

# A novel spontaneous mutation in the TAP2 gene unravels its role in macrophage survival

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doi:10.1111/imm.12694

Received 14 September 2016; revised 10  
November 2016; accepted 10 November  
2016.

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## Summary

We report a new mouse strain with a single point mutation in the type 2 transporter associated with antigen processing (TAP2). This strain randomly arose in one of our C57BL/6J mouse colonies and was initially discovered because of the lack of CD8<sup>+</sup> T cells in the periphery. Following our observation, we subsequently revealed a lack of cell surface MHC-I expression, derived from TAP2 protein deficiency. Our strain, named *eightless*, has a C to T substitution in exon 5 resulting in a glutamine to stop codon substitution at position 285 in the TAP2 protein. Interestingly, in addition to the expected lack of CD8<sup>+</sup> T cell phenotype, *eightless* mice have a diminished number of macrophages in their peritoneum. Moreover, following peritoneal inflammation, elicited *eightless* macrophages showed impaired survival both *in vivo* and *ex vivo*. Our study describes the first ever TAP2 complete knockout mouse strain and provides a possible explanation for why patients with TAP2 deficiency syndrome present clinical manifestations that would suggest a phagocyte defect rather than a lack of CD8<sup>+</sup> T cells.

**Keywords:** immunodeficiency diseases; major histocompatibility complex; T cells.

## Introduction

Bare lymphocyte syndrome (BLS) is characterized by a severe down-regulation of MHC class I (MHC-I) and/or class II molecules.<sup>1</sup> In type 1 BLS, the defect is confined to MHC-I, a complex consisting of a highly polymorphic membrane-spanning heavy chain associated with a light chain, the  $\beta_2$ -microglobulin ( $\beta_2m$ ). The MHC-I complex binds peptides that are 8–11 amino acids long and are generated from the cytosolic proteasome-dependent degradation of cellular proteins.<sup>2</sup> After proteolysis, these peptides are transferred to the lumen of the endoplasmic reticulum (ER) by the transporter associated with antigen presentation (TAP) and finally are loaded onto the MHC-I complex and relocated to the cell surface.<sup>3</sup> TAP, a member of the ATP-binding cassette (ABC) superfamily transporters, is a heterodimer consisting of two protein subunits, TAP1 and TAP2.<sup>4</sup> Deletion of or mutation in TAP1 and/or TAP2 severely affects the translocation of peptides into the ER as MHC-I molecules are retained within the ER, a phenomenon that can be reverted *in vitro* by cellular incubation at 26°C.<sup>5</sup>

MHC-I molecules are expressed on the cell surface of all nucleated cells and in the thymus are crucial for the presentation of self-peptides to developing CD8<sup>+</sup> thymocytes undergoing positive selection.<sup>6</sup> In the periphery, they support naive CD8<sup>+</sup> T cell survival,<sup>7</sup> cytotoxic T-cell generation and activation<sup>8</sup> and are able to modulate  $\gamma\delta$  T and natural killer (NK) cells by interacting with MHC-I binding receptors.<sup>9</sup> The generation of mutants carrying gene deletions within the MHC-I complex processing and antigen presentation pathway, such as  $\beta_2m^{-/-}$  and TAP1<sup>-/-</sup> mice,<sup>10–12</sup> has been useful to characterize the mechanisms behind these pathways and to provide information about CD8<sup>+</sup> T and NK cell development.<sup>13,14</sup> Surprisingly, despite being low in number, CD8<sup>+</sup> T cells were clearly detectable in these mice.<sup>15–17</sup>

Patients with TAP1 or TAP2 deficiency have been included within a specific BLS-I subgroup in which reduced HLA-I surface expression is associated with recurrent bacterial infections of the upper and lower airways eventually evolving to bronchiectasis, often associated with necrotizing granulomatous skin lesions.<sup>18,19</sup> Inexplicably, severe viral infections do not occur.

Clinicians prefer to refer to this condition as TAP deficiency syndrome rather than BLS-I.<sup>20</sup> Indeed percentages of CD8<sup>+</sup> T cells in these patients vary from more or less severe reductions to normal or even elevated values,<sup>21–23</sup> while there is an expansion of T cell receptor- $\gamma\delta^+$  (TCR- $\gamma\delta^+$ ) T cells<sup>18,23</sup> and NK cells,<sup>18</sup> which were found to be autoreactive in four patients studied.<sup>18,24</sup> However, functional tests revealed that NK cells from TAP-deficient patients have no cytotoxic activity towards HLA-I-deficient targets.<sup>24</sup>

These clinical manifestations and immunological features are difficult to interpret if we consider the biological role of TAP, so the physiopathology of BLS-I still remains mysterious.<sup>25,26</sup>

The mutation that we identified in our mouse strain, which we named *eightless*, very closely resembles the genetic lesions reported in humans, as BLS-I patients harbour a TAP2 premature stop codon, resulting in a truncated and non-functional protein.<sup>23</sup> We therefore decided to use this model in order to elucidate the physiopathology of human TAP deficiency syndrome.

In our study we found that these mice contain fewer peritoneal resident macrophages and when we induced peritoneal inflammation we noted that the newly generated macrophages died prematurely.

## Materials and methods

### Development of mouse strain

The *eightless* strain randomly arose in our colony from C57BL/6J mice that were purchased from Jackson Laboratories (Bar Harbor, ME) and were routinely screened by flow cytometry for CD4 and CD8 ratios in the blood. Outlying mice or their littermates were bred to confirm heritability of the trait, and to establish the strain. Both males and females (8–12 weeks) were used to define the described phenotypes. The entire work was approved by the Animal Ethics Committee, and carried out in accordance with institutional guidelines.

### Identification of the *eightless* mutation

Spleen RNA was extracted by Trizol reagent (Thermo Fisher Scientific, Waltham, MA) and cDNA generated by ProtoScript first-strand cDNA synthesis kit (New England BioLabs, Ipswich, MA).  $\beta 2m$ , *Tap1* and *Tap2* cDNAs were then amplified by PCR and sequenced using 16–20 bp complementary primers (sequences available on request).

### Flow cytometry

Blood, lymph node, thymus and spleen samples, as well as peritoneum lavage cells, were depleted of erythrocytes

by incubation with red blood cell lysis buffer (10 mM KHCO<sub>3</sub>, 150 mM NH<sub>4</sub>Cl, 0.1 mM EDTA, pH 8.0), re-suspended in FACS buffer (PBS, 2% fetal bovine serum, 3 mM EDTA) and stained with fluorochrome-conjugated antibodies (BioLegend, San Diego, CA). For survival assay after staining and wash, the samples were re-suspended in 10  $\mu$ g/ml propidium iodide (Sigma-Aldrich, St Louis, MO) FACS buffer. Samples were collected on a Gallios cytometer (Beckman Coulter, Brea, CA) and the data were analysed using KALUZA FLOW ANALYSIS SOFTWARE v1.2 (Beckman Coulter).

