The immunodominant influenza matrix T cell epitope recognized in human induces influenza protection in HLA-A2/K^b transgenic mice


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Abstract

The protective efficacy of the influenza matrix protein epitope 58–66 (called M1), recognized in the context of human HLA-A2 molecules, was evaluated in a HLA-A2/K^b transgenic mouse model of lethal influenza infection. Repeated subcutaneous immunizations with M1 increased the percentage of survival. This effect was mediated by T cells since protection was abolished following in vivo depletion of all T lymphocytes, CD8^+ or CD4^+ T cells. The survival correlated with the detection of memory CD8^+ splenocytes able to proliferate in vitro upon stimulation with M1 and to bind M1-loaded HLA-A2 dimers, as well as with M1-specific T cells in the lungs, which were directly cytotoxic to influenza-infected cells following influenza challenge. These results demonstrated for the first time that HLA-A2-restricted cytotoxic T cells specific for the major immunodominant influenza matrix epitope are protective against the infection. They encourage further in vivo evaluation of T cell epitopes recognized in the context of human MHC molecules.

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Introduction

Influenza viruses are ubiquitous pathogens which infect both animals and humans. They cause annual worldwide epidemics of respiratory illness, associated with excess morbidity and mortality in susceptible people (Falsey et al., 1995). In addition, they have a considerable economic burden consecutive to related treatments, hospitalizations, and work absenteeism (Levy, 1996).

Influenza vaccines, prepared with inactivated whole viruses or subvirion components, are well-tolerated. They appear to be highly efficient in preventing natural infection and/or in reducing the severity of illness caused by antigenically matched strains of viruses (Carman et al., 2000; Edwards et al., 1994; Gross et al., 1995; Potter et al., 1997). Neutralizing Abs, directed primarily to the hemagglutinin (HA) and the neuraminidase (NA), represent the major host defense mechanism induced upon immunization. Binding to virions can occur at a number of different steps prior to cell attachment, as well as at events following cell attachment, thereby reducing the amplification and the spread of the virus and attenuating the infection.

However, antibody responses are highly strain-specific, while the major surface glycoproteins of influenza viruses are subjected to frequent, accumulating mutations, called antigenic shifts and drifts (Kilbourne et al., 1990). These genetic variations allow the virus to escape from neutralization by preexisting antibodies (Kuwano et al., 1989; Laver et al., 1979; Moss et al., 1980). They impose updating and readministration of influenza vaccines every year.

Evidence from animal models indicated that if the antibodies play a major role in prevention of the disease, T cells are both necessary and sufficient for recovery from influenza (Bender and Small, 1992; Whitton and Oldstone, 1996). In particular, CD8^+ cytotoxic T cells can act as a second line of defense by eliminating any cell in the lungs which became infected despite the presence of neutralizing antibodies (Cerwenka et al., 1999a,b). They are important to
terminate infection, as demonstrated by the difficulty of influenza-infected CD8+ T cell-deficient mice to clear the virus from their lungs (Eichelberger et al., 1991; Wells et al., 1981).

CTLs recognize peptides in the form of 8 to 11 amino acids derived from degraded proteins which are either synthesized intracellularly or introduced directly into the cytoplasm (Bjorkman et al., 1987; Townsend et al., 1986). The peptides bind to class I MHC molecules shortly after they are synthesized. The complex is transported to the cell surface for recognition by a specific TCR.

In humans, the CTL response is directed to numerous surface and internal influenza proteins, with multiple epitopes being potentially recognized, depending on the expression of class I MHC molecules (Jameson et al., 1998; Gianfrani et al., 2000; Robbins et al., 1997). However, the specific immune responses detected after virus infection are primarily directed against a small fraction of the potential determinants, a phenomenon that is called immunodominance (Belz et al., 2000; Yewdell and Bennink, 1999). Accordingly, the immunodominant epitope recognized by HLA-A*0201-restricted human CTLs is composed of a fragment of the influenza matrix protein, called M1, comprising amino acid residues 58 to 66 (Gotch et al., 1987).

