

# Thymoquinone, the active ingredient of *Nigella sativa* seeds, enhances survival and activity of antigen-specific CD8-positive T cells *in vitro*

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## Introduction

Adoptive cell transfer (ACT) represents an important advance in cancer immunotherapy. The success of ACT depends on the functional quality of the cells transferred.<sup>1</sup> Adoptive transfer of autologous tumour-reactive T cells in the clinical setting involves harvesting T cells from the patient's peripheral blood, draining lymph nodes or the tumour bed (tumour infiltrating lymphocytes [TIL]), followed by cell expansion *in vitro* using polyclonal stimulation with anti-CD3 and anti-CD28 monoclonal antibodies (mAbs) in the presence of interleukin-2 (IL-2) as a growth factor. The cells are then re-infused to the patient together with the administration of IL-2 to improve cell engraftment and survival.<sup>2</sup>

Most early clinical studies of adoptive T-cell therapy are based on the use of T cells expanded *in vitro* for several cycles before adoptive transfer.<sup>3-7</sup> Although the use of these cells for adoptive transfer can mediate melanoma regression with an objective response rate of about 34%, the response rates in these studies were no better than that obtained using cytokines or cancer vaccines.<sup>8</sup> This low rate might be explained by the finding of recent studies demonstrating that the adoptively transferred T cells that had expanded *in vitro* for several cycles of expansion (late effectors) are considered terminally differentiated (exhausted) and show poor survival, trafficking and persistence, and anti-tumour responses *in vivo*. In contrast, antigen stimulation of T cells *in vitro* for a short period (early effectors) can generate early effector cells with greater anti-tumour responses *in vivo*.<sup>9,10</sup>

The greater anti-tumour responses of the early effectors have been attributed to higher expression of the CD62L homing molecule.<sup>2,11</sup> This facilitates T-cell trafficking to lymph nodes and crosstalk with antigen-presenting cells,<sup>12,13</sup> resulting in the differentiation of T cells into effector cytolytic cells capable of combating cancer. Importantly, the type of cytokine added to T cells during antigen stimulation

## ABSTRACT

Recent preclinical and clinical studies provide evidence that adoptive transfer of *in vitro* activated T cells can result in significant antitumour responses *in vivo* upon acquisition of certain survival and homing properties during *in vitro* activation. Based on recent studies showing *in vivo* antioxidant effects of thymoquinone (TQ), the active ingredient of *Nigella sativa* seeds, this study aims to determine whether or not TQ can increase survival and sustain the expression of the homing receptor CD62L in antigen-specific T cells *in vitro*. The results showed that stimulation of OT-1 (transgenic CD8<sup>+</sup>) T cells with OVA antigen resulted in activation, as shown by a decrease in the surface expression of CD62L which coincided with significant apoptosis measured three and five days after antigen stimulation. Addition of low concentrations of TQ during CD8<sup>+</sup> T-cell activation resulted in enhanced survival of the activated T cells and sustained expression of CD62L. These effects coincided with enhancement in the capability of CD8<sup>+</sup> T cells to produce the effector cytokine interferon- $\gamma$  (IFN $\gamma$ ). These results suggest that TQ has a beneficial effect in conditioning T cells *in vitro* for adoptive T-cell therapy against cancer and infectious disease.

KEY WORDS: Antigen, CD62L. Antigen, CD8. Apoptosis. Cytokines. *Nigella sativa*. Thymoquinone.

*in vitro* can shape the early versus late effector phenotype. For example, it has been demonstrated that IL-12<sup>14</sup> and IL-15 favour generation of T cells with greater CD62L expression compared to those stimulated in the presence of IL-2.<sup>11</sup> Therefore, approaches aimed at the generation of early effector populations with their favourable phenotype would improve the survival of antigen-stimulated T cells and their application in adoptive T-cell therapy.