### Real-time PCR

Total RNA from cultured macrophages was extracted by Trizol reagent (Thermo Fisher Scientific) and cDNA was synthesized using ProtoScript First Strand cDNA Synthesis Kit (New England BioLabs, Ipswich, MA). Quantitative real-time PCR was then performed using QuantStudio 12K Flex Real-Time PCR system with a Power SYBR green PCR master mix kit (Applied Biosystems, Foster City, CA). The primers sequences used in this study have been described elsewhere.<sup>27</sup>

### Exogenous addition of peptide and splenocytes culture

Spleens from euthanized mice were cut in small fragments with a blade and then smashed with a syringe plunger on a 40- $\mu$ m cell strain, filtered on a 50-ml Falcon tube, pelleted and red blood cells were lysed. Erythrocyte-depleted splenocytes were then incubated for 3–9 hr at 37° in medium (RPMI-1640, 10% fetal bovine serum, 1% peptomycin-streptomycin, 50 nM 2-mercaptoethanol) either untreated or with 1–2.5  $\times 10^{-6}$  M synthetic SIIN-FEKL peptide. Alternatively, the splenocytes were incubated overnight at 26° without peptide. After this time, cells were stained with anti-H-2K<sup>b</sup>-phycoerythrin (clone AF6 88.5) and the corresponding isotype control in FACS buffer and analysed by flow cytometry.

### Peritoneal macrophage culture

Residential peritoneal macrophages were directly collected from anaesthetized mice by lavage with 5 ml PBS using a 5-ml syringe with 19G needle. When elicited macrophages were needed, the mice were injected intraperitoneally with 1 ml 4% thioglycollate at least 36 hr in advance. Then, 5  $\times 10^5$  erythrocyte-depleted lavage-derived cells were plated on a tissue culture treated polystyrene 24-well plate (Corning, NY) in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 1% peptomycin-streptomycin, 50 nM 2-mercaptoethanol. After 2 hr, medium was removed, plate wells were washed twice in PBS and 1 ml media was added to remove unattached cells and select for macrophages. Cells were

cultured for 24–48 hr in a humidified incubator with 5% CO<sub>2</sub>. For survival assay macrophages were detached using Enzyme-Free Cell Dissociation Buffer (Gibco, Grand Island, NY), counted by microscopy and immediately processed for flow cytometry analysis as previously described.

#### Statistical analysis

All data were analysed using unpaired *t*-tests and were represented as mean ± SEM. All statistical analyses were carried out using PRISM software (GraphPad Software, San Diego, CA) and the  $\alpha$  level was set at 0.05 for all tests.

## Results

### Diminished CD8<sup>+</sup> T cells in *eightless* mutant mice

The recessive *eightless* phenotype was initially randomly detected in our C57BL/6J mouse colony among mice with very low CD8<sup>+</sup> T cell proportions (about 0.5%) in the blood. However, these mice had normal total leucocyte numbers in the spleen, thymus, lymph nodes and blood.

To further characterize the immunological defect caused by the *eightless* mutation, we immunophenotyped cells from spleen, lymph node and thymus by flow cytometry. Low percentages and absolute numbers of CD8<sup>+</sup> T cells were evident in the lymph nodes (Fig. 1a,b), spleen (Fig. 1c,d) and thymus (Fig. 1f,g) relative to those of wild-type mice. In addition, relative to wild-type mice, *eightless* mice were found to have a significantly increased percentage and absolute number of CD4<sup>+</sup> T cells but equal quantities of B cells in both spleen and lymph nodes (Fig. 1a–d). Finally, a slight increment in the number of NK but not NKT cells was found in *eightless* spleen (Fig. 1d) and, compared with the wild-type, *eightless* NK cells expressed substantially higher Ly49C/I levels (Fig. 1e).

### *Eightless* mice lack MHC-I cell surface expression

Reduced numbers of the CD8<sup>+</sup> T cell population can be caused by either lack of CD8 expression or reduced MHC-I expression on the cell surface. As CD8 expression was found to be normal on CD4<sup>+</sup> CD8<sup>+</sup> double-positive thymocytes (Fig. 1e) we excluded CD8 protein expression as the cause for the *eightless* phenotype.

To test the involvement of MHC-I expression in the phenotype, MHC-I cell-surface expression was detected in splenocytes by flow cytometry using an anti-H-2K<sup>b</sup> antibody. *Eightless* mice were found to express substantially lower levels of MHC-I than wild-type mice, with levels comparable to the isotype-matched control antibody (Fig. 2a,b).

In addition, as expected for C57BL/6J mice, H-2D<sup>b</sup> molecules were not expressed (data not shown), though

other non-classical molecules that might depend on TAP transport, such as Qa or M3, were not tested.

### Identification of the *eightless* mutation

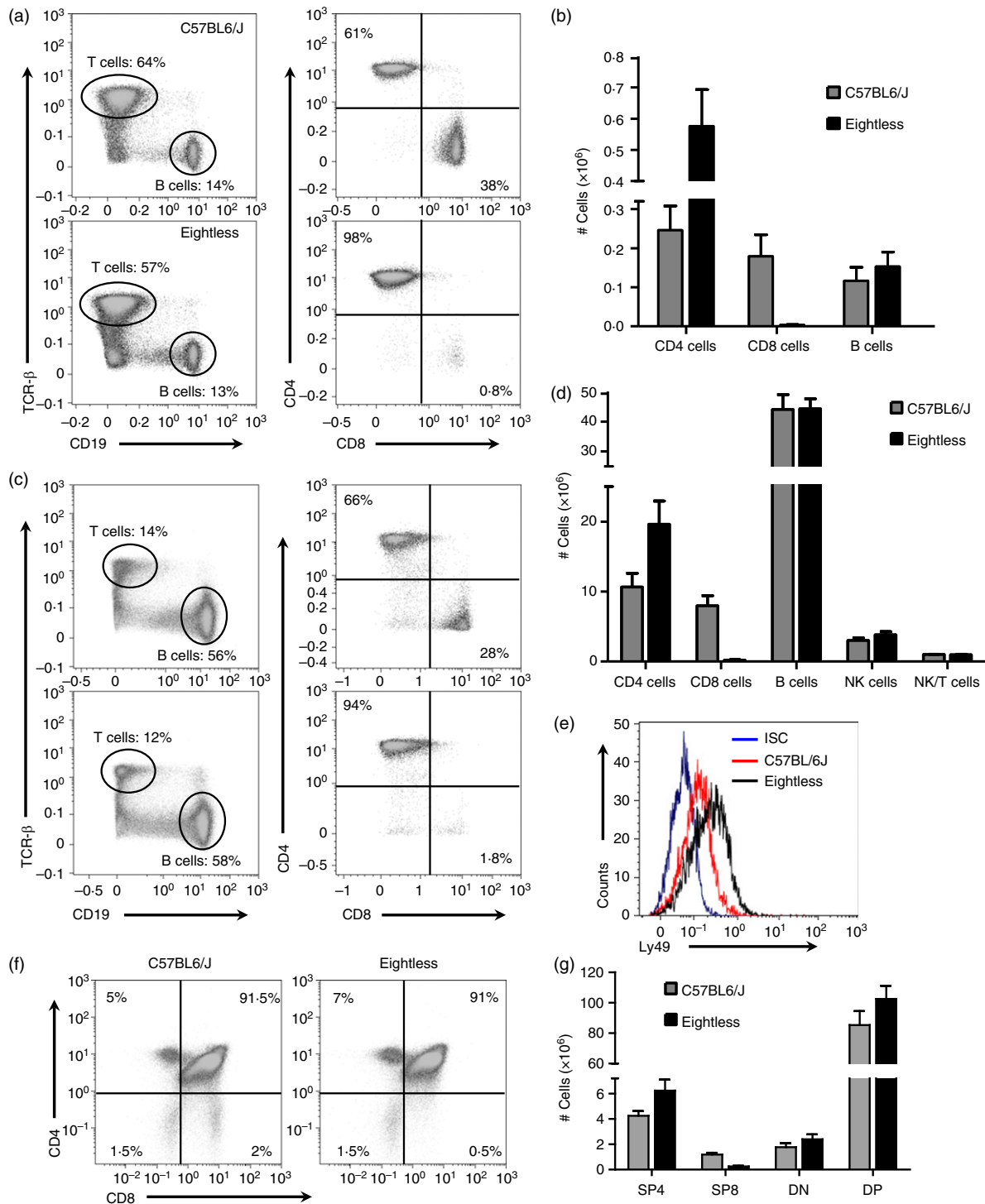
Several genes have been reported to have a role in the cell surface expression of the MHC-I complex. Among these genes are  $\beta$ 2m, TAP1, TAP2 and the MHC-I encoding genes themselves.