Immunization with the exact peptide sequence corresponding to a CTL epitope introduced intramuscularly by DNA immunization (Bot et al., 1998; Fomsgaard et al., 1999) or subcutaneously (s.c.) emulsified with a T helper cell epitope in incomplete Freund’s adjuvant was successfully employed in mice to raise cellular responses which afford a high degree of controllability (Gianfrani et al., 2000; Valmori et al., 1994). In that respect, HLA-transgenic mouse models provide the unique opportunity to investigate the immunogenicity of potential CTL epitopes and the biological relevance of these responses.

Our aim was to investigate whether M1 can induce an antiviral protective immune response in HLA-A*0201/Kb transgenic mice. The results showed for the first time that M1 induced a T cell-dependent influenza protection in HLA-A2/Kb transgenic mice. This effect correlated with the generation of M1-specific memory T cells detectable in the spleen 3 weeks after the last immunization and the presence of influenza-specific cytotoxic T cells in the lungs after influenza challenge.

Results

T cell-dependent protection of HLA-A2/Kb transgenic mice after immunization with M1

To test the protective efficacy of M1 against virus challenge, a lethal model of influenza infection was set up using the mouse-adapted A/PR8/34 H1N1 virus, HLA-A2/Kb transgenic, and C57BL/6 (H-2b) mice. These mice consistently suffered a progressive and dramatic weight loss resulting in death within 2 weeks following intranasal (in) instillation of the LD100 of H1N1 virus (data not shown). HLA-A2/Kb transgenic and C57BL/6 mice were immunized s.c. at the base of the tail two or three times with peptides M1 or ELA as control.

Peptides were mixed with TTPp30, an immunodominant T helper cell epitope of tetanus toxin (Valmori et al., 1992), and IFA, as described under Materials and methods, to improve the induction of the T cell responses (Valmori et al., 1994). Control mice for survival were treated by i.n. instillation with a sublethal dose of H1N1 virus. Three weeks after the last immunization, antibody responses were investigated by ELISA. None of the mice developed detectable influenza antibodies in the serum (Ab titers < 1.95 log10/ml) with the exception of the influenza-primed mice (3.74 ± 0.72 log10/ml). All the mice demonstrated the characteristic signs of morbidity during the first week after challenge with the LD100 of H1N1 virus. However, the weight of the control influenza-primed mice was first stabilized and increased thereafter (data not shown). All the influenza-primed mice survived, as demonstrated in Fig. 1A, while the mice previously treated with ELA went on losing weight (data not shown) and died within 10 days (Fig. 1A).

In contrast, at this time, most of the HLA-A2/Kb transgenic mice immunized with M1 also survived. This effect was dependent on the expression of HLA-A2 molecules, since it was observed with the HLA-A2/Kb transgenic but not with the C57BL/6 mice (not shown). In addition, the protective effect was improved with the numbers of immunizations: at day 10 postchallenge, approximately 70% of the mice survived after two immunizations versus 90% after three immunizations; at day 15, 50% of the mice survived after two immunizations versus 70% after three immunizations. Thus, immunizations with M1 elicited a protective effect against the morbidity and the mortality caused in HLA-A2/Kb transgenic mice by a lethal H1N1 virus challenge.

Since influenza antibodies were never detected in the serum of M1-primed mice, the role of T cells in influenza protection was evaluated by in vivo T cell depletion. HLA-A2/Kb transgenic mice were immunized twice with M1, as mentioned above. Control mice received a sublethal dose of H1N1 virus or were injected with the irrelevant peptide. Three weeks later, the M1-immunized mice were separated in four groups: one group untreated, and three other groups injected with antibodies to deplete, respectively, CD8+, CD4+, or both types of lymphocytes. A previous experiment demonstrated that these treatments did not affect the survival of uninfected mice or of influenza-challenged mice which were previously immunized with Immugrip (an inactivated influenza virus preparation which induces protective influenza antibodies, data not shown). As demonstrated in Fig. 1B, while approximately 50% of the M1-primed/untreated mice survived the lethal challenge at day 15, all the mice depleted of CD8+, CD4+, or both T cell subsets died within 13 days, comparing favorably with the mice
primed with the irrelevant peptide. These results indicated that both T lymphocyte populations were required for the protection of HLA-A2/Kb transgenic mice against a lethal influenza infection.

