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Suppression of blastogenesis and proliferation of activated CD4$^+$ T cells: intravenous immunoglobulin (IVIg) *versus* novel anti-human leucocyte antigen (HLA)-E monoclonal antibodies mimicking HLA-I reactivity of IVIg

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Summary

Activated CD4$^+$ T cells undergo blastogenesis and proliferation and they express several surface receptors, including β2-microglobulin-free human leucocyte antigen (HLA) heavy chains (open conformers). Intravenous immunoglobulin (IVIg) suppresses activated T cells, but the mechanism is unclear. IVIg reacts with HLA-Ia/Ib antigens but its reactivity is lost when the anti-HLA-E Ab is adsorbed out. Anti-HLA-E antibodies may bind to the peptides shared by HLA-E and the HLA-I alleles. These shared peptides are cryptic in intact HLA, but exposed in open conformers. The hypothesis that anti-HLA-E monoclonal antibodies (mAbs) that mimic HLA-I reactivity of IVIg may suppress activated T cells by binding to the shared peptides of the open conformers on the T cell surface was tested by examining the relative binding affinity of those mAbs for open conformers coated on regular beads and for intact HLA coated on iBeads, and by comparing the effects on the suppression of phytohaemagglutinin (PHA)-activated T cells of three entities: IVIg, anti-HLA-E mAbs that mimic IVIg [Terasaki Foundation Laboratory (TFL)-006 and (TFL)-007]; and anti-HLA-E antibodies that do not mimic IVIg (TFL-033 and TFL-037). Suppression of blastogenesis and proliferation of those T cells by both IVIg and the anti-HLA-E mAbs was dose-dependent, the dose required with mAbs 50–150-fold lower than with IVIg. TFL-006 and TFL-007 significantly suppressed blastogenesis and proliferation of activated CD4$^+$ T cells, but neither the non-IVIg-mimicking mAbs nor control antibodies did so. The suppression may be mediated by Fab-binding of TFL-006/TFL-007 to the exposed shared peptides. The mAb binding to the open conformer may signal T cell deactivation because the open conformers have an elongated cytoplasmic tail with phosphorylation sites (tryosine^{320}/serine^{335}).

Keywords: activated T cells, blastogenesis, intravenous immunoglobulin (IVIg), open conformers, shared peptides

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Introduction

Therapeutic preparations of intravenous immunoglobulin (IVIg) are known to suppress activated CD4$^+$ T lymphocytes, and their production of proinflammatory cytokines, when the cells are activated by various stimuli *in vitro* [1–11]. The mechanism of suppression remains speculative and has not been elucidated because IVIg is purified from plasma pooled from thousands of donors and contains several undefined antibodies. Nevertheless, IVIg is administered post-transplantation to reduce T cell activation and proliferation (blastogenesis) in allograft recipients to suppress antigen-presenting T cells involved in the production of antibodies targeting allograft antigens, biopsy-proven T cell-mediated allograft rejection and T lymphoproliferative disorders emanating after transplantation.

Recently, we showed that different therapeutic preparations of IVIg contained IgG antibodies reacting to a wide array of human leucocyte antigen (HLA) class Ia molecules (HLA-A, HLA-B and HLA-Cw) and HLA class Ib molecules

(HLA-E, HLA-F and HLA-G) [12]. Most importantly, the therapeutic preparations of IVIg differed in their reactivity to two different kinds of beads: iBeads™ coated with HLA heterodimers, i.e. HLA associated with β2-microglobulin (β2m), known as 'intact HLA' and regular beads coated with intact HLA, but also with more of the β2m-free heavy chain (HC) of HLA, known as 'open conformers' [13]. All preparations of IVIg showed greatly reduced reactivity for iBeads, suggesting that they recognize the open conformers of all HLA class I alleles much better than they recognize intact HLA.

There was a further consideration, however. The HLA reactivity of IVIg was lost when the HLA-E antibody reactivity of IVIg was adsorbed out [12], suggesting that IVIg may bind to the peptide sequences shared between HLA-E and other HLA alleles (HLA-A, -B,-Cw, -F and -G). This hypothesis was verified when the HLA-E and HLA-Ia reactivity of anti-HLA-E polyreactive monoclonal antibodies (mAbs) was inhibited by a series of shared peptide sequences on HLA-E (e.g. ^{117}AYDGKDY^{123} and ^{126}LNEDLRSWTA^{135}), but not by HLA-E-specific peptides [14–18]. These shared peptide sequences not only inhibited binding of the anti-HLA-E mAbs to HLA-E, but also inhibited the entire HLA-Ia reactivity, confirming that they are indeed peptide sequences shared by all HLA class I alleles. However, the shared peptide sequences remain cryptic in their native conformation due to the presence of β2m, as was well illustrated in a previous report [Fig. 4 in (12)]. Until the late 1980s it was believed that HLA class I molecules occurred on cell surfaces only as heterodimers, but it is now recognized that the HCs of HLA-I molecules occur on cell surfaces – like those of activated T cells – as open conformers [13].

Interestingly, there have been a few reports that, as with IVIg, mAbs raised against HLA-Ia alleles also suppressed T cell proliferation [19–22], T cell activation [20], interleukin (IL)-2 and IL-2R synthesis [22], and were capable of inducing apoptosis [23]. These reports did not identify the specific epitopes or amino acid sequences recognized by the anti-HLA-I mAbs. However, we hypothesized that some anti-HLA-E mAbs are not only capable of replicating the HLA class I reactivity of IVIg, but may also recognize the shared peptides on the open conformers specifically over-expressed on the cell surface of activated CD4+ T lymphocytes [24–32].

To test this hypothesis, mAbs directed against the exposed shared epitopes of open conformers common to all HLA-Ia and -Ib molecules were generated by immunizing mice with the open conformers of HLA-E. After ascertaining the reactivity of these mAbs to HLA-Ia and -Ib alleles and confirming their reactivity to regular beads and iBeads, they were added to culture wells containing T cells activated by PHA-P (phytohaemagglutin-*Phaseolus*). In this comparative study, the effects of different preparations of IVIg and different kinds of anti-HLA-E mAbs on PHA-P-activated T cells were examined to elucidate the possible mechanism underlying the suppression of activated CD4+ T cells.

Materials and methods

Therapeutic preparations of IVIg

The therapeutic IVIg preparations from three sources were used. They included GamaSTAN™ S/D (15–18 protein %; Talecris Biotherapeutics, Inc., Research Triangle Park, NC, USA), Octagam® (6 gr%, lot A913A8431; Octapharma Pharmazeutika, Lachen, Switzerland) and IVIGlob EX (5 gr%; VHB Life Sciences Limited, Mumbai, India). All IVIg preparations were serially diluted with phosphate-buffered saline (PBS) (pH 7·2); the protein concentrations after dilution may vary depending upon the original protein concentration of therapeutic preparations of IVIg.

Monoclonal antibodies used for *in-vitro* suppression of activated T cells

These mAbs were produced by immunization with β2m-free heavy chains (open conformers) of two different HLA-E alleles (HLA-E^{R107} and HLA-E^{G107}). The recombinant peptide heavy chains [10 mg/ml in 2-(N-morpholino)ethanesulphonic acid (MES) buffer] were obtained from the Immune Monitoring Laboratory (Fred Hutchinson Cancer Research Center, Seattle, WA, USA). Each antigen was immunized in two different mice, as detailed elsewhere [12]. The monoclonal antibodies, called 'TFL' mAbs in this study, were formerly called the 'PTER' series [12]. Three different kinds of anti-HLA-E mAbs were used. As shown in Table 1a, eight types of anti-HLA-E mAbs with differing reactivity for different HLA class Ia alleles (HLA-A, -B and -Cw) and HLA class Ib alleles (HLA-E, -F and -G) were generated. Of these, we used three different types: the one comprising TFL-033 (type 1), which is monospecific for HLA-E (the peptide-binding domain of this mAb is identified by inhibiting the mAb by HLA-E-restricted peptide sequences located on the α1 and α2 helices ^{65}RSARDTA^{71} and ^{143}SEQKSNDASE^{152}) [33]; one comprising TFL-037 (type 5), which reacts with HLA-E, but not with HLA-F or HLA-G, and also with the classical HLA class Ia alleles; and one comprised of TFL-006 and TFL-007 (type 8) which, like IVIg, reacts with all the classical HLA class Ia and non-classical HLA class Ib alleles (the peptide binding domain of this group's mAbs is identified by the inhibition of the mAb by peptide sequences of HLA-E shared with several HLA class Ia alleles, e.g. ^{117}AYDGKDY^{123} and ^{126}LNEDLRSWTA^{135}), but not by other peptide sequences [14–16]. These earlier reports show that the polyreactivity is not targeted at other motifs. Figure 1a,b shows that the shared peptide sequences are masked by β2m.

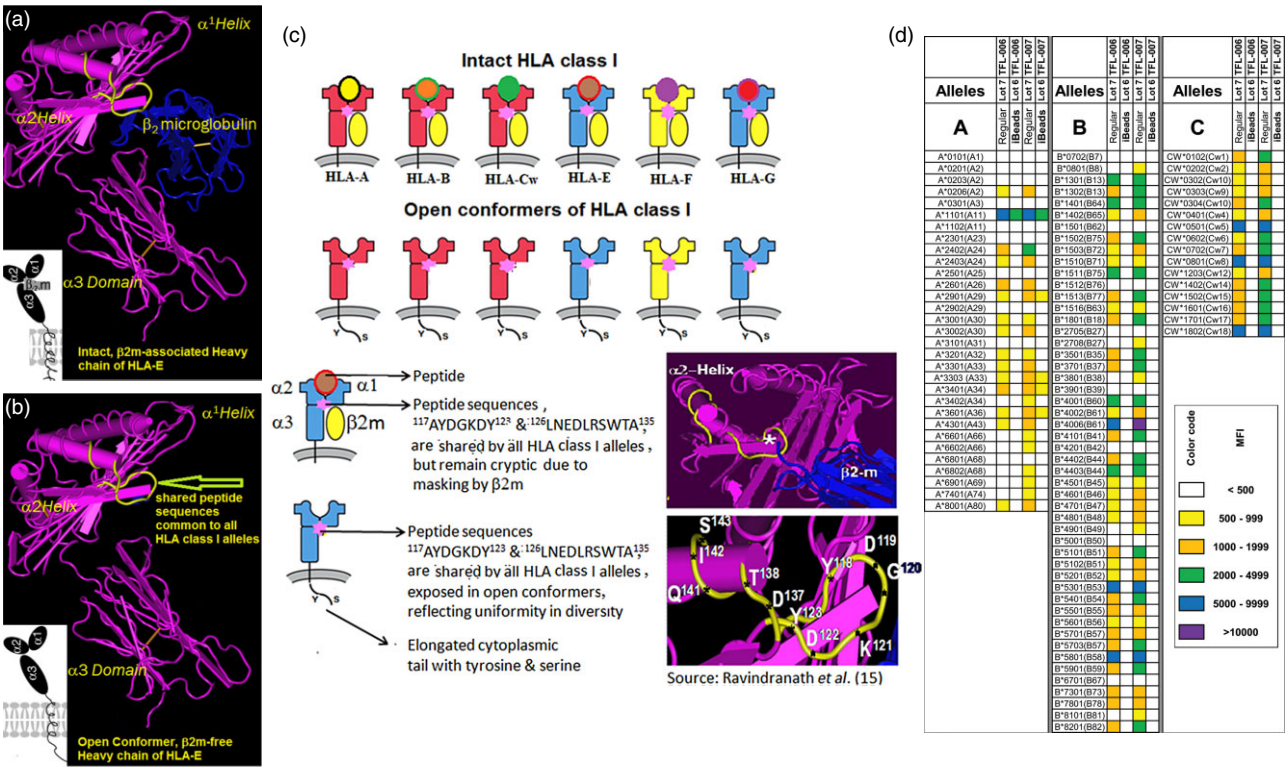

Fig. 1. Dual expression of human leucocyte antigen (HLA) class I molecules on the cell surface of activated T lymphocytes. (a) An intact HLA class I occurs as HLA heavy chain (HC) polypeptides associated with β2-microglobulin (β2m). (b) On the cell surface of activated lymphocytes, HLA class I molecules also occur as open conformers or β2m-free HC. All HLA class I alleles share certain amino acid sequences, notably ^{117}AYDGKDY^{123} and ^{126}LNEDLRSWTA^{135}. Although the shared peptides are present in the HC (shown in yellow), they are cryptic in native conformation due to masking by β2m. In open conformers, the shared peptide sequences are exposed to interact with other cell surface ligands and antibodies. (c) Diagrammatic comparison of intact and open conformers of different HLA class Ia (classical) and Ib (non-classical) alleles. (d) Anti-HLA-E monoclonal antibodies (mAbs) Terasaki Foundation Laboratory (TFL)-006s and TFL-007s recognized [as determined by mean fluorescence intensity (MFI)] regular HLA beads coated with both β2m-associated and β2m-free HCs of HLA (open conformers) but not iBeads, on which β2m-associated intact HLA predominated. These mAbs did not recognize intact HLA (as shown in Fig. 1a,c), but only β2m-free open conformers. The only HLA allele recognized by the mAbs on the iBeads was A*1101. We ascribe that recognition to the presence of more open conformers of HLA on the regular Beads than β2m-associated HLA. Intravenous immunoglobulin (IVIg) also recognized regular beads more readily than it did iBeads, but different therapeutic preparations differed in their MFI intensity [12]. As negative control, we used culture media that was negative for both regular beads and iBeads.

Two preparations of each mAb were used to suppress activated T cells. The first consisted of culture supernatants from the clones cultured in a medium containing RPMI-1640 w/L-glutamine and sodium bicarbonate (cat. no. R8758; Sigma-Aldrich, St Louis, MO, USA), 15% fetal calf serum (FCS) 0·29 mg/ml L-glutamine/Penn-Strept (cat. no. 400-110; Gemini-Bio, MedSupply Partners, Atlanta, GA, USA) and 1 mM sodium pyruvate (cat. no. S8636; Sigma). Five hundred μl aliquots of the supernatants stored at −20°C were used in the investigation. Isotypes of all the mAbs were characterized, and no IgM antibodies were detected. The second preparation consisted of ascitic fluid; the ascites were produced in Balb/c mice. Four million cells were injected per mouse into the peritoneal cavity of each mouse, with the ascites recovered after 10 days. Approximately 5–6 ml of ascites were collected, centrifuged and stored at −20°C as 500 μl aliquots.