It is unlikely that the phenotype is caused by mutations in MHC-I genes, as three separate mutations would be required to inactivate all three mouse MHC-I genes (H-2K, H-2D and H-2L). Since  $\beta$ 2m, TAP1 and TAP2 proteins are all crucial for efficient MHC-I complex formation, peptide loading and subsequent valid cell surface expression, we decided to sequence these genes. A single point mutation was detected in TAP2 consisting of a C to T transition of base 314 in exon 5 (Fig. 3a). This shift generated a glutamine to stop codon substitution at position 285 in the TAP2 protein (Fig. 3b).

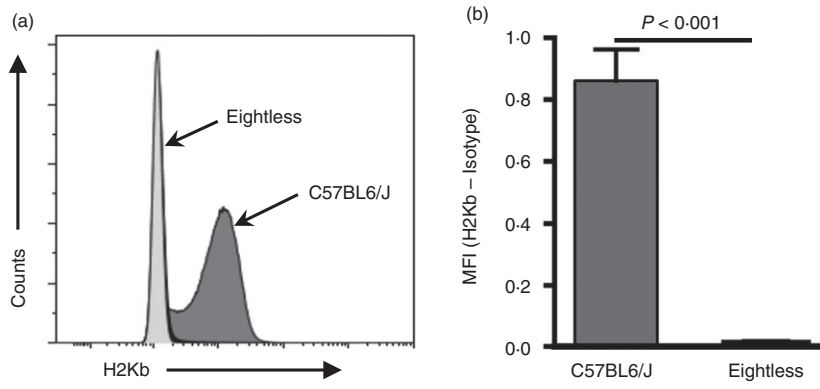
### Exogenous peptide or cell incubation at 26° rescues MHC-I surface expression

As mutation in TAP2 severely affects the translocation of peptides into the ER, *eightless* mice clearly have a defect in the transport of antigenic peptides into the ER. Therefore, it could be expected that the rest of the MHC-I antigen presentation pathway would be intact. TAP-deficient cell lines exogenously incubated with MHC-I-binding peptides or incubated at 26° partially restored their surface MHC-I expression.<sup>5,28</sup>

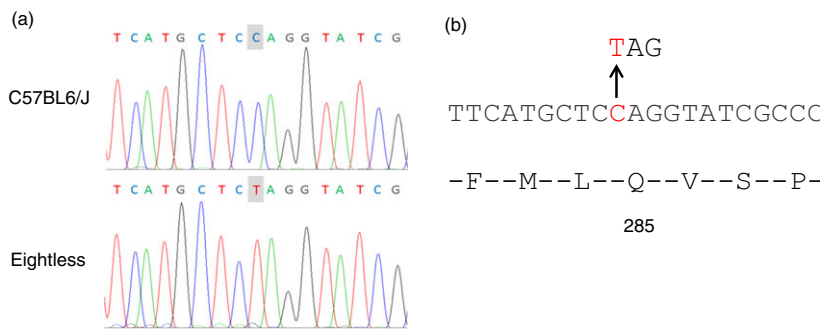
Therefore, we next tested the ability of splenocytes from *eightless* mice cultured with an H-2K<sup>b</sup>-binding ovalbumin-derived peptide (SIINFEKL) to increase levels of surface H-2K<sup>b</sup> measured by flow cytometry. As predicted for cells with a sole defect in TAP activity, cell culture with the peptide partially restored MHC-I expression in splenocytes from *eightless* mice (Fig. 4a), whereas MHC-I expression was only minimally affected in wild-type cells. Moreover, we found that the increase in expression was strongly dependent on the duration of incubation with the peptide, and less so on the dosage (Fig. 4a). The relevance of incubation time is emphasized by the evidence that during the time of our culture (3–9 hr), normal splenocytes tended to lose surface MHC-I (Fig. 4b) whereas MHC-I expression in *eightless* cells kept increasing (Fig. 4a). These data are compatible with the idea that several empty MHC-I complexes are normally present on the surface of TAP2-deficient cells but these constantly undergo rapid turn-over unless stabilized by the addition of exogenous peptide. In fact, kinetic studies have shown that empty MHC-I molecules are quickly internalized from the cell surface.<sup>29</sup>