It has been reported that the beneficial effects of the oil of the *Nigella sativa* seed and its active ingredient, thymoquinone (TQ), reduce the toxicity induced by the anti-cancer drug cyclophosphamide through enhancement of antioxidant mediator levels.<sup>15</sup> It has also been shown that *N. sativa* oil can induce potent antiviral effects associated with enhanced T-cell responses.<sup>16</sup> Other studies have also shown that the antitoxic effects of TQ in different disease models are associated with inhibition of 5-lipoxygenase products during inflammation,<sup>17</sup> and with enhanced radical scavenging (antioxidative) activity.<sup>18</sup>

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Production of high levels of oxygen radicals during T-cell activation can induce programmed death (apoptosis) in activated T cells. In view of the antioxidant effects of TQ, this study aims to determine whether or not conditioning of T cells with TQ during antigen stimulation *in vitro* can induce these cells to acquire a favourable phenotype and also aid survival.

## Materials and methods

### Mice

OT-1 T-cell receptor (TCR) transgenic mice on a C57Bl/6 (B6) background were purchased from Jackson Laboratory (Bar Harbor, ME, USA). CD8<sup>+</sup> T cells (OT-1) from these mice can recognise the major histocompatibility (MHC) class I peptide from ova albumin (OVAp). All animals were housed under specific pathogen-free conditions in accordance with institutional and federal guidelines at the Medical University of South Carolina, USA.

### Antibodies and reagents

Anti-CD16/CD32 and fluorescein isothiocyanate (FITC)-, phycoerythrin (PE)-, allophycocyanin (APC)- and cychrome-conjugated mAbs were purchased from Pharmingen (San Diego, CA, USA). The MHC class I SIINFEKL OVAp (American Peptide Company, Sunnyvale, CA, USA) was dissolved in 10% dimethyl sulphoxide (DMSO; Sigma, St Louis, MO, USA) and diluted in phosphate-buffered saline (PBS). Thymoquinone (2-isopropyl-5-methyl-1,4-benzoquinone; Sigma-Aldrich) was initially dissolved in DMSO and then diluted in PBS as required.

### Antigen-induced T-cell activation

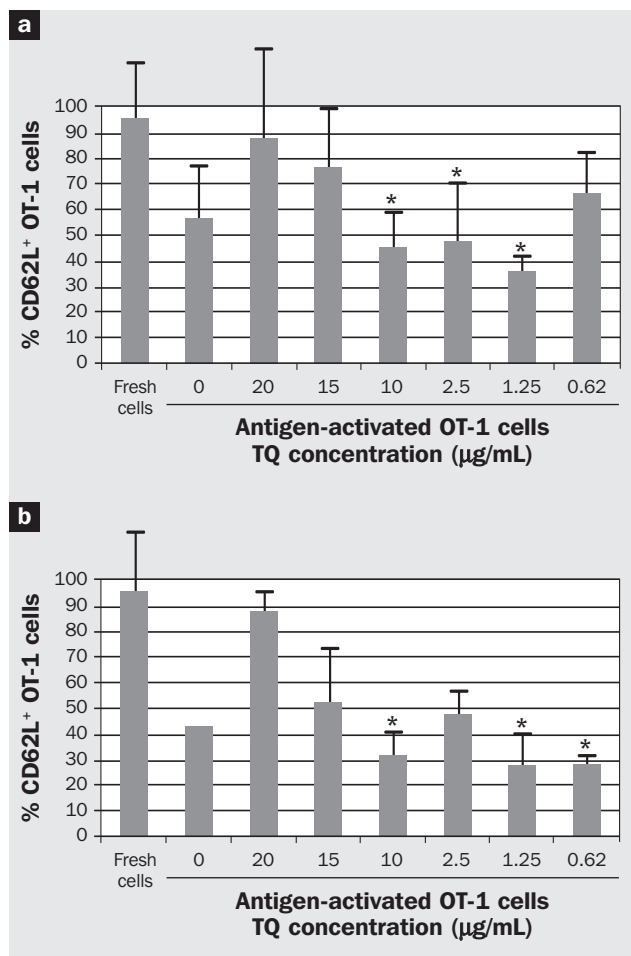
OT-1 cells were prepared and cultured, as previously described.<sup>19</sup> In brief, one or two OT-1 mice were sacrificed and spleen and peripheral (cervical, auxiliary, brachial and inguinal) and mesenteric lymph nodes were harvested and pooled. A single cell suspension was prepared in PBS, washed (x2) and resuspended in RPMI medium. Unfractionated OT-1 cells ( $1 \times 10^5$ ) were cultured in triplicate in six-well plates for three days in RPMI medium containing 1 µg/mL MHC class-I OVAp and TQ at the dose indicated in each experiment. Cells were harvested and washed (x2) and were freshly processed for activation phenotype, and apoptosis was assessed by flow cytometry. Supernatant was collected 24 h later and interferon- $\gamma$  (IFN $\gamma$ ) and tumour necrosis factor- $\alpha$  (TNF $\alpha$ ) levels were measured by a cytometric bead array (Pharmingen).

