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Specific immunotherapy of experimental myasthenia gravis *in vitro* and *in vivo*: The Guided Missile strategy

W. Sun [a], R.N. Adams [b], A. Miagkov [c], Y. Lu [d], H.-S. Juon [e], D.B. Drachman [b,*]

[a] Dept. of Microbiology and Immunology, Univ. of Maryland School of Medicine, Baltimore, MD 21201, United States
[b] Dept. of Neurology, Johns Hopkins School of Medicine, Meyer 5-119, 600 No. Wolfe St., Baltimore, MD 21287-7519, United States
[c] Novascreen Biosciences Corp., 7170 Standard Dr., Hanover, MD 21076, United States
[d] Sirnaomics, Inc., 401 Professional Dr., Suite 130, Gaithersburg, MD 20879, United States
[e] Dept. of Health, Behavior & Society, Johns Hopkins Bloomberg School of Public Health, 624 No. Broadway, 704, Baltimore, MD 21205, United States

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ABSTRACT

Current immunotherapy of myasthenia gravis (MG) is often effective, but entails risks of infection and neoplasia. The "Guided Missile" strategy described here is designed to target and eliminate the individual's unique AChR-specific T cell repertoire, without otherwise interfering with the immune system. We genetically engineered dendritic cells to present AChR epitopes and simultaneously express Fas ligand in an ongoing EAMG model. In both *in vitro* and *in vivo* experiments, these engineered cells specifically killed AChR-responsive T cells without otherwise damaging the immune system. AChR antibodies were markedly reduced in the treated mice. Translation of this method to treat human MG is possible.

1. Introduction

Myasthenia gravis (MG) is an autoimmune disorder characterized by weakness and fatigability of skeletal muscles. The pathogenesis of MG involves a reduction of the available acetylcholine receptors (AChRs) at neuromuscular junctions (Fambrough et al., 1973). Autoantibodies directed against AChRs are present in the great majority of cases (Lindstrom et al., 1976; Engel et al., 1979), or less commonly against muscle specific kinase (MuSK), another related neuromuscular junction protein (Hoch et al., 2001; Sanders et al., 2003). The autoantibodies reduce the available AChRs by several mechanisms (Kao and Drachman, 1977; Toyka et al., 1977; Drachman et al., 1980; Cole et al., 2010; Viegas et al., 2012). Although treatment of MG with general immunosuppressive agents is usually effective (Drachman, 2008), it has important drawbacks, including overall suppression of the immune system, with risks of infection or rarely neoplasia, as well as other adverse side effects of the medications (Berger and Houff, 2009). Treatment must usually be continued indefinitely, and patients are rarely "cured". Ideally, treatment of MG should eliminate the specific pathogenic autoimmune response without otherwise suppressing the immune system or producing other adverse side effects, and the results should be permanent, long-lasting or readily repeatable. Although autoantibodies are directly responsible for the loss of AChRs at neuromuscular junctions, therapeutic strategies directed at AChR-specific B cells are not practicable in ongoing disease (Drachman, 1994; Lindstrom, 1999). However, the AChR antibody response is T cell dependent (Newsom-Davis et al., 1989; Ragheb and Lisak, 1998), and immunotherapy directed at T cells can abrogate the B cells' autoantibody responses, with resulting clinical benefit (Christadoss and Dauphinee, 1986; Wraith et al., 1989; McIntosh et al., 1998). In designing specific immunotherapy for MG, a critical challenge is the marked heterogeneity of the T cell responses (Brocke et al., 1988; Hohlfeld et al., 1988; Newsom-Davis et al., 1989; Melms et al., 1992; Conti-Fine et al., 1998; Jung et al., 2008). Each myasthenic individual's repertoire of AChR-responsive T cells interacts with multiple AChR epitopes, and there are significant differences in the patterns of epitopes to which different individuals' T cells respond both in MG in humans and in EAMG in animals (Yeh and Krolick, 1987; Yang et al., 1998). Since a given patient's own antigen presenting cells (APCs) present the entire spectrum of AChR epitopes that are relevant to that individual's AChR-specific T cells, it should be possible to coopt the APCs for therapeutic use to target and eliminate those T cells. In this study, we have tested the ability of genetically engineered antigen presenting dendritic cells (DCs) to target and destroy AChR-specific T cells, in an experimental mouse model of ongoing MG (EAMG). Our findings show that DCs engineered to present AChR epitopes and Fas ligand (FasL) simultaneously can specifically target and kill AChR-responsive T cells both *in vitro* and *in vivo*, resulting in a marked reduction of both T cell responses to AChR and anti-AChR antibodies. Although technically challenging, this strategy could be adapted for the treatment of patients with MG.