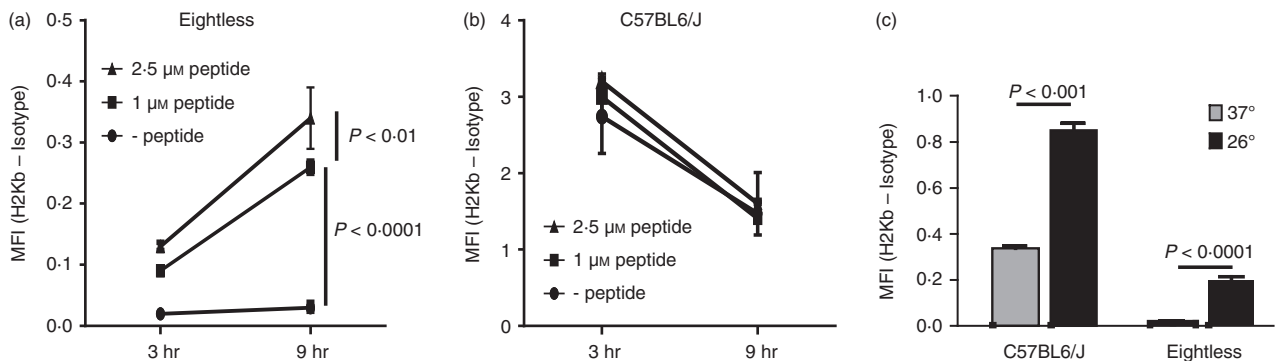
**Figure 1.** Diminished CD8<sup>+</sup> T cells in *eightless* mutant mice. (a). Flow cytometry analysis of the expression of CD19 versus T cell receptor- $\beta$  (TCR- $\beta$ ) by cells from two inguinal lymph nodes (left panels), and CD8 versus CD4 (right panels, gated on TCR- $\beta^+$  cells), from wild-type or *eightless* mice. Numbers in quadrants indicate per cent cells in each. (b) Graph presenting absolute numbers of CD4 T, CD8 T and B cells in the spleen of wild-type and *eightless* mice. (c) Same as in (a) using cells from the spleen. (d) Absolute numbers of CD4 T, CD8 T, natural killer (NK), NKT and B cells in the spleens of wild-type and *eightless* mice. (e) Histogram plot of Ly49 expression level on NK cells (gated on NK1.1<sup>+</sup> TCR- $\beta^-$  cells) from wild-type (red line) or *eightless* (black line) mice. ISC-isotype control (blue line). (f) Flow cytometry analysis of the expression of CD8 versus CD4 by thymocytes from wild-type or *eightless* mice. (g) Absolute numbers of CD4 and CD8 T double-negative, double-positive and either CD4 or CD8 single-positive thymocytes in the thymus of wild-type and *eightless* mice. ( $n = 6$  mice, a two tailed unpaired  $t$ -test was used for statistical analysis). Scale bars represent  $\pm$  SEM. [Colour figure can be viewed at [wileyonlinelibrary.com](http://wileyonlinelibrary.com)]



**Figure 2.** *Eightless* mice lack MHC-I cell surface expression. (a) Representative histogram of MHC-I expression (H2K<sup>b</sup>) on the cell surface of total splenocytes from *eightless* (light grey) and wild-type (dark grey) mice. (b) Graph summarizing the results presented in (a). Normalized values (H2K<sup>b</sup>-Isotype) of wild-type and *eightless* splenocyte MHC-I mean fluorescence intensity (MFI) ( $n = 6$  mice, a two-tailed unpaired *t*-test was used for statistical analysis). Scale bars represent  $\pm$  SEM.



**Figure 3.** Identification of the *eightless* mutation. (a) Genomic sequence from part of the TAP2 gene reveals a C > T point mutation in *eightless* mice. (b) Diagram of the TAP2 amino acids and nucleotide sequences demonstrating the nonsense mutation in *eightless* leading to the conversion of glutamine in position 285 to a stop codon. [Colour figure can be viewed at [wileyonlinelibrary.com](http://wileyonlinelibrary.com)]



**Figure 4.** Exogenous peptide or incubation at 26° rescues *eightless* MHC-I surface expression. (a, b) MHC-I cell surface expression on splenocytes from *eightless* (a) or wild-type (b) mice cultured with or without SIINFEKL peptide for 3 and 9 hr. (c) MHC-I cell surface expression on splenocytes from *eightless* or wild-type mice incubated overnight *in vitro* at 26° (black bars) or 37° (grey bars). ( $n = 3$  mice, a two-tailed unpaired *t*-test was used for statistical analysis). Scale bars represent  $\pm$  SEM.

The presence of empty MHC-I complexes on the cellular surface was strongly enhanced by overnight incubation at 26° in cells derived from both wild-type and *eightless* mice (Fig. 4c). The MHC-I expression rescue we detected in the mutated samples implies that *eightless* cells are genuinely TAP defective. In fact it is known that some peptide-receptive MHC-I molecules commonly escape ER retention to reach the cell surface and this flux can be enhanced by lowering temperature.<sup>5</sup> Incubation at 26° also enhanced surface expression of MHC-I molecules in

wild-type splenocytes, but the proportional enhancement of K<sup>b</sup> expression was less dramatic, most probably because these cells had higher baseline levels of pre-existing cell surface K<sup>b</sup> molecules.

### Reduced residential peritoneal macrophages in *eightless* mice

Given that patients with TAP syndrome clinically present with symptoms typical of an innate immune defect and

that the peritoneal cavity contains various immune cell populations crucial for the innate immune response, we decided to inspect the cellular composition of the peritoneum of *eightless* mice. Whereas the total number of CD45<sup>+</sup> cells recovered from lavage was similar in wild-type and *eightless* mice (data not shown), the proportion of the macrophage population out of total CD45<sup>+</sup> cells in *eightless* mice was reduced (Fig. 5a–c) and this difference resulted in a much lower absolute number of macrophages in *eightless* compared with wild-type mice (Fig. 5d). Also, residential peritoneal macrophage viability in *eightless* mice was slightly reduced (Fig. 5e,f).

#### Thioglycollate-elicited *eightless* macrophages are recruited normally into the peritoneum but are short-lived

As *eightless* mice have a reduced number of residential peritoneal macrophages, we decided to investigate whether this might be a consequence of reduced precursor recruitment or due to a defect in the mature macrophage itself. To distinguish between these two possibilities, we challenged mice with 1 ml intraperitoneal 4% thioglycollate and analysed their blood, bone marrow, spleen and peritoneal lavages at serial intervals from 36 hr up to 12 days (Fig. 6a–d). No significant differences in the number of recruited inflammatory monocytes and neutrophils were observed in the blood and spleen of *eightless* compared with wild-type mice, at all time-points tested (Fig. 6a,b). In addition, we observed no accumulation of monocyte and neutrophil precursors in the bone marrow of *eightless* mice (Fig. 6c). However, starting from around 4 days from thioglycollate injection, a significant reduction in both number and viability of macrophages was evident in the peritoneum cavity of *eightless* mice compared with that of wild-type mice (Fig. 6d,e). This tendency was conserved throughout the successive time-course of analysis and by the 12th day, most *eightless* macrophages had already disappeared (Fig. 6d).

These results suggest that following thioglycollate challenge, monocytes are recruited into the *eightless* peritoneum normally, and there they differentiate into macrophages. However, these cells seem to have a reduced survival capability.

#### Thioglycollate-elicited *eightless* macrophages show reduced survival *in vitro*

To demonstrate that in *eightless* mice inflammatory macrophages that develop in the peritoneum following thioglycollate challenge are more susceptible to cell death, we cultured these cells and checked their survival over time *in vitro*. For this purpose we used macrophages collected 5 days after thioglycollate injection, as we noted

that their number peaked on the fifth day and because up to the fourth day there was no evident difference in the number or viability of these cells (Fig. 6d,e). After 24 hr in culture, *eightless* macrophages (F4/80<sup>+</sup> CD45<sup>+</sup>, Fig. 7a) had a lower forward-scatter–side-scatter profile compared with those from wild-type mice (Fig. 7b). In addition, the percentage of *eightless* propidium iodide-negative macrophages decreased after 24 hr in culture (Fig. 7d). We collected cells after either 24 or 48 hr of culture and we found no difference between these two time-points (Fig. 7d). Therefore *in vitro*, some peritoneal macrophages die during the first 24 hr of culture whereas the remainder survive.

