such as OplAP, affects T-lymphocyte function and in turn, the T-cell-mediated immune response. Although IAPs are well known for their antiapoptotic function, OplAP expression in T lymphocytes appears not to affect cell survival during development, since OplAP tg mice have normal cell numbers and T-lymphocyte repertoire in lymph nodes. In addition, survival of *in vitro*- and *in vivo*-activated T lymphocytes does not appear to be altered in OplAP tg mice. The expression of OplAP in nonstimulated tg T lymphocytes leads to a marked increase in NF- κ B nuclear levels, similar to that reported for developing OplAP tg thymocytes.⁸ Moreover, nuclear NF- κ B levels in resting OplAP tg lymphocytes were similar to those of wt lymphocytes after *in vivo* challenge with an antigenic peptide. NF- κ B DNA-binding activity nevertheless increased further in tg T lymphocytes compared to wt after *in vivo* TCR triggering; these data concur with the OplAP-mediated increase in NF- κ B activity in Jurkat TCR-triggered cells. Although OplAP expression did not induce NF- κ B activation in transfected T cells in the absence of TCR triggering, unstimulated T lymphocytes expressing OplAP showed elevated nuclear NF- κ B levels, which were nonetheless insufficient to promote proliferation or activation. This suggests that OplAP-mediated NF- κ B nuclear translocation sensitizes T lymphocytes for greater activation and proliferation in response to TCR/CD3 stimulation. The requirement for TCR/CD3 signals to induce activation and proliferation in OplAP T lymphocytes suggests that additional NF- κ B activation signals are required, and/or that activation of other transcription factors is necessary to integrate the TCR/CD3 signaling pathways in the T lymphocyte.

OplAP expression in T lymphocytes thus elevates nuclear NF- κ B levels, which appear to reinforce the TCR signals, increasing T-cell activation and proliferative response. This idea is supported by data obtained in tg mice expressing a mutant I κ B α form that cannot be degraded, leading to defective NF- κ B activation. In these mice, blockade of NF- κ B activation is associated with a defect in CD3-mediated T-lymphocyte activation and proliferation.^{24,25} An increase in NF- κ B DNA-binding activity could lead to an opposite phenotype, as we found in OplAP tg mice. The elevated expression of the CD25 activation marker observed in TCR-activated tg lymphocytes can be explained by NF- κ B transcriptional regulation of the CD25 gene.¹⁵ In addition, the *in vitro* and *in vivo* cytokine overproduction described in OplAP tg T lymphocytes may be due to increased NF- κ B DNA-binding activity. This may lead to upregulation of IL-2 and IFN- γ genes, which are also targets of this transcription factor.¹⁵

The mechanism by which OplAP affects nuclear translocation of NF- κ B is unknown, although by inducing ubiquitination, OplAP may induce I κ B α degradation by the proteasome similar to that described for XIAP in human endothelial cells.⁷ Since the IAP RING motif contains ubiquitin ligase activity in other IAPs,^{3,26,27} we suggest that the OplAP RING motif has similar activity. OplAP could act *in vivo* by regulating NF- κ B activity through inducing I κ B α degradation by ubiquitination. Nonetheless, the fact that I κ B α ubiquitination depends on and

requires prior phosphorylation²⁸ renders this hypothesis unlikely. We therefore speculate on a more probable, ubiquitination-related OplAP-dependent mechanism that would implicate the IKK kinase complex, which is responsible for I κ B α phosphorylation. A recent study reported the regulation of IKK activity by a kinase that is activated by ubiquitination,²⁹ demonstrating a mechanism that induces ubiquitination-mediated kinase activation rather than degradation. OplAP-mediated ubiquitination may thus induce kinase activation upstream of IKK, which would in turn activate this complex, leading finally to NF- κ B nuclear translocation. A recent report showed that cultured T cells expressing the African swine fever virus IAP homologue induced NF- κ B activation, which was associated to elevated IKK activity.⁹ Further studies are required to determine the exact mechanism by which OplAP induces NF- κ B translocation to the nucleus.