### Assessment of CD62L expression

Fresh single-cell suspensions were prepared and  $1 \times 10^6$  cells were treated with anti-CD16/CD32 for 5 min on ice. Cells were then stained with an anti-CD62L mAb for 30 min on ice. The cells were washed (x2) and resuspended in 0.3 mL 0.5% bovine serum albumin (BSA) and 0.02% sodium azide solution. Cells were analysed by flow cytometry using the Cell Quest software package (Becton Dickinson, San Jose, CA, USA), as previously described.<sup>20</sup>

### Assessment of apoptosis

Cells were prepared and stained as above, washed (x2) with annexin-V binding buffer, and then 5 µL annexin-V was



**Fig. 1.** Optimal concentration of TQ on the activation of antigen-specific CD8<sup>+</sup> T cells. OT-1 (CD8<sup>+</sup>) T cells were harvested from spleen and LN of naïve TCR transgenic mice and  $1 \times 10^6$  cells were cultured for three (a) and five (b) days in RPMI medium and stimulated with MHC class I peptide (SIINFEKL) at different concentrations of TQ. Cells were harvested to assess the cell activation by analysing CD62L expression by flow cytometry.

added and the cells were incubated at room temperature in the dark for 15 min. Cells were then washed (x2) with annexin-V binding buffer, and resuspended in the same buffer prior to flow cytometry using the Cell Quest software package (Becton Dickinson), as previously described.<sup>14</sup>

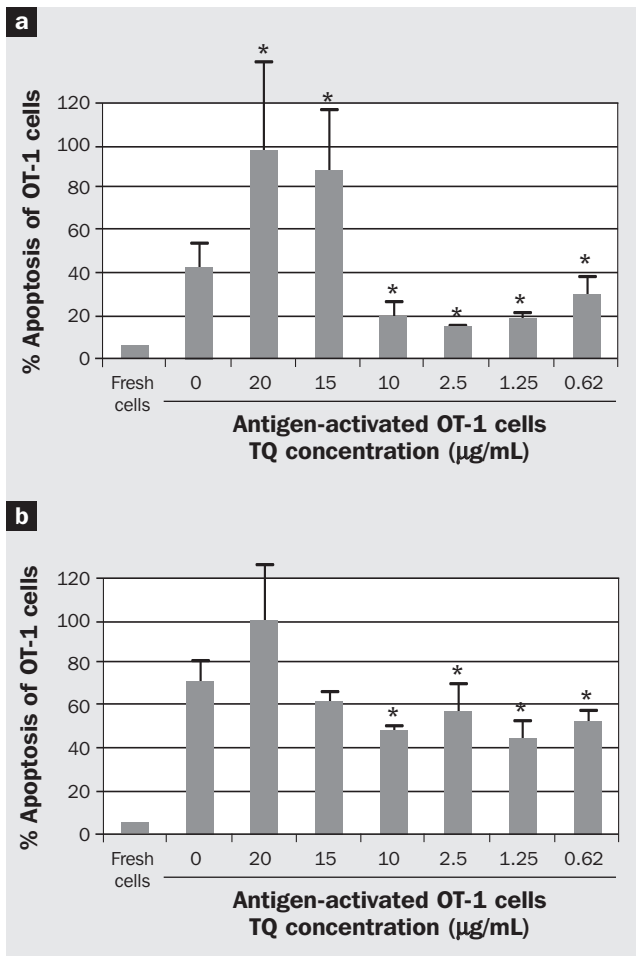
### Assessment of intracellular expression of IFN $\gamma$

Cells stained for surface markers as above were fixed, permeabilised and then stained with an anti-IFN $\gamma$  mAb to detect intracellular levels of this cytokine. Cells were analysed by flow cytometry using the Cell Quest software package (Becton Dickinson). All analyses were performed on gated Ly5.1<sup>+</sup>, CD8<sup>+</sup> T cells, which represent antigen-specific OT-1 (CD8<sup>+</sup>) T cells.<sup>19</sup>