M1-specific memory T cells in HLA-A2/Kb transgenic mice after immunization with M1

To investigate whether M1-specific memory T cells were induced after immunization, spleen cells were harvested from HLA-A2/Kb transgenic mice 3 weeks after two s.c. injections of M1 and tested for their ability to proliferate upon in vitro stimulation. Control mice were immunized twice with ELA prepared, as described for M1, with TTP30 and IFA. A significant proliferation of spleen cells was induced for most of the M1- and ELA-immunized mice in the presence of TTP30 (not shown). This proliferation was due to CD4+ T cells since it was maintained after CD4+ T cell depletion (Fig. 2B), but it was prevented by elimination of CD8+ T lymphocytes (Fig. 2A). No proliferation occurred with ELA, except when the mice were previously primed with this peptide (not shown). Thus, M1 induced specific CD8+ memory T cells which were detectable in the spleen of HLA-A2/Kb transgenic mice 3 weeks after the last distant immunization.

To investigate the frequency of the M1-specific T lymphocytes, spleen cells from HLA-A2/Kb transgenic mice immunized one, two, or three times were harvested, numerated, and stained with anti-CD8 antibodies and M1-loaded HLA-A2 dimers. Similar numbers of CD8+ T cells were found in the spleens in the different groups, as shown in Fig. 3. However, the percentage of M1-immunized mice with CD8+ memory T cells which were detectable in the spleen of HLA-A2/Kb transgenic mice 3 weeks after the last distant immunization.

Fig. 1. Protective effects of M1-immunization in HLA-A2/Kb transgenic mice HLA-A2/Kb transgenic mice were immunized by two or three s.c. injections of M1 (M1 2x and M1 3x, respectively) or ELA (ELA 3x) with TTP30 and IFA. Control mice for survival were primed i.n. with a sublethal dose of H1N1 virus (H1N1). (A) Three weeks after the last immunization, the mice were challenged with the LD100 of H1N1 virus. The results of a representative experiment out of three show the survival rate in each group containing 8 to 12 mice. (B) Three weeks after the last immunization, M1-immunized mice were depleted of CD4+ (M1-CD4), CD8+ (M1-CD8), or CD4+ and CD8+ (M1-CD4/8) T cells as described under Materials and methods. The survival rate was followed in each group (7–9 mice/group) after i.n. challenge with the LD100 of H1N1 virus.
M1-specific memory cytotoxic T cells in the lungs of HLA-A2/Kb transgenic mice following influenza challenge

Since lung CD8+ T cells are critical in recovery from lethal influenza challenge (Cerwenka et al., 1999a,b), we investigated whether such cells and memory lymphocytes were present in the lungs of M1-primed mice after influenza infection. HLA-A2/Kb transgenic mice were immunized s.c. two or three times with M1 or ELA and challenged 3 weeks later with H1N1 virus. Lung-infiltrating cells were isolated 9 days after challenge. Those of unprimed/unchallenged mice were used as control. The numbers of CD8+ T cells increased dramatically in the lungs of the infected mice, as compared with unprimed/unchallenged animals (approximately by six- and sevenfolds in the ELA and the M1-primed groups, respectively; Fig. 4). In addition, the cells varied considerably in their level of activation and expression of surface markers specific for naive and memory T lymphocytes. Unlike the unprimed/unchallenged mice, the mice immunized with M1 or ELA showed a high percentage of activated CD4+ (around 75 versus 10% in unprimed/unchallenged mice, not shown) and of CD8+ T cells (80 versus 5% in unprimed/unchallenged mice; Fig. 4). Interestingly, the numbers and the percentage of activated CD8+ T cells were significantly increased in the mice immunized with M1, as compared with those immunized with ELA (P < 0.05). In addition, the decrease in the percentage of naive T cells (CD3+CD62Lhigh cells) and the increase in the percentage of memory T lymphocytes (CD3+CD49d+ cells) were significantly amplified in the M1 as compared with the ELA-primed group (P < 0.05). Thus, a more efficient recruitment and/or proliferation of CD8+ and memory T cells occurred in the lungs of M1-primed mice.