As a result of the *in vivo* T-lymphocyte activation and proliferation enhancement, OplAP tg mice developed a severe T-cell-mediated CHS inflammation in which cytokines are crucial for cell activation and migration.²³ IFN- γ and TNF α , which were elevated in OplAP tg mice after *in vivo* T-cell activation, are implicated in eliciting the inflammatory response in CHS.^{23,30} Increased cytokine production by OplAP tg T lymphocytes associated with the increased hapten-induced proliferative response could thus explain the exacerbated CHS reaction, especially in the inflammatory phase. The elevated nuclear NF- κ B levels observed in OplAP tg T lymphocytes may explain the increased inflammation observed in CHS, as NF- κ B is a key regulator of cellular inflammatory responses.¹³

Our findings indicate that the IAP family member OplAP affects T-lymphocyte function *in vivo*. The results constitute the first evidence of IAP involvement in T-cell activation and proliferation associated to increased NF- κ B nuclear presence. This study consequently indicates the importance of IAPs in the immune system, not only in suppressing apoptosis, but also for regulating other cellular processes such as mature T-lymphocyte activation and proliferation. It would be of interest to determine whether the susceptibility to activate, proliferate and produce cytokines by OplAP tg T lymphocytes leads to the development of autoimmune disease or lymphoproliferative alterations in tg mice.

Materials and Methods

Mice

Tg mice expressing OplAP were generated in our laboratory⁸ and were extensively backcrossed to C57BL/6 mice. OplAP cDNA was subcloned into the *Bam*HI site of the p56^{lck} human growth hormone (hGH) vector. A 6.2 kb *Not*I fragment containing the *lck* proximal promoter OplAP cDNA and an hGH sequence were prepared by gel purification and diluted in injection buffer (10 mM Tris pH 7.5, 0.12 mM EDTA). Tg mice were generated by pronuclear microinjection of fertilized oocytes from (C57BL/6 \times CBA) F₁ mice; microinjected eggs were transferred at the two-cell stage to recipient pseudopregnant OF1 females. Offspring were analyzed by PCR and transgenesis confirmed by Southern blot analysis. For *in vivo* T-cell activation, we used F5TCR mice (kindly provided by Dr D Kioussis) crossed with OplAP tg mice to obtain double tg F5TCR/OplAP tg mice.

Flow cytometry analysis and cell sorting

Single-cell suspensions were prepared from control and tg mice by mincing lymph nodes through a 150 μ m nylon mesh; viable cells were counted by trypan blue exclusion. Lymphocytes were cultured (24 h, 37°C) on plates precoated with anti-CD3 ϵ (2C11) antibody (Pharmingen, San Diego, CA, USA) at 5 μ g/ml, and then stained with saturating antibody concentrations at 4°C for flow cytometry analysis. Directly conjugated mAb were used against CD4-(PE), CD8-(tricolor) and CD25-(FITC) (Pharmingen and Southern Biotechnologies, Birmingham, AL, USA). Flow cytometry and multi-parameter analysis were performed on a Coulter Epics XL cytometer (Coulter, Miami, FL, USA). Dead cells were excluded by gating on forward- and side-light scatter. Anti-CD3-stimulated lymph node cell culture samples were stained with PI (Coulter DNA-Prep) and cells with apparent DNA content $<2n$ as determined by flow cytometry analysis were considered apoptotic. Cell suspensions from tg lymph nodes were stained with anti-CD3-(FITC) mAb and sorted by FACS (Coulter Epics Altra).

Western blot analysis

Cytosolic extracts of cell suspensions from total lymph node and CD3⁺-sorted T lymphocytes were prepared by incubation (30 min, 4°C) in extraction buffer (50 mM Tris-HCl pH 7.6, 150 mM NaCl, 0.5 mM EDTA, 10 mM NaH₂PO₄, 1% Nonidet-P40, 0.4 mM Na₃VO₄, 1 mM PMSF, 10 μ g/ml aprotinin and 10 μ g/ml leupeptin). Cell lysates were centrifuged to deplete nuclei. Cytosolic extracts (30 μ g/lane) were resolved in 10% SDS-PAGE and transferred to nitrocellulose membranes (Bio-Rad, Hercules, CA, USA). Western blot was performed using rabbit anti-OplAP serum and developed with horseradish peroxidase (HRP)-conjugated anti-rabbit IgG antibody (Dako, Glostrup, Denmark; 1 : 1000 dilution). Anti-mouse β -actin Ab (Sigma, St. Louis, MO, USA), developed with HRP-conjugated anti-mouse IgG antibody (Dako), was used to confirm protein loading.