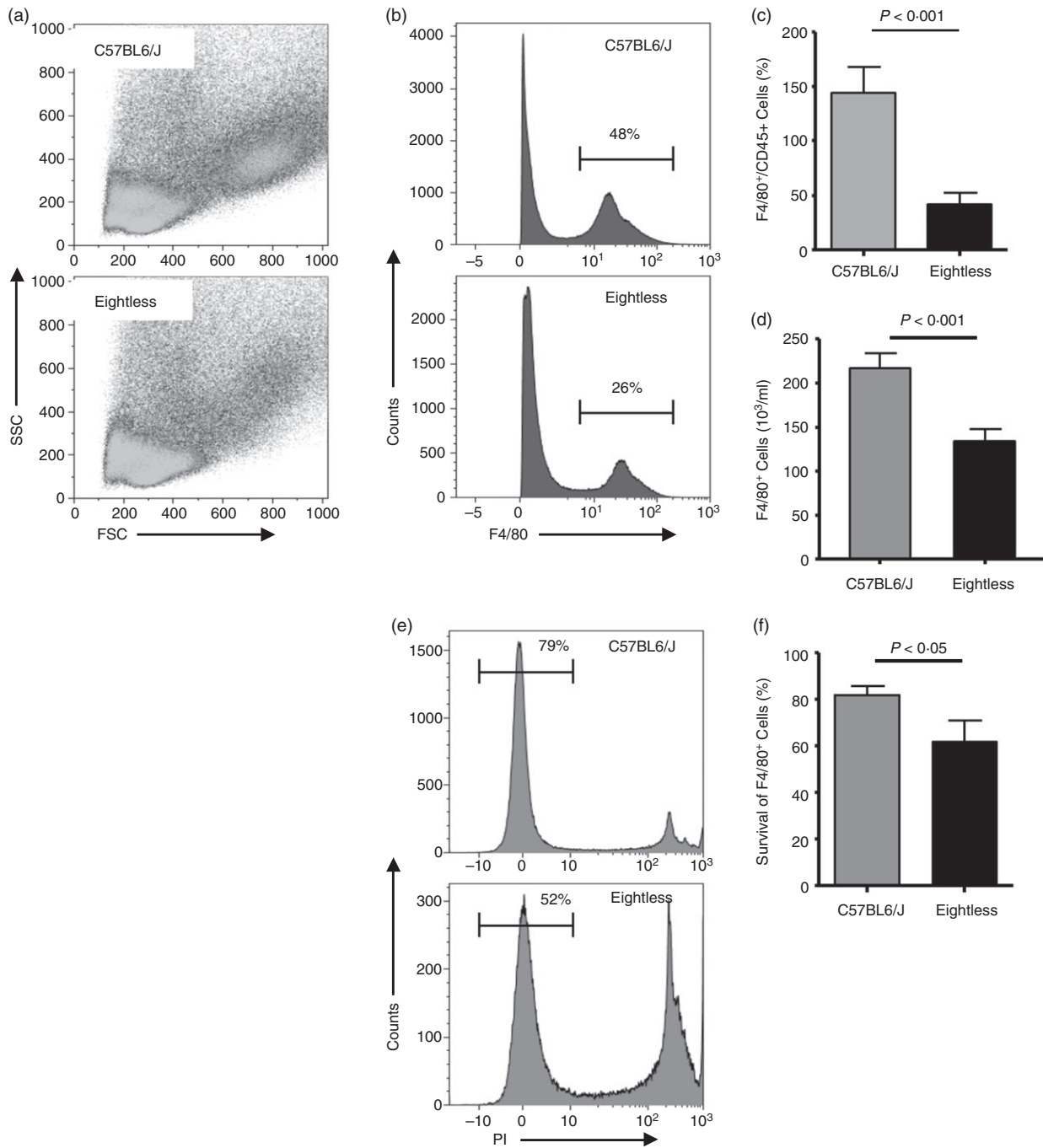
These data support our notion that during inflammation, most of the newly generated TAP-deficient macrophages are defective and therefore die prematurely.

#### Thioglycollate-elicited *eightless* macrophages show intact ER stress response

TAP deletion or mutation, severely affects the translocation of peptides into the ER and as a result MHC-I molecules are retained within the ER. The accumulation of MHC-I proteins in the ER raises the possibility that the death of macrophages observed in *eightless* mice is the result of an ER stress response. Specifically, the development of chronic, unresolved ER stress can lead to induction of cellular death, primarily through the activation of the transcription regulators, CHOP or XBP1s.<sup>30</sup> Therefore to examine whether the ER stress response is activated in *eightless* macrophages, the expressions of ER stress genes and splicing of XBP1 mRNA were measured in peritoneal macrophages. For this, the peritoneal macrophages from wild-type and *eightless* mice were tested under steady-state conditions and upon Toll-like receptor 4 activation by lipopolysaccharide treatment. To positively control the experiment, the expressions and splicing of XBP1 mRNA were also measured in tunicamycin-treated macrophages from wild-type mice. Interestingly, ER stress response genes were not found to be elevated in *eightless* macrophages in comparison with wild-type cells (Fig. 8a–d). Similarly, the ratio between the spliced and unspliced transcripts of XBP1 was elevated in both wild-type and *eightless* lipopolysaccharide-treated macrophages to the same level (Fig. 2e). These results indicate that the ER stress response is not elevated in *eightless* macrophages and therefore suggest that chronic ER stress can be excluded as the mechanism governing *eightless* macrophage cellular death.