Fig. 2. M1-specific T cells in the spleen of HLA-A2/Kb transgenic mice. HLA-A2/Kb transgenic mice were immunized twice s.c. with M1 (mouse 1 to 5) with TTP30 and IFA and sacrificed after 3 or 4 weeks. Spleen cells were harvested and cultured in vitro after depletion of CD8+ (A: CD4+ T cells) or CD4+ (B: CD8+ T cells) T cells, in medium alone (0), or with 10 μg/ml of M1, TTP30, or ELA. Thymidine incorporation was measured after 72 h of culture. The results of a representative experiment out of three show the mean cpm ± SD calculated from quadruplicate wells in each culture with the responding mice.

Fig. 3. M1-specific HLA-A2-restricted T cells in the spleen of HLA-A2/Kb transgenic mice. HLA-A2/Kb transgenic mice were immunized once (1×), twice (2×), or three (3×) times s.c. with M1 or ELA with TTP30 and IFA and sacrificed after 3 or 4 weeks. Spleen cells were isolated and stained with anti-CD8 Abs and M1-loaded HLA-A2 dimers. The results of a representative experiment out of three show (A) the mean total numbers ± SD of CD8+ T cells and (B) the mean % ± SD of these cells stained with M1-loaded HLA-A2 dimers in the spleen calculated with 5–6 mice in the different groups. *P < 0.05, as compared with the ELA group.
after influenza challenge than in mice immunized with the irrelevant peptide.

Lung infiltrating cells were further tested for their cytotoxic activity. Lymphocytes were incubated, without in vitro restimulation, with mouse target cells such as EL4 (H-2Kb) and P815 (H-2Kd) or with T2 cells (HLA-A*0201). No cytotoxic activity was ever detected without a previous influenza challenge (not shown), nor after challenge, when murine cell lines, EL4 or P815 cells, were used as targets, either untreated, infected with H1N1 virus, or pulsed with M1 or ELA (data not shown). In contrast, as reported in Table 1, lymphocytes of M1 (but not of ELA)-immunized mice were directly lytic to T2 cells previously pulsed with M1 (but not with ELA) or infected with H1N1 virus. In addition, the percentage of mice with cytotoxic T cells in lungs increased with the number of immunizations: approximately 50% of M1-primed mice had cytotoxic cells after two immunizations, and 80% had cytotoxic cells after three immunizations (Table 1). These data correlated favorably with the above reported percentages of mice showing a proliferative cellular response upon stimulation in vitro with M1 and having a prolonged survival after two and three immunizations. Thus, the T cell-dependent influenza protection of HLA-A2/Kb transgenic mice correlated with the detection of M1-specific memory T cells, in the spleen 3 weeks after the last immunization, and, after influenza challenge, in the lungs, where they became activated and directly cytotoxic to influenza-infected cells.

Discussion

Analysis of the T cell repertoire developed in HLA-A2 people after influenza infection consistently pointed out the predominance of a CTL response generated against the matrix protein epitope encompassing amino acid residues 58 to 66 (Gianfrani et al., 2000; Gotch et al., 1987; Jameson et al., 1998). However, the importance of this response in recovery from infection was completely unknown. In addition, it remained particularly difficult to investigate without the possibility of isolating it from concomitant responses (due to other viral antigens and other immune mechanisms) and investing its effects on the course of an influenza infection. Taking advantage of HLA-A2 transgenic mice, we demonstrated for the first time that M1 induced a specific T cell response with a protective activity against influenza infection.

The HLA-A2 molecule is the most common allele in humans and many viral or tumoral peptides able to stabilize this class I MHC molecule as well as to induce HLA-A2-restricted CTLs have been described (Bharadwaj et al., 2001; Keogh et al., 2001; Zhou et al., 2001). Differences may be observed between the strength and the hierarchy of the responses generated to the different epitopes in transgenic mice compared to those seen in human following natural infection (Bharadwaj et al., 2001). However, a peptide which is recognized by human memory cytotoxic T cells was generally also immunogenic in the corresponding
transgenic mice. On the other hand, HLA-transgenic mice provided an interesting model for the identification of otherwise unidentified human CTL epitopes (Man et al., 1995).