The IgG in the culture supernatant (400 μl) and ascites (150 μl mixed with 150 μl of PBS pH 7·0) were purified by passing each aliquot of supernatant or ascites through a Protein G Spin Kit (0·2 ml, cat. no. 89949; Thermo Scientific, Waltham, MA, USA). The TFL IgG mAbs were purified with both commercial and in-house buffers. The commercial buffer contained 0·02% sodium azide, so the mAbs were either used after extensive dilution, as recommended [34–36] in the initial experiments, or the interference of sodium azide was avoided completely by using in-house buffers (binding buffer, 50 mM sodium acetate, pH 7; elution buffer, 100 mM glycine, pH 2·8; and neutralizing buffer, 1 M Tris-HCl, pH 8·5) without any NaN_3.

The purified antibodies were lyophilized overnight at 37°C, using Speedvac (Thermo Scientific). The purified culture supernatants were tested for HLA reactivity. The protein concentrations of the mAbs were measured with a

BioPhotometer (Eppendorf, Hauppauge, NY, USA). In particular, the purified mAbs were diluted with AIM-V® medium (cat. no. 12055-083; Life Technologies-Gibco, Grand Island, NY, USA) with 1% HEPES, with an optimal pH range between 7·2 and 7·5. Sodium azide-free purified and lyophilized ascites and purified culture supernatants were used to study the immunomodulation of T cells.

Assessment of HLA-reactivity of anti-HLA-E mAbs

Multiplex Luminex®-based immunoassay was used [12,14–18] to measure the mean fluorescent intensity (MFI) of culture supernatants, ascites, IVIg and purified anti-HLA-E mAbs (the latter two at different dilutions). Using dual-laser flow cytometry (Luminex xMAP® multiplex technology), the single antigen assays were carried out for data acquisition and analysis of anti-HLA-Ia and anti-HLA-E antibodies. The LABScreen® Single Antigen assay (One Lambda, Inc., Canoga Park, CA, USA) consists of a panel of colour-coded microspheres (single antigen beads), coated with HLA antigens to identify antibody specificities. The single recombinant HLA-Ia antigens in the lot used contained 31 HLA-A, 50 HLA-B and 16 HLA-Cw allelic molecules. The array of HLA alleles on the beads is listed in the product information at http://www.onelambda.com. The HLA-I microbeads have built-in control beads: positive control beads coated with human IgG and negative control beads coated with serum albumin. Mean and standard deviation (s.d.) of MFI for each allele were recorded as .csv files.

Distinguishing β2m-associated intact HLA from β2m-free open conformers

The beads supplied by the manufacturer have two categories of proteins attached to them: β2m-free HLA heavy-chain polypeptide and heavy-chain polypeptide in association with β2m. Realizing the heterogeneity of proteins, the manufacturer has recently developed iBeads (provided to us as Felix beads for in-house experimental use), which are regular HLA-Ia antigen-coated microbeads subjected to proprietary enzymatic treatment to remove or reduce the amount of open conformers, referred to by the manufacturer as 'denatured antigens'. The MFI values in Fig. 1d reflect the correction of the experiments' trimmed mean values against negative control after normalization.

Isolation and processing of T lymphocytes

On the day of the experiments, 60 ml of whole blood from a healthy normal male donor was collected after obtaining the donor's consent and Institutional Review Committee approval. Human T lymphocytes were isolated from the whole blood, using Ficoll™-Hypaque (GE Healthcare Bio-Sciences Corp., Piscataway, NJ, USA) to recover peripheral blood mononuclear cells (PBMCs), and using Lympho-Kwick® (One Lambda) to recover lymphocytes. The isolated lymphocytes were separated into two lots, one treated with phytohaemagglutinin (PHA) (cat no. L-1800-100; EY Laboratories Inc., San Mateo, CA, USA) at a final concentration of 2·25 μl/ml, the other with no PHA, and both cultured in 96-well tissue culture plates containing AIM-V medium with 1% HEPES, optimal range of pH between 7·2 and 7·5. IVIg or mAbs were added to cells in culture within 2 h of adding PHA (the total volume was adjusted to 200 μl), based on an earlier report that evaluated the effects of time difference in the addition of IVIg and other toxins after adding PHA [5]. The timing of adding PHA is important, as a kinetic and stereological study on activation of human T lymphocytes established that [^{3}H]-leucine uptake started as early as 12 h after culture initiation [37]. Furthermore, the uptake is associated with protein synthesis and accumulation, a process that occurs during the second phase of T cell activation after the change in membrane lipid and cytoskeleton of T cells [38–40].

The number of cells after PHA activation or exposure to IVIg or mAbs was measured by flow cytometry after staining the cells with PE-labelled anti-CD4 and peridinin chlorophyll (PerCP)-labelled anti-CD3 and anti-CD8 mAbs. Fig. 2a presents a typical profile of CD3+ T lymphocyte categories and numbers in cultures after 72 h of exposure to PHA-P or only AIM-V medium (1% HEPES); this shows the population of activated CD4+ T lymphocytes. Both lots were exposed to IVIg or anti-HLA-E mAbs.

Measurement of blastogenesis and proliferation of PHA-activated T cells

Blastogenesis of PHA-activated T cells was determined by counting the lymphoblasts after culturing purified lymphocytes from donors for 72 h with or (as control) without PHA. Lymphoblasts were recognized by flow cytometry based on size (side-scatter) and granularity (forward-scatter). In the tables and figures of flow cytometric profiles, three groups of T cells were identified for CD4+ and CD8+ T lymphocytes: groups 1 and 2, which comprise subsets of resting lymphocytes, and group 3, which comprises lymphoblasts.

The proliferation assay is based on labelling the purified lymphocytes during PHA activation with the intracellular fluorescent dye carboxyfluorescein succinimidyl ester (CFSE: $C_{25}H_{15}NO_9$; mol. mass: 473·39 g/mol) and, using flow cytometry, measuring mitotic activity by the successive twofold reductions in fluorescent intensity of the T cells placed in culture for 72 h [38]. CFSE is cell-permeable, and is retained for long periods within cells by covalently coupling by means of its succinimidyl group to intracellular molecules. Due to this stable linkage, once incorporated within cells, CFSE is not transferred to adjacent cells, but remains in the cell even after several mitotic divisions. After 72 h, the labelling of the cells was measured: PHA-treated T

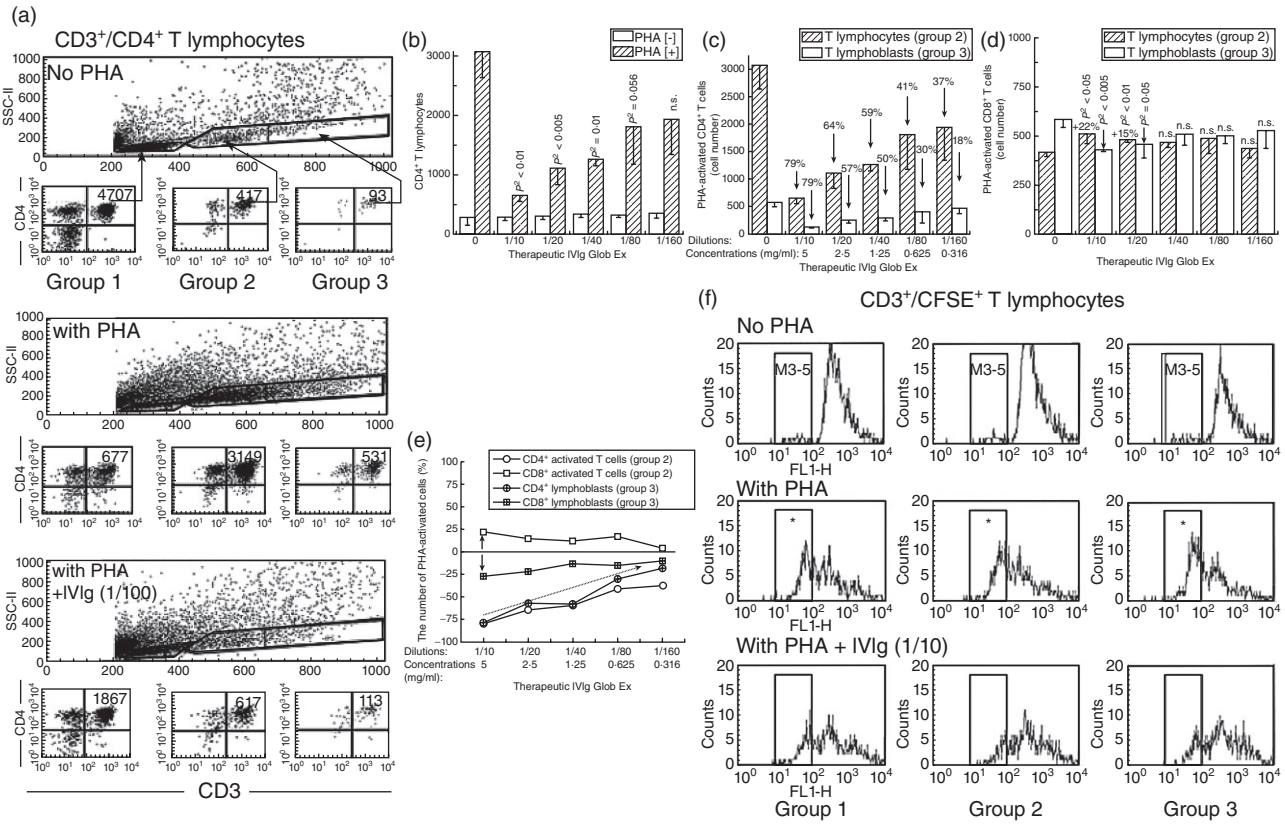

Fig. 2. Dose-dependent inhibition of phytohaemagglutinin (PHA)-activated CD3$^+$/CD4$^+$ T cells *in vitro* by a therapeutic preparation of intravenous immunoglobulin (IVIg) (GlobEx). (a) Flow cytometric profile of CD3$^+$ T cells and gating of CD4$^+$ T cells from a normal non-alloimmunized male (donor R). The lymphocytes were untreated as control, and treated as noted. The three sets of one large panel with three smaller panels show the profiles of CD3$^+$/CD4$^+$ T lymphocytes 72 h after culture without PHA (top set), after treatment with PHA (middle set) and after exposure to a therapeutic preparation of IVIg (GlobEx) and PHA (lower set). In each set, the large rectangular box shows the overall distribution patterns of CD3$^+$/CD4$^+$ T lymphocytes, which are divided into three groups based on CD4$^+$ staining and size of cells to illustrate the differences in the CD4$^+$ T cell population and number of events. Groups 1 and 2 are resting populations of CD4$^+$ lymphocytes, whereas group 3 comprises lymphoblasts based on size and granularity. Note that the number of cells increased in both groups 2 and 3 after PHA treatment, reflecting their activation by PHA treatment. The bottom set of panels illustrates the impact of IVIg treatment on PHA-activated T cells. A comparison of the number of cells in groups 2 and 3 – between treatment with only PHA and with both PHA and IVIg – shows a marked decline in the number of CD4$^+$ T cells after IVIg treatment. (b) Dose-dependent suppression of the activated total population of CD3$^+$/CD4$^+$ T lymphocytes by a therapeutic preparation of IVIg (GlobEx) in cultures with and without PHA. Examination was in triplicate after 3 days in the presence or absence of IVIg at different dilutions. The number of PHA-activated CD4$^+$ T lymphocytes – but not the number of those control cells unexposed to PHA – decreased significantly (*P*-values above the bars) with the IVIg dilutions shown, except that the decrease with 1/160 dilution was not significant. Little change was observed in the control cells treated similarly with IVIg. (c) Dose-dependent suppression of both PHA-activated CD4$^+$ T lymphocytes (group 2) and CD4$^+$ T lymphoblasts (group 3) by the same therapeutic preparation of IVIg. (d) The therapeutic preparation of IVIg suppressed PHA-activated CD8$^+$ T lymphoblasts (group 3) significantly (*P*-values indicated) only at dilutions of 1/10 and 1/20 but did not affect group 2 T lymphocytes. The population of CD8$^+$ T cell cultures with and without PHA was examined in triplicate after 3 days in the presence or absence of IVIg at different dilutions. (e) A comparison of changes in the number of PHA-activated CD4$^+$ and CD8$^+$ cells among the T lymphocytes and T lymphoblasts showed that the effect of IVIg on CD4$^+$ was more striking than on CD8$^+$ T cells. (f) The same therapeutic preparation of IVIg suppressed proliferation of PHA-activated CD3$^+$ T lymphocytes. Proliferation of carboxyfluorescein succinimidyl ester (CFSE)-labelled cells was examined in triplicate 3 days after treatments. The three rows provide a representative profile of the changes that occurred after incubating cells with CFSE when the cells were treated as noted. The IVIg was GlobEx. CFSE permeates the cells and is retained for more than 72 h. The left side in each box is indicated by a column marked in the upper row as 'M3/4' for mitoses 3 and 4, with mitosis 5 to its left; the right side of each box represents mitoses 1 and 2 (M1/2). The number of cells in each column was measured and compared for each treatment and group of T lymphocytes. Group 1 comprises resting T cells; group 2, T lymphocytes; and group 3, T lymphoblasts. In the top row (no PHA), the number of cells in the M3/4 column is very meagre in all three controls. The middle row (the PHA-treated groups) shows a quite opposite picture: cell numbers in in all three M3/4 columns are high, suggesting that cell proliferation occurred in all three T cell populations. The bottom row shows the impact of adding IVIg: the number of cells in the M3/4 column is highly reduced compared with that in the corresponding PHA-only columns, suggesting suppression of proliferation by IVIg. Decrease in the number of cells number in the right, M1/2, side of the boxes is a consequence of proliferation of CFSE-labelled cells; therefore, their migration to the left represents cells that underwent mitosis. All told, the data in this figure show that IVIg at the tested dilutions suppresses cell proliferation of PHA-activated CD3$^+$ T lymphocytes and lymphoblasts.

cells undergo, on average, four to six divisions. Most importantly, the suppressive effects of the agents used can be visualized by cessation of progression of mitotic activity, as measured by the successive twofold reductions in the fluorescent intensity after 72 h of treatment.