## Discussion

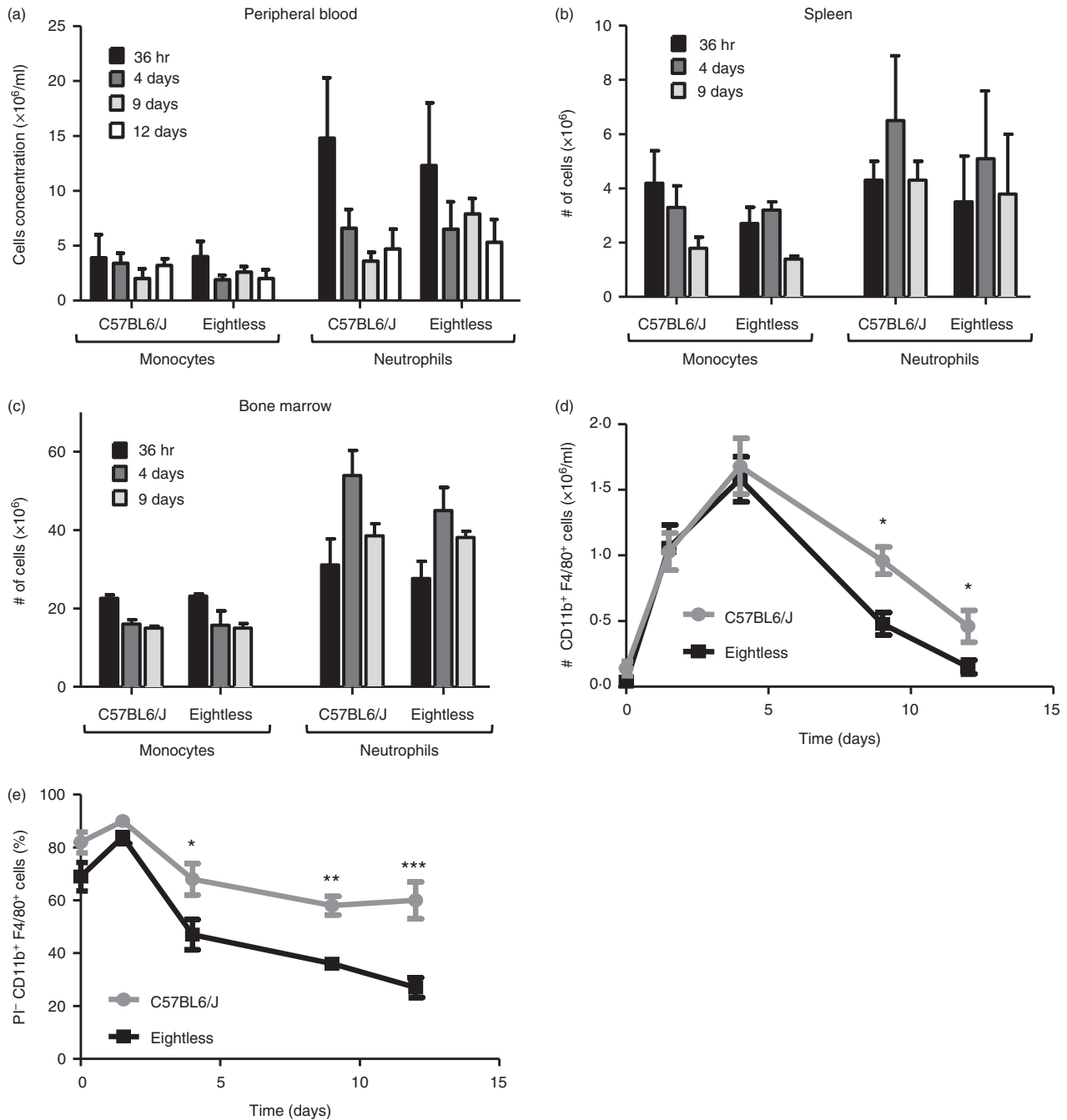
The lack of CD8<sup>+</sup> T cells in *eightless* mice closely recapitulates the phenotype reported in other MHC-I-deficient mice.<sup>10–12,31</sup> Recently a chemically induced point mutation in TAP2 gene, *jasmine*, was generated. Mice



**Figure 5.** Reduced peritoneal resident macrophages in *eightless* mice. (a) Flow cytometry density plots of forward scatter versus side scatter of total residential immune cells (gated on CD45<sup>+</sup> population) from the peritoneal cavity of wild-type (upper panel) and *eightless* (lower panel) mice. (b) Representative histogram plots of F4/80 staining of peritoneal CD45<sup>+</sup> cells from wild-type (upper panel) or *eightless* (lower panel) mice. Numbers above bracketed lines indicate per cent macrophages. (c) Percentage of peritoneal resident macrophages in wild-type (grey bar) or *eightless* (black bar) mice. (d) The absolute numbers of peritoneal resident macrophages in wild-type (grey bar) or *eightless* (black bar) mice. (e) Representative histogram plots of propidium iodide staining of peritoneal-resident macrophages from wild-type (upper panel) or *eightless* (lower panel) mice. Numbers above bracketed lines indicate per cent of live macrophages. (f) Bar graph summarizing the data presented in (e). (c, d and f  $n = 10$  mice, a two-tailed unpaired *t*-test was used for statistical analysis). Scale bars represent  $\pm$  SEM.

homozygous for this mutation showed a severe reduction of the TAP2 protein expression.<sup>31</sup> To our knowledge, *jasmine* is the only existing mouse model besides *eightless*

harbouring a TAP2 defect. However, in *jasmine* TAP2 expression is only reduced, whereas in *eightless* this gene is fully disrupted. Therefore, an animal model harbouring