In the case of influenza, M1 was shown to represent, as in humans, a major determinant for the murine CTL response in HLA-A2 transgenic mice, following either influenza infection or immunization with the whole virus (Engelhard et al., 1991; Vitiello et al., 1991). Thus, it appeared particularly convenient to use these mice to generate M1-specific responses and to assess their influence on the course of influenza infection. M1 immunization significantly reduced the morbidity and increased the survival rate after a lethal influenza challenge. These effects correlated with the detection of CD8+ memory T cells able to recognize the peptide upon in vitro stimulation, and to bind M1-loaded HLA-A2 dimers in the spleen, 3–4 weeks after the last distant immunization. In accordance with these results and the known properties of M1, CD8+ T lymphocytes were confirmed to be critical, since survival was abolished after in vivo depletion of these cells.

Interestingly, elimination of CD4+ T cells also resulted in mouse death, indicating that this cell subset was also important. Helper T cells play a key role in antiviral immunity with pleiotropic effects, such as cytokine secretion and help for antibody production. They are critical for the generation of memory CD8+ CTL responses, both in vitro and in vivo (Ostankovich et al., 1997; BenMohamed et al., 2000; Fernando et al., 2002; La Gal et al., 2002). In addition, CD4+ CTLs have been described in humans (Engelhard et al., 1991; Linnemann et al., 2000), as well as in mice lacking CD8+ cytotoxic T cells (Bot et al., 1998; Epstein et al., 2000; Sherman et al., 1992). However, the in vivo contribution of CD4+ CTLs against infectious organisms remains controversial. In our experiments, immunization with M1 resulted in the generation of CD4+ memory T cells which were specific for TTP30 (the helper peptide used during immunizations), but not for M1. TTP30-specific CD4+ T cells were also detected in ELA-primed mice (not shown), although no protective effect was observed. Thus, the death of M1-primed mice following depletion of CD4+ T cells was likely to reflect the need of these cells in amplification of the CD8+ T cell response rather than a direct antiviral role. This requirement agrees with previous observations made by others (Fernando et al., 2002; La Gal et al., 2002; BenMohamed et al., 2000) and correlates with the necessity of using TTP30 during immunizations to generate a detectable T cell response against M1. It is further reinforced by the notion that antiviral protection might be more difficult to achieve without appropriate amplification of the immune response, after immunization with a single CD8+ T cell epitope than after immunization, with a mixture of several ones.

As pulmonary lymphocytes are particularly relevant to respiratory virus clearance (MacKenzie et al., 1989; Flynn et al., 1998; Cerwenka et al., 1999a and b), we focused our attention on them. Flow cytometry analysis showed that influenza challenge resulted in activation of lung infiltrating lymphocytes, as well as in the presence of large numbers of cells with a memory phenotype. These modifications were seen in both ELA- and in M1-primed mice, indicating, in accordance with earlier observations (Flynn et al., 1999; Hogan et al., 2001), that massive recirculation, recruitment, and/or proliferation of T cells with a memory phenotype occurred following influenza infection. However, the percentages of activated and memory T cells were further increased after immunization with M1, as compared with ELA, suggesting that a fraction of lung lymphocytes was specifically reactive to influenza antigens. This hypothesis was verified by the detection in M1-primed mice only, of lung-infiltrating T cells able to lyse, without in vitro re-stimulation, target cells which were either pulsed with M1 or infected with H1N1 virus. Thus, M1 provided a stimulation which was antigenically authentic relative to native influenza antigens. This priming favored the recruitment and/or the amplification and the reactivation of M1-specific memory T cells at the site of influenza infection. This is fully consistent with the previously documented migration