## Results

### Effect of TQ on activation

Fresh, unfractionated splenocytes ( $1 \times 10^6$ ) containing CD8<sup>+</sup> OT-1 T cells were stimulated with 1 µg/mL peptide for three days in RPMI medium in the presence or absence of



**Fig. 2.** Optimal concentration of TQ and its effect on apoptosis of antigen-specific CD8<sup>+</sup> T cells. Cells were harvested to assess the cell apoptosis by analysing the expression of annexin-V by flow cytometry. See legend to Figure 1 for further details.

different concentrations of TQ. CD8<sup>+</sup> T cells were harvested on day 3 and day 5 after stimulation *in vitro* with OVA<sub>p</sub> to assess cell activation and apoptosis. Day 3 analysis showed that 57% antigen-stimulated CD8<sup>+</sup> T cells exhibited CD62L<sup>+</sup> expression, compared to 95% unstimulated CD8<sup>+</sup> T cells. Addition of 20, 15 or 0.62 µg/mL TQ to CD8<sup>+</sup> T cells during antigen stimulation increased the numbers of CD62L<sup>+</sup>-expressing cells from 57% to 87, 76 and 66%, respectively. However, addition of 10, 2.5 or 1.25 µg/mL TQ during stimulation of CD8<sup>+</sup> T cells stimulated with antigen decreased CD62L<sup>+</sup> expression from 57% to 45, 47 and 35%, respectively (Fig. 1a). Overall expression of CD62L on CD8<sup>+</sup> T cells on day 5 was much lower than on day 3. The numbers of CD62L<sup>+</sup>-expressing CD8<sup>+</sup> T cells stimulated with antigen alone for five days was 42%. Addition of 20 µg/mL and 15 µg/mL during the five-day culture increased CD62L expression from 42% to 87% and 52%, respectively, while addition of 10, 2.5, 1.25 or 0.62 µg/mL TQ changed the level of CD62L expression in the antigen-stimulated CD8<sup>+</sup> T cells from 42% to 30%, 48%, 27% and 27%, respectively (Fig. 1b).

#### Effect of TQ on apoptosis

Measuring apoptosis on day 3 (Fig. 2a) showed that stimulation of CD8<sup>+</sup> T cells with antigen alone induced 43% of the cells to undergo apoptosis, compared to 5% of the

unstimulated cells. Treatment of CD8<sup>+</sup> T cells with higher concentrations of TQ (20 and 15 µg/mL) increased the number of apoptotic cells to 97% and 88%, respectively. Interestingly, addition of lower concentrations of TQ (10, 2.5, 1.25 or 0.62 µg/mL) induced 19%, 14%, 18% and 29%, respectively, of the cells to undergo apoptosis. On day 5 (Fig. 2b), stimulation of the cultured CD8<sup>+</sup> T cells with antigen alone resulted in rapid apoptosis of the CD8<sup>+</sup> T cells (72%), compared to unstimulated CD8<sup>+</sup> T cells (5%). Treatment of CD8<sup>+</sup> T cells with 20 µg/mL TQ increased the number of apoptotic cells by approximately 100%. Lower concentrations of TQ (15, 10, 2.5, 1.25 and 0.62 µg/mL) resulted in less apoptosis, compared to the antigen-stimulated CD8<sup>+</sup> T cells (62%, 49%, 58%, 45% and 53%, respectively).

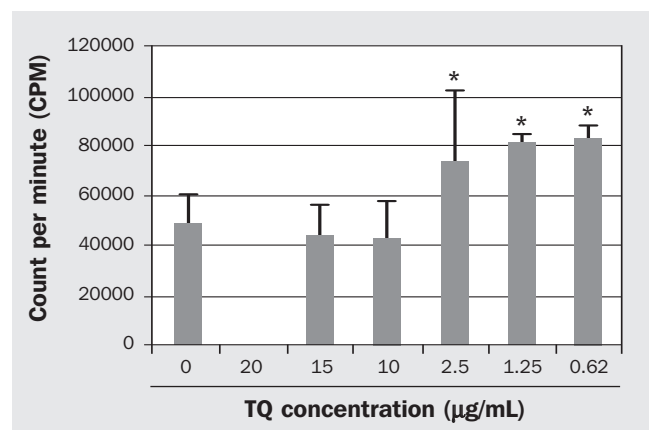
#### Effect of TQ on *in vitro* proliferation

