μl/ml; Sigma-Aldrich) was added at the same time to block cytokine secretion. The cells were washed after 5 hours and resuspended at 10^7 cells/ml in staining buffer (Dulbecco's PBS without Mg^{2+}or Ca^{2+}, 1% heat-inactivated FCS, w/v 0.09% sodium azide; pH adjusted to 7.4–7.6). The cells were then incubated for 15 min with purified 2.4 G2 antibody 10 μg/ml (PharMingen) to block nonspecific staining by fluorochrome-conjugated antibodies via Fc receptors. The cells were washed twice with staining buffer, and 10^6 cells were stained with 1 μg FITC-labeled anti-mouse CD8a and PerCP-labeled anti-mouse CD3e (PharMingen) in 50 μl staining buffer at 4°C for 30 min. The cells were again washed twice with staining buffer, followed by fixation and permeabilization with Perm/Wash (Cytofix/Cytoperm Kits; PharMingen) for 20 min at 4°C. The cells were washed twice and resuspended in 50 μl of the same solution. PE-conjugated anti-interferon (IFN)-γ monoclonal antibody (0.5 μg/10^6 cells; PharMingen) was added and the suspension was incubated for 30 min at 4°C. The cells were then washed twice with Perm/Wash solution and resuspended in staining buffer.

The FACScan was used to analyze the percentage of intracellular IFN-γ-containing cells among the $CD3^+$ and $CD8^+$ cells. Isotype controls for anti-CD3a, anti-CD8e, and anti-IFN-γ (PharMingen) were subtracted from the acquired data.

ELISPOT analysis of IFN-γ production by immune spleen cells

The procedure for the ELISPOT technique was obtained from PharMingen, whose reagents were used whenever possible. The wells of an ELISPOT plate (CTL Immunospot plate; Cellular Technology Ltd, Cleveland, OH, USA) were coated overnight at 4°C with 100 μl anti-mouse IFN-γ (2 μg/ml; PharMingen) diluted at 1:200 in coating buffer (PBS, pH 7.2). The coated wells were washed with blocking solution (R10S medium), fresh blocking solution was added to each well, and then the plate was incubated for 2 hours at room temperature. The blocking solution was then discarded and 100 μl A2L2 cells (2×10^5-1×10^6) was added to each well of the ELISPOT plate.

Immune spleens were dissected from vaccinated mice and were prepared as already described for tetramer analysis. Increasing numbers of spleen cells (5×10^6 - 2×10^7) in 100 μl R10S medium were added to the wells, and the plate was incubated for 24 hours at 37°C in a 5% CO_2 atmosphere at 99% humidity. Wells containing only spleen cells served as negative controls, and spleen cells from VRP-*neu*-vaccinated mice cultured overnight with 5 μg/ml concanavalin A (Sigma-Aldrich) served as a positive control. The cell suspension was aspirated, and the wells were washed twice with deionized water and were then soaked with deionized water for 5 min. The wells were washed three times with wash buffer I (PBS containing 0.05% Tween-20).

The detection antibody, biotinylated anti-mouse IFN-γ (PharMingen), was diluted to 2 μg/ml in dilution buffer (PBS containing 10% FBS), and 100 μl was added to each well. The plate was incubated at room temperature for 2 hours, and the wells were washed three times with buffer I. Avidin–horseradish peroxidase reagent (PharMingen) was diluted to 1:100 in dilution buffer, and 100 μl was added to each well, which was then incubated at room temperature for 1 hour. The wells were washed four times with wash buffer I and twice with wash buffer II (PBS). A stock solution containing 100 mg 3-amino-9-ethyl-carbazole (Sigma) dissolved in 10 ml *N*, *N*-dimethylformamide (Sigma-Aldrich) was prepared. The final substrate solution was made by adding 333 μl 3-amino-9-ethyl-carbazole stock solution to 10 ml of 0.1 M sodium acetate (pH 5.0), followed by filtering through a 0.45-μm filter. Five microliters of 30% H_2O_2 was added to the substrate solution immediately before use.

One hundred microliters of the final substrate solution was added to each well, and the plate was incubated in the dark for 5–60 min at room temperature. The reaction was stopped by washing the wells with deionized water. The plate was air-dried overnight at room temperature in the dark and sent to ZellNet Consulting, Inc. http://www.zellnet.com, where the spots were enumerated automatically using an ImmunoSpot Series I analyzer (BD Biosciences). If overlapping spots (confluence) were present in the wells, the number of spots in a nonconfluent area of that well was determined. To estimate the total number of spots in each well with confluence, the following equation was used: total spot number = spot count + 2 × (spot count × % confluence / [100% - % confluence]).

Statistical analysis

Student's *t* test was performed using Prism 4.0 Graphpad Software (San Diego, CA, USA). Statistical analysis, power analysis and the sample size per group were evaluated and found to be statistically acceptable by Dr Lyle Broemling (Associated Professor of Biostatistics, The University of Texas MD Anderson Cancer Center).

Results

Induction of antigen-specific IgG by vaccination with VRP-neu

Groups of Balb/c mice ($n = 5$ per group) were vaccinated once subcutaneously in one hind leg footpad with either 10^6 IU VRP-*neu* or 10^6 IU VRP-HA suspended in PBS. The HER2/*neu*-specific humoral response of serum pooled from mice in each group was measured 14 days later by flow cytometry using A2L2 cells. Compared with the mice vaccinated with VRP-HA, the mice vaccinated with VRP-*neu* had a strong IgG response (Fig. 1). Pre-immune sera for both groups were nonreactive with A2L2 cells, and immune sera from both vaccinated groups were nonreac-