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<th>Table 1: Cytotoxic activity of lung infiltrating cells</th>
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*Note.* HLA-A2/Kb transgenic mice were immunized with two or three s.c. injections of M1 (M1 3× and M1 2×, respectively) or the irrelevant peptide (ELA 3×) in TTP30 and IFA and challenged after 3 weeks with the LD100 of H1N1 virus. Lung infiltrating cells were isolated upon enzymatic digestion 9 days later and assessed without in vitro restimulation for their cytotoxic activity against HLA-A2+ T2 target cells alone (T2), previously infected with H1N1 virus (T2-H1N1), pulsed with M1 (T2-M1) or with ELA (T2-ELA). The results of a representative experiment out of three show the percentage of specific lysis obtained at effector/target cell ratio of 60:1, after 6 h of incubation in the presence of lung infiltrating cells of 5 to 10 mice in each group. nd, not done.
patterns of memory T cells (Weninger et al., 2001); it is in accordance with the role of lung CD8⁺ T cell infiltration in recovery from lethal influenza challenge (Cerwenka et al., 1999b).

It was noteworthy that the CTL detected in M1-primed mice were lytic to HLA-A2⁺ targets but completely inefficient against cells expressing murine MHC molecules. Thus, influenza protection induced upon M1-immunization in the HLA-A2/Kb transgenic mice was solely mediated by HLA-A2-restricted CD8⁺ T cells.

In conclusion, we showed that HLA-transgenic mice can be used not only, as previously, to explore the human T cell repertoire developed against viruses in the context of a particular human MHC molecule, but also to evaluate in vivo the protective efficacy of individual epitopes. In the case of influenza, it is formerly assumed that most of the cross-protection observed against different strains of viruses relies on the activity of cytotoxic T cells (Sambhara et al., 2001). It would thus be particularly interesting to identify the epitopes derived from internal proteins which are less abundant to the infected cell surface but also less variable than HA or NA, to design a vaccine. This vaccine could provide a broader and more lasting immunity. Also, a similar strategy could be applied for identification of protective epitopes for other virus infections and for tumor immunotherapy.

Materials and methods

Mice

Specific pathogen-free 7- to 8-week-old female C57BL/6 mice (H-2b) were purchased from IFFA Credo (L’Arbresle, France); HLA-A2/Kb transgenic mice (HLA-A*0201/Kb; 6-A2KB H-2B) (Sherman et al., 1992) were acquired from Harlan (U.S.) or from breeding cages of less than four generations. The mice were housed and manipulated according to French and European guidelines. They were kept under specific pathogen-free conditions, fed rat and mouse maintenance diet A04 (UAR, Villemoissin-sur-Orge, France), and given water ad libitum.

Peptides Flu-M1 and TTp30 syntheses, purification, and characterization

The peptide chains corresponding to influenza A matrix M1 58-66 (GILGFVFTL) and a 21-mer tetanus toxin-derived synthetic peptide (amino acid residues 948-968: NNTVSVFLRVPKVSASHLEQ) called TTp30, containing an immunodominant T cell epitope for both human and mice were synthesized by solid-phase method on an Applied Biosystems 433A synthesizer using FMOC/tBu chemistry. The side-chain protecting groups were trityl (Trt) for His, Asn, and Gln; tbutyl (tBu) for Ser, Thr, and Asp; Pentamethylchromansulfonyl (Pmc) for Arg, and tert-butyloxycarbonyl (tBoc) for Lys and Trp. The crude peptides, cleaved off from resin with trifluoroacetic acid (TFA) in the presence of scavengers, were lyophilized and purified by preparative reverse phase-high performance liquid chromatography (RP-HPLC) as previously described (Plotnicky-Gilquin et al., 2000). The peptides were characterized by electrospray-mass spectroscopy (ES-MS), their purity was higher than 95%, as indicated by RP-HPLC. ELA (ELA-GIGILTV), a Melan-A/MART-1 antigen immunodominant peptide analogue (Rivoltini et al., 1999), was synthesized as previously described (Beck et al., 2001). It was used throughout the experiments as an irrelevant peptide.

Virus

The mouse-adapted A/PR8/34 (H1N1) virus was purchased from the American Type Culture Collection (ATCC VR95). It was propagated for 3 days at 35°C in the allantoic cavity of day 10 fertilized eggs and purified on a continuous sucrose gradient according to the method of Bachmann et al. (1999). It was resuspended in PBS (2564 hemagglutination Units/ml) and stored in small aliquots at −80°C until use. The lethal doses (LD₁₀₀) corresponding to 100% of death were determined for each strain of mice.