CD8<sup>+</sup> T cells ( $1 \times 10^6$ ) were cultured for three days in RPMI medium and stimulated with OVA<sub>p</sub> in the presence or absence of different concentrations of TQ, and cell proliferation was assessed. Addition of 20 µg/mL TQ completely suppressed the proliferative activity of antigen-stimulated CD8<sup>+</sup> T cells. Addition of 15 or 0.62 µg/mL TQ did not significantly alter the proliferation of CD8<sup>+</sup> T cells. Addition on 10, 2.5 or 1.25 µg/mL TQ markedly increased the proliferative response of CD8<sup>+</sup> T cells (Fig. 3).

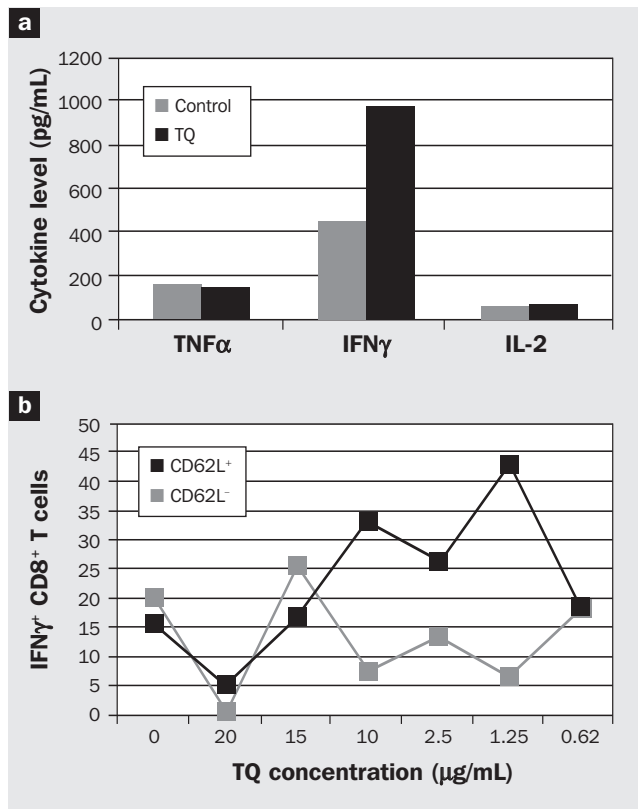
#### Effect of TQ on cytokine production

Fresh, unfractionated splenocytes from OT-1 transgenic mice ( $1 \times 10^6$ ) were cultured for three days in RPMI medium and stimulated with 1 µg/mL OVA<sub>p</sub> in the presence or absence of 2.5 µg/mL TQ. Supernatant was collected to measure levels of IL-2, TNFα, and IFNγ by an enzyme-linked immunosorbent assay (ELISA). Although addition of TQ did not show an obvious effect on TNFα or IL-2 production, it markedly increased the IFNγ production (Fig. 4a).

To confirm that this IFNγ was produced by antigen-specific CD8<sup>+</sup> T cells, the experiment was repeated to measure the intracellular expression of IFNγ in CD62L<sup>+</sup> and CD62L<sup>-</sup> CD8<sup>+</sup> T-cell subsets in the presence of different concentrations of TQ. Addition of 20 µg/mL TQ significantly decreased intracellular expression of IFNγ both in CD62L<sup>+</sup> and CD62L<sup>-</sup> subsets (Fig. 3). Addition of 15 µg/mL TQ did not markedly alter intracellular expression of IFNγ in either



**Fig. 3.** Conditioning CD8<sup>+</sup> T cells *in vitro* with TQ enhanced proliferation. Cells were harvested to assay cell proliferation by flow cytometry. See legend to Figure 1 for further details