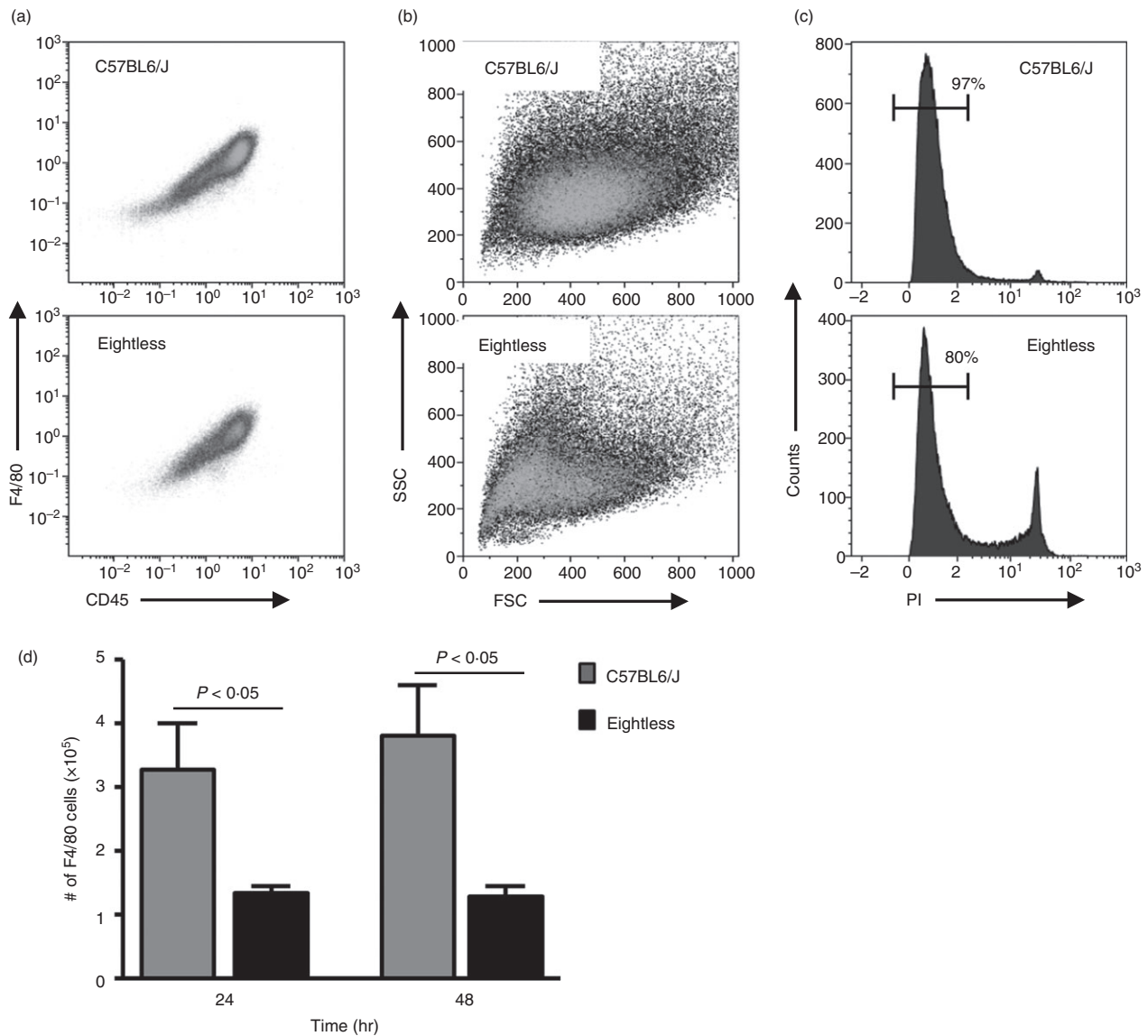


**Figure 6.** Thioglycollate-elicited *eightless* macrophages are recruited normally into the peritoneum but are short-lived. (a–c) Absolute numbers of neutrophils ( $\text{CD11b}^+ \text{Ly6C}^{\text{low}} \text{Ly6G}^+$ ) and monocytes ( $\text{CD11b}^+ \text{Ly6C}^{\text{hi}} \text{Ly6G}^-$ ) in the peripheral blood (a), spleen (b) and bone marrow (c) of *eightless* or wild-type mice, at different time-points after thioglycollate injection ( $n = 3$  mice, a two-tailed unpaired *t*-test was used for statistical analysis; none of the differences are statistically significant). Scale bars represent  $\pm$  SEM. (d) Absolute number of macrophages ( $\text{CD11b}^+ \text{F4/80}^+$ ) from mouse peritoneum at different time-points after thioglycollate injection. Some differences between *eightless* and wild-type are significant. (e) Percentages of live macrophages (propidium iodide-negative,  $\text{CD11b}^+ \text{F4/80}^+$ ) from mouse peritoneum cavity at different time-points after thioglycollate injection. (e, f,  $n = 3$  mice for each time-point, a two-tailed unpaired *t*-test was used for statistical analysis). Scale bars represent  $\pm$  SEM, \* $P < 0.05$ , \*\* $P < 0.01$  and \*\*\* $P < 0.001$

a genuinely disrupted version of the TAP2 gene, such as *eightless*, should be extremely useful to address a series of unresolved questions regarding TAP complex functionality.

Our results confirm that, similarly to TAP1, TAP2 has a non-redundant role in peptide loading of MHC-I molecules indicating that the TAP complex truly works as a heterodimer. Although this confirms previous *in vitro*



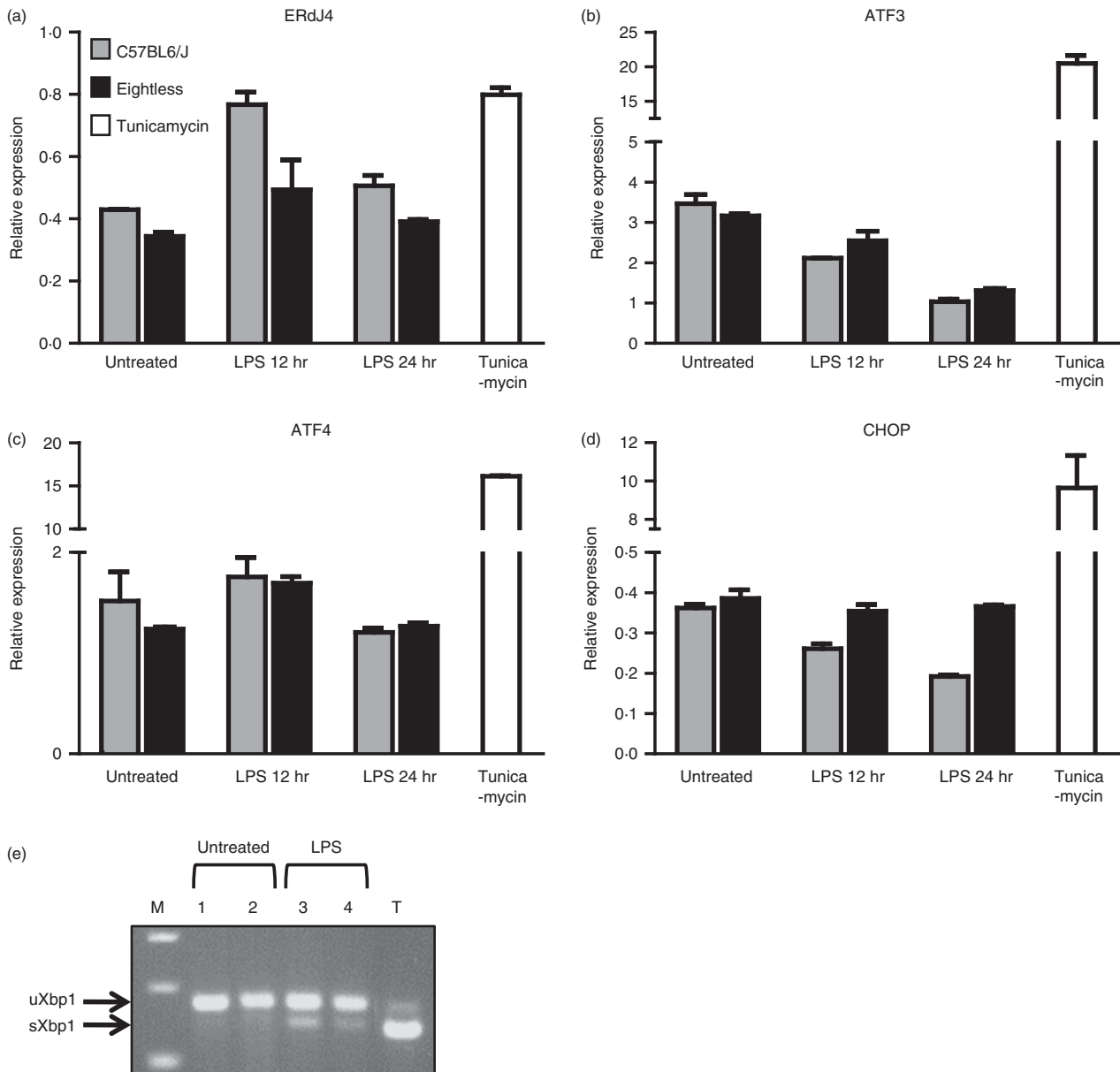


**Figure 7.** Thioglycollate-elicited *eightless* macrophages show reduced survival *in vitro*. (a–c) Flow cytometric analysis of wild-type or *eightless* peritoneal macrophages 5 days after thioglycollate that were incubated *in vitro* for 24 hr. (a) CD45 versus F4/80 staining. (b) Forward/side scatter profile of cells presented in (a). (c) propidium iodide staining of the cells presented in (a). Numbers above bracketed lines indicate per cent of live macrophages. (d) Graph bar summarizing the numbers of F4/80<sup>+</sup> cells from peritoneal macrophages 5 days after thioglycollate that were incubated *in vitro* ( $0.5 \times 10^5$  per well) for 24 or 48 hr ( $n = 8$  mice, a two-tailed unpaired *t*-test was used for statistical analysis). Scale bars represent  $\pm$  SEM.