Proliferation assays

Lymphocytes were cultured in complete medium (RPMI 1640 with 10% fetal calf serum, 10 μ M mercaptoethanol, 100 mM L-glutamine, 10 mM HEPES and antibiotics), then plated (4×10^5 cells/well) in 96-well flat-bottom microtiter plates precoated overnight with PBS or anti-CD3 ϵ antibody at 5 μ g/ml or at suboptimal doses (Figure 2). Antigen-induced lymphocyte proliferation was triggered using DNBS (50 μ g/ml; Sigma) in cultures of lymphocytes from wt and OplAP tg mice, 5 days after DNFB sensitization. Stimulation was also induced in the presence of PMA (10 ng/ml) plus ionomycin (1 μ g/ml). Triplicate samples were cultured (48 h, 37°C) for all assays. ³[H]thymidine (1 μ Ci/well) was added, cells harvested 8 h later and radioisotope incorporation into DNA determined.

ELISA assays

In vitro IL-2, IFN- γ and TNF α production were measured in ELISA in culture supernatants from T lymphocytes, unstimulated or stimulated with anti-CD3 (1 μ g/ml), on plates precoated with anti-mouse antibodies (IL-2 and IFN- γ , Endogen, Boston, MA, USA; TNF α , R&D Systems, Minneapolis, MN, USA). Quantification was by comparison to standards supplied. *In vivo* cytokine production was measured from mouse serum 90 min after antigenic peptide injection. ELISA was performed as described above.

Nuclear protein extraction and EMSA

To obtain nuclear extracts, T lymphocytes from control (PBS) and F5 antigen peptide-injected mice were incubated (10 min, 4°C) in buffer A

(10 mM HEPES pH 7.6, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1% NP40) before centrifugation to pellet nuclei. Nuclei were lysed by incubation (30 min, 4°C, with agitation) in buffer B (20 mM HEPES pH 7.6, 20% glycerine, 420 mM NaCl, 1.5 mM MgCl₂, 0.2 mM EDTA). After centrifugation, supernatants containing nuclear proteins were separated and frozen at -70°C. EMSA was performed using a ³²P-labeled, double-stranded oligonucleotide bearing the human IL-2 promoter κ B site (5'-GATCGGGATTTCACCT-3'). DNA-binding reactions (12.5 μ l) containing 5 μ g nuclear extracts, 2 μ g poly (dl-dC) and 2.5 mM EDTA, 2.5 mM DTT, 2.5% glycerol in 50 mM Tris (pH 8) were incubated (10 min, 4°C); 10⁵ cpm of radiolabeled oligonucleotide probe were added and incubated (30 min, 4°C). The resulting nucleoprotein complexes were resolved by electrophoresis in native 4% polyacrylamide gels (in 0.5 \times TBE; 120 V, 2.5 h), then visualized and quantified by phosphoimaging (Molecular Dynamics, Sunnyvale, CA, USA). The background was subtracted using the local median algorithm of the ImageQuant program (v.1.2. Molecular Dynamics).

Luciferase assay

JHM12 cells were transiently cotransfected with the *Renilla* luciferase vector pRL-TK, an inducible vector containing the luciferase reporter gene, and (i) pEF-Bos (pEF) vector (control), (ii) pEF bearing the OplAP sequence, or (iii) the pFC active MEKK1 domain (positive control). The reporter gene was under the control of a basic promoter with NF- κ B-binding sites bearing enhancer elements, or of the hIL-2 promoter, which contains NF- κ B-binding domains (PathDetect NF- κ B *cis*-Reporting System; Stratagene, La Jolla, CA, USA). At 48 h post-transfection, cells were cultured for 14 h in anti-CD3-coated plates and a luciferase assay was performed. Luciferase inducible activity was normalized using the *Renilla* luciferase constitutive activity in transfected cells.

CHS assay and immunohistochemistry

Mice were sensitized by topical application of 50 μ l DNFB (0.25%; Sigma) in acetone/olive oil (4 : 1) to the shaved abdomen. After 5 days, 10 μ l of 0.2% DNFB in the same solution was applied to both sides of the right ear and solvent alone (control) to the left ear. Ear thickness was measured with a micrometer (Mitutoyo Corp., Kawasaki, Japan) before challenge and each day after challenge. Ear swelling was determined by subtracting pre- from postchallenge values and any swelling detected in the challenged control ear. For histopathological analyses, treated ears were frozen to prepare ear sections (10 μ m), which were then hematoxylin/eosin stained and examined by light microscopy.

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