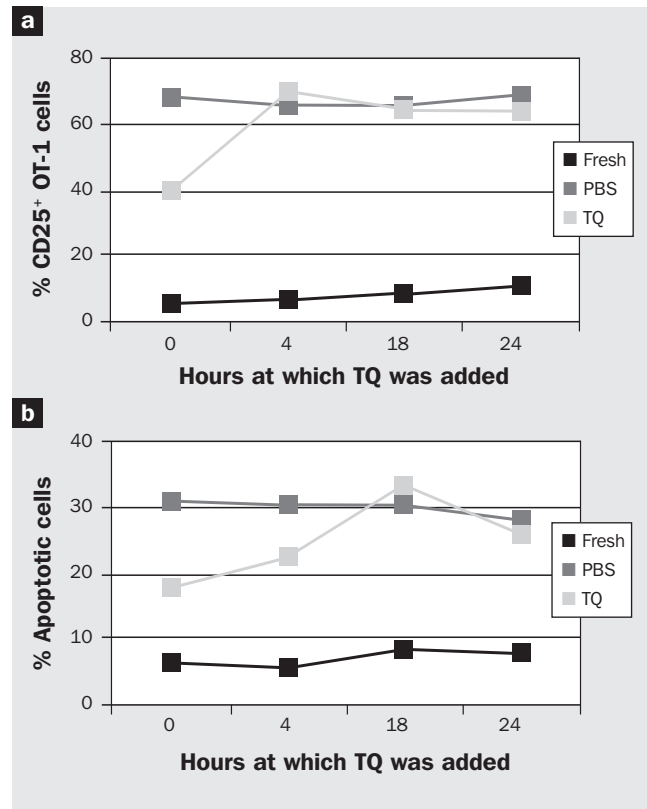
**Fig. 4.** Conditioning CD8<sup>+</sup> T cells *in vitro* with TQ enhanced their cytokine production. Cells were harvested to assay the intracellular expression of IFN $\gamma$  in CD62L<sup>+</sup> and CD62L<sup>-</sup> subsets (a) and cytokine production (b) by flow cytometry. See legend to Figure 1 for further details

subset. Consistent with IFN $\gamma$  production (Fig. 4a), addition of 10, 2.5 or 1.25  $\mu$ g/mL TQ markedly increased intracellular expression of IFN $\gamma$  in the CD62L<sup>+</sup> subset, but decreased expression in the CD62L<sup>-</sup> subset (Fig. 4b).

#### Optimal timing of TQ addition

Unfractionated splenocytes from OT-1 mice were stimulated with 1  $\mu$ g/mL OVA<sub>p</sub> *in vitro* for three days. Then, 2.5  $\mu$ g/mL TQ was added either at the beginning of antigen stimulation (0 h) or after 4, 18, or 24 h. Phosphate-buffered saline was added to control cells. Cells were harvested on day 3 (i.e., 72, 68, 54 or 48 h following TQ addition) and activation was assessed by measuring CD25 expression. Cell apoptosis was assessed by measuring annexin-V expression. Unstimulated cells showed minimal CD25 expression. Stimulation of cells with antigen alone induced CD25 expression in 5% (unstimulated cells) to about 68%. Addition of TQ with peptide regulated CD25 expression in antigen-stimulated CD8<sup>+</sup> T cells, showing 40% expression compared to 68% in the control cells. Addition of TQ after 4, 18 or 24 h had no effect, showing 70%, 65%, and 65% CD25 expression, respectively, in the antigen-stimulated cells (Fig. 5a), indicating that addition of TQ at the beginning of antigen priming is critical to the induction of immunomodulatory effects.

Analysis of apoptosis showed that stimulation with antigen alone induced apoptosis in 28–30% CD8<sup>+</sup> T cells. Addition of TQ at the time of antigen stimulation (0 h) or shortly after antigen stimulation (4 h) significantly reduced



**Fig. 5.** Optimal timing of addition of TQ to activate antigen-specific CD8<sup>+</sup> T cells. Cells were harvested to assess cell activation by analysing the expression of CD25 (a) and apoptosis by analysing the expression of annexin-V (b) by flow cytometry. See legend to Figure 1 for further details

apoptosis to 17% and 22%, respectively. Later addition of TQ (18 or 24 h) had no effect on apoptosis, whereby 33% and 26% antigen-specific CD8<sup>+</sup> T cells, respectively, were affected (Fig. 5b).