* Corresponding author. Tel.: +1 410 955 5406; fax: +1 410 955 1961.
E-mail address: dandrac@aol.com (D.B. Drachman).

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2. Materials and methods

2.1. Mice and reagents

Female, 6–8 wk old, wild-type (wt) C57Bl/6 mice were purchased from the National Cancer Institute (Frederick, MD). Fas-deficient lpr/lpr mice were purchased from Jackson Laboratories (Bar Harbor, ME). C57Bl/6 transgenic mice expressing a high percentage of T cells with receptors specific for the immunodominant α subunit epitope of Torpedo AChR (Lobito et al., 2002) were bred in the Transgenic Facility at Johns Hopkins, and were used to produce T cell lines for *in vitro* experiments. Mice were kept under pathogen-free conditions at Johns Hopkins University, School of Medicine. All animal experiments were performed in accordance with protocols approved by the Animal Care and Use Committee of the Johns Hopkins School of Medicine.

2.2. Antigens

Acetylcholine receptor (AChR) was purified from the electric organs of *Torpedo californica* (Pacific Biomarine, Venice, CA) by affinity chromatography, using cobra toxin linked to Sepharose 4B beads, as previously described (Eldefrawi and Eldefrawi, 1973). Hemocyanin from Keyhole Limpets (KLH) was obtained from Sigma, St. Louis, MO.

2.3. Construction and generation of adenoviral vectors

2.3.1. Adenoviral production

Because the AdV5 used in these experiments is replication-incompetent, it must be "packaged" in a cell line (HEK293) that provides it with the E1 genes that are essential for the replication and transcription of the AdV viral DNA. However, HEK293 cells express Fas, and are therefore vulnerable to FasL-induced apoptosis. To protect the HEK293 cells from FasL-induced apoptosis, we stably transfected them with the baculoviral p35 gene driven by a CMV promoter. The p35 protein is a highly potent anti-apoptotic agent (Miagkov et al., 2004). Our HEK293-P35 line is resistant to FasL-induced apoptosis, and efficiently produces FasL-bearing AdV.

HEK293 cells or HEK293-P35 cells were infected with viruses at a multiplicity of infection (MOI) of 10 PFU/cell and harvested when all of the cells were rounded up and about 80% were detached. The cells were centrifuged, and the pellets were combined in a total of 10.0 ml sterile PBS. The cells were lysed by three freeze-thaw cycles, and centrifuged at 1000 rpm for 5 min to remove the debris. The supernatant, containing crude virus was purified by ultracentrifugation on a CsCl density gradient (Green and Loewenstein, 2006), and the AdV titers were determined using a Clontech titration kit, according to the manufacturer's instructions.

2.3.2. Insertion of genes into adenoviral vectors

We used the Adeno-X® cloning system (Clontech) for engineering of recombinant adenoviral vectors expressing the genes of interest. It utilizes two plasmids: (1) A destination (Ad-X) plasmid, which carries the adenovirus 5 genome with E1 and E3 segments deleted, producing a safe, replication-incompetent AdV vector, with space for >8 kb of foreign DNA. (2) A shuttle plasmid (pShuttle) for cloning of the DNA constructs of interest, and for ligating them into the Ad-X plasmid.