HLA molecular typing

DNA from the blood cells of donors was isolated by using the QIAamp DNA Mini Kit (Qiagen, Inc., Valencia, CA, USA). Extracted DNA was then PCR-amplified and typed using a LABType® SSO Typing Test Kit (One Lambda). Table 1b details the HLA typing of the T lymphocyte donors. The purpose of typing was to determine whether IVIg or anti-HLA-E mAbs (types 5 or 8) could recognize these typed alleles that occur as open conformers on activated T cells. Table 1b shows the donor HLA types that were recognized by TFL-037, TFL-006 and TFL-007 in square brackets ([ ]) after the alleles.

Results

Parallel diversity in HLA-Ia/-Ib reactivity of IVIg and anti-HLA-E mAbs TFL-006 and TFL-007

The HLA-Ia and -Ib reactivity of IVIg and two different anti-HLA-E mAbs were presented in our earlier report [12]. A comparison of the HLA reactivity of different therapeutic preparations of IVIg with that of mAbs TFL-006 and TFL-007 and other anti-HLA-E mAbs used in the present study are presented in Tables 1a,b. Table 1a provides a classification of mouse mAbs generated after immunizing the mice with β2m-free heavy chains (open conformers) of two different HLA-E alleles (HLA-E^{R107} and HLA-E^{G107}). Table 1b compares the number of HLA alleles recognized by the different preparations of IVIg and by the anti-HLA-E mAbs TFL-006, -007, -033 and -037; specific alleles were coated individually on microbeads.

In Table 1b, type 1 comprises monospecific mAbs, of which TFL-033 was studied in detail to show that it recognized HLA-E-restricted peptide sequences [33]. Neither the purified culture supernatants nor purified ascites of TFL-033 reacted with HLA-A,-B or -Cw or with HLA-F or -G at any of the dilutions tested. TFL-033 was inhibited by two HLA-E-restricted peptides, ^{65}RSARDTA^{71} and ^{143}SEQKSNDASE^{152}, but ^{143}SEQKSNDASE^{152} showed dose-dependent inhibition of TFL-033s binding to the HLA-E coated on the microbeads [33]. These two HLA-E-restricted peptide sequences were found on the surface of α1 (^{65}RSARDTA^{71}) and α2 (^{143}SEQKSNDASE^{152}) helices on the HC of intact HLA-E (Fig. 1a).

In striking contrast, type 8, which comprised the purified culture supernatants and purified ascites of mAbs TFL-006 and TFL-007, reacted with HLA-A, -B, -Cw, -E, -F and -G, suggesting that the mAbs recognized peptide sequences shared between HLA-E and HLA-A, -B, -Cw, -F and -G, as seen in the β2m-free HC of HLA-E (shown in yellow, Fig. 1b and as pink star in Fig. 1c). We reported earlier the inhibition of polyspecific HLA-E mAbs by these shared peptide sequences. The peptide binding domain of this group's mAbs was identified by inhibiting the mAb by peptide sequences of HLA-E shared with several HLA class Ia alleles, e.g. ^{117}AYDGKDY^{123} and ^{126}LNEDLRSWTA^{135} ([14–16]; see insert in Fig. 1c). In the present investigation, we compared the relative affinity of the TFL mAbs that mimic IVIg for regular beads and for iBeads. The HLA-Ia alleles on regular beads occur both as intact HLA (with β2m) and as β2m-free HCs, i.e. open conformers. Intact HLA predominates on iBeads, and they are considered to be restricted to intact HLA, the presence of open conformers so scant it can essentially be ignored. Figure 1d shows that both TFL-006 and TFL-007 had distinctly different reactivity to regular beads and iBeads. Strikingly, both of the anti-HLA-E mAbs showed reactivity to the regular beads and absence of reactivity to iBeads, except for reduced reactivity to HLA-A*1101, establishing the affinity of both TFL-006 and TFL-007 for the open conformers of all HLA-Ia.

The mAb TFL-037 is in a different category that, while also polyreactive, did not react with HLA-F or HLA-G, and therefore may not bind to the same shared peptide sequence as do TFL-006 and TFL-007. The peptide sequence of mAb TFL-037 remains to be defined.

Table 1c compares the HLA reactivity of the four mAbs with that of different therapeutic preparations of IVIg. Two different preparations of IVIg (GlobEx and Octagam) and the four different mAbs were used in this investigation to suppress proliferation of PHA-activated CD4+ T lymphocytes from two normal non-alloimmunized human males, donors R and J, R being about 30 years older than donor J.

IVIg suppressed blastic transformation of PHA-activated T cells

The efficacy of IVIg (GlobeEx) in suppressing PHA-activated CD4+ T lymphocytes is shown in Fig. 2a–f. Three major subpopulations of CD4+ T cells can be seen in fluorescence activated cell sorter (FACS) analysis (Fig. 2a), based on the forward- and side-scatters of the lymphocytes, which essentially distinguish the size of the cell population. The three subpopulations are designated group 1 (resting), group 2 (resting and possibly naive T lymphocytes) and group 3 (T lymphoblasts). In all groups, the density of cell population changed markedly depending on treatment. The number of group 2 T lymphocytes and T lymphoblasts increased after PHA treatment, the number of CD4+ T lymphoblasts increasing five- to sixfold over those that were not treated with PHA, and decreasing fivefold after treatment with IVIg. The number of activated T lymphocytes, but not of those unexposed to PHA, decreased significantly in a dose-dependent manner when treated with IVIg

Table 1. Monoclonal anti- human leucocyte antigen (HLA)-E monoclonal antibodies (mAbs) ($n > 150$) generated after immunizing the mice with open conformer of HLA-E.

(a) Based on the differences in their HLA class Ia and Ib reactivity, the anti-HLA-E mAbs are categorized into eight types. Type 1 [Terasaki Foundation Laboratory (TFL)-033] reacted only with HLA-E and not with any other HLA class I alleles; type 5 (TFL-037) did not recognize HLA-F and HLA-G; and type 8 (TFL-006 and TFL-007) reacted with all HLA class Ia and Ib alleles. The common shared peptide sequences are exposed in the open conformer in the absence of β2-microglobulin [15]

	Non-classical HLA-Ib			Classical HLA-Ia			
Types of anti-HLA-E mAbs	HLA-E	HLA-F	HLA-G	HLA-A	HLA-B	HLA-Cw	Names of mAbs
Type 1	+	−	−	−	−	−	TFL-033
Type 2	+	+	−	−	−	−	
Type 3	+	−	+	−	−	−	
Type 4	+	+	+	−	−	−	
Type 5	+	−	−	+	+	+	TFL-037
Type 6	+	+	−	+	+	+	
Type 7	+	−	+	+	+	+	
Type 8	+	+	+	+	+	+	TFL-006 & TFL-007

(b) Reactivity (determined by mean fluorescent intensity, MFI) of the undiluted culture supernatants of the mAbs on different human leucocyte antigen (HLA) class I alleles. Note that TFL-033 and TFL-037 did not recognize the heavy chains of HLA-F and HLA-G. The differences in MFI among the mAbs are critical to an understanding of the possible binding site of the mAbs on the cell surface of activated T lymphocytes

	TFL-033	TFL-037	TFL-006	TFL–007		TFL-033	TFL-037	TFL-006	TFL–007		TFL-033	TFL-037	TFL-006	TFL-007
Isotype	IgG1	IgG2b	IgG2a	IgG2a		IgG1	IgG2b	IgG2a	IgG2a		IgG1	IgG2b	IgG2a	IgG2a
Neg	3	8	15	7	B*0702(B7)			1 331	841	CW*0102(Cw1)		674	7 242	3 268
Pos	71	103	88	85	B*0801(B8)			2 092	1 033	CW*0202(Cw2) [J]		2 662	10 690	6 084
HLA-E	24 411	13 025	22 522	21 618	B*1301(B13)		3 684	5 654	3 979	CW*0302(Cw10)		1 321	5 917	3 062
HLA-F			12 650	11 035	B*1302(B13)		1 568	2 237	1 426	CW*0303(Cw9)		2 212	7 114	4 250
HLA-G			7 193	2 670	B*1401(B64)		9 268	11 319	8 767	CW*0304(Cw10)		1 846	6 584	3 891
A*0101(A1)			2 395	1 037	B*1402(B65)		1 492	4 414	2 558	CW*0401(Cw4)			2 843	1 272
A*0201(A2)			856		B*1501(B62) [R]			1 097		CW*0501(Cw5)		10 015	16 131	13 096
A*0203(A2)			1 095		B*1502(B75)		5 150	6 256	4 497	CW*0602(Cw6)		1 060	9 396	4 274
A*0206(A2)			1 494	843	B*1503(B72)		1 016	2 831	1 926	CW*0702(Cw7)		753	12 251	6 919
A*0301(A3)			818		B*1510(B71)			2 616	1 470	CW*0801(Cw8)		7 709	13 456	10 733
A*1101(A11) [J,R]		7 062	10 190	8 476	B*1511 (B75)		5 715	9 041	5 902	CW*1203(Cw12)			5 055	2 102
A*1102(A11)			860		B*1512(B76)		956	1 624	996	CW*1402(Cw14)		2 129	8 727	4 936
A*2301(A23)			614		B*1513(B77)		4 650	5 326	3 365	CW*1502(Cw15) [R]		1 574	6 030	3 225
A*2402(A24) [J]		1 287	3 133	2 011	B*1516(B63)		3 591	5 614	3 443	CW*1601(Cw16)		1 109	8 462	4 364
A*2403(A24)		1 005	3 151	1 967	B*1801(B18)		4 185	6 990	4 890	CW*1701(Cw17)		1 433	13 521	9 069
A*2501(A25)			1 230	692	B*2705(B27)		702	2 591	1 576	CW*1802(Cw18)		15 389	17 918	15 207
A*2601(A26)			3 368	1 638	B*2708(B27)		1 438	4 437	2 671	Cw* alleles	0	14	16	16
A*2901(A29)		1 465	3 194	2 256	B*3501(B35)		8 235	10 205	8 594					
A*2902(A29)			2 235	1 136	B*3701(B37)		3 039	6 472	4 338					
A*3001(A30)			2 229	1 237	B*3801(B38)			3 844	1 820					
A*3002(A30)		994	3 353	2 211	B*3901(B39)		4 068	7 093	5 304					
A*3101(A31)			858		B*4001(B60)		2 518	5 743	3 758					
A*3201(A32)		1 053	2 237	1 508	B*4002(B61)		4 032	6 118	4 675					
A*3301(A33) [R]		928	2 791	1 627	B*4006(B61)		14 399	15 643	13 758					
A*3303(A33)		2 215	4 212	2 961	B*4101(B41)		3 721	7 191	5 277					

A*3401(A34)		1 643	6 268	3 968	B*4201(B42)		636	
A*3402(A34)			1 399	893	B*4402(B44)	1 342	7 062	4 059
A*3601(A36)		1 694	5 806	3 826	B*4403(B44)	4 459	7 256	5 638
A*4301(A43)		789	4 420	2 364	B*4501(B45)	5 725	9 535	7 646
A*6601(A66)			3 644	1 526	B*4601(B46)	3 924	6 491	4 130
A*6602(A66)			1 395	789	B*4701(B47)	2 152	6 528	3 895
A*6801(A68)			1 314	859	B*4801(B48)	1 254	4 365	2 716
A*6802(A68)			2 078	1 276	B*4901(B49)			
A*6901(A69)			1 964	917	B*5001(B50)		741	
A*7401(A74)			723		B*5101(B51)	1 634	6 205	3 724
A*8001(A80)			2 841	1 430	B*5102(B51)	2 082	5 251	3 579
A* alleles	0	11	32	24	B*5201(B52)	1 538	4 524	2 728
					B*5301(B53)	6 270	8 807	7 323
					B*5401(B54)	3 122	5 556	4 153
					B*5501(B55)	1 326	2 829	1 887
					B*5601(B56)		1 386	777
					B*5701(B57)			
					B*5703(B57)		1 229	600
					B*5801(B58) [R]	7 525	10 160	8 047
					B*5901(B59)	1 211	5 646	3 001
					B*6701(B67)		675	
					B*7301(B73)	1 271	3 347	2 171
					B*7801(B78)	3 373	6 089	4 597
					B*8101(B81)		1 352	729
					B*8201(B82)	2 195	4 367	3 069
					B* alleles	0 37	48	44

Letters J and R after HLA class I alleles refers to the typing of the donors who have provided T cells for this study.

(c) The number of HLA-A, HLA-B, HLA-Cw alleles recognized by type 8 mAbs TFL-006 and TFL-007 compared with the number of HLA alleles recognized by different therapeutic preparations of intravenous immunoglobulin (IVIg)

	Reactivity of different HLA class I antigens					
	Classical HLA-Ia alleles			Non-classical HLA-Ib		
IVIgs *versus* TFL mAbs	HLA-A	HLA-B	HLA-Cw	HLA-E	HLA-F	HLA-G
IVIg (GamaStan, USA)	31	49	16	+	+	+
IVIg (Octagam, Mexico)	30	47	16	+	+	+
IVIg (GlobEx, India)	20	39	16	+	+	+
TFL-006 (formerly PTER006)	31	48	16	+	+	+
TFL-007 (formerly PTER007)	26	44	16	+	+	+
TFL-037 (formerly PTER037)	11	37	14	+	0	0
TFL-033 (formerly PTER033)	0	0	0	+	0	0

Table 2. Dose-dependent inhibition of phytohaemagglutinin (PHA)-activated CD4$^+$ and CD8$^+$ T lymphoblasts *in vitro* by a therapeutic preparation of intravenous immunoglobulin (IVIg) (Octagam).