#### Kinetic analysis of TQ effects on activation and apoptosis

Unfractionated splenocytes from OT-1 mice were stimulated with 1  $\mu$ g/mL OVA<sub>p</sub> *in vitro* for three days in the presence or absence of 2.5  $\mu$ g/mL TQ, and the cells were harvested after one, two and three days of culture to assess activation and apoptosis by measuring expression of CD25 and annexin-V, respectively. Analysis of cell activation of cells harvested after 24 h showed that addition of TQ did not alter the expression of CD25. By day 2, however, expression was markedly increased, while by day 3 it was decreased, compared to levels in the antigen-stimulated CD8<sup>+</sup> T cells with no TQ (Fig. 6a). Analysis of apoptosis showed that addition of TQ did not significantly alter apoptosis measured on days 1 or 2 after stimulation (Fig. 6b). CD8<sup>+</sup> T cells stimulated with antigen in the presence of TQ showed less apoptosis on day 3 compared to cells stimulated with antigen but with no TQ (Fig. 5b).

## Discussion

CD8<sup>+</sup> T cells are a critical component of the cellular immune response where they play an important role in the control of viral infection<sup>21</sup> and in eliminating cells with malignant



potential.<sup>14</sup> Previous studies have focused on improving the survival and trafficking phenotypes of CD8<sup>+</sup> T cells and their functionality *in vitro* for *in vivo* adoptive immunotherapy.<sup>14,22</sup> The results of the present study show that addition of low concentrations of TQ during *in vitro* stimulation of CD8<sup>+</sup> T cells with cognate antigen can result in acquisition of a better survival phenotype in CD8<sup>+</sup> T cells, and enhanced functional activity. These results highlight the beneficial effects of TQ on T cells *in vitro* and the use of these cells in adoptive T-cell therapy.

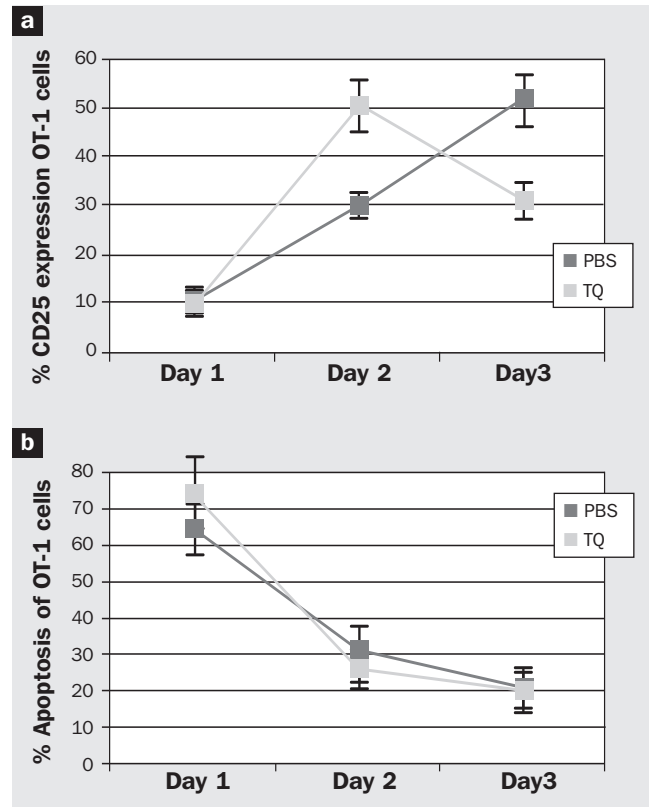
Stimulation of CD8<sup>+</sup> T cells with OVAp alone resulted in significant activation, and addition of TQ (20 or 15 µg/mL) induced an approximate eight-fold increase in apoptosis, while low concentrations of TQ (10, 2.5, 1.25 or 0.62 µg/mL) resulted in many fewer cells undergoing apoptosis. Addition of low concentrations of TQ (10, 2.5 or 0.62 µg/mL) markedly increased proliferation and IFN $\gamma$  production by CD8<sup>+</sup> T cells, in particular by CD62L<sup>+</sup>, CD8<sup>+</sup> T-cell subset. This could be due to the toxic effects of TQ at high concentration and its stimulatory effect at low concentrations.