Immunization procedures and challenge

Mice were immunized at the base of the tail once, twice, or three times at 3-week intervals by s.c. injections of a 200 µl PBS preparation containing 100 µg M1 or ELA, mixed with 100 µg TTp30 and 50% v/v of incomplete Freund’s adjuvant (IFA, Sigma). Peptides were previously solubilized in DMSO at 2 mg/ml. Control mice for protection were immunized by i.n. instillation of 0.1 HAU of purified H1N1 virus in 50 µl PBS. Three or 4 weeks after the last immunization, mice were bled to determine H1N1 virus-specific serum antibody titers. They were anesthetized with 2.5 ml/kg of a 4/1 mixture (v/v) of ketamine (Imalgene 500, Rhône Mérieux, Lyon, France) and xylazine (Rompun at 2%, Bayer, Puteaux, France) before i.n. instillation of 50 µl containing the LD₁₀₀ of H1N1 virus and then followed for survival and body masses.

In vivo depletion of T cells

Depletions of CD4⁺ and/or CD8⁺ T cells were performed as previously described (Plotnicky-Gilquin et al., 2000). Mice were injected intraperitoneally with mAbs GK1–5 (anti-CD4) and H35 17.2 (anti-CD8) 1 day before challenge and again on day 2 and 3 thereafter. More than 98% of the targeted T lymphocytes were eliminated as monitored by flow cytometry.
ELISA
Serum influenza antibody titers were determined by ELISA, as previously described (Power et al., 1997), except that the plates were coated overnight at +4°C with 50 μl per well of Immugrip (inactivated influenza strains 1999/2000; Pasteur Merieux Serums et Vaccins, Lyon, France) adjusted to 0.5 μg HA protein/ml in PBS. The serum of HLA-A2/Kb transgenic mice, immunized three times ip with 100 μl Immugrip diluted 1/3 in PBS, was used as a positive control. Optical densities (OD) were measured at 450 nm.

Preparation of lung infiltrating cells and FACS analysis
Mice were anesthetized and exsanguinated by cardiac puncture 9 days after challenge with the LD100 of H1N1 virus. Lung infiltrating cells were isolated upon enzymatic digestion, numerated, and stained as reported (Plotnicky-Gilquin et al., 1999). Cells were incubated with combinations of fluorescein (FITC)- and phycoerythrin (PE)-conjugated mAbs to lymphocyte surface molecules CD3, CD4, CD8, CD69, CD62L, and CD49D (Caltag, San Francisco, CA), at previously determined optimal concentrations. They were analyzed for two color stains using a FACSVantage (Becton–Dickinson, Erembodegem, Belgium) after removing debris and dead cells by gating on forward and side scatter parameters. The total numbers of CD8+ T cells were determined for each mouse by multiplying the percentage of stained cells by the number of nucleated cells isolated from the lungs.

T cell proliferation assay
Splenocytes were harvested 3 or 4 weeks after the last immunization. CD4+ or CD8+ T cells were depleted (>95%) using a magnetic cell separator (MACS, Miltenyi Biotex, Bergisch Gladbach, Germany), according to the manufacturer’s instructions. Cells were washed, numerated, and resuspended at 10^6 cells/ml in culture medium consisting of RPMI 1640, 10% heat-inactivated fetal bovine serum, 2 mM L-glutamine, 100 U gentamicin per milliliter, and 5 × 10^{-5} M mercaptoethanol (all from Gibco, Cergy Pontoise, France). They were stimulated at 5 × 10^5 cells/well in 96-well round-bottom plates (Costar, Brumath, France), for 72 h at 37°C, 5% CO2, with TTp30, M1, or ELA (1 mg/10 μl DMSO), at a final concentration of 10 μM/μl. All cultures included wells stimulated with 5 μg/ml of concanavalin A (ConA, Sigma) as positive controls (not shown). Eighteen hours before the end of the culture, the cells were pulsed with 0.25 μCi methyl 3H-thymidine (Amersham, Les Ulis, France). They were harvested onto filters using a semi-automatic harvester (Skaton, Lier, Norway) and the incorporated 3H-thymidine was determined using a COBRA II auto gammacounter (Packard Instruments, Meriden, CT). The results were expressed in counts per minute and were calculated from the geometric means of quadruplicate cultures.