Octagam-IVIg	T lymphoblasts							
	Blastogenesis			Proliferation				
				M1/2		M3–5		
	Mean	s.d.	P^2	Mean	s.d.	Mean	s.d.	P^2
CD4								
No PHA	46	8		73	17	1	1	
With PHA only	1685	89	**<0·0001**	338	15	1486	95	**<0·0001**
PHA + IVIg (1/10)	945	87	**0·0005**	405	5	675	68	**0·0003**
PHA + IVIg (1/20)	1365	100	**0·019**	432	23	1105	71	**0·005**
PHA + IVIg (1/40)	1796	81	n.s.	464	31	1540	43	n.s.
CD8								
No PHA	53	13		80	20	4	7	
With PHA only	1951	171	**<0·0001**	329	15	1751	193	**<0·0001**
PHA + IVIg (1/10)	1134	13	**0·001**	431	115	893	9	**0·002**
PHA + IVIg (1/20)	1717	198	n.s.	380	17	1596	46	0·005
PHA + IVIg (1/40)	2280	127	n.s.	419	61	2036	137	n.s.

Significant *P*-values are indicated in bold.

The lymphocytes were from a normal non-alloimmunized male (donor J). The values represent the mean of triplicate analyses ± standard deviation (s.d.) and two-tailed *P*-values. The table shows the flow cytometric profile of the number of CD4$^+$ and CD8$^+$ T lymphoblasts when untreated (as control), and when treated as noted. The 'Blastogenesis' column shows the number of CD4$^+$ or CD8$^+$ T lymphoblasts 72 h after culture. Note that IVIg significantly suppressed PHA-activated CD4$^+$ T lymphoblasts in a dose-dependent manner, whereas it suppressed PHA-activated CD8$^+$ T lymphoblasts only at a 1/10 dilution. The 'Proliferation' column shows the M1/2 and M3–5 numbers of CD4$^+$/carboxyfluorescein succinimidyl ester (CFSE)$^+$ and CD8$^+$/CFSE$^+$ T lymphoblasts with the same treatment pattern as above after exposure for 72 h. Note that IVIg significantly suppressed proliferation (as seen by the M3–5 numbers) of both PHA-activated CD4$^+$ and CD8$^+$ T lymphoblasts in a dose-dependent manner; n.s. = not significant.

(Fig. 2b). The significant dose-dependent decrease is seen most distinctly in group 2 (CD4$^+$ T lymphocytes) and group 3 (CD4$^+$ T lymphoblasts) (Fig. 2c). Interestingly, no such dose-dependent decrease is seen with the same groups of CD8$^+$ T cells (Fig. 2d) except for the CD8$^+$ T lymphoblasts of group 3, which showed a decrease only at higher concentrations of IVIg (dilutions of 1/10 and 1/20). The differences in the dosimetric effects of IVIg on CD4$^+$ and CD8$^+$ in group 2 (T lymphocytes) and group 3 (T lymphoblasts) are summarized in Fig. 2e. It is noteworthy that only PHA-activated CD4$^+$ T cells were suppressed significantly in a dose-dependent manner by IVIg.

IVIg suppressed proliferation of PHA-activated T cells

PHA activation involves proliferation of all subpopulations of CD3$^+$ T lymphocytes as seen in the shift of CFSE$^+$ cells from M1/2 to M3/5 (Fig. 2f). More than 95% of the CFSE$^+$ cells not treated with PHA remained in M1/2 and did not migrate to the M3/4 zone, whereas PHA treatment resulted in their migration (see the M3/4-zone boxes marked with asterisks in Fig. 2f), indicating that proliferation is the outcome of PHA activation. IVIg inhibited proliferation, as shown by the fewer number of cells in the M3/5 zone (Fig. 2f). At the same time, and in contradistinction for subpopulations not treated with PHA, the number of undivided cells (M1/2) did not remain high but declined sharply, suggesting that the cells had undergone cell death.

To validate these observations, we used another therapeutic preparation of IVIg (Octagam) to examine in triplicate analyses whether the proliferation of CD4$^+$ and CD8$^+$ T lymphoblasts was suppressed by IVIg in a dose-dependent manner (Table 2). Indeed, significant dose-dependent suppression of the number of PHA-activated CD4$^+$ T lymphoblasts occurred. There was also suppression of PHA-activated CD8$^+$ T lymphoblasts at a higher concentration of IVIg, but not dose-dependent suppression. PHA treatment resulted in significant migration of CFSE$^+$ CD4$^+$ and C8$^+$ T lymphoblasts to the M3/4 zone. Indeed, IVIg significantly inhibited both PHA-activated CD4$^+$ and CD8$^+$ T lymphoblasts.

Anti-HLA-E mAbs (TFL-006 and TFL-007) suppressed the blastic transformation of PHA-activated T lymphocytes

These mAbs that mimic the HLA-I reactivity of IVIg suppressed both PHA-activated CD4$^+$ and CD8$^+$ T lymphocytes. Suppression by TFL-007 is evident from a comparison in Fig. 3a–d of the flow cytometric profiles of CD4$^+$ and CD8$^+$ T lymphocytes treated only with PHA, and treated with PHA and TFL-007s at 1/10 and 1/100 dilutions. Note that TFL-007s at both dilutions significantly suppressed the number of both CD4$^+$ and CD8$^+$ T lymphoblasts (see the statistical profile on the sides of Fig. 3a), while no such decrease in cell population was

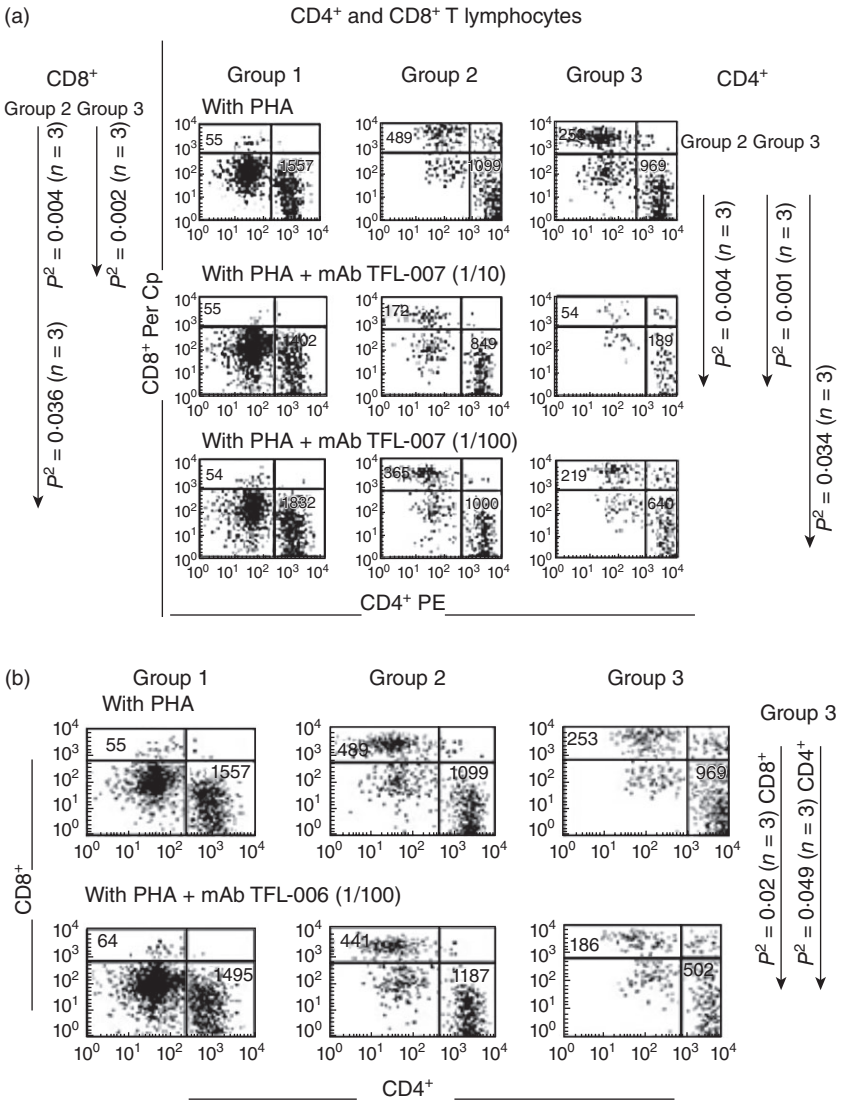

Fig. 3. Dose-dependent inhibition of phytohaemagglutinin (PHA)-activated CD4+/CD8+ T cells *in vitro* with two human leucocyte antigen (HLA) class I polyreactive anti-HLA-E monoclonal antibodies (mAbs), Terasaki Foundation Laboratory (TFL)-007s and TFL-006s; 's' = culture supernatants. The T cells were stained with phycoerythrin (PE)-labelled anti-CD4 mAbs (*x*-axis) and peridinin chlorophyll (PerCP)-labelled anti-CD8 mAbs (*y*-axis). The profile is divided into three groups based on staining and size of cells to illustrate the differences in the CD4+ and CD8+ T cell populations and number of events. Group 1 comprises resting CD4+ and CD8+ lymphocytes, group 2 resting CD4+ and CD8+ lymphocytes and group 3 CD4+ and CD8+ lymphoblasts. (a) Flow cytometric profiles of PHA-treated CD4+ T cells (lower right of the boxes) and CD8+ T cells (upper left) from a normal non-alloimmunized donor (R) after treatment with mAb TFL-007s. Comparison of groups 1, 2 and 3 in the top row (treated only with PHA) shows that the number of CD4+ T cells in all three groups and the CD8+ T cells in groups 2 and 3 were high. The middle row (PHA and mAb TFL-007s at 1/10 dilution) shows that the number of both CD4+ and CD8+ T cells have decreased significantly in groups 2 and 3 (note the statistical profile on the right side). In comparison, the bottom row with the same treatment, but at 1/100 dilution, shows a dose-dependent decrease in the number of PHA-activated CD4+ and CD8+ T lymphocytes. Figure represents one analysis. (b) Flow cytometric profiles of PHA-treated CD4+ T cells (lower right of the boxes) and CD8+ T cells (upper left) from the same donor after treatment with mAb TFL-006s. The top row (only PHA) shows that the number of CD4+ T cells and CD8+ T cells in groups 2 and 3 were higher than when the cells were treated with PHA and mAb TFL-006s at 1/100 dilution. Indeed, with that treatment, the number of both CD4+ and CD8+ T cells in group 3 decreased significantly (see the statistical profile). Figure represents one analysis. (c) The density of the population of CD4+ T cell cultures with and without PHA, and after adding TFL-037s at 1/10 dilution, TFL-006s (1/10) and TFL-007s (1/10 and 1/100) to the PHA-treated CD4+ T cells. The values are expressed as the mean of triplicate analyses ± standard deviation (s.d.) and two-tailed *P*-values. The two-tailed *P*-values, if significant, are indicated by a horizontal line connecting the two groups. Groups 1 and 2 comprise resting CD4+ lymphocytes, and group 3 CD4+ lymphoblasts. Note that all groups of the PHA-activated CD4+ T lymphocytes remained unaffected by treatment with mAb TFL-037s, and that only group 3 decreased because of treatment with mAb TFL-006s. Both PHA-treated groups 2 and 3 decreased significantly after treatment with mAb TFL-007s at 1/10, but only PHA-treated group 3 showed significant decrease after treatment with mAb TFL-007s at 1/100 dilution. Clearly, both anti-HLA-E mAbs TFL-006s and TFL-007s significantly suppress PHA-activated CD4+ T lymphoblasts, although the dosimetric suppression byTFL-007s is more obvious. (d) The density of the population of CD8+ T cell cultures with and without PHA, and after adding TFL-037s at 1/10 dilution, TFL-006s (1/10) and TFL-007s (1/10 and 1/100). The values are expressed as in (c) (triplicate analyses), with division into the same three groups, but of CD8+ cells. Note that all groups of the PHA-activated CD8+ T lymphocytes remained unaffected by treatment with mAb TFL-037s or mAb TFL-006s, but that the cell populations of both groups 2 and 3 decreased significantly after by treatment with mAb TFL-007s at both 1/10 and 1/100. The anti-HLA-E mAbs TFL-007s showed significant dose-dependent suppression of PHA-activated CD8+ T lymphoblasts.

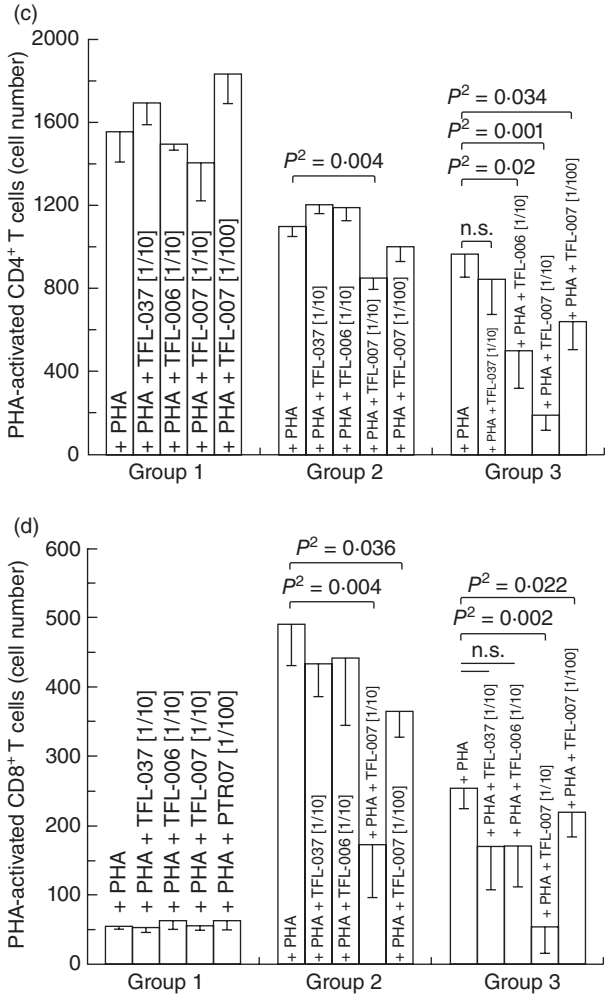

Fig. 3. *Continued*

observed for the resting cells. Importantly, the CD8$^+$ T lymphocytes showed significant dose-dependent decrease (Fig. 3d). Although the group 2 T lymphocytes also decreased, the decrease was significant only at 1/10.