data, we cannot rule out that TAP1 and TAP2 might also have distinct functions as suggested by other *in vitro* studies.<sup>32–35</sup>

In line with previous data, *eightless* mice were shown to have residual CD8<sup>+</sup> T cells.<sup>10,11,31</sup> In both TAP1<sup>-/-</sup> and  $\beta 2m^{-/-}$  mice, this limited population has the ability to recognize allo-antigens *in vitro*, though the level of reactivity among the two strains is different.<sup>36</sup> Several possible explanations have been proposed to clarify this phenomenon. The explanation most supported by both researchers and clinicians is that very limited amounts of

MHC-I molecules are expressed on the surface of TAP-deficient cells, an idea supported by the detection of a polyclonal CD8<sup>+</sup> T cell repertoire displaying both diversity and peptide specificity in TAP1<sup>-/-</sup> mice and in TAP2-deficient patients.<sup>37,38</sup> However, the occurrence of a minor population of functional CD8<sup>+</sup> T cells even in H-2K<sup>b-/-</sup>/D<sup>b-/-</sup> mice, animal models which by definition are completely deficient of classical MHC-I molecules,<sup>39</sup> would imply that there could be an additional developmental pathway entirely independent of the H-2K and D locus. The different amount of CD8<sup>+</sup> T cells in humans



**Figure 8.** Thioglycollate-elicited *eightless* macrophages show intact endoplasmic reticulum (ER) stress response. (a–d) RT-PCR analysis for ERDJ4 (a), ATF3 (b), ATF4 (c) and CHOP (D) mRNA relative expression normalized to the UBC housekeeping gene from treated (12–24 hr) and untreated wild-type (grey bar) or *eightless* (black bar) peritoneal macrophages ( $n = 3$ ). Tunicamycin-treated wild-type peritoneal macrophages served as a positive control for ER stress (white bar). (e) Representative results of unspliced and spliced *Xbp1* in wild-type (lanes 1 and 3) or *eightless* (lanes 2 and 4) macrophages untreated or treated with lipopolysaccharide (12 hr). T-Tunicamycin (4 hr) treated wild-type peritoneal macrophages. M-DNA marker.

and mice can be easily explained by a more extensive exposure to pathogens in humans compared with laboratory animals. Nevertheless, the presence of a residual number of CD8<sup>+</sup> T cells in mouse strains defective for MHC-I expression is still not fully understood. *Eightless* cells were able to increase their MHC-I expression on their surface following incubation with exogenous peptide in a timely dependent manner. In addition, a strong increase in K<sup>b</sup> expression was detected after incubation of *eightless* splenocytes at 26°. These results support the

notion that a limited amount of empty MHC-I molecules is constantly present and continuously recruited on the *eightless* cell surface. Interestingly, TAP1<sup>-/-</sup> macrophages have been reported to express lower levels of pre-existing, cell-surface, peptide-receptive MHC-I molecules compared with wild-type and these molecules also have a reduced half-life, because they do not include complexes bound to easily dissociable and replaceable low-affinity peptides.<sup>40</sup> This scenario is also explained by the fact that empty and fully conformed MHC-I complexes use

distinct recycling routes resulting in a higher rate of open MHC-I conformer internalization.<sup>41</sup> Therefore TAP-deficient cells would essentially harbour particularly unstable truly empty MHC-I molecules that can be readily stabilized by encountering exogenous peptide, which might be sufficient to support CD8<sup>+</sup> T cell survival and activation.

Patients affected by TAP syndrome usually suffer from recurrent bacterial infections of the upper respiratory tract such as chronic purulent rhinitis, sinusitis and otitis media during the first 6 years of life.<sup>20–22</sup> Interestingly, necrotizing granulomatous lesions, which strictly resemble Wegener's granulomatosis, are often detectable in paranasal sinuses of these patients.<sup>18</sup> Involvement of the lower respiratory tract typically manifests in the second decade of life with recurrent spastic bronchitis, bronchiolitis, bacterial pneumonia and eventually bronchiectasis leading to a clinical scenario that resembles chronic granulomatous disease. Severe viral infections are noticeably absent and normal titres of antibodies against viruses are present in the blood.<sup>19–22</sup> These clinical manifestations would imply a B cell or phagocyte rather than CD8<sup>+</sup> T-cell defect. As in TAP syndrome titres of antibodies towards common viruses and total immunoglobulin levels are typically normal, or even increased,<sup>20,22</sup> a humoral defect can be reasonably excluded. Here we showed that the number of *in situ* macrophages in *eightless* mice is reduced, and that during inflammation monocyte precursors are recruited normally into their peritoneum, where the newly generated macrophages die prematurely. These results indicate that the clinical symptoms of TAP2 deficiency can result from a macrophage survival defect. To our knowledge any specific alteration in the phagocyte cellular system in patients or in animal models of TAP deficiency has never been previously reported.

Nevertheless, the mechanisms behind the limited survival of these cells are unknown. Here we tested the possibility that the death of macrophages observed in *eightless* mice is mediated by unresolved ER stress response due to the accumulation of improperly conformed MHC-I molecules in the ER. Our results indicate comparable ER stress responses in *eightless* and wild-type macrophages, both under steady state and upon activation. Therefore, chronic ER stress can be excluded as the mechanism governing TAP-deficient macrophages cellular death. Other possible explanations would be that the TAP complex directly supports macrophage survival, or that the cytosolic deposition and accumulation of peptides that cannot be transferred to the ER might be toxic to these cells.

To conclude, we report here for the first time the existence of a specific survival defect in TAP2-deficient macrophages. In addition, we present a novel unique animal model for human TAP syndrome consisting of a TAP2 gene stop codon that results in disruption of this

gene. Finally, this study implies that a better comprehension of TAP syndrome should include consideration of the innate branch of the immune system.

## Acknowledgements

This work was supported by grants from the Israel Science Foundation grant No. 1275/12, Israel Cancer Research Fund grant No. 13/726/RCDA, Marie Curie People grant No. 322006 and Concern Foundation.

## Disclosures

The authors declare no conflict of interest.

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