Previous studies have shown that TQ exhibits an antioxidant effect at low concentration versus pro-oxidant properties at high concentration.<sup>23,24</sup> A recent study also showed antiproliferative and cytotoxic effects of TQ at high concentration (20 µmol/L) in hepatocyte primary culture as demonstrated by a significant increase in the percentage of necrotic cells.<sup>24</sup> Similarly, TQ is reported to be a pro-apoptotic agent in the human colon cancer cell line HCT-116.<sup>25,26</sup>

It is suggested that TQ activates p53 and reduces the anti-apoptotic protein concentration of Bcl-2.<sup>27</sup> Moreover, the antiproliferative effects of TQ have been observed at concentrations between 25 µmol/L and 100 µmol/L, associated with induction of apoptosis at a concentration of 100 µmol/L.<sup>28</sup> In contrast, normal cells and primary mouse keratinocytes are resistant to the apoptotic effect of TQ, and no significant alteration to their morphology and proliferation has been observed, confirming the selectivity of TQ for cancer cells.<sup>29,30</sup> Therefore, the dose-limiting effects of TQ on immune cells *in vivo* should be considered during its application.

The role of reactive oxygen species (ROS) in this process has been studied in response to stimulation by antigen, superantigen<sup>31</sup> or antibodies to the T-cell receptor (TCR) complex.<sup>32</sup> Several studies have shown that antioxidants (e.g., vitamin E, *N*-acetylcysteine, *Mangifera indica* polyphenol) prevent activation-induced cell death (AICD).<sup>33-35</sup> Other studies have examined the effects of antioxidants on TCR-stimulated death of T cells, and show that exposure to antioxidants inhibits AICD of T cells in cell lines and in primary T cells.<sup>36</sup>

Although the stimulatory effects of the low concentrations of TQ on T-cell survival and function could be attributed to different mechanisms, one could be its antioxidant effect. Although this study did not measure production of ROS in the cell culture, the authors have reported recently the capability of TQ to increase the antioxidant mechanisms *in vivo*.<sup>15</sup> The antioxidant activity of TQ has been reported previously, in which TQ was found to act as a scavenger of superoxide, hydroxyl radical and singlet molecular oxygen.<sup>37-41</sup> Collectively, these data indicate that ROS produced by T cells following TCR stimulation can be regulated by exposure of T cells to antioxidants such as TQ. Furthermore, treatment with TQ was found to prevent the increase in ROS formation caused by exposure of Jurkat T



**Fig. 6.** Kinetic analysis of TQ effects on the activation and apoptosis of antigen-specific CD8<sup>+</sup> T cells. Cells were harvested to assess cell activation by analysing the expression of CD25 (a), and apoptosis by analysing the expression of annexin-V (b) by flow cytometry. See legend to Figure 1 for further details

cells to n-hexane, coinciding with decreases in the induced changes in cell proliferation.<sup>42</sup>

The strong antioxidant properties of TQ may be related to the redox properties of the quinone structure of the TQ molecule, and its unrestricted crossing of morphological barriers, gaining easy access to subcellular compartments and facilitating the ROS scavenging effect.<sup>39</sup> Although it is not clear how pharmacologic antioxidants, including TQ, modulate T-cell function, it has been shown that antioxidants such as rosmarinic acid, a natural phenolic product with antioxidant activity, selectively inhibits TCR-induced activation of phospholipase C- $\gamma$  and inducible T-cell kinase without affecting extracellular signal-regulated kinase or activated protein 1 activation.<sup>43</sup> It has also been shown that addition of pharmacological antioxidants during T-cell activation *in vitro* can augment activation of extracellular signal-regulated kinase, coinciding with decreases in T-cell apoptosis.<sup>44,45</sup>

In conclusion, the present results indicate that the activation phenotype, survival and function of antigen-specific T cells can be differentially modulated by TQ concentrations. At low concentration, TQ not only directly modulates the expression of CD62L on activated CD8<sup>+</sup> T cells but also selectively promotes the survival and function of activated CD8<sup>+</sup> T cells that express high levels of CD62L. This may be attributed to the antioxidant activity of TQ. These data have significant application in the clinical setting as this natural compound can be used to condition T cells before adoptive immunotherapy. □

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