The effect of anti-HLA-E mAb TFL-006s on PHA-treated T lymphocytes from the same donor was somewhat similar to that of TFL-007s (Fig. 3b–d). TFL-006s at 1/10 (Fig. 3c) and at 1/100 (Fig. 3b) dilution decreased the number of PHA-activated CD4$^+$ and CD8$^+$ T lymphoblasts. Further examination compared the effect of TFL-006s with that of TFL-006a, and found that both significantly suppressed both PHA-activated CD4$^+$ and CD8$^+$ T lymphoblasts in a dose-dependent manner (Table 3).

To validate these observations, we replicated the above experiments in triplicate with lymphocytes from another normal non-alloimmunized male (donor J) to study the effect on CD4$^+$ and CD8$^+$ T lymphoblasts by TFL-006a and TFL-007a after purifying IgG from the ascites (Table 4) As with donor R's T lymphocytes, the number of CD4$^+$ T lymphoblasts increased after PHA treatment, and when the PHA-activated CD4$^+$ T lymphoblasts were exposed to the TFL mAbs at the dilutions listed in the table, a significant dose-dependent suppression was observed. Again, no such suppression was evident for group 1. Note that this donor's group 2 subpopulation did not respond to PHA.

Anti-HLA-E mAbs (TFL-006 and TFL-007) suppressed proliferation of PHA-activated T cells

In Fig. 4a, CFSE-labelled lymphocytes cultured for 3 days without PHA were compared with those treated with PHA, those treated with PHA and mAb TFL-006s, and those treated with PHA and TFL-007s. Analysis was in triplicate. Proliferation was inferred when the number of cells decreased for mitoses 1 and 2 (M1/2) with a concomitant increase for mitoses 3–5 (M3–5). While the number of cells after M3–5 is very meagre when untreated with PHA, it is very high for M3–5 in PHA-treated groups, indicating that PHA significantly promoted T cell proliferation in all groups of CD4$^+$ T lymphocytes. In fact, treatment with PHA and TFL-007s, or with PHA and TFL-006s, reduced the number of CD4$^+$ T lymphoblasts in M3–5, indicating suppression of PHA-induced proliferation of CD4$^+$ T lymphoblasts. No such decrease was observed for groups 1 and 2 T lymphocytes. A decrease in the CD4$^+$ M1/2 cell population after treatment with TFL-007s or TFL-006s suggests possible cell death of T lymphoblasts after the addition of mAb. The suppression of CD4$^+$ T lymphoblast proliferation caused by mAb TFL-007s was significant and dose-dependent, as shown in Fig. 4b, which compares the number of CD4$^+$ T lymphoblasts that were not treated with PHA with their number when treated with PHA only, and when treated with PHA and TFL-007s at 1/10 and at 1/100 dilution. The mAb TFL-006s also suppressed proliferation of CD4$^+$ T lymphoblasts significantly (Fig. 4c) and in a dose-dependent manner (data not shown). This suppression of proliferation was observed for group 2 T lymphocytes after treatment with TFL-007s but not with TFL-006s. Anti-HLA-E mAb TFL-006s and TFL-007s also suppressed proliferation of PHA-activated CD8$^+$ T lymphoblasts (Fig. 5).

These experiments with T lymphocytes from another donor confirmed that proliferation of CD4$^+$ T lymphocytes and T lymphoblasts was indeed affected by anti-HLA-E mAbs TFL-006 and TFL-007. The increase observed in M3–5 after PHA activation shows that PHA activation involves proliferation. Examination of the effects of TFL-006a and TFL-007a confirmed that these mAbs suppressed proliferation of PHA-activated CD4$^+$ T lymphocytes and T lymphoblasts. Two-tailed *P*-values confirmed that both mAbs decreased the number of PHA-activated CD4$^+$ lymphoblasts, thereby suppressing proliferation significantly and in a dose-dependent manner.

Table 3. Dose-dependent inhibition of phytohaemagglutinin (PHA)-activated CD4+ and CD8+ T lymphoblasts *in vitro* by anti-human leucocyte antigen (HLA)-E monoclonal antibodies (mAbs) Terasaki Foundation Laboratory (TFL)-006s and TFL-006a.

		CD4+ lymphoblasts			CD8+ lymphoblasts		
TFL mAbs	Concentration	Mean	s.d.	P^2	Mean	s.d.	P^2
Culture supernatant							
No PHA		192	14		78	11	
PHA only		1190	91	0·002	364	59	0·01
PHA + serum free AVIM medium		1075	94	n.s.	376	27	n.s.
PHA + murine IgG (1/100)		1033	92	n.s.	332	64	n.s.
PHA + TFL-006s (1/10)		231	59	0·0003	70	25	0·007
PHA + TFL-006s (1/20)		320	79	0·003	117	32	0·008
PHA + TFL-006s (1/40)		575	63	0·004	204	20	0·03
PHA + TFL-006s (1/80)		894	73	0·02	298	26	n.s.
PHA + TFL-006s (1/160)		904	91	0·02	275	29	n.s.
Ascites							
No PHA		190	3		70	3	
PHA only		1243	106	0·003	403	31	0·003
PHA + murine IgG (1/100)		1330	166	n.s.	422	37	n.s.
PHA + TFL-006a (1/100)	8·870 μg/ml	478	193	0·008	176	75	0·02
PHA + TFL-006a (1/200)	4·435 μg/ml	568	173	0·008	191	72	0·02
PHA + TFL-006a (1/400)	2·218 μg/ml	588	195	0·01	207	67	0·02
PHA + TFL-006a (1/800)	1·109 μg/ml	786	127	0·009	248	16	0·005
PHA + TFL-006a (1/1600)	0·555 μg/ml	1499	158	n.s.	477	52	n.s.

The lymphocytes were from another normal non-alloimmunized male (donor R). The top set shows results with anti-HLA-E mAb TFL-006s in culture supernatant without purifying immunoglobulin (IgG). The bottom set shows results with a fresh batch of ascites without purifying IgG. The top two rows of cell numbers in both sets are with and without adding PHA, the following rows with PHA and various dilutions of TFL-006s/a. Note that the 's' dilutions are from 1/10 to 1/160 and the 'a' dilutions from 1/100 to 1/1600. In the experiments involving ascites, the concentration of the stock solution of mAb TFL-006a was 887 μg/ml, which was appropriately diluted in 200 μl of medium in the culture wells. The concentrations at different dilutions show that the optimal concentration needed for significant suppression is about 1 μg/ml. All experiments were performed in triplicate. The significant values are expressed as mean ± standard deviation (s.d.) with two-tailed *P*-values. In both experiments, the mAb-treated PHA-activated CD4+ or CD8+ lymphoblasts decreased significantly in a dose-dependent manner. Clearly, both the anti-HLA-E mAbs TFL-006s and TFL-006a significantly suppress PHA-activated CD4+ and CD8+ T lymphoblasts dosimetrically. Purified mAb were diluted in 200 μl of culture media per well; n.s. = not significant.

HLA-E or HLA-I mAbs that do not mimic HLA-reactivities of IVIg did not suppress blastic transformation and proliferation of PHA-activated CD4+ T lymphocytes

The anti-HLA-E mAbs TFL-006 and TFL-007 bound to several HLA-A, -B, -Cw, -E, -F and -G alleles in a manner strikingly similar to that of different therapeutic preparations of IVIg (Table 1c). In marked contrast, another anti-HLA-E mAb (TFL-033) proved monospecific, binding only to HLA-E and not to any other HLA alleles. TFL-033 recognized a peptide sequence different from those recognized by TFL-006 and TFL-007. Yet another anti-HLA-E mAb, TFL-037, bound to HLA-E and HLA-A, -B and -Cw but not to HLA-F or -G, suggesting that this mAb did not recognize the peptide sequences recognized by TFL-006 and TFL-007. These findings, involving different kinds of HLA-E mAbs, helped to determine whether suppression of proliferation of activated CD4+ T lymphocytes mediated by TFL-006 and TFL-007 is due to the binding of these mAbs to shared peptide sequences of HLA-I. In Table 5, the suppressive effects of HLA-E monospecific mAb TFL-033 and of anti-HLA-E mAb TFL-037, which is non-reactive to HLA-F and -G, are compared with those of IVIg-mimetic polyreactive anti-HLA-E mAbs TFL-006 and TFL-007, as well as control antibodies – human IgG1 isotype control mouse antibody and anti-HLA class Ia allelic mAbs ×2124, ×9123 and ×9133. While both TFL-006a and TFL-007a decreased the number of PHA-activated CD4+ T lymphoblasts significantly, neither the control mAbs nor TFL-033 nor TFL-037 suppressed those cells (Table 5, Figs 3d and 6a,b).

The fact that the anti-HLA-E mAb TFL-037 did not suppress proliferation of CD4+ T lymphocytes or lymphoblasts was evident after CFSE-labelling (Fig. 6a,b).

Anti-HLA-E mAbs TFL-006 and TFL-007 had no effect on non-activated T lymphocytes

Every set of experiments on PHA-activated T lymphocytes included, as controls, purified T lymphocytes that were not exposed to PHA. Table 6 provides a typical profile of the effect of TFL-006 and TFL-007 on a donor's non-activated T cell population. The T lymphoblasts remained very few in number and were unaffected by the anti-HLA-E mAbs or by

Table 4. Dose-dependent suppression of both blastogenesis and proliferation of CD4+ T lymphoblasts by anti- human leucocyte antigen (HLA)-E monoclonal antibodies (mAbs) Terasaki Foundation Laboratory (TFL)-006a and TFL-007a, as compared with Groups 2 and 3. The lymphocytes were obtained from donor J.

		Resting (group 1)						Naive (group 2)						Lymphoblasts (group 3)							
		Blastogenesis		Proliferation				Blastogenesis		Proliferation				Blastogenesis			Proliferation				
		Mean	± s.d.	M1/2		M3–5		Mean	± s.d.	M1/2		M3–5		Mean	± s.d.		M1/2		M3–5		
CD4	Concentration			Mean	± s.d.	Mean	± s.d.			Mean	± s.d.	Mean	± s.d.			$^{**}P^2$	Mean	± s.d.	Mean	± s.d.	$^{**}P^2$
TFL-006a	(stock: 887 μg/ml)																				
No PHA		3457	38	3432	22	89	18	3429	36	3480	37	6	6	39	6		53	13	1	1	
With PHA		2584	108	1044	124	1685	38	783	138	656	132	147	10	1977	62	<0·0001	320	3	1850	64	<0·0001
1/100	8·870 μg/ml	2745	33	2371	24	486	38	1894	33	1869	34	57	14	934	69	<0·0001	506	30	576	51	<0·0001
1/200	4·435 μg/ml	2583	55	1873	46	823	93	1440	55	1366	52	98	9	1259	105	<0·0001	470	22	954	118	0·0003
1/400	2·218 μg/ml	2476	123	1592	181	1017	56	1194	139	1110	142	104	12	1437	30	0·0002	485	27	1162	57	0·0002
1/800	1·109 μg/ml	2493	5	1310	73	1327	68	1003	102	896	91	129	18	1660	110	0·01	404	20	1450	120	0·007
1/1600	0·555 μg/ml	2542	50	1187	31	1494	86	880	49	773	27	131	20	1851	72	n.s.	390	21	1669	75	0·03
TFL-007a	(stock: 627 μg/ml)																				
No PHA		3752	21	3686	25	129	10	3726	18	3767	15	7	3	34	5		40	6	3	3	
With PHA		2856	133	1150	71	1814	169	850	118	750	102	122	29	2198	224	<0·0001	333	15	2021	221	<0·0001
1/100	6·270 μg/ml	2984	74	2524	101	550	47	2006	138	1975	137	56	11	1050	67	0·001	462	27	714	60	0·0006
1/200	3·135 μg/ml	2886	68	2111	64	876	5	1587	87	1520	71	92	14	1372	106	0·0050	473	35	1072	38	0·0020
1/400	1·568 μg/ml	2820	108	1735	29	1194	115	1164	36	1101	42	82	6	1789	132	0·05	521	71	1471	142	0·02

**The *P*-values were obtained for comparison of two parameters. First values obtained after phytohaemagglutinin (PHA) only is compared with values obtained with no PHA; secondly, the values obtained after TFl-006a or TFL-007a treatment were compared with values obtained with PHA only; purified mAb were diluted in 200 ml of culture media per well. The table shows the results after treatment with anti-HLA-E mAbs TFL-006a, then TFL-007a, both obtained from fresh batches of ascites after purifying IgG. The results shown are after treatment of cultures with and without PHA, and then after the PHA-treated cells were exposed to TFL mAbs at the listed dilutions. Note the change in number of cells in each group after PHA activation and the changes from M1/2 to M3–5. The increase in the M3–5 phase only after PHA-activation indicates that activation induces proliferation; these groups were examined for the effect of TFL mAbs in suppressing proliferation, with all experiments in triplicate. The numbers are expressed as mean ± standard deviation (s.d.) two-tailed *P*-values. Both TFL-006a and TFL-007a significantly decreased the number of PHA-activated CD^+ lymphocytes, suppressing proliferation in a dose-dependent manner. No such suppression during either blastogenesis or proliferation was observed for the other groups. As in Table 3, the concentration of the stock solution of mAb TFL-006a was 887 μg/ml and that of TFL-007a was 627 μg/ml, both appropriately diluted in 200 μl of medium in the culture wells. The concentrations at different dilutions show that the optimal concentration needed for significant suppression by TFL-006a is 1–1·5 μg/ml, and that of TFL-007a is 5 μg/ml.