Staining of cells with HLA-A2 dimers
A stock peptide solution was prepared at 100 μg/ml in DMSO. M1 was incubated overnight at 37°C together with HLA-A2 dimers (HLA-A2: Ig protein, Pharmingen) at a 50 molar excess, in the presence of β2-microglobulin (stock solution at 250 μg/ml), i.e., 1 μg dimer, 0.23 μg M1, and 0.5 μg β2-microglobulin for each test sample. Spleen cells (10^9) were incubated in 100 μl RPMI 1640–10% FCS with 10 μl human IgG (stock solution: 200 μg/ml) for 10 min at RT. After one wash, 10 μl RPMI containing M1-loaded dimers, or empty dimers as control, were added to the cell pellet and samples were incubated for 60 min at 4°C, under agitation. After two washes in PBS–0.2% BSA–50 μM EDTA, cells were resuspended in 100 μl PBS–1% bovine serum albumin–0.01% NaN3 and incubated for 30 min at 4°C with 0.5 μg of a PE-conjugated rat anti-mouse IgG1 Ab (Pharmingen). After washes in PBS–0.2% BSA–50 μM EDTA, cells were resuspended in 100 μl PBS–1% BSA–0.01% NaN3 and incubated for 30 min at 4°C with 0.5 μg of FITC-conjugated rat anti-mouse CD8 Abs (Pharmingen). After washes, samples were transferred into FACS tubes and analyzed on a FACSVantage SE flow cytomter (Becton–Dickinson). Fifty to 100 thousand CD8+ cells were analyzed for each sample, after gating lymphocytes on FCS-SSC parameters. The total numbers of CD8+ T cells in the spleens were calculated by multiplying the percentage of cells stained with the anti-CD8 Abs by the total numbers of nucleated cells in the spleen. The percentage of A2 dimer/ M1-positive CD8+ T cells were corrected by subtraction of the percentage of cells stained with empty dimers.

Cytotoxicity assay
Cytotoxic activities were measured using a 51Cr release assay. Human T2 (Tap−/−, HLA-A*0201) or murine EL4 (H-2Kb) and P815 (H-2Kk) cells were used as targets, either untreated or after infection with H1N1 virus, or incubated overnight at 37°C with peptides at 50 μg/ml in OptiMEM-1 (Life Technology, Gibco, no.; 31985-047). The expression of viral antigens was confirmed by staining of the cells with a mouse anti-H1N1 virus serum. The fixation of peptides on T2 cells was revealed by stabilization of class I MHC molecules on the cell surface after staining with an anti-HLA class I Ab (MA2.1, no. HB54, ATCC). Target cells were washed, numerated, and labeled by a 2-h incubation, with 100 μCi Na51CrO4 (Amersham) per 10^6 cells. After washes, target cells were mixed with lung infiltrating cells at an effector/target cell ratio of 60:1 in 96-well plates and incubated at 37°C, 5% CO2, for 6 h. Spontaneous and maximum 51Cr releases were obtained in wells containing the target cells in medium or with 100 μl Triton X-100 diluted 1/100 in water, respectively. After plate centrifuga-
tion, a 100-μl sample of supernatant from each cell mixture was counted in a Packard 1900CA-Tricarb betacounter to determine the amount of 51Cr released from target cells. The percentages of specific lysis were calculated as follows: % specific lysis = (experimental release − spontaneous release)/(total release − spontaneous release) × 100.

The spontaneous release for T2 and murine target cells in medium alone never exceeded 15 and 20%, respectively.

Statistical analyses

Statistical analyses were done using the t-test and Kolmogorov–Smirnov test (for low sample numbers) of the Statigraphic software program (Manugistics, Rockville, MD).

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References