In order to assure that each "Guided Missile" dendritic cell (DC) simultaneously presents the antigen AChR, and expresses the warhead FasL, we utilized a bidirectional inducible promoter system, delivered by the AdV vector. The bidirectional system results in transcription of both genes and expression of both proteins. The bidirectional system has two "minimal" (enhancer-deficient) CMV promoters facing in opposite directions, with a tetracycline-responsive element (TRE) between them. Each of the two genes of interest is cloned downstream from each of the CMV promoters. To activate the promoters and thereby trigger simultaneous expression of the two genes, a "tetracycline-controlled transactivator protein" (tTA) must be provided and must bind to the TRE. The tTA itself is delivered to the DC by co-infection with a separate AdV vector that constitutively expresses tTA. The two gene constructs used in these experiments are as follows: (1) Fas ligand (FasL). We have engineered a non-cleavable FasL mutant (Tanaka et al., 1998), with EGFP fused to its N-terminus to monitor its expression (see below). This was cloned in the counter-clockwise position in the bidirectional promoter system. (2) The AChR presentation construct (sig-AChR α1-210-LAMP) was cloned in the clockwise position and is referred to as "AChR" hereafter. tTA is required for the expression of the two genes, and gene expression is relatively silent in its absence.

2.3.2.1. Fas ligand (FasL). Naturally-occurring FasL can be enzymatically cleaved, releasing soluble FasL, which competitively inhibits the FasL-mediated cytotoxic effect. We therefore constructed a more potent non-cleavable mutant FasL (lacking the aa 110–133 cleavage site), by a PCR method, as previously described (Tanaka et al., 1998) for use in these studies.

2.3.2.2. AChR presentation. For AChR presentation, we utilized a construct consisting of cDNA for the extracellular sequence of the Torpedo AChR α-subunit (amino acids 1–210), linked to signals of the endosomal/lysosomal associated membrane protein (mouse LAMP-1) that directs the protein to the MHC Class II trafficking pathway (Chen et al., 1988; Guarnieri et al., 1993; Valentin et al., 2009). The LAMP-1 signal sequence (sig) is at its 5′ end, and the LAMP-1 transmembrane and cytoplasmic tail (LAMP-1 Tm/Cyt) is at its 3′ end.

The adenoviral vectors used in these experiments are named according to the genes that they expressed, as follows: 1) Ad.EGFP (control vector with fluorescent tag); 2) Ad.AChR/EGFP; 3) Ad.AChR/FasL; and 4) Ad.FasL/EGFP.

2.3.3. Bone marrow-derived dendritic cells

Primary bone marrow DCs were generated from mouse bone marrow precursors as described (Inaba et al., 2009). In brief, bone marrow cells were flushed with PBS from femurs and tibias of either wild type C57Bl/6 mice or lpr/lpr mice on a C57Bl/6 background. Erythrocytes were lysed with 0.9% ammonium chloride. After 3 washes, cells were seeded in 6-well plates at 1×10^6/ml in 3 ml RPMI 1640 containing 10% heat-inactivated FBS, 20 mM HEPES, 2 mM glutamine, 1 mM non-essential amino acids, sodium pyruvate, 0.1 mM 2-mercaptoethanol and 20 ng/ml recombinant murine GM-CSF. On day 2, nonadherent cells (granulocytes) were gently removed and fresh media was added. On day 4, loosely adherent DC aggregates were dislodged and re-plated. On day 6 of culture, released, nonadherent cells, which had the typical morphological features of DCs were harvested. The purity of DCs, as measured by a panel of antibodies (BD PharMingen, San Diego, CA) to the surface markers CD11c, MHC II, CD80, CD86 and CD40, indicated that more than 85% of cells were authentic DCs.

2.3.4. Adenovirus transduction of DCs

Immature DCs were transduced at 37 °C for 4 h, at an MOI of 200 for each adenovirus, except for Ad.tTA which was transduced at an MOI of 100. DCs were thoroughly washed 3 times with culture medium, and re-plated in complete medium with 20 ng/ml recombinant mouse GM-CSF and 20 ng/ml IL-4 for an additional 24 h. The transduction efficiency was determined by flow cytometry of the cell surface expression of FasL or EGFP depending on the AdV vector used.

3. Experimental Protocols

Each of the following *in vitro* experiments was repeated five times, with closely similar results.