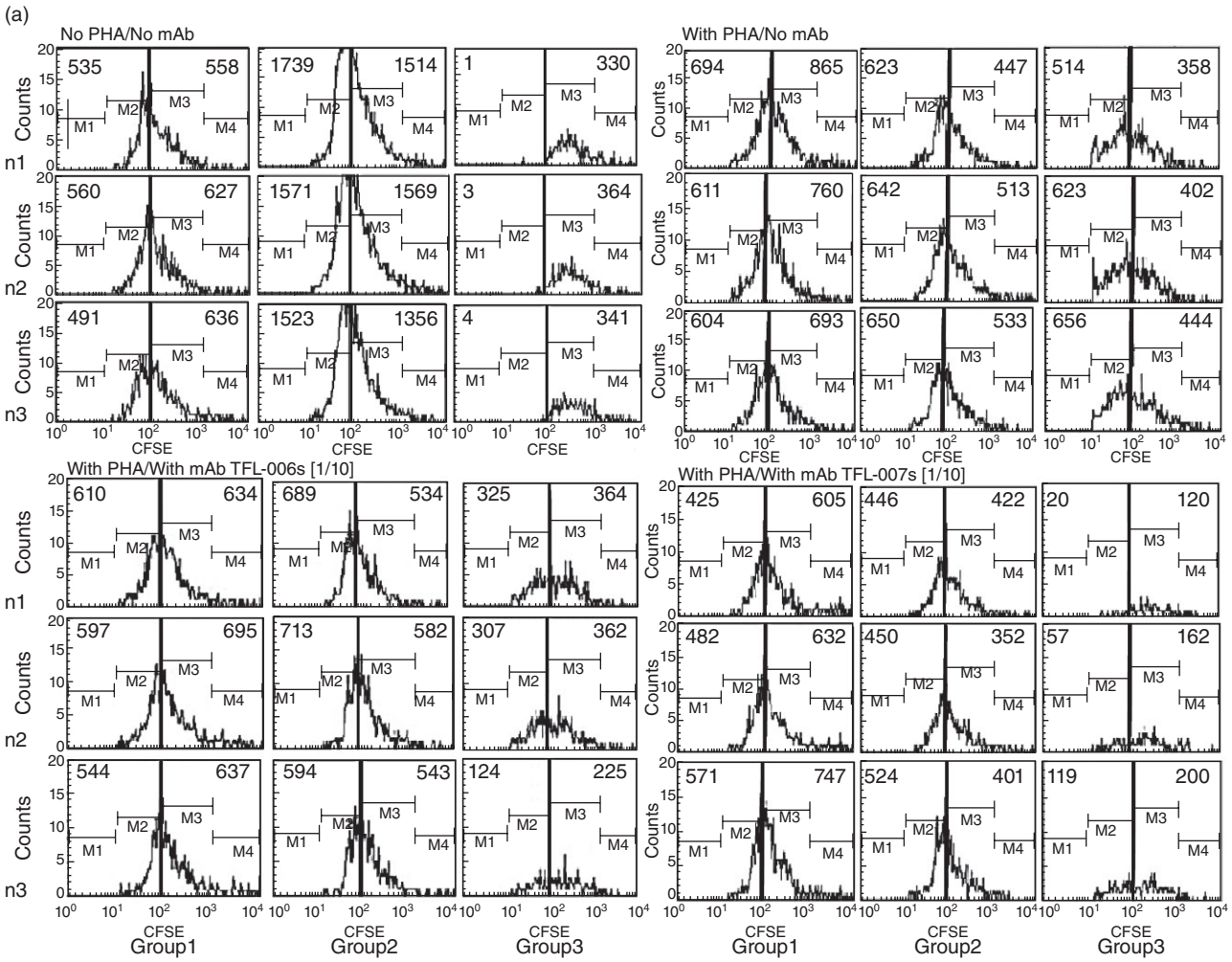

Fig. 4. Suppression of *in-vitro* proliferation of phytohaemagglutinin (PHA)-activated CD4+ and CD8+ T lymphocytes by anti-human leucocyte antigen (HLA)-E monoclonal antibodies (mAbs) mimicking human leucocyte antigen (HLA)-I reactivity of intravenous immunoglobulin (IVIg). The carboxyfluorescein succinimidyl ester (CFSE)-labelled lymphocytes were cultured with or without PHA or with PHA and mAb Terasaki Foundation Laboratory (TFL)-006s or PHA and mAb TFL-007s, both mAbs at 1/10 dilution. Three days after culture, cells were labelled with fluorescent dye-conjugated anti-CD4+ or anti-CD8+ antibodies before analysis. CFSE labelling allowed us to gauge and show cell proliferation: when the CFSE-labelled cell population undergoes mitosis, after 72 h it has migrated from the right to the left side of each rectangular box in the figure depending on the number of mitoses. The distance moved shows the number of cell divisions. (a) Effect of anti-HLA-E mAb TFL-006s and TFL-007s on proliferation of CD4+/CFSE+ T lymphocytes. After incubating cells with CFSE, the cells were treated as noted. Each box in the figure is divided by a vertical line into two sub-boxes, the right for mitoses 1 and 2 (M1/2) (parent lymphocytes) and the left for mitosis 3 to 5 (M3–5) (the progeny). The number of cells after each treatment (including 'no PHA') of each T lymphocyte population was counted and compared, the number shown in each sub-box. Note that with no PHA the number of cells in the M3–5 sub-box is very meagre for all groups of CD4+ T lymphocytes. With PHA-only treatment, the very high number of cells for M3–5 indicates that proliferation has occurred in all three groups. The impact of treatment with PHA and TFL-007s or PHA and TFL-006s is unmistakable: the number of cells in the M3–5 sub-box is reduced in all groups, indicating suppression of proliferation. No such decrease was observed with resting or naive T lymphocytes. (b) Effect of TFL-007s on proliferation of CD4+/CFSE+ T lymphoblasts after incorporation of CFSE. The values represent the mean of triplicate analysis, with treatment as indicated in the bars. Two-tailed *P*-values are indicated by a horizontal line connecting the two groups. (c) Effect of TFL-006s on proliferation of CD4+/CFSE+ T lymphoblasts after incorporation of CFSE. As with (c), the values represent the mean of triplicate analysis, with treatment as indicated in the bars. Two-tailed *P*-values are indicated by a horizontal line connecting the two groups.

isotype control antibodies. It is interesting that anti-HLA-E antibodies, isotype control and, notably, AIM-V with 1% HEPES media control, *per se*, increased the number of CD8+ T cells in all three groups. Therefore, the increase observed in culture with different dilutions of mAb TFL-007 was due to AIM-V media rather than the mAb itself.

Discussion

The results of this investigation show the differential effects on the suppression of PHA-activated T lymphocytes by IVIg, by anti-HLA-E mAbs that mimic the HLA class Ia and Ib reactivity of IVIg (TFL-006 and TFL-007), and by anti-

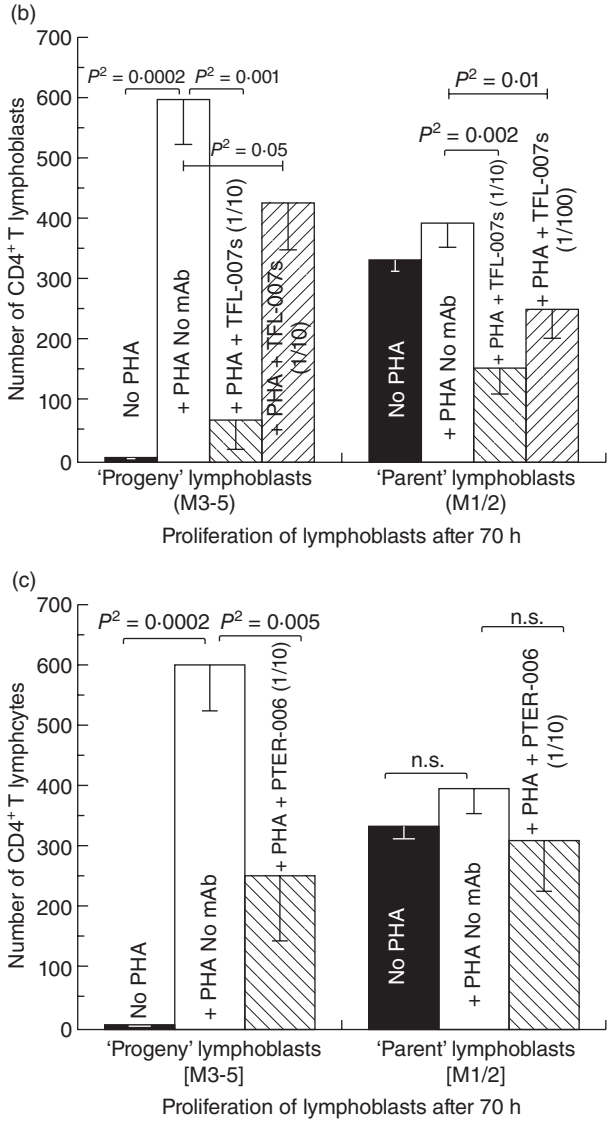

Fig. 4. *Continued*

HLA-E mAbs that do not mimic that reactivity (TFL-033 and TFL-037). The mAbs TFL-006 and TFL-007 proved to be more potent suppressors of blastogenesis and proliferation of activated $CD4^+$ T lymphocytes than IVIg. The concentration of anti-HLA-E mAbs required for this suppression was as much as 150-fold lower than the required concentration of IVIg. Neither IVIg nor the mAbs TFL-006 and TFL-007 affected the non-activated $CD4^+$ T cell population (Fig. 2b, Table 6).

T cell activation occurs in three successive steps [39]. The first involves changes in the cell's lipids (metabolism of arachidonic acid) and cytoskeleton. These changes modify the physical properties of the T cell membranes to modulate the activity and expression of membrane proteins [40]. The second step involves initiation of transcription leading to the production of lymphokines with an increase in RNA and protein content (mitotic phase G1). Consequently, cytoplasmic volume increases and the nuclear/cytoplasmic ratio decreases. The final step involves DNA replication (phase S–G2) leading to mitosis and proliferation. Strikingly, the entire event occurs within 12 h and reaches maximum activation by 48 h [38].

Both blastic transformation and proliferation result in transitory cell-surface expression of several molecules. These include: IL-2R [41–43]; Fc receptors for IgG (FcγRI/ CD64, FcγRII/ CD32 and FcγRIII/CD16 [44]; IgE (FcεRII)/ CD23) [45]; insulin receptors; insulin-like growth factor 1R and IL-2R [46]; alpha-fetoprotein and transferrin receptors [47]; a non-disulphide-linked heterodimer of polypeptide chains 33 kDa and 38 kDa called 'Me14/D12' [48]; MICA [49]; HLA class II antigens HLA-DR, -DP and -DQ [50–52]; and, most importantly, the over-expression of β2m-free heavy chains of HLA class I, called 'open conformers' [19–21,25].

Our observations of IVIg dose-dependent suppression of PHA-activated human $CD4^+$ T lymphocytes accord with previous reports. Such suppression was observed in separate *in-vitro* cell cultures after activation by PHA, by anti-CD3 antibody, by tetanus toxoid pokeweed mitogen or by allogenic mixed cells [3,53]. Similar IVIg-mediated suppression of proliferation of activated T cells was observed *in vivo* [8,54]. Although suppression of proliferation of activated T cells was noticed in patients with multiple sclerosis, IVIg did not cause cell death as determined by caspase activation, DNA fragmentation or CD95 blockade or Bcl-2 [8]. This leads to the inference that the therapeutic preparations of IVIg down-regulated activated T cells through suppression of blastogenesis and proliferation rather than by modulation of cell death.

Our earlier report [12], that IVIg HLA-reactivity was lost when the HLA-E Ab reactivity of IVIg was adsorbed out, led us to develop mAbs against HLA-E that might mimic the HLA-Ia and HLA-Ib reactivity of IVIg. Of the many anti-HLA-E mAbs developed, some (e.g. TFL-006 and TFL-007) indeed mimicked the HLA-I reactivity of IVIg; others were either monospecific, in that they recognized only HLA-E (e.g. TFL-033), or they recognized only a few HLA class I alleles (e.g. TFL-037). Inhibition of the binding of the anti-HLA-E mAbs TFL-006 and TFL-007 to HLA-E-coated beads by the most common and accessible shared peptide sequences[1] located on the α2 helix – HLA-E (^{117}AYDGKDY^{123} and ^{126}LNEDLRSWTA^{135}) [14–16,18] (Fig. 1c; Table 7) – confirmed that these anti-HLA-E mAbs recognized the shared amino acid sequences or epitopes on other HLA class I molecules. The ability of TFL-006 and TFL-007 to bind to regular beads but not to iBeads, which are coated mainly with intact HLA-Ia, and the suppression

[1]Extensive search for the peptides at http://blast.ncbi.nlm.nih.gov/Blast.cgi?%20PROGRAM=blastp&%20PAGE_TYPE%20=BlastSearch&LINK%20LOC=blasthome revealed that the peptides are restricted only to MHC class I heavy chains.

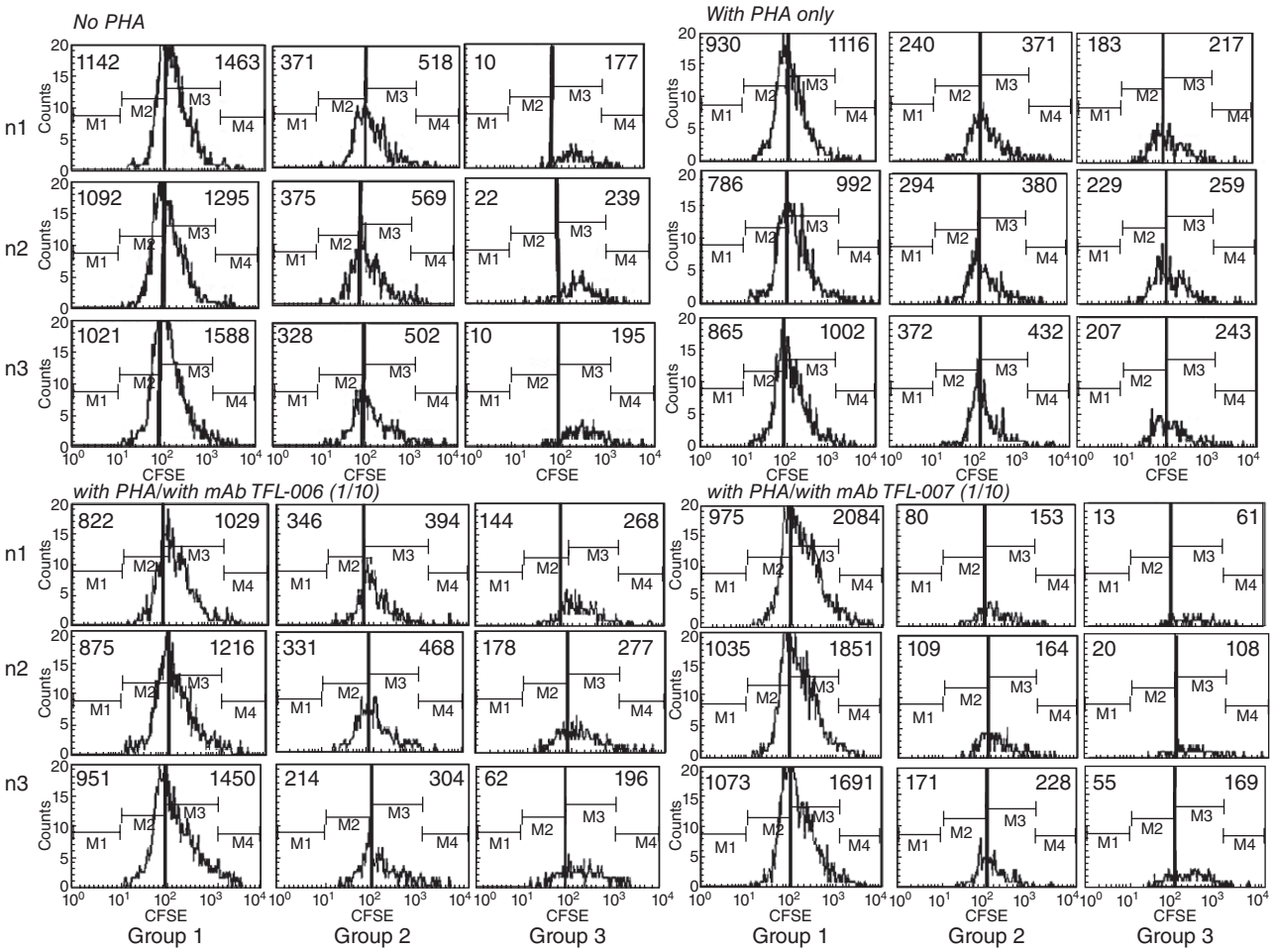

Fig. 5. Effect of Terasaki Foundation Laboratory (TFL)-006s and TFL-007s on proliferation of CD8+ T lymphocytes. The details are the same as in Fig. 4a, but for CD8+ T lymphocytes.

of blastogenesis and proliferation by TFL-006 and TFL-007, but not by mAbs TFL-033 or TFL-037, which do not bind to shared peptide sequences of open conformers, perhaps because they do not recognize the shared peptide sequences recognized by TFL-006 and TFL-007, further confirmed that TFL-006 and TFL-007 bind to the shared peptide domains on open conformers. Furthermore, failure of IVIg (Fig. 2b) or TFL-006 and TFL-007 (Table 6) to influence the number of non-activated CD4+ T cells, which do not over-express open conformers [19–21,25], lends support to the hypothesis that IVIg and TFL-006 and TFL-007 suppress activated T cells by binding to the open conformers. Figure 1a–c illustrates that the shared peptide sequences of HLA class Ia and Ib (shown by the yellow line in the structure, Fig. 1a,b, and by the pink star on all HLA alleles in Fig. 1c) are masked by β2-microglobulin in intact HLA but are exposed on the open conformers of HLA class I. These figures are key to understanding the binding site of anti-HLA-E mAbs that mimic IVIg. Previous studies reported that T cells, upon activation, over-express open conformers on the cell surface [19–21,25]. The HLA class I open conformers generated on the surface of activated T lymphocytes carry the binding site for IVIg as well as for anti-HLA-E mAbs, i.e. the peptide sequences shared by HLA-A, -B, -Cw, -E, -F and -G (Fig. 1c).

From the finding that TFL-006 and TFL-007 suppressed the blastogenesis and proliferation of PHA-activated CD4+ T cells, but that TFL-033 and TFL-037 did not, we inferred that the suppression may involve binding of the Fab portion of those anti-HLA-E mAbs that mimic the HLA class I reactivity of IVIg or, of course, those present in IVIg itself, to the shared peptide epitopes exposed on the T cells' open conformers. The same suppressive mechanism would apply to IVIg, although previous investigators attributed the suppression of activated T cells by IVIg to the Fc portion of the antibodies binding to Fcγ receptors that are generated on the cell surface of T cells upon activation [54–57]. The fact that neither the mAbs TFL-033 and TFL-037 nor the mouse isotype control IgG mixture of antibodies suppressed blastogenesis and proliferation of T lymphocytes does not support the contention that interaction between the Fc portion of Abs and Fcγ receptors contributes to the sup-

Table 5. Comparison of the effect of control antibodies [mouse "anti-human IgG" antibody (isotype control)], anti-human leucocyte antigen (HLA) class Ia allele Abs, anti-HLA-E monospecific mAb Terasaki Foundation Laboratory (TFL)-033, and anti-HLA-E mAb TFL-037 (HLA-F- and G-non-reactive) *versus* intravenous immunoglobulin (IVIg)-mimetic anti-HLA-E monoclonal antibodies (mAbs) TFL-006s and TFL-007s on suppression of blastogenesis.

CD4	Mean	SD	P^2 value
Negative control mAbs ($n = 3$)			
No PHA			
Naive	3454	106	
T lymphoblasts	263	14	
With PHA			
Naive	1099	48	<0·0004
T lymphoblasts	969	117	0·0005
mouse "anti-human IgG" antibody (isotype control)			
Naive	1024	131	n.s.
T lymphoblasts	1758	84	n.s.
Iλ HLA-Ia (A11, A43) mAb ×2124 (source: Nadim/TFL)			
Naive	924	11	0·02
T lymphoblasts	1758	84	n.s.
Iλ HLA-Ia mAb ×9123 (source: Nadim/TFL)			
Naive	950	117	n.s.
T lymphoblasts	1435	276	n.s.
Iλ HLA-Ia mAb ×9133 (source: Nadim/TFL)			
Naive	1032	54	n.s.
T lymphoblasts	1511	162	n.s.
TFL HLA-E mAbs ($n = 3$)			
No PHA			
Naive	3055	195	
T lymphoblasts	254	18	
With PHA			
Naive	1099	48	0·0001
T lymphoblasts	969	117	0·0005
PHA +/TFL-007 (1/100)			
Naive	1000	65	n.s.
T lymphoblasts	640	137	0·03
PHA +/TFL-006 (1/100)			
Naive	1187	65	n.s.
T lymphoblasts	502	184	0·02
PHA +/TFL-037 (1/100)			
Naive	1169	72	n.s.
T lymphoblasts	911	54	n.s.
PHA +/TFL-033 (HLA-E monospecific mAb) (1/100)			
Naive	401	38	n.s.
T lymphoblasts	509	78	n.s.

All antibodies were obtained from culture supernatants, the $CD4^+$ T lymphocytes from donor R. Treatment of cultures was with and without adding phytohaemagglutinin (PHA) (first two rows). The number of cells is shown for group 2 (T lymphocytes) and group 3 (T lymphoblasts) after exposing PHA-treated cells to the various control antibodies, and then to the TFL mAbs at 1/100 dilution. The values are expressed as mean ± standard deviation (s.d.) ($n = 3$) with two-tailed *P*-values for treatment with only PHA compared with no PHA, and with PHA plus mAb dilutions compared with only PHA. Note that both TFL-006a and TFL-007a decreased the number of PHA-activated T cells significantly while neither the control mAbs nor TFL-033, an HLA-E monospecific mAb, suppressed the blastogenesis. The data establish the importance of the Fab portion of the antibody in binding to the shared peptide sequences exposed on the open conformers of HLA class Ia and Ib alleles. The Fc portions of these and other mAbs did not suppress proliferation. These data also establish that open conformers of HLA class I may act as inhibitory ligands for activated T cells. See Table Ib for HLA-reactivities of the TFL-mAbs. 1λ refers to the original source of mAb as One Lambda, Inc.

pression of PHA-activated T cells. Another study cited '[i]nhibition of immunoglobulin production in vitro by F(ab′)2 fragments, but not by the Fc portion of a monoclonal antibody' [58]. Furthermore, while addressing the questions of whether IVIg-mediated inhibition is a result of purification processing of IgG, of donor pooling or of the intrinsic down-regulatory activity of IgG, MacMillan *et al.* compared the effect of the Fab fragment of IgG with that of IgG isolated from single-donor plasma on T cell proliferation by IVIg [9]. The addition of Fab fragments of IgG to

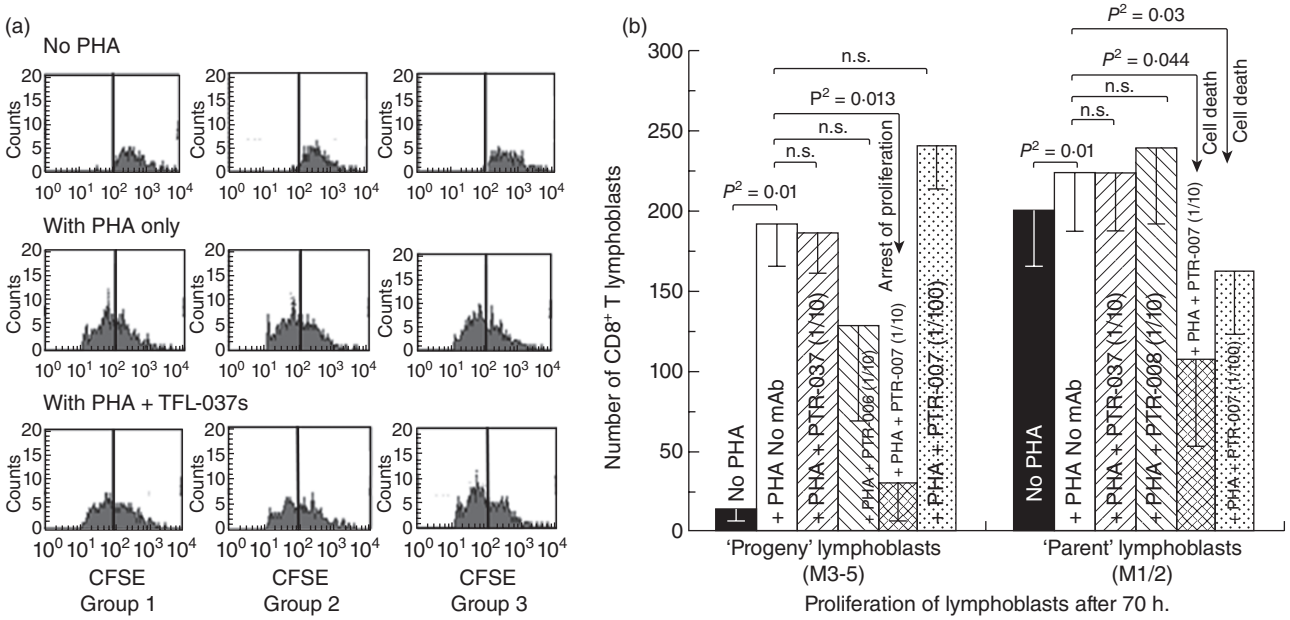

Fig. 6. Anti-human leucocyte antigen (HLA)-E mAb Terasaki Foundation Laboratory (TFL)-037s, which did not mimic the HLA reactivity of intravenous immunoglobulin (IVIg) in that it did not recognize HLA-F and HLA-G, although it recognized the peptide sequences not shared by HLA-F and HLA-G, did not suppress phytohaemagglutinin (PHA)-induced proliferation of T lymphocytes. (a) TFL-037s did not affect PHA-induced proliferation of $CD4^+$ subpopulations of T lymphocytes. After incubating cells with carboxyfluorescein succinimidyl ester (CFSE), treatment was as indicated above each row. The three groups of T cells are the same as described in Fig. 2. Note that in the upper row (no PHA) all cells are present in the right side of the sub-box and there are no lymphocytes in left sub-box (M3–5). With PHA only (second row), there is a very high number of $CD4^+$ T lymphocytes in the M3–5 sub-boxes, indicating that proliferation had occurred in all groups. Addition of anti-HLA-E mAb TFL-037s did not affect the number of $CD4^+$ T cells, suggesting that TFL-037s does not affect their proliferation. (b) Neither TFL-037 nor TFL-006 suppressed PHA-activated $CD8^+$ T lymphoblasts, whereas TFL-007s reduced proliferation significantly in M3–5 (progeny lymphoblasts) and M1/2 (parent lymphoblasts).

IgG-depleted plasma in culture reduced T cell proliferation significantly, establishing that the Fab region is sufficient to mediate this inhibition. It appears that the Fab portion of an antibody is capable of arresting different signalling pathways of lymphocytes. However, the investigators did not specify the Fab portion of any particular kind of IgG, such as that of Fab reacting to the shared peptide sequences of HLA class I alleles. In another study, the presence of IVIg's anti-CD4 activity was also implicated in the inhibition of T cell activation and functions [59].

Based on previous literature, the events leading to activation are illustrated by Fig. 7a–c. They show the transformation of resting T cells (Fig. 7a) to activated T cells (Fig. 7b) under the influence of PHA. PHA activation initiates phosphorylation of the cytoplasmic domain of CD3, resulting in signal transduction leading to activation of transcription factors [60], transcription and production of cell surface molecules such as IL-2Rα [41–43] and open conformers of HLA class I [19–21,25] (Fig. 7b,c). If the binding by mAbs TFL-006 and TFL-007 is responsible for the suppression of blastogenesis and proliferation, it may involve reversal of phases of activation of T lymphocytes, mediated possibly by signal transduction. In support of such an inference, several reports have clearly documented the elongation of the cytoplasmic tail of the HLA class I open conformers and the exposure of otherwise cryptic tyrosine^{320} [61–64] and serine^{335} [65] with a provision for phosphorylation (Fig. 7c). Although serine^{335} is generally considered the primary site of phosphorylation in this tail, phosphorylation of tyrosine^{320} has been indicated as that site by others [64,66]. Either way, what is most important is that the open conformers in activated normal human T cells are associated with tyrosine phosphorylation and are capable of enabling *cis* interactions with cell surface receptors or other signalling molecules [65–69]. The phosphorylation mediated by TFL mAbs may result in dephosphorylation of the cytoplasmic tails of CD3 molecules by activating phosphatases (Fig. 6c), leading to the arrest of transcription factors and synthesis of the proteins involved in blastogenesis and mitosis. These events, which are portrayed in Fig. 7 based on reports by other investigators, suggest that suppression of activation of T cells could be due to the binding of anti-HLA-E mAbs that mimic the HLA-reactivity of IVIg to the shared amino acid sequences exposed on the open conformers of HLA class I. This activation can involve any or all molecules of HLA class I open conformers expressed on the surface of T lymphocytes, as illustrated in Fig. 1c. If so, then the anti-HLA antibodies

Table 6. Effect of anti- human leucocyte antigen (HLA)-E monoclonal antibodies (mAbs) Terasaki Foundation Laboratory (TFL)-006s and TFL-007s, and of media and mouse isotype control antibodies on $CD4^+$ and $CD8^+$ T lymphocytes not activated by phytohaemagglutinin (PHA).

	Group 1, resting						Group 2, naive						Group 3, lymphoblasts					
	CD4 ($n = 3$)			CD8 ($n = 3$)			CD4 ($n = 3$)			CD8 ($n = 3$)			CD4 ($n = 3$)			CD8 ($n = 3$)		
	Mean	s.d.	P^2	Mean	s.d.	P^2	Mean	s.d.	P^2	Mean	s.d.	P^2	Mean	s.d.	P^2	Mean	s.d.	P^2
No PHA	3669	110		739	18		3487	99		669	19		192	12		78	9	
Experimental																		
TFL-006s 1/10	3280	61	**0·012**	1071	48	**0·001**	3068	51	**0·006**	961	41	**0·001**	235	23	**n.s.**	117	7	**0·008**
TFL-006s 1/20	3258	218	**n.s.**	944	86	**0·03**	3033	182	**0·036**	861	87	**0·037**	241	40	**n.s.**	89	12	**n.s.**
TFL-006s 1/40	3394	225	**n.s.**	1012	36	**0·001**	3175	244	**n.s.**	933	33	**0·001**	236	20	**n.s.**	88	8	**n.s.**
TFL-006s 1/80	3415	199	**n.s.**	990	49	**0·002**	3243	174	**n.s.**	927	43	**0·002**	187	30	**n.s.**	67	9	**n.s.**
TFL-006s 1/160	3637	30	**n.s.**	982	32	**0·001**	3464	22	**n.s.**	924	27	**0·0004**	188	16	**n.s.**	62	10	**n.s.**
TFL-007s 1/10	2881	47	**0·001**	858	20	**0·004**	2723	33	**0·0005**	792	32	**0·01**	176	16	**n.s.**	71	10	**n.s.**
Controls																		
Media control	3621	12	**n.s.**	1181	67	**0·0004**	3325	13	**n.s.**	1040	50	**0·0003**	315	10	**0·0003**	152	15	**0·002**
Mouse IgG control 1/100	3294	132	**0·03**	914	59	**0·01**	3111	122	**0·02**	829	49	**0·007**	198	14	**n.s.**	205	14	**n.s.**

Significant *P*-values are indicated in bold.

T lymphoblasts were not affected by the mAbs at any dilution, whereas the $CD8^+$ T cell populations of groups 1 and 2 showed marked increase after mAb treatment as well as with media and isotype controls. AIM-V culture medium with 1% HEPES may be responsible for the increase in media control as well as in isotype and mAbs that were diluted in AIM-V culture medium with 1% HEPES. At higher concentrations of TFL mAbs (1/10 and 1/20), a marginal decrease in $CD4^+$ lymphocytes was observed in groups 1 and 2 and also in the isotype controls.

Table 7. Amino acid sequences of human leucocyte antigen (HLA)-E that are E-restricted and those shared by one or more HLA-Ia and Ib alleles (extracted and modified from references [14,16,70]).

Peptide sequences of two HLA-E alleles		Number of HLA alleles					
		Classical class Ia			Non-classical class Ib		
HLA-E^{107G} & HLA-E^{107R}	No. of amino acids	A	B	Cw	F	G	Specificity
^{65}RSARDTA^{71}	7	0	0	0	0	0	E-restricted
^{90}AGSHTLQW^{97}	8	1	10	48	0	0	Polyspecific
^{102}ELGPD*R(G)*RF ^{109}	8	0	0	0	0	0	E-restricted
^{115}QFAYDGKDY^{123}	9	1	104	75	0	0	Polyspecific
^{117}AYDGKDY^{123}	7	491	831	271	21	30	Polyspecific
^{126}LNEDLRSWTA^{135}	10	239	219	261	21	30	Polyspecific
^{137}DTAAQI^{142}	6	0	824	248	0	30	Polyspecific
^{137}DTAAQIS^{143}	7	0	52	4	0	30	Polyspecific
^{143}SEQKSNDASE^{152}	10	0	0	0	0	0	E-restricted
^{163}TCVEWL^{168}	6	282	206	200	0	30	Polyspecific

Position 107 may have either [R] or [G]; only peptides with six or more amino acids are shown in the Table.

that recognize the shared peptides on the open conformers, and that occur naturally in the sera of normal, healthy and non-alloimmunized males [15,70], may play a novel role in regulating T lymphocytes that are activated *in vivo* during infection, inflammation, autoimmune diseases and cancer. The implications of our study also suggest a novel functional role for the natural anti-HLA antibodies occurring in healthy individuals that recognize the shared peptide sequences on the open conformers of HLA in regulating T lymphocytes activated *in vivo*.

To sum up, anti-HLA-E mAbs TFL-006 and TFL-007, which mimic the HLA-I reactivity of IVIg, significantly suppressed both blastogenesis and proliferation of activated T lymphocytes *in vitro* in a dose-dependent manner. Furthermore, their suppressive ability was superior to that of IVIg because the mAbs required concentrations as much as 150-fold lower than the concentrations IVIg required for suppression. These findings, together with the recent report ([71]) that the anti-HLA-E mAb TFL-007 is also capable of suppressing allo-HLA IgG production *in vitro* much more efficiently than the therapeutic preparations of IVIg, strengthens the contention that the TFL anti-HLA-E mAbs can serve as IVIg mimetics.

It is most likely the monoclonality of the mAbs TFL-006 and TFL-007 and their F(ab′)2 binding to the shared peptide domains of the open conformers of HLA-I that account for their suppressing T cell proliferation better than IVIg polyclonal antibodies, which are admixed with several other antibodies, anti-idiotypic antibodies and antigens, and whose binding mechanism (hence suppressive mechanism) remains unclear.

In conclusion, suppression of antigen-specific activated T cells is highly desirable with autoimmune diseases, with transplant recipients and patients waiting for allografts. Thus, anti-HLA-E mAbs TFL-006 and TFL-007, humanized or chimerized, may prove a better suppressive agent than IVIg. Clinical trials can elucidate the question and help to establish that the anti-HLA-E mAbs reacting to HLA-I open conformers as a substitute for IVIg.

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Author contributions

M. H. R. designed the entire study, performed the experiments, collected and validated extensive data, developed and characterized the anti-HLA-E mAb TFL-007, analysed the HLA-reactivity of the sera of the subject involved, formulated the hypothesis, periodically monitored the experimental outcome, analysed the data and performed statistical analysis, prepared the figures, discussed and wrote the paper. P. I. T. was instrumental in undertaking this investigation, contributed to designing the study, examining the results and providing interpretations. T. P. assisted in carrying out the experiments and provided continuous support for data analyses and discussion. V. J. assisted in carrying out the experiments, was involved in data analysis and in discussion of the results. S. K. assisted in carrying out the experiments, was involved in the data analysis and in discussion of the results.

Disclosure

None of the authors have any conflicts of interest or financial interests.

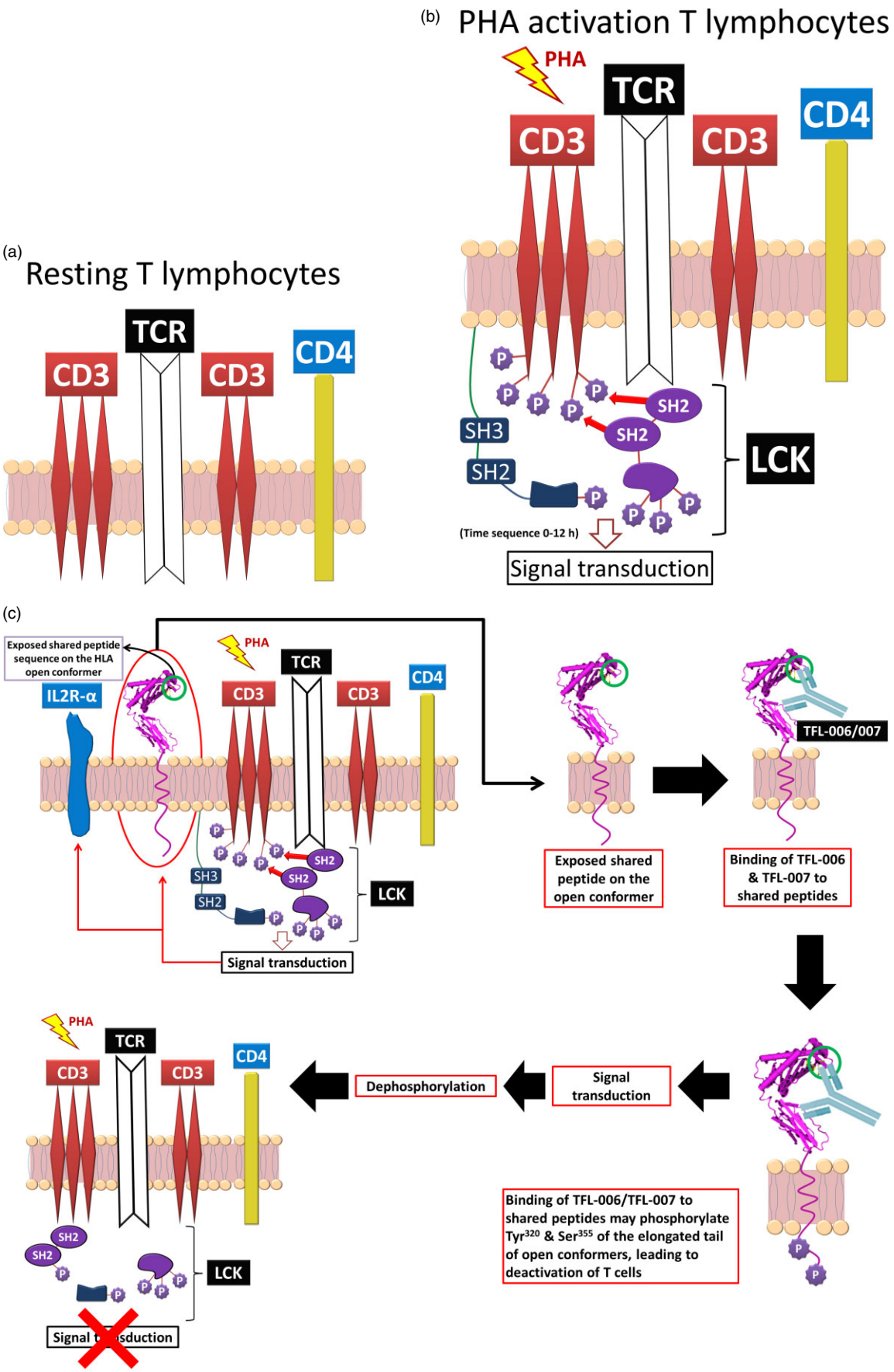

Fig. 7. A model (developed based on Ref. 60) illustrating the possible mechanism underlying phytohaemagglutinin (PHA)-activation of T cells and the suppression of activated T cells mediated by polyspecific anti-human leucocyte antigen (HLA)-E monoclonal antibodies (mAbs) (Terasaki Foundation Laboratory (TFL)-006 and TLF-007) and possibly by intravenous immunoglobulin (IVIg). (a,b) Based on a model proposed by Mustelin, Vang and Bottini [60] for T cell activation. (a) Illustrates the structure of CD3/T cell receptor (TCR)/CD4 on the lipid raft (pink zone) of the bi-layered lipid membrane in the non-phosphorylated non-activated $CD4^+$ T cells. Non-activated $CD4^+$ T cells are least affected by either IVIg (Fig. 2b) or TFL-006 and TFL-007 (Table 6). (b) Protein tyrosine kinase exemplified by lymphocyte-specific protein tyrosine kinase (LCK)-induced phosphorylation of tyrosine-based activation in the cytoplasmic domain of CD3, which leads to activation of transcription factors and transcription of cell surface molecules such as interleukin (IL)-2Rα and open conformers of HLA class I. SH-1, SH-2 and SH-3 represent family members of Src homology; they are involved in mediating the cytoplasmic domain of CD3. Further activation of the tyrosyl-phosphorylated motifs then interact with SH-1 domains within the protein kinase LCK, leading to further signaling function [60,69]. (c) Cell surface expression of IL-2Rα [43] and open conformers of HLA class I [13,24–32]. Importantly, the exposure of shared amino acid sequences of all the HLA open conformers is indicated by a circle. It is this site that is recognized by TFL-006 and TFL-007. Possible interaction and consequences of recognition of the shared peptide sequences by the anti-HLA-E antibodies are illustrated in three steps: first, the exposure of the shared peptide sequence on the open conformer; secondly, recognition of the shared epitopes on the open conformer by anti-HLA-E mAbs; thirdly, possible phosphorylation of the elongated cytoplasmic tail of open conformers. That elongation results in exposure of cryptic tyrosine (Tyr^{320}) [54–57] and serine (Ser^{355}) [65] residues in the cytoplasmic tail. It may be the binding of anti-HLA-E mAbs to the shared peptide sequences that initiates the phosphorylation leading to signal transduction. A final step involves initiation of dephosphorylation of the cytoplasmic domain of CD3, resulting in arrest of activation or suppression. That seems plausible, as the phosphorylation is known to be reversible.

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